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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
16 September 2010 (16.09.2010)

PCT

(10) International Publication Number
WO 2010/103488 A1

(51) International Patent Classification:
A61K 39/12 (2006.01) *C12N 7/04* (2006.01)
C07K 14/18 (2006.01)

(21) International Application Number:
PCT/IB2010/051072

(22) International Filing Date:
12 March 2010 (12.03.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2,658,259 12 March 2009 (12.03.2009) CA

(71) Applicants *(for all designated States except US)*: **INSTITUT PASTEUR** [FR/FR]; 28, Rue du Docteur Roux, F-75015 Paris (FR). **HKU-PASTEUR RESEARCH CENTRE** [CN/CN]; 8 Sassoon Road Dexter HC Man Building POKFULAM, Hong Kong Sar (CN).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **WANG, Peigang** [CN/CN]; Flat 18 13/F Kwai Yick Bldg, Phase I 432, des voeux RD West, Hong Kong (CN). **ALTMEYER, Ralf, Marius** [DE/SG]; #19 Chirnhill Circle The Light #17-06, Singapore 229768 (SG). **NAL-ROGIER, Béatrice, Thérèse, Marie** [FR/CN]; 140 Pokfulam Road Middleton Towers Block, 1, 3/F, Flat 5 POKFULAM, Hong Kong Sar (CN). **KUDELKO, Mateusz** [FR/CN]; Flat A 1/F 115 Tai Peng New Village Yung, Shue Wan Lamma Island, Hong Kong (CN). **DESPRES, Philippe** [FR/FR]; 18 place de la Liberté, F-92250 La Garenne Colombes (FR).

(74) Agent: **CABINET ORES**; 36 rue de St Pétersbourg, F-75008 Paris (FR).

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

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

Published:

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

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2010/103488 A1

(54) Title: DENGUE VIRUS-LIKE PARTICLE AND USES THEREOF

(57) Abstract: The present invention relates to the field of dengue virus and more particularly to polynucleotides encoding dengue VLPs and their use in compositions and methods for eliciting a specific anti-dengue immune response against Dengue-associated diseases or infections.

DENGUE VIRUS-LIKE PARTICLE AND USES THEREOF

FIELD OF THE INVENTION

The present invention relates to the field of dengue virus and more particularly to polynucleotides encoding dengue VLPs for the four serotypes and their use in compositions and for eliciting a specific anti-dengue immune response against Dengue-associated diseases or infections, and as a source of serospecific pure and native antigens for diagnosis.

BRIEF DESCRIPTION OF THE PRIOR ART

Dengue has emerged as the most important vector-borne viral disease in tropical areas. The four serotypes of dengue virus each cause human disease and are transmitted by Aedes mosquitoes. Epidemics with a high frequency of a severe, life-threatening illness known as dengue hemorrhagic fever (DHF) continue to expand geographically. The disease burden is estimated to be up to 100 millions of cases every year, including over 500,000 cases of DHF and about 25,000 fatal cases, mainly in children under the age of 15. Despite the increased health and economic impact of dengue, there are as yet no specific preventive or therapeutic interventions. There is an urgent need for reliable, rapid diagnostic and therapeutic tools for people at risk of DV infection.

Dengue belongs to flaviviruses. These viruses have two structural envelop proteins, E and M, the later being expressed as an immature precursor protein prM. The E glycoprotein is responsible for receptor binding and membrane fusion, while prM glycoprotein is responsible for the correct folding of E. During the maturation of virus particle, the prM protein will be cleaved by furin to form M and soluble pr proteins and this cleavage will improve dimerization of E proteins (Mukhopadhyay et al., 2005). Assembly of infectious particles requires both glycoproteins and nucleocapsid. However, prM and E proteins from several flaviviruses, such as JEV and TBEV, were found to be able to form virus-like particles (VLPs) in the absence of any other viral component (Ferlenghi et al., 2001; Hunt, Cropp, and Chang, 2001).

Recently, the prME driven VLP formation was also observed in dengue virus (Chang et al., 2003; Konishi and Fujii, 2002).

DV1 VLPs were reported to be secreted by human HeLa and 293 epithelial cell lines using a recombinant vaccinia virus vector which drive expression of prM and E structural proteins (Fonseca et al., 1994) or cells transfected with a recombinant plasmid containing the prM-E gene (Raviprakash et al., 2000), respectively. In these studies, the authors did not described formation and secretion properties of DV1 VLPs in details but focused on their immunogenicity to propose VLPs as a candidate vaccine. Others have described assembly and secretion of DV2 VLPs (Konishi and Fujii, 2002; Pryor et al., 2004). Konishi and Fujii have developed a stable CHO cell line which produces DV2 VLPs (Konishi and Fujii, 2002). In this study, the authors had to mutate the furin cleavage site on prM to avoid cell-cell fusion between host cells. As a consequence, DV2 VLPs produced by this cell line expressed envelope glycoproteins in an immature state where E is non-fusogenic. In another study, Pryor and colleagues described production of DV2 VLPs in COS cells and reported that mutation of the histidine at M39 does not affect assembly of heterodimers in the ER but secretion of VLPs and replication of infectious virus (Pryor et al., 2004). More recent studies by Gwong-Jen J. Chang and colleagues have shown that DV1 and DV2, unlike DV3 and DV4 RSPs, were not secreted efficiently by a Chinese hamster ovary (CHO) cell line when full length E was used (Chang et al., 2003; Purdy and Chang, 2005). The authors had to replace the carboxy-terminal 20% region of DV1 and DV2 E genes with the corresponding sequence of JEV to observe significant VLP secretion. The authors mapped the sequence responsible for intracellular retention onto the E-H1 alpha-helix domain of DV2 E protein and more precisely shown the involvement of amino-acids 398, 401 and 412 (Purdy and Chang, 2005). Recently, a endoplasmic reticulum retention signal was described in the stem-anchor region of DV2, stronger than the one in JEV, which could contribute to the inefficient production of DV2 VLPs (Hsieh et al., 2008). Although this DV/JEV chimeric strategy provides a method to

efficiently generate VLPs, it may affect the DV budding process or/and the antigenicity of DV VLPs.

SUMMARY OF THE INVENTION

The present invention provides a polynucleotide comprising a codon-optimized dengue prME nucleotide sequence, a cloning or expression vector comprising the polynucleotide of the invention, and a host cell comprising same.

The present invention also provides a dengue virus-like particle (VLP) produced by the host cell of the invention, and its use in composition and a method for generating an immune response in a host, and in a method for treating or preventing a dengue-associated disease.

The present invention further provides a method for producing strain-specific dengue virus-like particle (VLP), comprising the steps of:

a) introducing an optimized dengue prME sequence into a host cell;

b) incubating said host cell under conditions to produce VLPs; and

c) harvesting the produced VLPs.

The present invention also provides a method of screening for an inhibitory dengue cell-binding agent, comprising the steps of:

a) contacting a VLP as defined above and a candidate agent with a Dengue susceptible host cell under suitable condition to allow binding of the VPL to said cell; and

b) evaluating the capacity of the agent to inhibit the formation of a VLP/cell complex.

The present invention also provides a method of screening for a dengue virus production inhibitory agent, comprising the steps of:

a) getting into the cell the produced VLP as defined above; and

b) evaluating the capacity of the agent to inhibit the production of VLP by producer cells.

The present invention also provides a method for treating or preventing a dengue-associated disease, comprising the step of administering an inhibitory agent as defined above to a host in need thereof.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A, 1B, 1C, 1D, and 1E: Optimization of codon usage of DEN1 prME gene increases expression level in mammalian cells. Flow cytometry data on permeabilized cells using 4E11 monoclonal antibody against E. Figure 1A: mock-transfected cells; Figure 1B: cells transfected with native prME gene; Figure 1C: cells transfected with optimized prME gene. Figures 1D and 1E: Panels show the codon usage of native and optimized prME gene.

Figure 2: Subcellular localization of E protein in HeLa-prME cells. E protein is mainly localized in endoplasmic reticulum where it colocalizes with erp72, an endoplasmic reticulum resident protein. E do not concentrates in ergic and Golgi compartments where it does not colocalize with ergic-53 and golgin-97 markers, respectively.

Figures 3A and 3B: Characterization of DEN 1 VLP produced by the stable HeLa-prME cell line. A) Analysis of E profile by Western blotting on cell lysate and supernatant of three stable clones of HeLa-prME. Right panel: Monomeric E protein was present in cell lysate (CL) of clones B3, B7 and D4 of HeLa-prME cells.

Figure 3A: Detection of E protein in concentrated supernatant (SN) and cell lysate (CL) from B3, B7 or D4 cells. Both homodimers and monomers are detected by Western blotting in concentrated supernatant.

Figure 3B: Analysis of E and prM expression in cell lysate and supernatant of HeLa-prME cells. The cell lysate (CL) or supernatant (SN) of HeLa-prME cells were analyzed by SDS-PAGE followed by Western blotting and hybridization using anti-E mAb 4E11 (a, b) or anti-DV1 mouse serum from Philippe Buchy – Institut Pasteur of Cambodia - (c, d) or by silver-staining (e). Using anti-E mAb 4E11, the monomeric E protein could be detected in SN and CL (a). The homodimeric E protein was also detected in SN, but was absent when the sample was heated in the presence of DTT (SN^R) (b). Using anti-DV1 mouse polyclonal antibody, the prM protein could be detected in CL but not in SN (c). When the cell line was treated with NH4Cl, which could inhibit the cleavage of

prM, the uncleaved prM protein was detected in SN (SN^{NH4Cl}, d). The presence of prM and M protein was also confirmed by silver-staining (e), in which M protein or prM protein were detected in SN or SN^{NH4Cl}, respectively. The parent HeLa cells were used as control (e, C lane).

Figure 4: Sucrose gradient analysis of DEN1 VLP. The concentrated supernatant from HeLa-prME cells was centriguged in a 20 to 60% discontinuous sucrose gradient at 28,000 rpm (Beckman SW-41Ti rotor) for 2.5 hours at 4°C. Fractions of 0.5 ml were collected and measured using the 4E11 mAb and Chemical luminescence dot-blot. The control VLP (VLP in PBST) was treated with 0.5% Triton X-100 for 1 hour before it was subjected to sucrose gradient.

Figure 5: Estimation of E protein quantities in supernatant (SN), ultra-centrifuge concentrated supernatant (CSN), or cell lysate (CL) of transiently transfected 293T cells. SN, CSN and CL samples were collected after 48 hours of culture from 10cm-diameter dishes (~78 cm^2) containing 25ml culture medium (number of cells: ~ 10 million cells). SN, CSN and CL samples were loaded on SDS-PAGE. Quantities of E proteins in each sample were estimated by Western-blotting with 4E11 monoclonal antibody and densitometry. Signal intensities were compared to signals obtained for a serial dilution of soluble E protein of know concentration. Results from one 10cm-diameter dish in this experiment: total E protein in cellular supernatant is **92.8 ug** (3.7ug/ml), and after concentration is 79.7ug (265.8ug/ml). About **86%** E protein can be recovered after ultra-centrifuge concentration.

Figures 6A, 6B, 6C, 6D, 6E and 6F: Production of VLPs for all 4 dengue serotypes. Codon usage of DEN2, 3 and 4 prME genes were optimized according to the same criteria used for DEN1. 293T cells were transiently transfected with constructs coding for optimized prME genes for DEN1, 2, 3 and 4. At 48 hours post transfection, supernatants were harvested and analyzed by western blot using anti-E mAb 4E11 (Figure 6A), a cocktail of four sera from Cambodian patients seropositive for DV1, DV2, DV3 or DV4 (Figure 6B), or individual sera from Cambodian patients seropositive for DV1, DV2, DV3 or DV4 (Figure 6C). Intensities of signals detected by Western blotting

were quantified by densitometry. Figure 6D:. Relative intensity of signals for the E protein detected with dengue patient sera for the four dengue serotype VLP preparations. Figure 6E: Relative intensity of signals for the prM protein detected with dengue patient sera for the four dengue serotype VLP preparations. Figure 6F: Relative intensity of signals for the E protein detected with the anti-E 4E11 monoclonal antibody for the four dengue serotype VLP preparations.

Figure 7: VLP of DEN1 to 4 sediment in fractions 6-11 in 20-60% sucrose gradients. VLP concentrated by ultracentrifugation was subjected to 20-60% sucrose gradient fractionation. 24 fractions were collected and analyzed by Western blotting with specific anti-E antibodies. Intensity of signals was quantified by densitometry. VLP levels are presented as the percentage of E protein in each fraction.

Figures 8A, 8B, 8C, 8D, and 8E: Production of luciferase-DV1 VLP and binding to target cells. The optimized DV1 prME gene was modified by replacement of the C-terminal transmembrane domain of the E protein by the cDNA sequence coding for the luciferase protein (prMEluc). Figure 8A: The prMEluc or a combination of prMEluc and prME or a control empty vector were transiently transfected into 293T cells and the luciferase activity in supernatant was measured 48 hours later. Figure 8B: The luciferase activity in ultracentrifugation-concentrated supernatant (Conc. SN) was much higher than that in supernatant (SN), suggesting association with sedimentable VLPs. Figure 8C: The E-luciferase protein (Eluc) could be detected in concentrated supernatant by western-blot using the anti-E antibody 4E11. Figure 8D: The binding ability of luciferase DV1 VLP to Vero cells could be blocked by pre-incubation with heparin. Figure 8E: The binding ability of luciferase DV1 VLP to human macrophages could be enhanced by pre-incubation with the anti-E antibody 4E11.

Figures 9A and 9B: Treatment of DV VLP with 0.5% Triton-X100 is required for microplate coating and quantification by ELISA. Ultracentriguge-concentrated DV1 VLP sample (100 times concentrated) was resolved in PBS or PBS + 0.5% TritonX100. For the coating of 96-well microplate, VLP were

serially diluted with PBS (Figure 9A) or + 0.5% Triton-X100 (Figure 9B) (1:2-1:1024). 50 µl of diluted VLP was added to each well and incubated at 4°C overnight. All samples were loaded in triplicate. For the ELISA, sample supernatants were discarded, 100 µl PBST, 5% milk was added to each well and incubated at 4°C overnight. Then, 50 µl of 1:2000 4E11 diluted in PBST + 5% milk was added into each well and incubated at RT for 1 hour. After three washes in PBST, 50 µl of 1:2000 HRP-conjugated secondary antibody diluted in PBST + 5% milk was added into each well and incubated at RT for 1 hour. After three washes in PBST, substrate was added and OD value measured.

Figure 10: Chemical Luminescence dot-blot approach to measure VLP production in medium-scale screens.

Figure 11: Application of HeLa-prMe cells in the screen of factors involved in dengue egress. HeLa-prME cells were transfected with a siRNA library (Dharmacon, Human Membrane Trafficking G-005500 Lot #06127) targeting to 122 membrane trafficking genes and the VLP in supernatant was measured using chemical luminescence dot-blot 96 hours later. Non-targetting siRNA was used as control. The siRNAs that significantly reduced VLP production were labelled in gray while those increased in dark. SiRNA targeting to prME (ER) or transfection reagent alone (TR) were also included as controls.

Figure 12: Confirmation of inhibition of DV1 VLP production by siRNA targeting of specific cellular factors identified by siRNA library screening. The stable HeLa prME cell line was transfected with siRNAs targeting various members of a family of cellular factors identified for their modulatory effects on DV1 VLP production in a siRNA library screening (figure 11). 72 hours post transfection, cell supernatants and cell lysates were prepared and analysed by western blot. Absence of E protein in supernatant of cells treated with the siRNA combination "H" indicates inhibition of VLP production.

Figure 13: Optimized prME sequence, dengue virus type 1, strain FGA/89 (SEQ ID NO: 1).

Figure 14: Optimized prME sequence, dengue virus type 2, strain FGA/02 (SEQ ID NO: 2).

Figure 15: Optimized prME sequence, dengue virus type 3, strain PaH881/88 (SEQ ID NO: 3).

Figure 16: Optimized prME sequence, dengue virus type 4, strain 63632 (SEQ ID NO: 4).

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have developed a new tool advantageously useful in the field of dengue virus. Such a tool finds a particular advantageous application in the field of diagnosis, drug screening, vaccine development and antibody production.

In this connection, the present invention relates to a new source of native dengue antigens following the development of Dengue virus-like (VLP) particles of the four serotypes DV1 to DV4. The present invention further relates to polynucleotides encoding dengue VLPs and their use in compositions and methods for eliciting a specific anti-dengue immune response against Dengue-associated diseases or infections.

A Dengue-associated disease or infection may be, for instance, dengue fever or dengue haemorrhagic fever. Dengue fever is a febrile illness (fever, severe headache, pain behind the eyes, muscle and joint pain, and rash) while dengue haemorrhagic fever is a potentially lethal complication (fever, abdominal pain, vomiting, bleeding). Dengue viruses are classified in four serotypes DV1 to DV4.

In this connection the invention further relates to a new source of serospecific native antigens (DV1, DV2, DV3 and DV4) and their use in compositions and methods in diagnosis of dengue virus infection.

In this connection the invention further relates to a new source of serospecific native antigens (DV1, DV2, DV3 and DV4) and their use in compositions and methods in diagnosis of dengue virus infection.

The inventors have developed a chemical luminescent dot-blot (CLDB)-based method to screen libraries of molecules for enhancement or inhibition of dengue VLP production by a stable DV1 VLP producer cell line. The method has been used by the inventors to screen a library of small interfering RNA.

In this connection the invention further relates to a new methodology to screen libraries of molecules for enhancement or inhibition of VLP production. Libraries of molecules include, for instance, libraries of chemical compounds, drugs, peptides, siRNAs, cDNAs.

The authors have demonstrated that the biochemical properties of DV VLPs are similar to the ones of dengue virus. In this aspect, the VLPs could be used to mimic the virus to study binding to and infection of host cells.

In this connection the invention further relates to a new source of serospecific dengue virus mimicry and their use in compositions and methods in study of dengue virus infection.

Definitions

The term "isolated" is meant to describe a nucleic acid construct or a polypeptide that is in an environment different from that in which the nucleic acid construct or the polypeptide naturally occurs.

The term "subject" refers to any subject susceptible to be infected by a *Dengue* strain. For instance, such a subject may be, but not limited to, a human.

The term "*Dengue* strain" refers to a strain of any serotypes/genotypes. For instance, a dengue virus type 1 strain may be strain FGA/89 (CRBIPvIMFH1); a dengue virus type 2 may be strain FGA/02; a dengue virus type 3 may be strain PaH881/88 (CRBIPvIMFH3); and a dengue virus type 4 may be strain 63632 (CRBIPvIMFH4).

The term "treating" refers to a process by which the symptoms of an infection or a disease associated with a dengue strain are alleviated or completely eliminated.

As used herein, the term "preventing" refers to a process by which symptoms of an infection or a disease associated with a dengue strain are obstructed or delayed.

The expression “immune response” refers to an *in vivo* or *in vitro* reaction in response to a challenge by an immunogen, such as Dengue VLPs as defined therein. An immune response is generally expressed by an antibody production (*e.g.*, neutralizing antibodies) and/or a cell-mediated immunity.

The expression "an acceptable carrier" means a vehicle for containing the compounds obtained by the method of the invention that can be administered to a subject host without adverse effects. Suitable carriers known in the art include, but are not limited to, gold particles, sterile water, saline, glucose, dextrose, or buffered solutions. Carriers may include auxiliary agents including, but not limited to, diluents, stabilizers (*i.e.*, sugars and amino acids), preservatives, wetting agents, emulsifying agents, pH buffering agents, viscosity enhancing additives, colors and the like.

Amino acid or nucleotide sequence "identity" is determined from an optimal global alignment between the two sequences being compared. An optimal global alignment is achieved using, for example, the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, J. Mol. Biol. 48:443-453) or the BIOEDIT v7.0.3 software. "Identity" means that an amino acid or nucleotide at a particular position in a first polypeptide or polynucleotide is identical to a corresponding amino acid or nucleotide in a second polypeptide or polynucleotide that is in an optimal global alignment with the first polypeptide or polynucleotide. By the statement "sequence A is n% identical to sequence B", it is meant that n% of the positions of an optimal global alignment between sequences A and B consists of identical amino acid residues or nucleotides.

The term “production of VLP” refers to all steps from assembly of viral protein prM and E into VLP, their intracellular trafficking and secretion by the producer cell.

The term “inhibit” refers to a reduction in the VLP production parameter being measured. For instance, the amount of such reduction is measured relative to a standard (control). "Reduction" is defined herein as a decrease of

at least around 25% relative to control, preferably at least around 50%, and most preferably of at least around 75%.

1. Polynucleotides of the invention

The inventors have thus designed and synthesized optimized dengue polynucleotides. It is therefore an object of the invention to provide an isolated polynucleotide which comprises a codon-optimized dengue prME nucleotide sequence. The dengue prME gene may be derived from, for instance, the dengue serotype 1, 2, 3 or 4 (*i.e.* DV1, 2, 3 and 4 prME genes). For instance, the polynucleotide contemplated by the present invention may comprise a nucleotide sequence substantially identical to SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, or 8. By "substantially identical", it will be understood that the polynucleotide of the invention preferably has a nucleic acid sequence which is at least 75 % identical, more particularly 85 % identical and even more particularly 95 % identical to part or all of the sequence shown in SEQ ID NO: 1 to and 5 to 8, but encodes for a dengue prME polypeptide having an amino acid sequence identical to the amino acid sequence of the native dengue prME protein from which the polynucleotide of the invention derives from.

By "codon-optimized", it is meant that the native prME nucleotide sequence has been modified to incorporate regulation sequences in order to provide adequate expression of the desired prME protein product encoded by the polynucleotide of the invention. For instance, but not limited to, such a modification may be to replace the signal sequence of the native prME nucleotide sequence by that from another source, such as the vesicular stomatitis virus G-protein (VSV-G). Another example of a regulation sequence may be a kozak sequence (*e.g.* GCCACC) that may be added to the 5 end of start codon ATG to enhance the expression. Another example of such a modification may be the replacement of codons rarely used in mammals by codons which are frequently used in mammals. It is understood that any other suitable regulation sequence or modification to optimize a sequence known to one skilled in the art may be used in accordance with the present invention. SEQ ID NO: 5 to 8 thus represent the respective codon-optimized prME sequences of Dengue virus serotypes 1 to 4, wherein codons of the native

prME sequence have been replaced by the corresponding mammalian codons, thereby allowing for an enhanced expression of prME in mammalian cells. SEQ ID NO: 1 to 4 respectively comprise SEQ ID NO: 5 to 8, to which have been added a restriction site, a Kozak sequence and a nucleic acid sequence encoding a VSV-G signal sequence in 5', as well as stop codons and a restriction site in 3'. In particular, the above-mentioned nucleotide sequence encoding a VSV-G signal sequence may itself be codon-optimized by replacement of native codons by codons allowing for an enhanced expression in mammalian cells, notably as described by Witko et al., 2010.

It will be understood that the polynucleotide of the invention may further comprises another sequence coding for a different protein, such as a marker protein (*e.g.*, luciferase).

2. Vector, cells and VLP production method of the invention

The present invention is also concerned with a vector comprising a polynucleotide of the invention. As used herein, the term "vector" refers to a polynucleotide construct designed for transduction/transfection of one or more cell types. Vectors may be, for example, "cloning vectors" which are designed for isolation, propagation and replication of inserted nucleotides, "expression vectors" which are designed for expression of a nucleotide sequence in a host cell, or a "viral vector" which is designed to result in the production of a recombinant virus or virus-like particle, or "shuttle vectors", which comprise the attributes of more than one type of vector. Such a vector may be one of those deposited at the CNCM on November 12, 2008, under accession numbers I-4084, I-4085, I-4086 or I-4087.

In a related aspect, the present invention provides a host cell comprising a vector as defined above. The term "host cell" refers to a cell that has a new combination of nucleic acid segments that are not covalently linked to each other in nature. A new combination of nucleic acid segments can be introduced into an organism using a wide array of nucleic acid manipulation techniques available to those skilled in the art. A host cell can be a single eukaryotic cell, or a single prokaryotic cell, or a mammalian cell. The host cell can harbor a vector that is extragenomic. An extragenomic nucleic acid vector

does not insert into the cell's genome. A host cell can further harbor a vector or a portion thereof that is intragenomic. The term intragenomic defines a nucleic acid construct incorporated within the host cell's genome. A host cell of the invention may be a cell from a cell line, such as HeLa and 293T. Besides, the host cell of the invention may express the nucleic acid segments or the vector transiently or stably. In particular, such a host cell of the invention may consist of a cell from the cell line deposited at the CNCM under accession number I-4083 on November 12, 2008. Such a HeLa-prME cell line advantageously produces, in particular stably, a dengue virus-like particle (VLP) according to the present invention.

It is therefore an aspect of the invention to provide a method for producing strain-specific dengue virus-like particle (VLP). Such a method comprises the steps of:

a) optimizing a dengue prME DNA sequence for optimal expression in a host cell;

b) introducing an optimized dengue prME sequence into a host cell;

b) incubating said host cell under conditions to produce VLPs; and

c) harvesting the produced VLPs.

It will be understood that by the expression “under conditions” when referring to VLP production in host cells, it is meant that the incubation step is carried out at a temperature and for a period of time sufficient to allow effective production of VLPs within said host cell.

As one skilled in the art may appreciate, the so produced VLPs may be advantageously be used for the production of antibodies (*e.g.* monoclonal antibodies) that are immunologically specific to the selected particular dengue strain. With respect to such contemplated antibodies, the term "immunologically specific" refers to antibodies that bind to one or more epitopes of a protein of said particular dengue strain, but which do not substantially recognize and bind other molecules in a sample containing a mixed population of antigenic biological molecules.

3. Compositions and methods of use

Another aspect of the present invention relates to compositions for treating and/or preventing a dengue-associated disease or to induce an immune response in a host. The composition of the present invention advantageously comprises at least one dengue VLP molecule as defined above. The composition of the invention further comprises an acceptable carrier. Preferably, the composition of the invention is a pharmaceutical composition, in particular a vaccine composition, comprising at least one dengue VLP molecule as defined above, in association to a pharmaceutically acceptable carrier.

In related aspects, the invention provides a method for treating and/or preventing a dengue-associated disease, such as dengue, and a method for generating an immune response in a host. The methods comprise the step of administering to a subject in need thereof a composition of the invention. Thus, the present invention also relates to a VLP molecule as defined above, or a pharmaceutical composition as defined above, for use for generating an immune response in a host and/or for preventing or treating a dengue-associated disease, such as dengue.

In another related aspect, the invention provides a method of screening for a dengue virus production inhibitory agent. Such a method comprises the step of contacting a cell that produces VLPs as defined above, in particular a cell from the cell line deposited at the CNCM under accession number I-4083 on November 12, 2008, and a candidate agent under suitable condition to allow VLP production and secretion by said producer cells.

The screening method of the invention further comprises a step b) of evaluating the capacity of the agent to inhibit the production of VLPs by producer cells.

In particular, evaluating the capacity of the agent to inhibit the production of VLPs by producer cells, involves:

(i) concentrating the supernatant from a culture of the producer cells on a membrane, in particular a PVDF membrane, thereby adsorbing VLPs on the membrane;

(ii) quantifying the E protein of the adsorbed VLPs on the membrane, in particular by contacting the membrane with a detection antibody, for instance conjugated with horseradish peroxidase, and by quantifying the detection antibodies attached to the membrane;

(iii) comparing the quantity of E protein of adsorbed VLPs with respect to a reference level, such as the quantity of E protein of adsorbed VLPs obtained from producer cells not contacted by the agent; and

(iv) deducing therefrom if the agent inhibits the production of VLPs by producer cells.

In another related aspect, the invention provides a method of screening for an inhibitory dengue cell-binding agent. Such a method comprises the step of contacting a VLP as defined above and a candidate agent with a Dengue susceptible host cell under suitable condition to allow binding of the VLP to said cell. By the expression "Dengue susceptible host cell" refers to any cell susceptible to be infected by a *Dengue* strain, such as (Vero cells, Macrophages*)*. It will be understood that by the expression "under suitable conditions" when referring to a VLP-host cell complex, it is meant that the contact between the VLP and the host cell is carried out at a temperature and for a period of time sufficient to allow effective binding between the VLP and the host cell.

The screening method of the invention further comprises a step b) of evaluating the capacity of the agent to inhibit the formation of a VLP/cell complex.

The candidate agent may be of any type. In particular, where the method of screening for a dengue virus production inhibitory agent is concerned, the candidate agent may be a siRNA.

EXAMPLES

The present invention will be more readily understood by referring to the following example. This example is illustrative of the wide range of applicability of the present invention and is not intended to limit its scope. Modifications and variations can be made therein without departing from the spirit and scope of the invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, the preferred methods and materials are described.

Example 1: Characterization of dengue 1 viral like particle and it intracellular trafficking

The dengue 1 viral-like particle (VLP) was generated by using the optimized prME gene and a VLP producing stable cell line was also established. The optimized gene significantly enhanced the expression of prME glycoproteins and therefore facilitated the generation of DV1 VLP. The present results showed that the VLP underwent the same maturation process and had the same surface structure as real virus. Glycoprotein prME was synthesized in ER and would take at least 3 hours from synthesis to be secreted. During the intracellular trafficking, prM protein was cleaved by furin convertase and followed by the rearrangement of E protein. E protein formed homodimer in matured VLP particle. The establishment of stable cell line provided safe and convenient tool to study prM/E interaction and assembly of empty virus-like particles.

Materials and methods

Cell lines, antibody and contrustion

HeLa cells were maintained in DMEM containing 10% FBS. The monoclonal antibody 4E11 was provided by Dr. Philippe Despres. Optimized prME gene was synthesized in Geneart Company and subcloned into pcDNA or retroviral vector pCHMWS-IRES-Hygromycin (provided by Dr. Rik Gijsbers) between BamHI and XhoI. To delivery the prME-opt into HeLa cells, the

pCHMWS-prME-opt-IRES-Hygromycin, pcDNA-VSV-G and p8.71 plasmids were co-transfected into 293T cells. The cell supernatant containing infectious particles was harvested 48 hours post-transfection and used to infect HeLa cells. Two days after infection, cells were selected in culture medium containing 100ug/ml hygromycin for 2 weeks. Surviral cells (HeLa-prME) were maintained in DMEM + 10% FBS.

Generation of VLP

To generate the VLP, the pcDNA-prME-opt (10ug) was transfected into 293T cells using calcium phosphate precipitate method. Supernatants were harvested 48 h later and cell debris was removed by centrifuge at 3000rpm for 15min and 10000rpm for 30min. Clarified supernatant was then concentrated by ultracentrifuge at 28000rpm for 2.5 hours. The pellet was resuspended in 100ul of PBS. For the production of immature VLP, 20mM NH4Cl was added to the culture medium 8 hours after the transfection.

For the sucrose gradient analysis, the resuspended VLP was centriguged in a 20 to 60% sucrose gradient at 28,000 rpm (Beckman SW-41Ti rotor) for 2.5 hours in 4°C. All sucrose solutions were prepared with HEPES buffer. Fractions of 0.5 ml were collected and measured using Chemical luminescence dot-blot (CLDB). In some experiments, the VLP was treated with 0.5% Triton X-100 for 1 hour before it was subjected to sucrose gradient.

Chemical Luminescence Dot Blot (CLDB)

A CLDB method was used to quantify the VLP. Briefly, the samples containing VLP were transferred to polyvinylidene difluoride (PVDF) membrane through a 96-well module so that the samples were arranged in 96-well format. The membrane was blocked overnight in 5% milk in PBST solution, incubated with anti-E antibody (4E11, 1:10000) for 1 hour and then incubated for 1 hour with a peroxidase-labeled goat anti mouse IgG polyclonal antibody (1:10000). Five time diluted ECL western blot detection reagents

(Invitrogen) were added to the membrane and the luminescence intersity was measure by Mecrobeta (PerkinElmer).

FACS

Cells were detached by incubation in 10mM EDTA at 37C for 10 min, fixed in 2% PFA, and then permeablized in 0.1% Triton X-100. After washing, the cells were incubated with anti-E antibody (4E11, 1:200) for 1 hour at 4C. Normal mouse were used as control. The cells were then washed and incubated for 30 min with diluted fluorescein isothiocyanate-labeled antiserum. Cells were analyzed using cytometer (BD Biosciences).

Gel electrophoresis, immunoblotting and silver staining

The VLP was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4~12% NuPAGE gene (Invitrogen). For immunodetection, proteins were blotted from gels onto PVDF membranes. The membrane was blocked overnight in 5% milk in PBST solution and then incubated with anti-E antibody (4E11, 1:1000) for 1 hour. After washes, the membrane was incubated for 1 hour at room temperature with a peroxidase-labeled goat anti mouse IgG polyclonal antibody. The membrane was finally visualized using ECL western blot detection reagents (Invitrogen). For silver staining, the gel was fixed for 30 min and incubated with Na Thiosulfate for 30 min at RT. After three times washes, the gel was incubated with Silver Nitrate for 40 min and developed for 15 min in Na carbonate solution. EDTA solution was used to stop the development finally.

Results

Establishment of prME expressing cell line

To establish a cell line that stably produces DV1 VLP, the inventors first delivered its prME gene into HeLa cells using a retroviral vector. Intracellular expression of dengue E protein was monitored by flow cytometry on permeabilized transduced HeLa cells. The 4E11 monoclonal antibody against

the dengue 1 E protein was used. Low level of E protein was detected (**Fig 1B**). The analysis of the prME gene sequence revealed that its natural codon usage is not optimal for expression in mammalian cells (**Fig 1D**). Therefore, the inventors decided to synthetize an optimized prME (prME-opt) gene, which consisted of codons preferentially used in mammalian cells (**Fig 1E**). More than 70% codons were modified for prME optimization without changing the amino acid sequence. In addition, the negative cis-acting sites (such as splice sites, poly(A) signals, etc) which might have negatively influenced expression were also removed. Nucleotides coding for the signal sequence of the vesicular stomatis virus (VSV) G envelop glycoprotein were inserted in frame upstream the prME optimized sequence.

Gene optimization resulted in significant increase of E expression levels in transduced HeLa cells (HeLa-prME) (**Fig 1C**). Without cell permeablization, E protein was hardly detected, indicating that E protein was intracellular but not expressed at cell surface.

To further characterize the intracellular distribution of E protein, HeLa-prME cells were fixed, permeabilized, and double stained for E protein and for cellular marker antigens. Erp72, ERGIC-53 and Golgin 97 are resident proteins of the endoplasmic reticulum (ER), ER-Golgi intermediary compartment (ERGIC) and Golgi respectively. The dengue E glycoprotein colocalized with the erp72 marker but not with ERGIC-53 and Golgin 97 (**Fig 2**). No staining was observed at the plasma membrane, confirming the previous cytometry data. Altogether, the present data demonstrate that the dengue 1 E glycoprotein is enriched in the endoplasmic reticulum in the HeLa-prME stable cell line.

Example 2: Dengue 1 virus-like particles are efficiently produced by the HeLa-prME stable cell line

The inventors have then tested secretion of VLPs by the HeLa-prME stable cell line. Cell lysate (CL) and clarified supernatant (SN) concentrated by ultra-centrifugation were analyzed by Western blotting and hybridization with either the anti-E monoclonal antibody 4E11 (**Figures 3A and 3B a,b**) or an

anti-DV1 serum from a human patient (**Figure 3B c,d**). Silver staining of polyacrilamide gel was also performed (Figure 3B e). In CL samples, the monomeric E glycoprotein could be clearly observed with an apparent molecular size of 50 kDa (**Figure 3B a and c**). Only traces of 100 kDa E homodimers were detected in CL, suggesting that the majority of viral proteins are localized in the early pre-Golgi secretory pathway in HeLa prME cells. This result is in agreement with the immuno-fluorescence data of this study, which show that E mainly distributes in the ER. Interestingly, high levels of 100 kDa E glycoprotein homodimers were detected in SN samples as well as 50 kDa E monomers (**Figure 3B a to d**). Detection of E monomers in SN most likely results from partial denaturation of protein complexes in SDS-PAGE. E homodimers were no longer detected when samples were heated in presence of dithiothreitol (SN^R) (**Figure 3B b**). The E monomers detected from SN samples migrated to a slightly higher position in the polyacrilamide gel than the ones of CL samples. This result illustrates the acquisition of complex N-glycans in the Golgi apparatus by secreted E glycoproteins. The anti-DV1 serum also allowed detection of the prM protein at an apparent molecular weight of 21 kDa in CL but not SN samples (**Figure 3B c**). Moreover, treatment of HeLa-prME cells with NH_4Cl, which inhibits acidification of the trans-Golgi compartment and, as a consequence, activity of furin protease, blocked prM cleavage (**Figure 3B d and e**). In these conditions, prM and E viral proteins were found in SN, and E homodimers were still detected (**Figure 3B d**). Homodimerization of E, acquisition of complex sugars and efficient cleavage of prM suggest that prM and E viral proteins have correctly assembled in the ER into VLPs which have trafficked through the secretory pathway before secretion into the cell medium. Altogether, these results suggest that expression of DV1 prM-Eopt allows assembly and secretion of DV1 VLPs by a mechanism similar than infectious dengue viruses.

To further confirm that viral proteins detected in medium of HeLa-prME cells were assembled into VLPs, the inventors performed sucrose gradient fractionation on cleared cell supernatant (**Figure 4**). Freshly passed stable HeLa-prME cells were grown for 24 hours in complete medium, medium was

harvested, cleared by low speed centrifugation and VLPs were pulled-down by ultracentrifugation at 28000 rpm for 3 hours. For flaviviruses, the glycoprotein E could be pulled down from lipid membrane by treatment with the non-ionic detergent Triton X-100 (Allison et al., 2003). The inventors analyzed distribution of E after fractionation of VLPs which had been pre-treated with 0.5% Triton-X 100 for 1 hour. Levels of E glycoprotein in each fraction were measured by a chemical luminescence dot-blot (CLDB) system developed by inventors. The CLDB has a wide linear range and is sensitive enough to quantify the E protein in small volumes without any concentration step. The fraction quantification showed that E glycoprotein in VLPs sedimented in fractions from 20% to 30% sucrose (**Figure 4, black bars**). However, in Triton-X100 treated VLPs, the E protein peak was shifted to the top of the gradient (**Figure 4, white bars**), showing that the E protein had been solubilized upon detergent treatment.

Example 3: Establishment of a stable cell line constitutively expressing DV 1 VLPs

Optimized DV1 prME gene was synthesized in Geneart Company and subcloned into a retroviral vector pCHMWS-IRES-Hygromycin (provided by Dr. Rik Gijsbers) between BamHI and XhoI. To delivery the prME-opt into HeLa cells, the pCHMWS-prME-opt-IRES-Hygromycin, pcDNA-VSV-G and p8.71 plasmids were co-transfected into 293T cells. The cell supernatant containing infectious particles was harvested 48 hours post-transfection and used to infect HeLa cells. Two days after infection, cells were selected in culture medium containing 100ug/ml hygromycin for 2 weeks. Surviral cells (HeLa-prME) were maintained in DMEM + 10% FBS. Single colony culture was applied to the survival HeLa-prME cells pool and three colonies were obtained (B3, B7 and D4). The intracellular expression of E protein and the presence of E protein in cellular supernatant of these cells was analyzed western-blot using anti-E 4E11 monoclonal antibody as primary antibody (**Fig 3A**).

Example 4: Efficient production of DV1 to 4 VLPs by mammalian cells

Dengue VLPs could also be generated by transient transfection in 293T cells. In this experiment, optimized DV prME genes were subcloned into pcDNA vector. The pcDNA-prME-opt (10ug) was transfected into 293T cells using calcium phosphate precipitate method. Supernatants were harvested 48 h later and cell debris was removed by centrifuge at 3,000rpm for 15 min and 10,000rpm for 30 min. Clarified supernatant was then concentrated by ultracentrifuge at 28,000rpm for 2.5 hours. The pellet was resuspended in 100ul of PBS. The concentrated supernatant was then tested by western-blot or subjected to sucrose gradient. Anti-E monoclonal antibody 4E11 or four sera from Cambodian patients seropositive for DV1, DV2, DV3 or DV4 were used in the western-blot. The E protein could be detected at a similar level, demonstrating that the VLPs for all four dengue viruses could be produced efficiently. Dimeric E protein was easily found in DV1, 3 and 4 but not in DV2 VLPs and further studies are required to elucidate this point (**Fig 6**).

For the sucrose gradient analysis, the resuspended VLP was centrifuged in a 20 to 60% sucrose gradient at 28,000rpm (Beckman SW-41Ti rotor) for 2.5 hours in 4°C. All sucrose solutions were prepared with HEPES buffer. Fractions of 0.5 ml were collected and analyzed by western-blot (**Fig 7**).

In addition, the inventors established HeLa-prME and 293T-prME cell lines of DV1, DV2 and DV3 using the same procedure described for DV1 HeLa-prME. The maturation of RSPs produced by both cell types was compared using SDS-PAGE and silver staining of the gel. Supernatants from parental HeLa and 293T cells were used as controls in the experiment. The inventors found that, whereas only a small fraction of prM was cleaved in the VLPs produced by 293T-prME cell lines, cleavage of prM was much more effective in VLPs from the HeLa-prME cell lines. This result indicates that efficacy of maturation is cell type dependent.

Example 5: Engineering of luciferase-DV VLPs and its application in cell binding assay

To make the DV1 prME-luciferase (prMEluc) construction, the luciferase gene was inserted to the 3 end of the first transmembrane domain of optimized DV1 prME gene and therefore the second transmembrane was deleted. The prMEluc or prMEluc + prME were transient transfected into 293T cells and the luciferase activity in clarified supernatant was measured 48 hours later. It was found that higher luciferase activity could be detected in supernatant of 293T cells co-transfected with prME-luc and prME (**Fig 8A**). The luciferase activity could be enhanced by ultra-centrifuge concentration (**Fig 8B**), indicating that the luciferase protein was integrated into the VLP particles and could be precipitated together. The presence of E-luciferase fused protein (Eluc) was further confirmed by western-blot (**Fig 8C**).

The inventors have proved that the VLP-luc could be used to study the binding ability of dengue susceptible cells such as Vero. The binding of VLP-luc to Vero cells could be blocked by heparin (**Fig 8D**), the analog of heparan sulfate, while it could be enhanced by anti-E antibody 4E11 on macrophage (**Fig 8E**).

Example 6: HeLa-prME cells producing VLPs can be used to study the interaction between DV and host cells

The obtained results show that the DV1 VLPs produced by HeLa-prME cell line mimic maturation and secretion of DV1, thus providing a useful tool to study the interaction between DV and host cells during viral egress.

To identify host factors that could either enhance or reduce production of DV VLPs, the inventors first developed a quantitative assay to relatively quantify levels of secreted particles in supernatant of HeLa-prME cells (**Fig. 10**).

The chemiluminescence dot-blot (CLDB) assay is based on the concentration of RSPs from cell supernatant on PVDF membranes, followed by detection of E with a specific horseradish peroxidase (HRP)-conjugated antibody and quantification of substrate-induced luminescence using a

luminometer. Data using purified E protein of known concentrations showed that, when ranging between 400 pg to 40 ng, E protein on PVDF membrane displayed a very good linear correlation with the luminescence density in CLDB assay.

The CLDB was first used to estimate the VLPs yield from HeLa-prME cell line, and it was found that the concentration of E protein in supernatant of HeLa-prME cell line was around 500–1000 ng/ml under the culture condition used for siRNA transfection.

A siRNA library that consisted of 122 genes which target cellular membrane trafficking was then screened using the HeLa-prME cell line. Non-targeting siRNA (NT) and siRNA targeting DV1 prME were added as controls. Library and control siRNAs were transfected in triplicates on 96-well plates. Levels of RSPs secreted by siRNA transfected HeLa-prME were measured by CLDB assay from 40 microliters of supernatant from each well. Levels of E protein in cell supernatant were expressed in relative luminescence units and ratios to that of NT controls are shown in Figure 11. T test was used to assess the statistical significance of differences between each sample and NT. Differences were considered statistically significant when $P<0.05$. A confirmation

A confirmation of inhibition of DV1 VLP production by siRNA targeting of specific cellular factors identified by siRNA library screening. The stable HeLa prME cell line was transfected with siRNAs targeting various members of a family of cellular factors identified for their modulatory effects on DV1 VLP production in the siRNA library screening. 72 hours post transfection, cell supernatants and cell lysates were prepared and analysed by western blot (**Fig. 12**). Absence of E protein in supernatant of cells treated with the siRNA combination "H" indicates inhibition of VLP production.

The inventors observed that targeting of 23 genes resulted in significant reduction of DV1 VLPs amounts in supernatants whereas targeting of 22 other genes induced a significant increase. For instance, the results showed that down-regulation of ADP-ribosylation factor 1 (ARF1), which regulates secretory membrane transport, resulted in 3 fold decrease of DV1 RSPs in cell

supernatant, suggesting its involvement in the secretion of DV. Some genes whose down-regulation enhanced levels of DV VLPs in the supernatant are involved in endocytosis, such as the three dynamins which show a 2 to 4 fold increase in dengue RSPs secretion. These proteins are involved in the budding process or in the transport of vesicles. Such an enhancement might have been due, at least in part, to a blockade in the re-internalization of secreted VLPs by HeLa-prME cells. Although further experiments are required to confirm the screening data, this study validates the use of DV VLPs-producing HeLa-prME cell line in combination with the CLDB-based quantification strategy as a promising system to facilitate the identification of cellular factors involved in DV secretion.

General Discussion

In these examples, an optimized DV 1 prME gene was used to establish a HeLa-prME cell line which could stably producing DV1 VLP. Optimized DV2, DV3 and DV4 were also used to produce VLP for the dengue serotypes 2, 3 and 4. In the present examples, the efficient production of DV VLP without any change in amino acid might attribute to the application of codon optimization, which has been proven to be an effective method to increase the express level of glycoprotein from various viruses (Haas et al., 1996; Nie et al., 2004). The optimized gene significantly enhanced the expression of prME glycoproteins and therefore facilitated the generation of DV VLP with native viral proteins. The establishment of VLP producing cell lines provides a system to study the late stages of dengue virus life cycle and provides a new tool for applied research in the field of dengue virus.

The DV1 VLP has been generated previously, but the author had to replace the transmembrane domain with that of JEV because it contained an ER retention signal (Purdy and Chang, 2005). In the present examples, the efficient production of DV1 VLP without any change in amino acid might attribute to the application of codon optimization, which has been proven to be an effective method to increase the express level of glycoprotein from various viruses (Haas, Park, and Seed, 1996; Nie et al., 2004). The optimized gene

significantly enhanced the expression of prME glycoproteins and therefore facilitated the generation of DV1 VLP. The establishment of VLP producing cell lines provided a system to study the budding process.

The VLP secreted by the HeLa-prME cells was characterized. Sucrose gradient results demonstrated that the VLP was sensitive to detergent treatment, showing it contained the lipid membrane. The presence of dengue glycoproteins was proved by western blot and silver staining. Glycoprotein E formed homodimer on secreted VLP and the homodimeration was important step of dengue virus maturation (Modis et al., 2004; Zhang et al., 2003). During the maturation of dengue virus, E protein first formed heterodimer with prM protein in host cells and then rearranged to homodimer when the VLP was secreted (Kuhn et al., 2002; Zhang et al., 2004). The rearrangement from heterodimer to homodimer required the prM protein to be cleaved by host cell's proprotein convertase furin to form M and soluble pr protein, and this step was important for maturation of dengue virus (Keelapang et al., 2004). Although the prM/E heterodimer was not found in our experiment, possibly because their binding was weak, the cleavage of prM to M protein was confirmed in the present study. Taken together, these results showed the VLP underwent the same maturation process as real virus.

The intracellular trafficking of VLP was also studied in the HeLa-prME cell line. By immunostaining, most E protein was localized to ER, where the dengue glycoproteins were synthesized (Lindenbach, 2001). A litter E protein was present in ERGIC and Golgi, indicating the pathway through it. The secretion pathway was also investigated by temperature or drug block experiments. Incubation in 15°C, which induce the block between the intermediate compartment and the cis-Golgi, or in 20°C to block the exit from the TGN, or BFA treatment for inhibition of exit from ER could all significantly reduce secreted VLP, outlined the places involved in the egress of DV1 VLP. The present results also showed that it would take at least 3 hours for VLP from synthesis to be released, a litter longer than TBEV (Lorenz et al., 2003).

Besides viral entry and replication, viral egress is another target site for anti-dengue drug development. Several factors have been found to be able to

affect this process but need further studies. The establishment of stable DV1 VLP producing cells provided a system to mimic the egress process of dengue virus. In combination with the sensitive quantification system CLDB, it offered a platform to screen the potential inhibitor for dengue virus secretion.

The dengue 1 viral-like particle (VLP) was generated by using the optimized prME gene and a VLP producing stable cell line was also established. The optimized gene significantly enhanced the expression of prME glycoproteins and therefore facilitated the generation of DV1 VLP. The present results showed that the VLP underwent the same maturation process and had the same surface structure as real virus. Glycoprotein prME was synthesized in ER and would take at least 3 hours from synthesis to be secreted. During the intracellular trafficking, prM protein was cleaved by furin convertase and followed by the rearrangement of E protein. E protein formed homodimer in matured VLP particle. The establishment of stable cell line provided a system to study the factors that are involved in the egress process of dengue virus.

REFERENCES

Allison, S.L., Y.J. Tao, G. O'Riordain, C.W. Mandl, S.C. Harrison, and F.X. Heinz. 2003. Two distinct size classes of immature and mature subviral particles from tick-borne encephalitis virus. *J Virol.* 77:11357-66.

Chang, G.J., A.R. Hunt, D.A. Holmes, T. Springfield, T.S. Chiueh, J.T. Roehrig, and D.J. Gubler. 2003. Enhancing biosynthesis and secretion of premembrane and envelope proteins by the chimeric plasmid of dengue virus type 2 and Japanese encephalitis virus. *Virology.* 306:170-80.

Ferlenghi, I., M. Clarke, T. Ruttan, S.L. Allison, J. Schalich, F.X. Heinz, S.C. Harrison, F.A. Rey, and S.D. Fuller. 2001. Molecular organization of a recombinant subviral particle from tick-borne encephalitis virus. *Mol Cell.* 7:593-602.

Fonseca, B.A., S. Pincus, R.E. Shope, E. Paoletti, and P.W. Mason. 1994. Recombinant vaccinia viruses co-expressing dengue-1 glycoproteins prM and E induce neutralizing antibodies in mice. *Vaccine*. 12:279-85.

Haas, J., E.C. Park, and B. Seed. 1996. Codon usage limitation in the expression of HIV-1 envelope glycoprotein. *Curr Biol*. 6:315-24.

Hsieh, S.C., I.J. Liu, C.C. King, G.J. Chang, and W.K. Wang. 2008. A strong endoplasmic reticulum retention signal in the stem-anchor region of envelope glycoprotein of dengue virus type 2 affects the production of virus-like particles. *Virology*. 374:338-50.

Hunt, A.R., C.B. Cropp, and G.J. Chang. 2001. A recombinant particulate antigen of Japanese encephalitis virus produced in stably-transformed cells is an effective noninfectious antigen and subunit immunogen. *J Virol Methods*. 97:133-49.

Keelapang, P., R. Sriburi, S. Supasa, N. Panyadee, A. Songjaeng, A. Jairungsri, C. Puttikhunt, W. Kasinrerk, P. Malasit, and N. Sittisombut. 2004. Alterations of pr-M cleavage and virus export in pr-M junction chimeric dengue viruses. *J Virol*. 78:2367-81.

Konishi, E., and A. Fujii. 2002. Dengue type 2 virus subviral extracellular particles produced by a stably transfected mammalian cell line and their evaluation for a subunit vaccine. *Vaccine*. 20:1058-67.

Kuhn, R.J., W. Zhang, M.G. Rossmann, S.V. Pletnev, J. Corver, E. Lenches, C.T. Jones, S. Mukhopadhyay, P.R. Chipman, E.G. Strauss, T.S. Baker, and J.H. Strauss. 2002. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. *Cell*. 108:717-25.

Lindenbach, B.D., and C. M. Rice. 2001. Fields virology. Lippincott Williams & Wilkins, Philadelphia, Pa. 991–1041 pp.

Lorenz, I.C., J. Kartenbeck, A. Mezzacasa, S.L. Allison, F.X. Heinz, and A. Helenius. 2003. Intracellular assembly and secretion of recombinant subviral particles from tick-borne encephalitis virus. *J Virol*. 77:4370-82.

Modis, Y., S. Ogata, D. Clements, and S.C. Harrison. 2004. Structure of the dengue virus envelope protein after membrane fusion. *Nature*. 427:313-9.

Mukhopadhyay, S., R.J. Kuhn, and M.G. Rossmann. 2005. A structural perspective of the flavivirus life cycle. *Nat Rev Microbiol.* 3:13-22.

Needleman, S.B., and C.D. Wunsch. 1970. A general method applicable to the search for similarities in the amino acid sequence of two proteins. *J Mol Biol.* 48:443-53.

Nie, Y., P. Wang, X. Shi, G. Wang, J. Chen, A. Zheng, W. Wang, Z. Wang, X. Qu, M. Luo, L. Tan, X. Song, X. Yin, M. Ding, and H. Deng. 2004. Highly infectious SARS-CoV pseudotyped virus reveals the cell tropism and its correlation with receptor expression. *Biochem Biophys Res Commun.* 321:994-1000.

Pryor, M.J., L. Azzola, P.J. Wright, and A.D. Davidson. 2004. Histidine 39 in the dengue virus type 2 M protein has an important role in virus assembly. *J Gen Virol.* 85:3627-36.

Purdy, D.E., and G.J. Chang. 2005. Secretion of noninfectious dengue virus-like particles and identification of amino acids in the stem region involved in intracellular retention of envelope protein. *Virology.* 333:239-50.

Raviprakash, K., T.J. Kochel, D. Ewing, M. Simmons, I. Phillips, C.G. Hayes, and K.R. Porter. 2000. Immunogenicity of dengue virus type 1 DNA vaccines expressing truncated and full length envelope protein. *Vaccine.* 18:2426-34.

Witko, S.E. et al. 2010. Refined methods for propagating vesicular stomatitis virus vectors that are defective for G protein expression. *J. Virol. Methods* in press

Zhang, Y., J. Corver, P.R. Chipman, W. Zhang, S.V. Pletnev, D. Sedlak, T.S. Baker, J.H. Strauss, R.J. Kuhn, and M.G. Rossmann. 2003. Structures of immature flavivirus particles. *Embo J.* 22:2604-13.

Zhang, Y., W. Zhang, S. Ogata, D. Clements, J.H. Strauss, T.S. Baker, R.J. Kuhn, and M.G. Rossmann. 2004. Conformational changes of the flavivirus E glycoprotein. *Structure.* 12:1607-18.

CLAIMS

1. A polynucleotide comprising a codon-optimized dengue prME nucleotide sequence.

2. The polynucleotide of claim 1, wherein the dengue prME gene is from dengue serotype 1, 2, 3 or 4.

3. The polynucleotide of claim 1, comprising the nucleotide sequence substantially identical to SEQ ID NO: 1, 2, 3, 4, 5, 6, 7 or 8.

4. The polynucleotide of claim 3, further comprising a sequence coding for a marker protein.

5. A cloning or expression vector comprising the polynucleotide of any one of claims 1 to 4.

6. The vector of claim 5, consisting of one of the plasmid deposited at the CNCM under accession number I-4084, I-4085, I-4086 or I-4087, on November 12, 2008.

7. A host cell comprising the polynucleotide of any one of claim 1 to 4 or a vector as defined in claim 5 or 6.

8. A cell line comprising the cell of claim 7, and deposited at the CNCM under accession number I-4083 on November 12, 2008.

9. A dengue virus-like particle (VLP) produced by the host cell of claim 7 or the cell line of claim 8.

10. A composition comprising the VLP of claim 9 and an acceptable carrier.

11. A method for generating an immune response in a host, comprising the step of administering a composition as defined in claim 10.

12. A method for treating or preventing a dengue-associated disease, comprising the step of administering the composition of claim 10 to a host in need thereof.

13. A method for producing strain-specific dengue virus-like particle (VLP), comprising the steps of:

a) introducing an optimized dengue prME sequence into a host cell;

b) incubating said host cell under conditions to produce VLPs; and

c) harvesting the produced VLPs.

14. A method of screening for an inhibitory dengue cell-binding agent, comprising the steps of:

a) contacting a VLP as defined in claim 9 and a candidate agent with a Dengue susceptible host cell under suitable condition to allow binding of the VPL to said cell; and

b) evaluating the capacity of the agent to inhibit the formation of a VLP/cell complex.

15. A method of screening for a dengue virus production inhibitory agent, comprising the steps of:

a) getting into the cell the VLP as defined in claim 9; and

b) evaluating the capacity of the agent to inhibit the production of VLP by producer cells.

16. Use of the VLP as defined in claim 9 or the composition of claim 11, to induce an immune response in a host.

17. Use of the VLP as defined in claim 9 or the composition of claim 11, for treating or preventing a dengue-associated disease.

18. Use of the inhibitory agents or derivatives obtained from the method of claim 14 or 15, for treating or preventing a dengue-associated disease.

19. Use of the VLP as defined in claim 9 or the composition of claim 10, for identifying an inhibitory dengue cell-binding agent.

20. Use of the VLP as defined in claim 10 or the composition of claim 11, for identifying a dengue production inhibitory agent.

21. A method for treating or preventing a dengue-associated disease, comprising the step of administering an inhibitory agent obtained by the method of claim 18 or 19 to a host in need thereof.

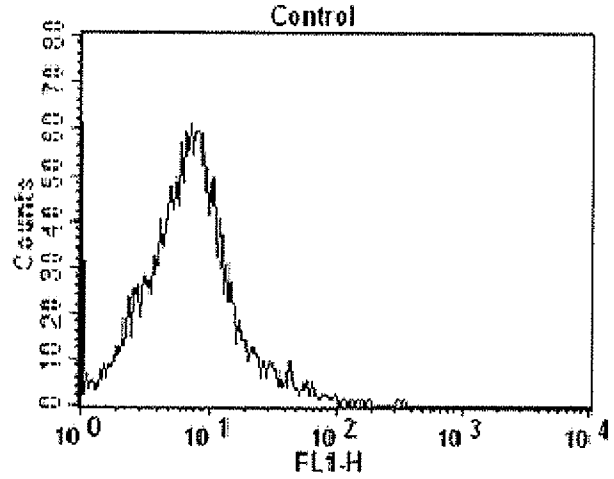

Figure 1A

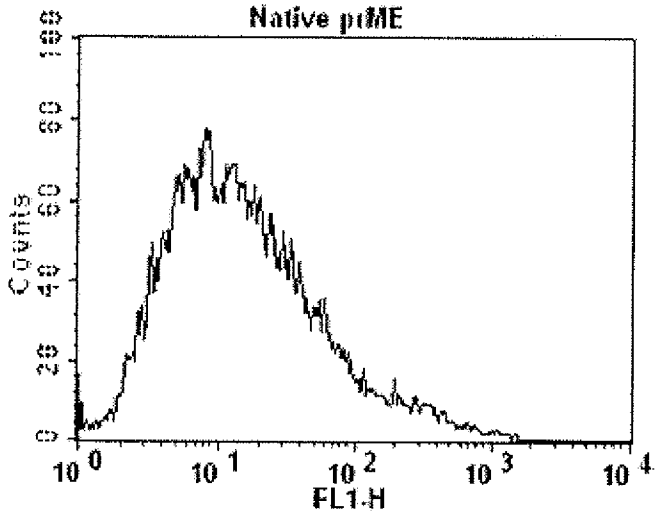

Figure 1B

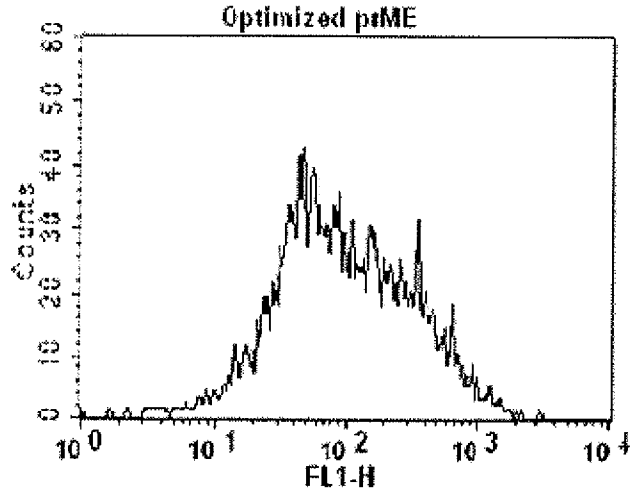

Figure 1C

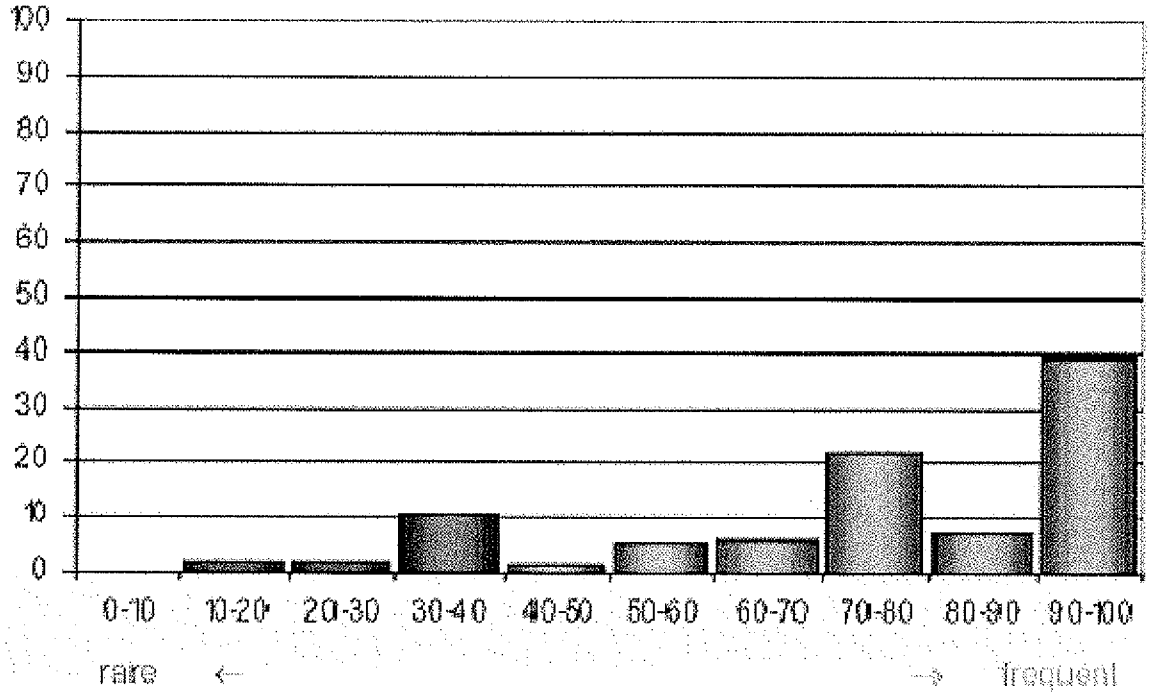

Figure 1D

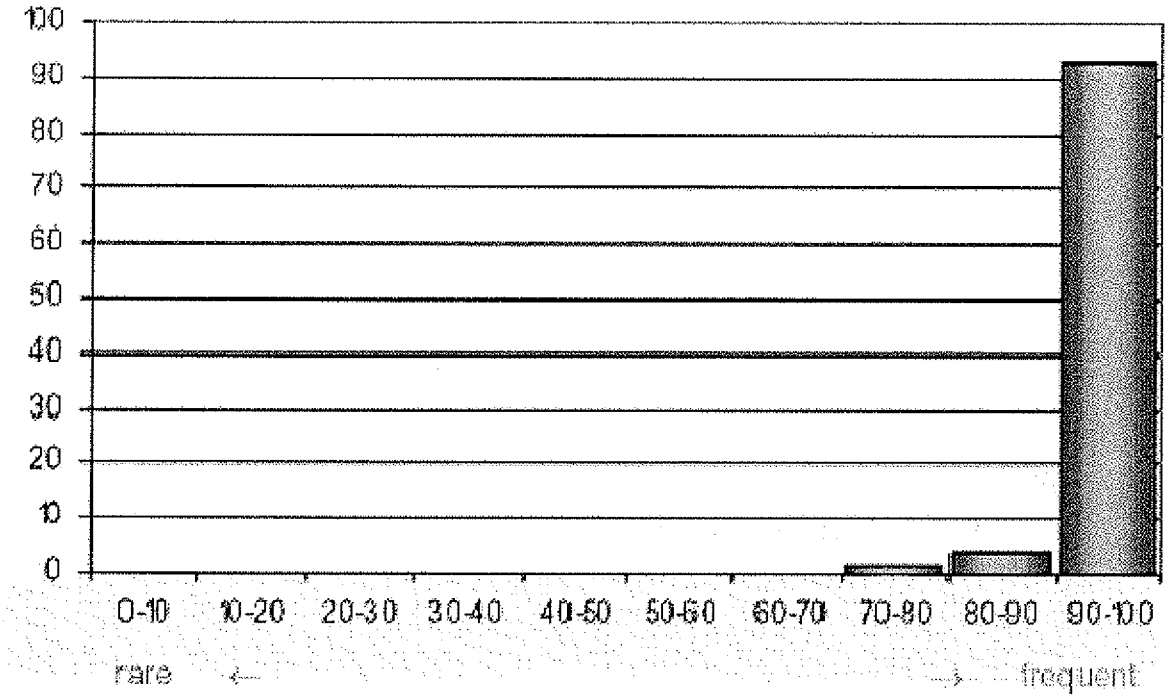

Figure 1E

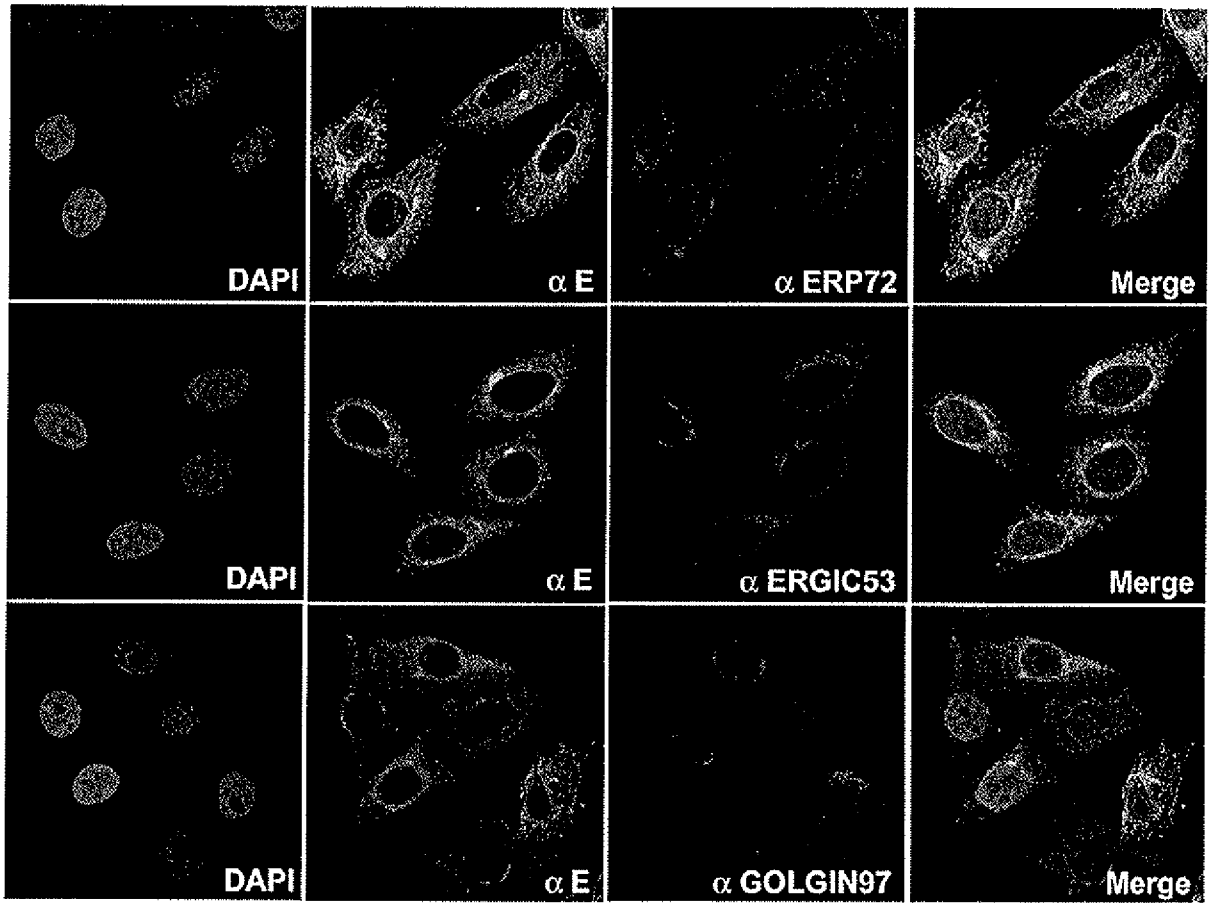
DAPI
α E
α ERP72
Merge
DAPI
α E
α ERGIC53
Merge
DAPI
α E
α GOLGIN97
Merge

Figure 2

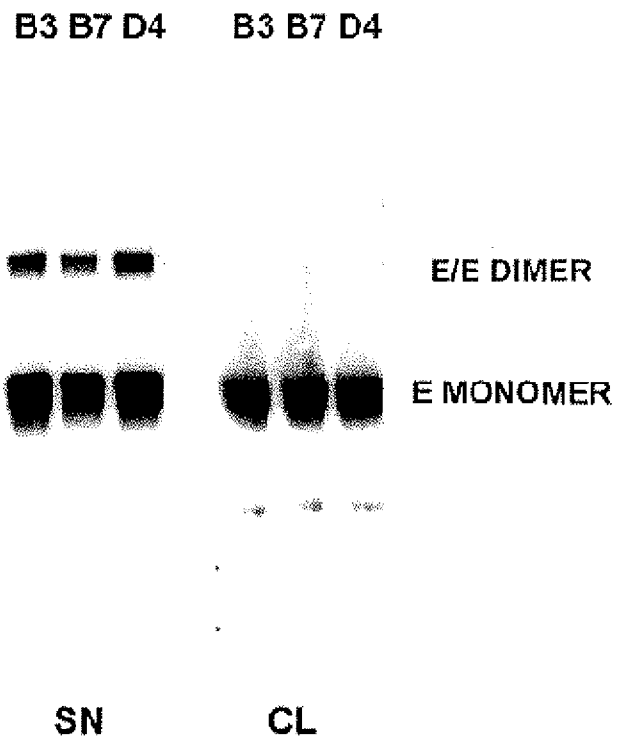

Figure 3A

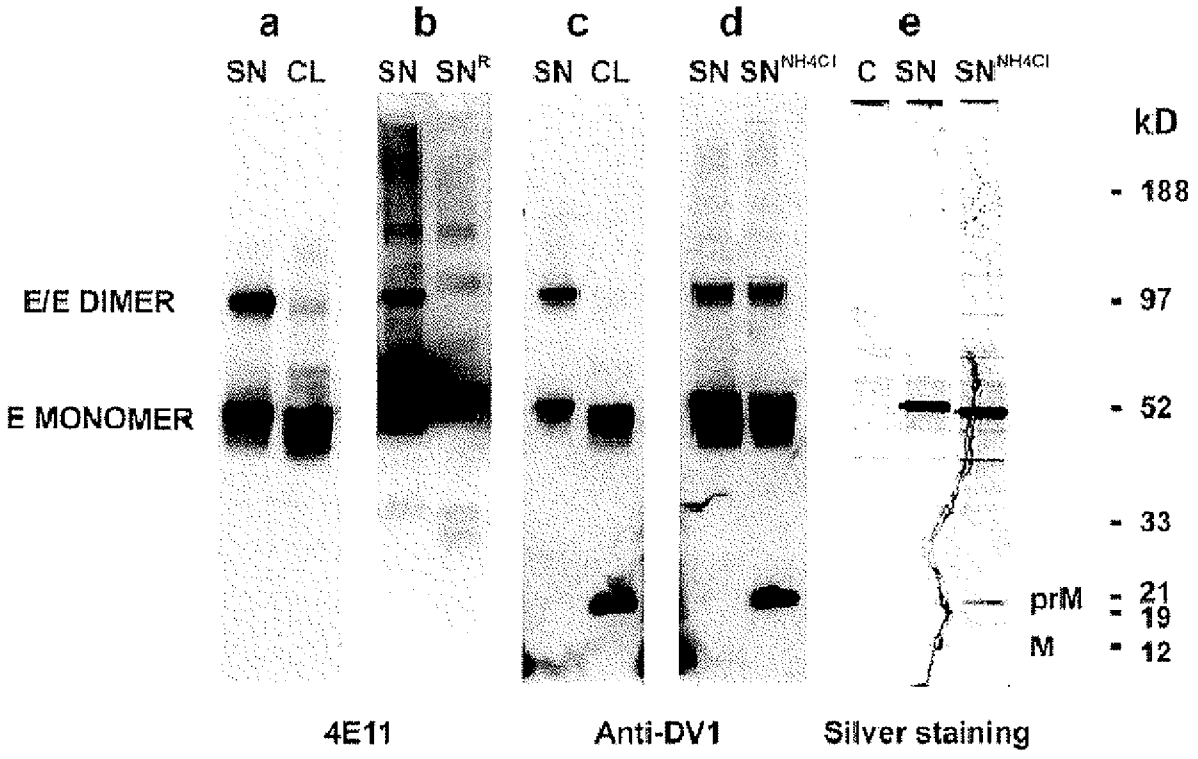

Figure 3B

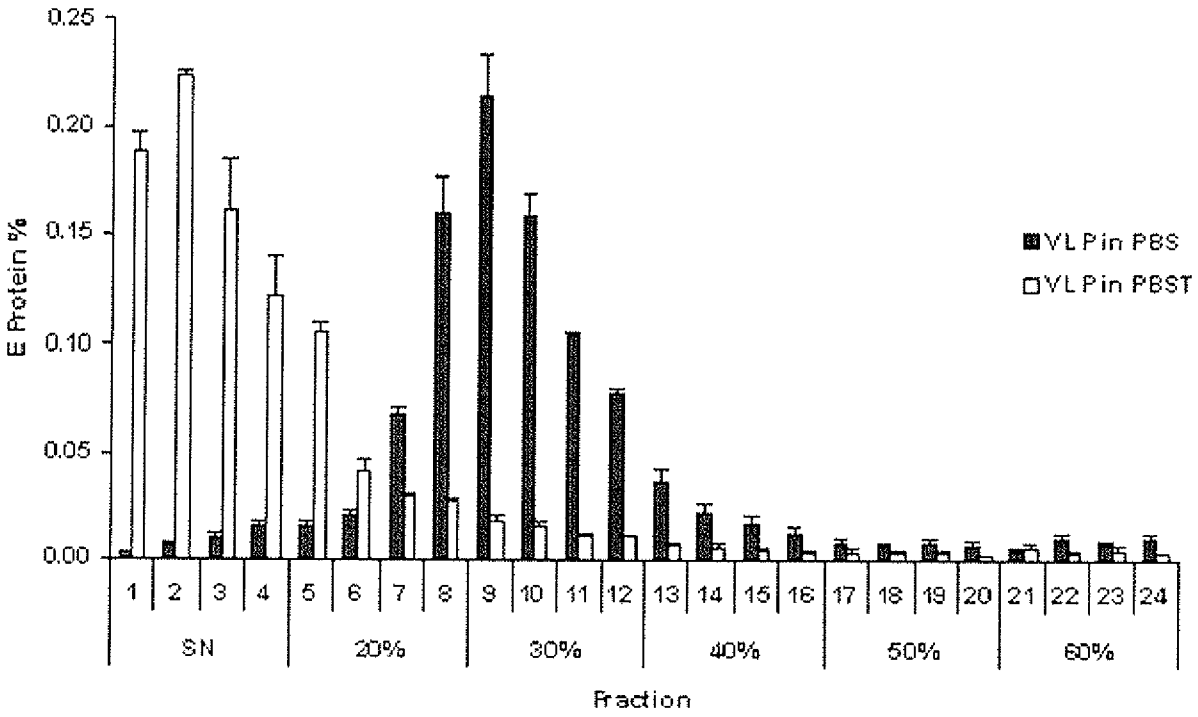

Figure 4

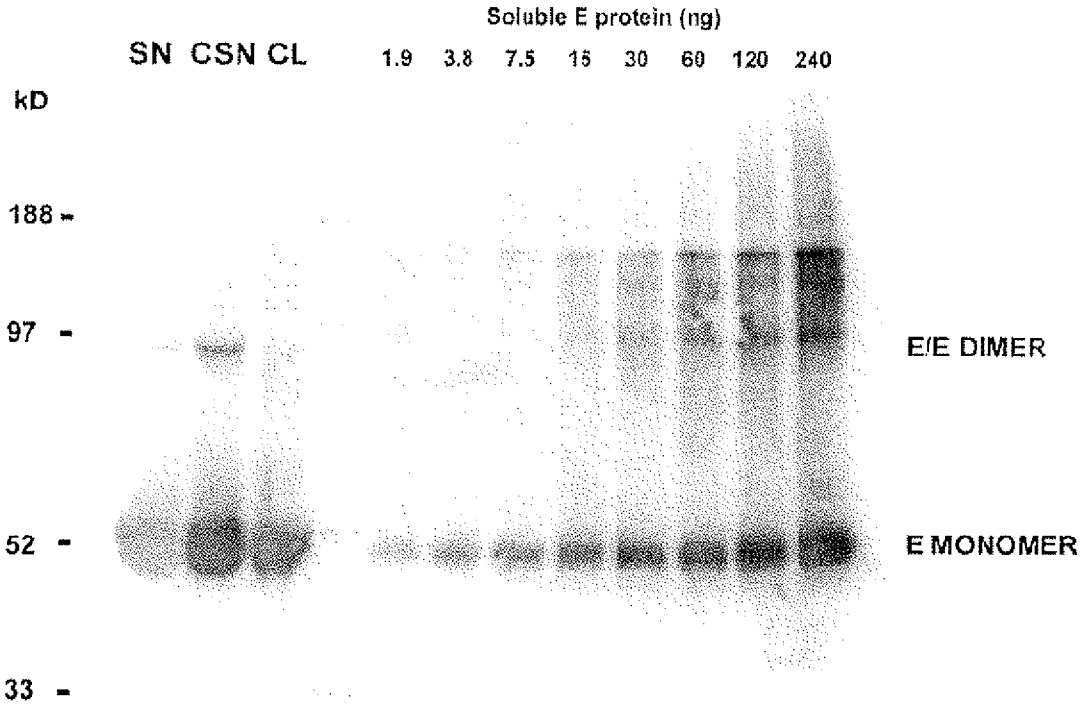

Figure 5

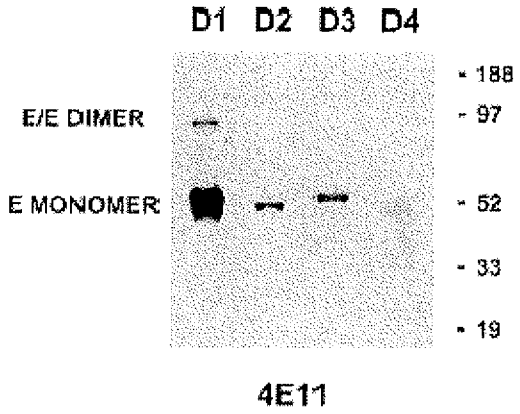

Figure 6A

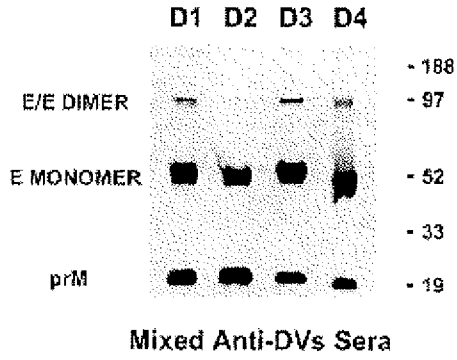

Figure 6B

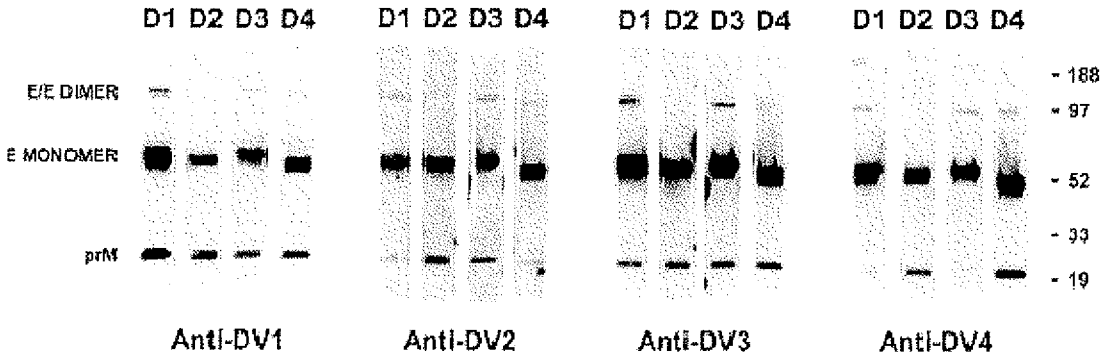

Figure 6C

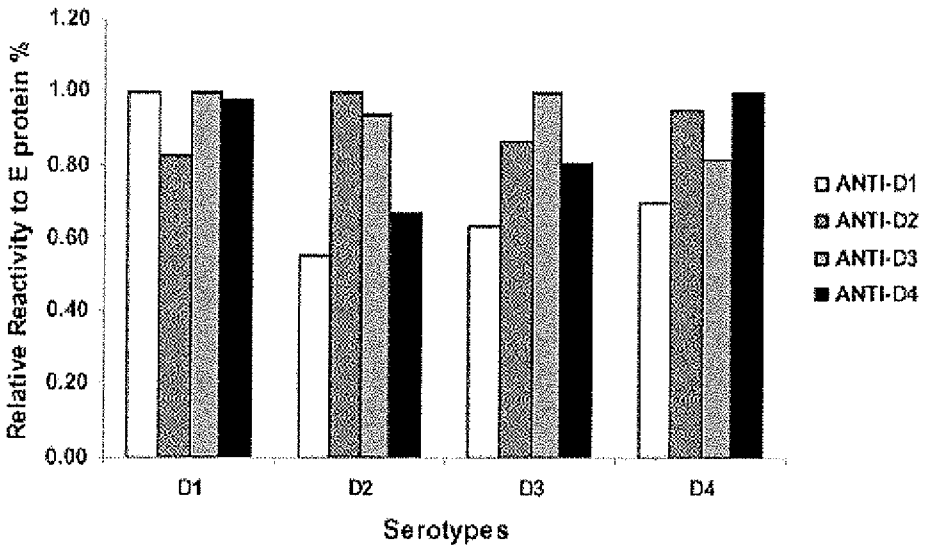

Figure 6D

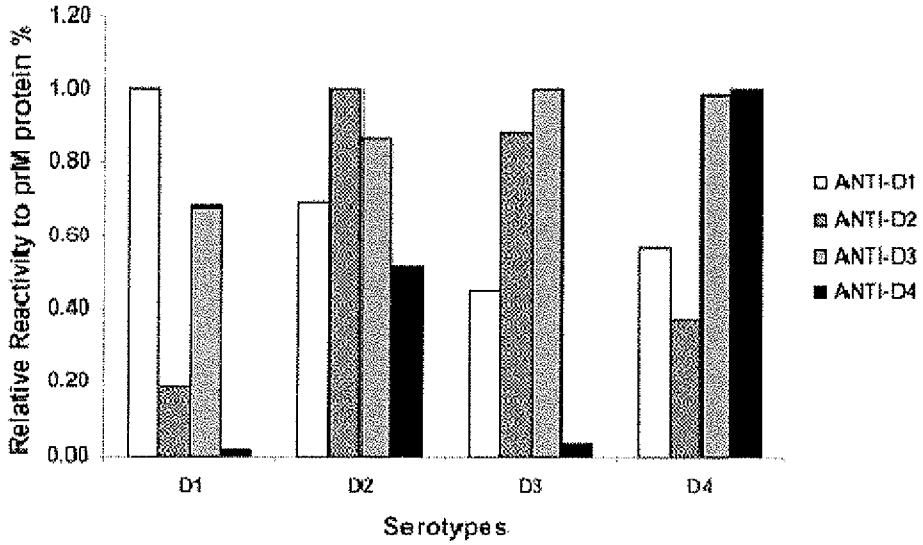

Figure 6E

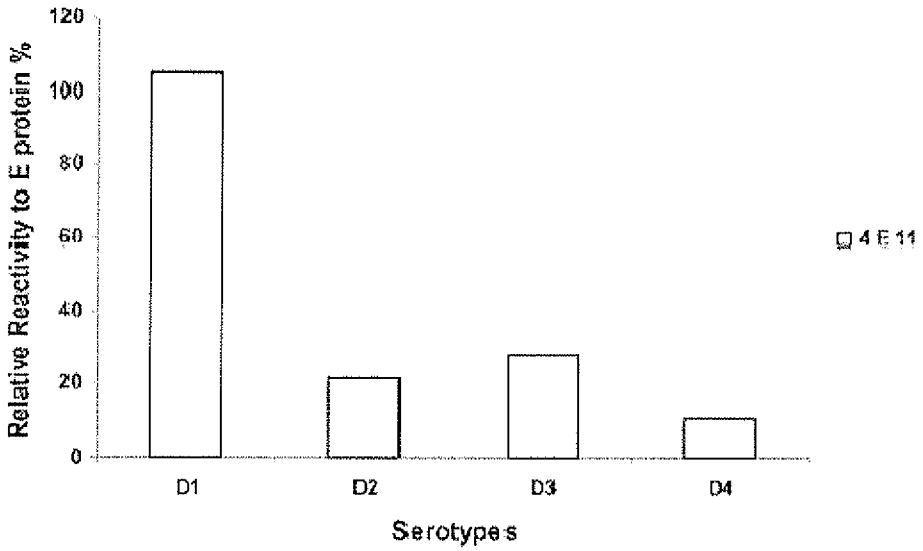

Figure 6F

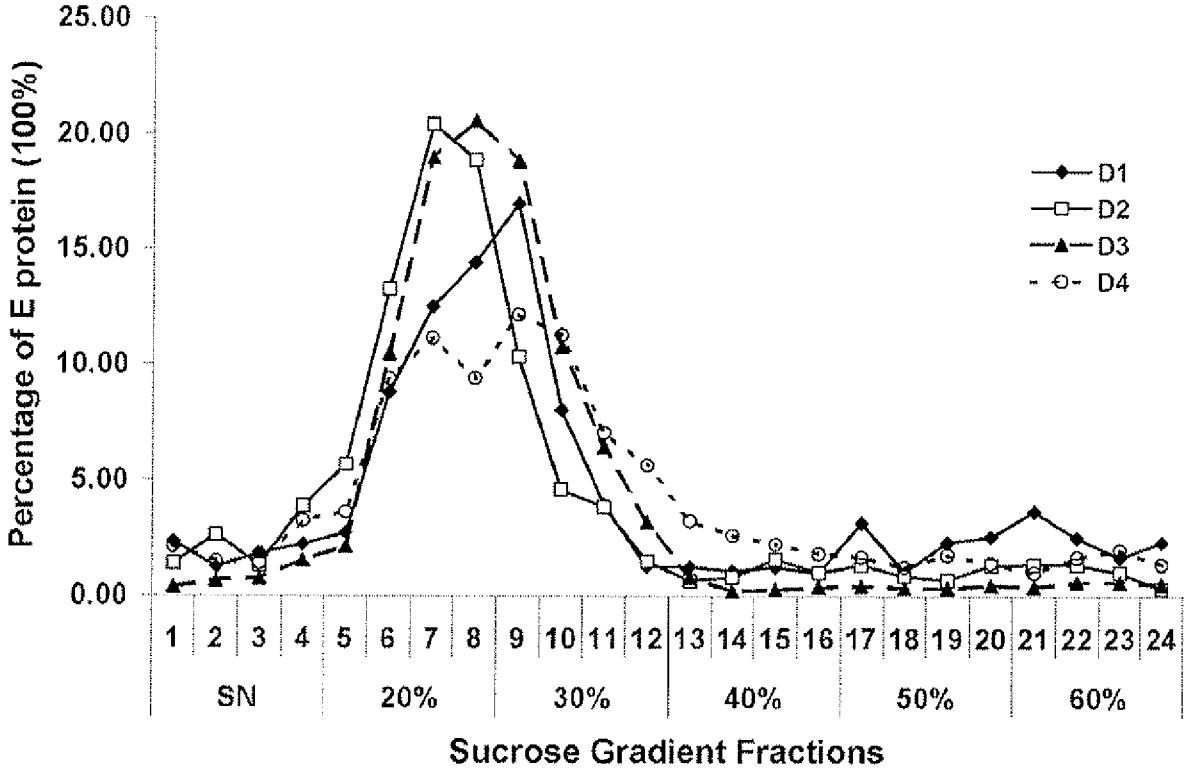

Figure 7

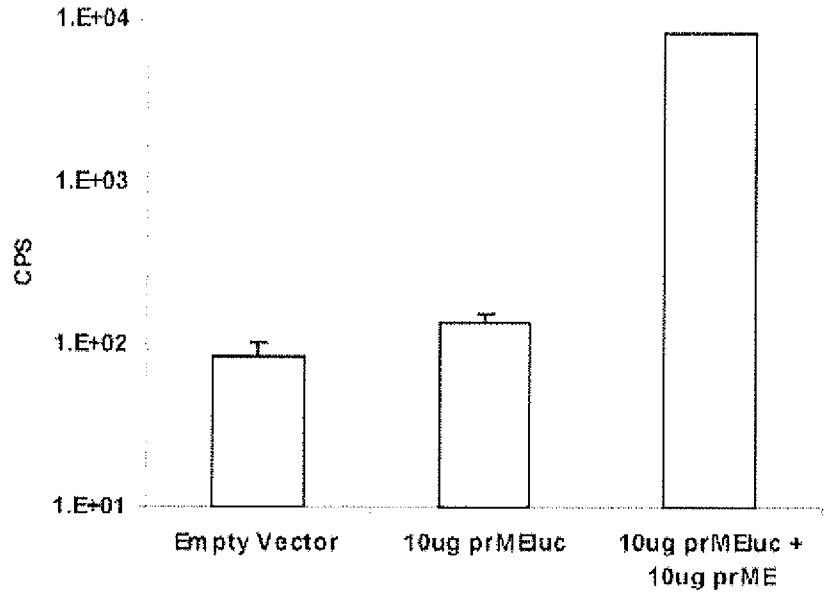

Figure 8A

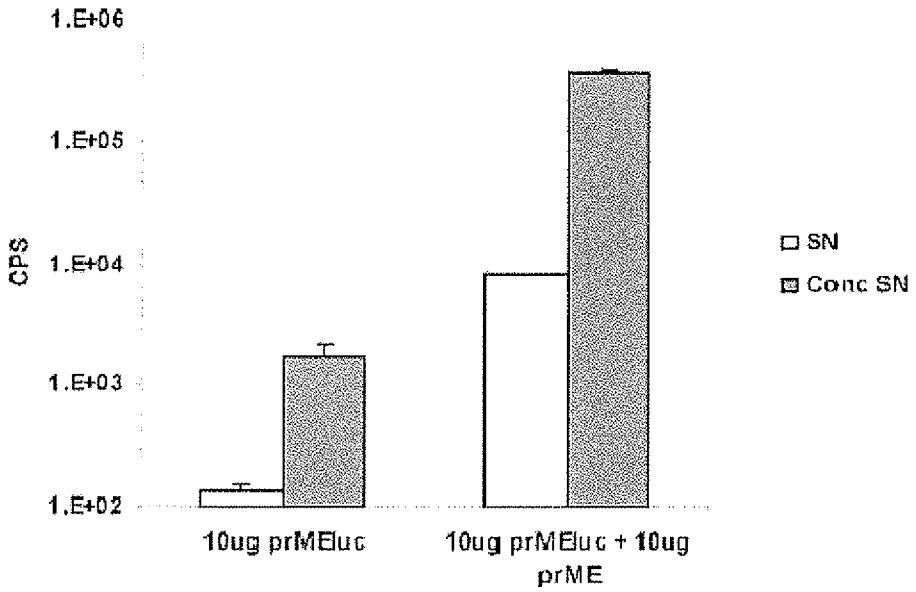

Figure 8B

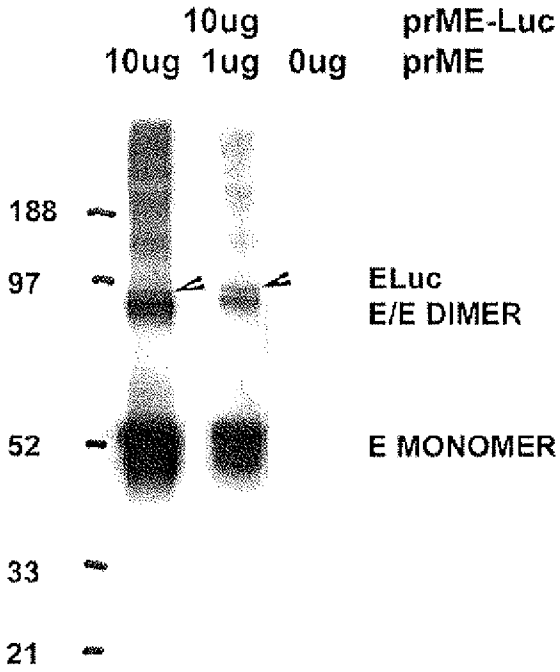

Figure 8C

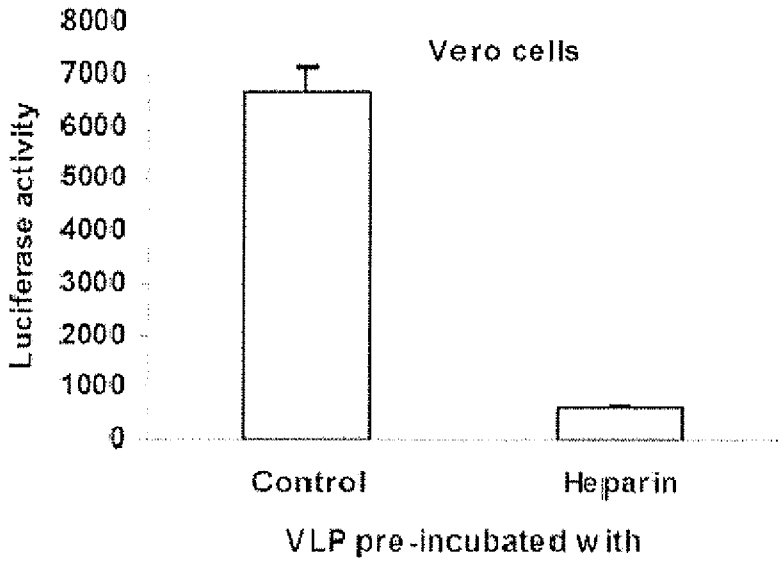

Figure 8D

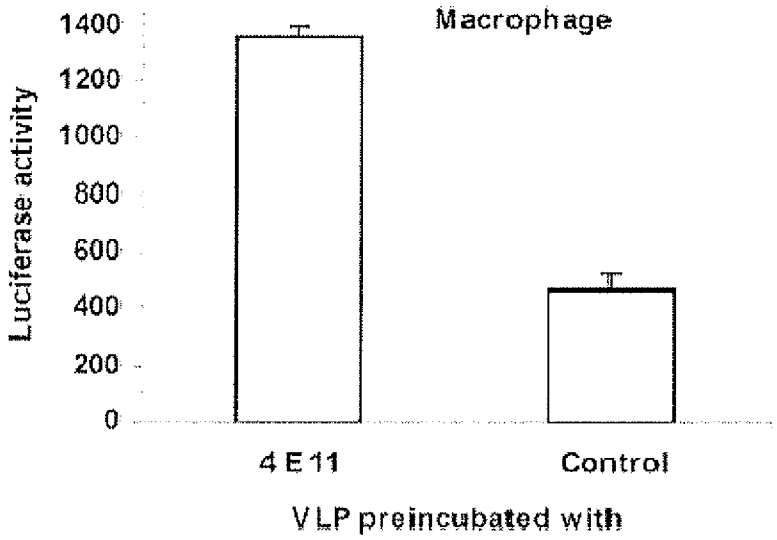

Figure 8E

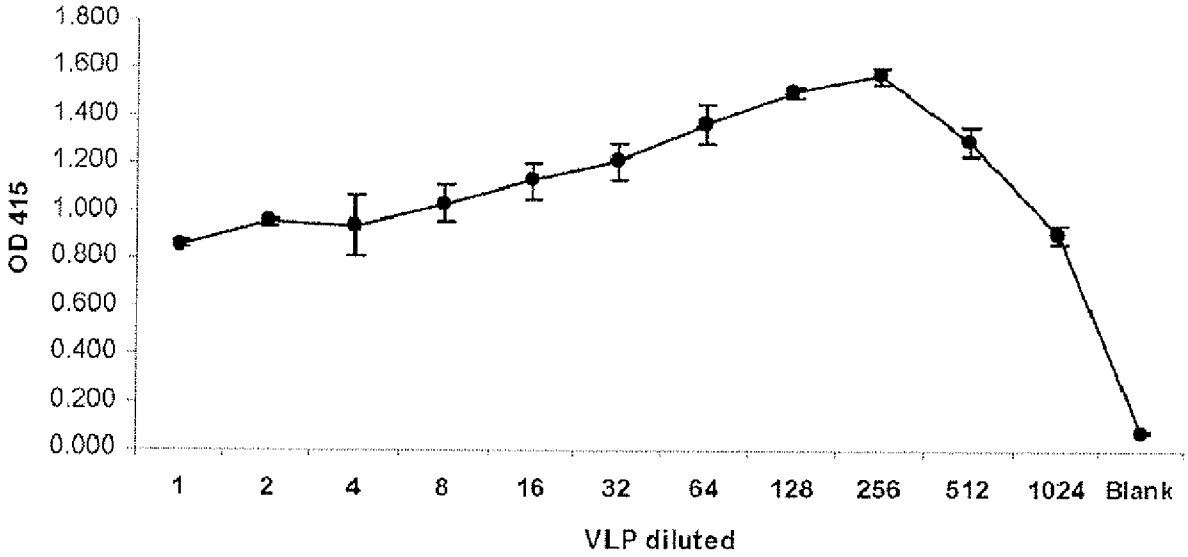

Figure 9A

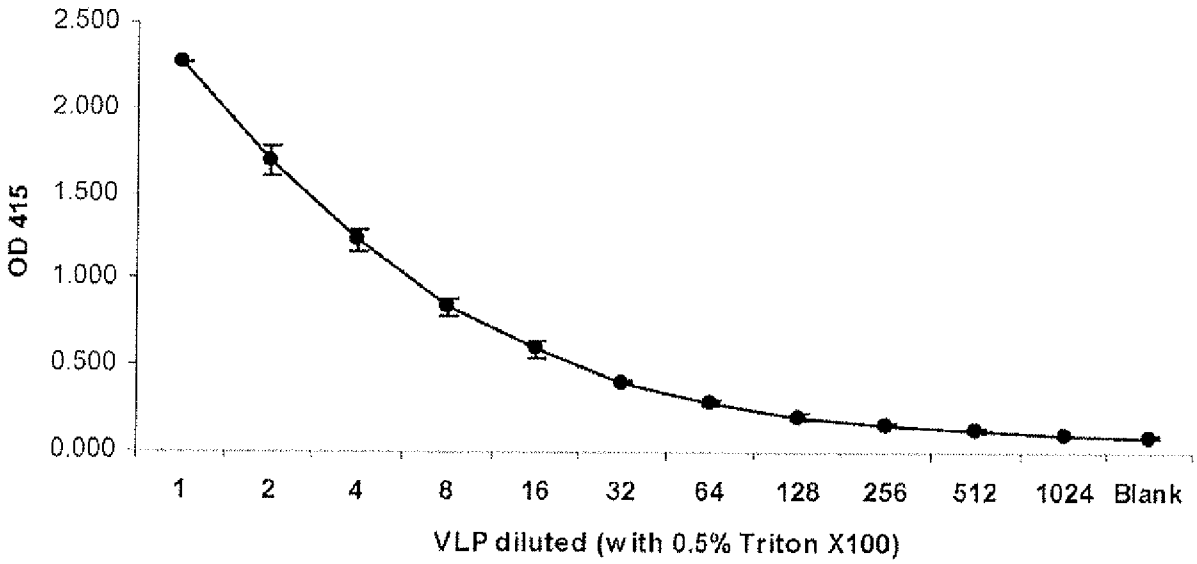

Figure 9B

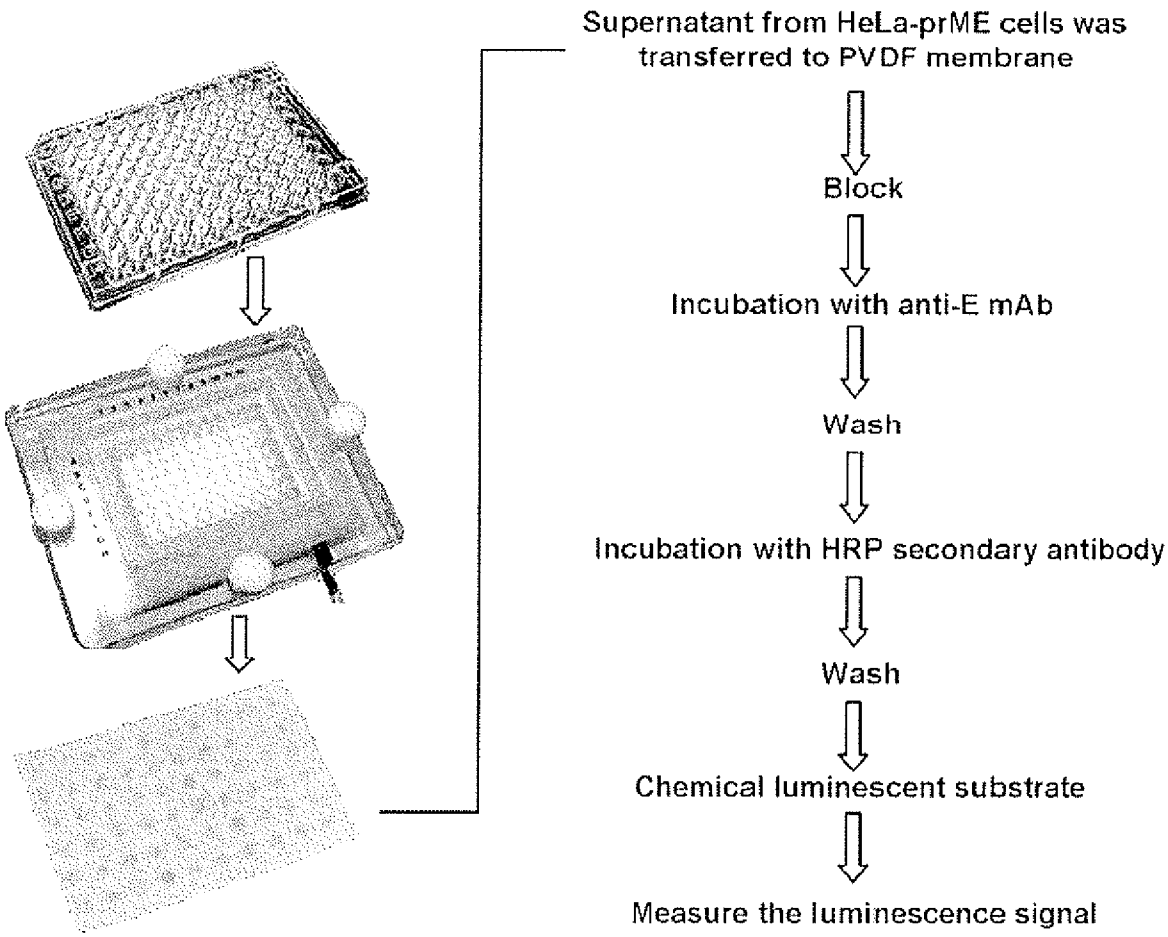
Supernatant from HeLa-prME cells was transferred to PVDF membrane
Block
Incubation with anti-E mAb
Wash
Incubation with HRP secondary antibody
Wash
Chemical luminescent substrate
Measure the luminescence signal

Figure 10

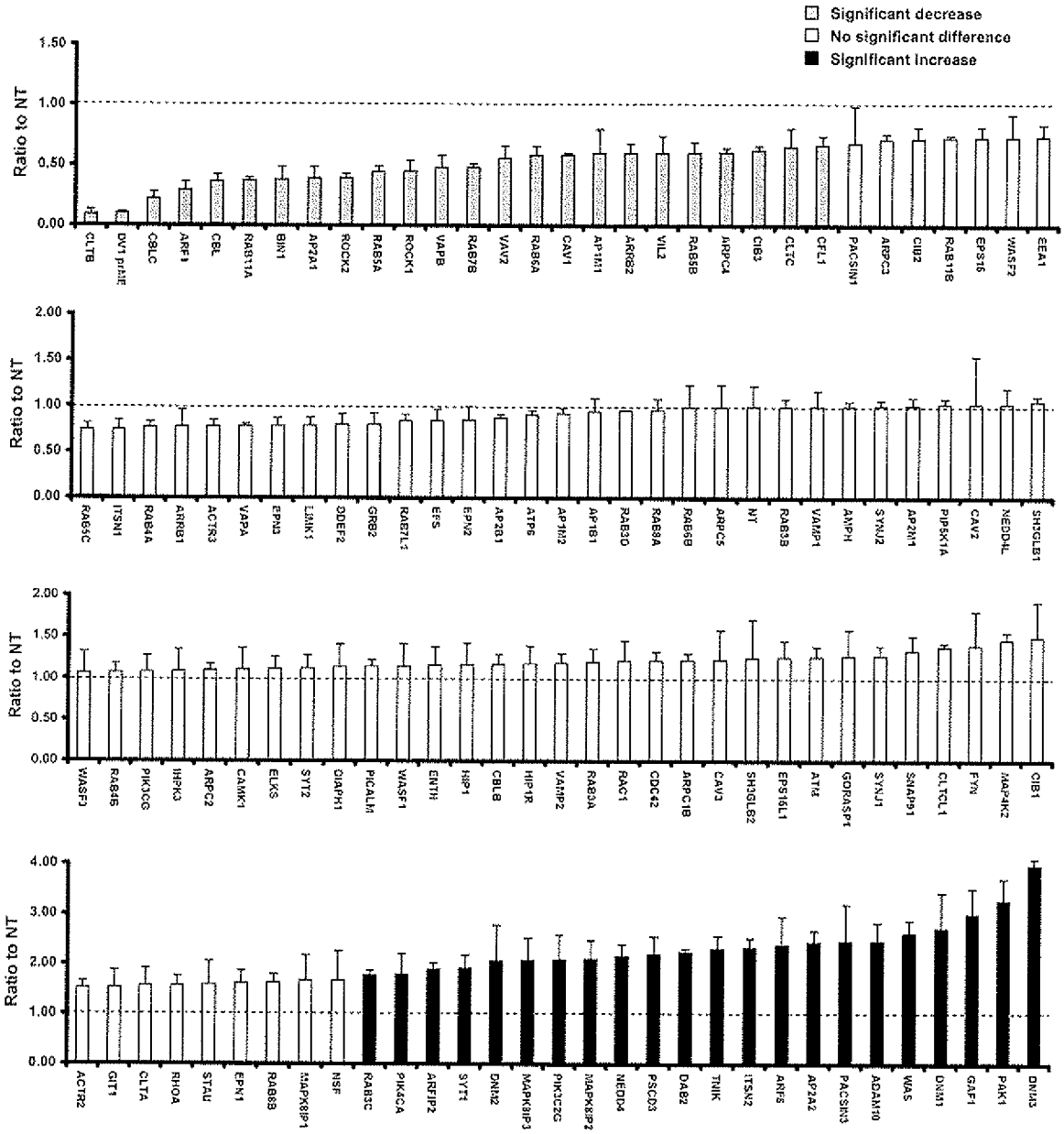
Significant decrease
No significant difference
Significant increase
Ratio to NT
1.50
1.00
0.50
0.00
CLTB
DVL1 protein
CBLC
ARF1
CBL
RAB11A
BIN1
AP2A1
ROCK2
RAB5A
ROCK1
VAPB
RAB7B
VAV2
RAB6A
CAV1
AP1M1
ARR82
VIL2
RAB8B
ARPC4
CIB3
CLTC
CFL1
PACSIN1
ARPC3
CIB2
RAB11B
EPS16
WASF2
EEA1
2.00
1.50
1.00
0.50
0.00
RAB5C
ITSN1
RAB4A
ARRB1
ACTR3
VAPA
EPN3
LRRK1
SDEF2
GRB2
RABEP1
EPS
EPN2
AP2B1
ATP6
AP1M2
AP1B1
RAB3D
RAB8A
RAB6B
ARPC5
NT
RAB3B
VAMP1
AMPH
SYNJ2
AP2M1
PIP5K1A
CAV2
NEDD4L
SH3GLB1
2.00
1.50
1.00
0.50
0.00
WASF3
RAB4B
PIK3CG
INPP4
ARPC2
CAMK1
ELKS
SYT2
DIAPH1
PICALM
WASF1
ENTH
HIP1
CBLB
HIP1R
VAMP2
RAB3A
RAC1
CDC42
ARPC1B
CAV3
SH3GLB2
EPS15L1
ATP2
GDRASP1
SYNJ1
SNAP91
CLTCL1
FYN
MAP4K2
CIB1
4.00
3.00
2.00
1.00
0.00
ACTR2
GIT1
CLTA
RHOA
STAU
EPN1
RAB8B
MAPK8IP1
NSF
RAB3C
PIK4CA
ARFIP2
SYT1
DNM2
MAPK8IP3
PIK3CZG
MAPK8IP2
NEDD4
PSCD3
DAB2
TNK
ITSN2
ARF6
AP2A2
PACSIN3
ADAM10
WAS
DNM1
GAP1
PAK1
DNM3

Figure 11

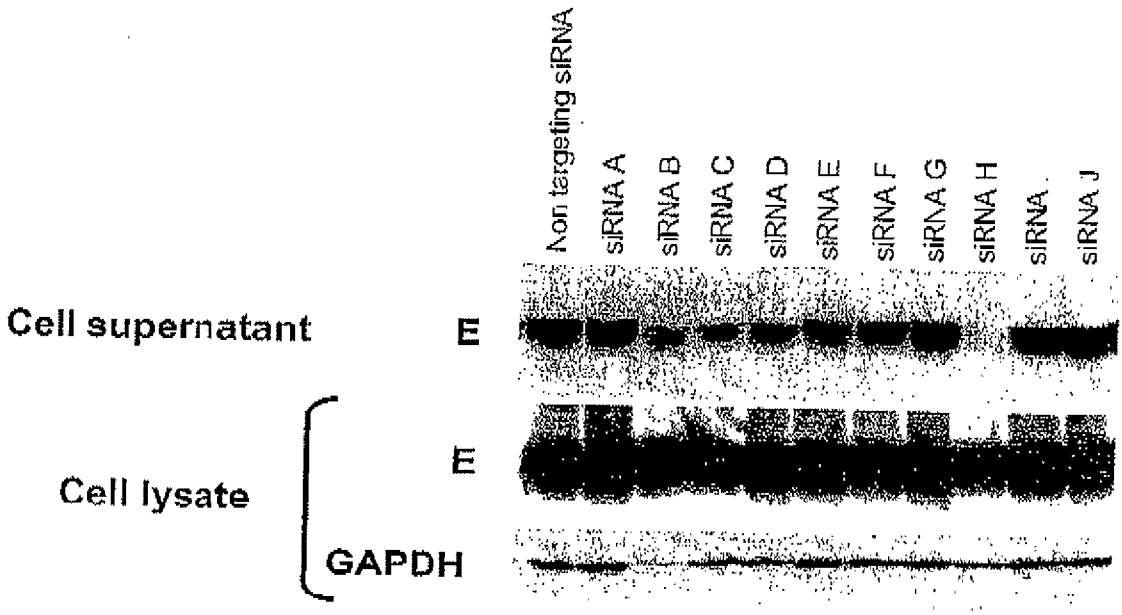
Non targeting siRNA
siRNA A
siRNA B
siRNA C
siRNA D
siRNA E
siRNA F
siRNA G
siRNA H
siRNA I
siRNA J
Cell supernatant
E
Cell lysate
E
GAPDH

Figure 12

DV1 opt prME (FGA):

GGATCCGCCACCATGAAGTGCCTGCTGTACCTGGCCTTCCTGTTCATCGGCGTGAACTGCTTCC
ACCTGACCACCAGAGGCGGCGAGCCCCACATGATCGTGTCCAAGCAGGAGAGAGGCAAGAGC
CTGCTGTTCAAGACCAGCGCCGGAGTGAACATGTGTACCCTGATCGCCATGGATCTGGGCGAG
CTGTGTGAGGACACCATGACCTACAAGTGCCCCAGAATCACCGAGGCCGAGCCCGACGACGT
GGACTGCTGGTGTAACGCCACCGATACCTGGGTGACCTACGGCACCTGTAGCCAGACCGGCG
AGCACAGGAGAGACAAGAGAAGCGTGGCCCTGGCCCCCCATGTGGGCCTGGGCCTGGAGACC
AGAACCGAGACCTGGATGAGCAGCGAGGGCGCCTGGAAGCAGATCCAGAAGGTGGAGACCT
GGGCCCTGAGACACCCCGGCTTCACCGTGATCGCCCTGTTCCTGGCCCACGCCATCGGCACCA
GCATCACCCAGAAGGGCATCATCTTCATCCTGCTGATGCTGGTGACCCCTAGCATGGCCATGA
GATGTGTGGGCATCGGCAACAGGGACTTCGTGGAGGGCCTGAGCGGCGCCACCTGGGTGGAC
GTGGTGCTGGAGCACGGCAGCTGTGTGACCACCATGGCCAAGAACAAGCCCACCCTGGACAT
CGAGCTGCTGAAAACCGAGGTGACCAACCCTGCCGTGCTGAGGAAGCTGTGTATCGAGGCCA
AGATCAGCAACACCACCACCGACAGCAGATGCCCCACCCAGGGCGAGGCCACCCTGGTGGAG
GAGCAGGACGCCAACTTCGTGTGTCGGAGGACCGTGGTGGACAGAGGCTGGGGCAACGGCTG
TGGCCTGTTCGGCAAGGGCAGCCTGCTGACCTGTGCCAAGTTCAAGTGTGTGACCAAGCTGGA
GGGCAAGATCGTGCAGTACGAGAACCTGAAGTACAGCGTGATCGTGACCGTGCACACCGGCG
ACCAGCACCAAGTGGGCAACGAGACCACCGAGCACGGCACCATCGCCACCATCACCCCCCAG
GCCCCTACCAGCGAGATCCAGCTGACCGATTACGGCACCCTGACCCTGGATTGTAGCCCTAGA
ACCGGCCTGGACTTCAACGAGATGGTGCTGCTGACCATGAAGGAGAAGAGCTGGCTGGTGCA
CAAGCAGTGGTTCCTGGACCTGCCCCTGCCCTGGACCAGCGGCGCCAGCACCTCCCAGGAGAC
CTGGAACAGACAGGACCTGCTGGTGACATTCAAGACCGCCCACGCCAAGAAGCAGGAGGTGG
TGGTGCTGGGCAGCCAAGAGGGCGCCATGCACACCGCCCTGACAGGCGCCACCGAGATCCAG
ACCAGCGGCACCACCACAATCTTCGCCGGCCACCTGAAGTGTCGGCTGAAGATGGACAAGCT
GACCCTGAAGGGCATGAGCTACGTGATGTGTACCGGCAGCTTCAAGCTGGAGAAGGAGGTGG
CCGAGACCCAGCACGGCACAGTGCTGGTGCAGGTGAAGTACGAGGGCACCGACGCCCCCTGT
AAGATCCCTTTCAGCACCCAGGATGAGAAGGGCGTGACACAGAACGGCAGACTGATCACCGC
CAACCCCATCGTGACCGACAAGGAGAAGCCCGTGAACATCGAGACCGAGCCCCCCTTCGGCG
AGAGCTACATCATTGTGGGAGCCGGCGAGAAGGCCCTGAAGCTGTCCTGGTTCAAGAAGGGC
AGCAGCATCGGCAAGATGTTCGAGGCCACCGCCAGAGGCGCCAGAAGAATGGCCATCCTGGG
CGATACCGCCTGGGACTTCGGCTCCATCGGCGGCGTGTTCACCTCTGTGGGCAAGCTGGTGCA
TCAGGTGTTCGGCACCGCCTACGGCGTGCTGTTCAGCGGAGTGAGCTGGACCATGAAGATCGG
CATCGGCATCCTGCTGACATGGCTGGGCCTGAATTCTAGAAGCGCCAGCCTGAGCATGACCTG
TATCGCTGTGGGCATGGTGACCCTGTACCTGGGCGTGATGGTGCAGGCCTGATGACTCGAG

Figure 13

DV2 opt prME (FGA02 French Guyana, Dussard):

GGATCCGCCACCATGAAATGTCTGCTGTACCTGGCCTTCCTGTTCATCGGCGTGAATTGTTTCC
ACCTGACCACCAGGAACGGCGAGCCCCACATGATCGTGAGCAGACAGGAGAAGGGCAAGAG
CCTGCTGTTCAAGACCGAGGACGGCGTGAACATGTGTACCCTGATGGCCATGGACCTGGGCG
AGCTGTGCGAGGACACCATCACCTACAAGTGTCCCTTCCTGAGGCAGAACGAGCCCGAGGAC
ATCGACTGTTGGTGTAACAGCACAAGCACCTGGGTGACCTACGGCACCTGCACCACCACCGGC
GAGCACAGAAGGGAGAAGAGGAGCGTGGCCCTGGTGCCCCACGTGGGCATGGGATTGGAAA

CCAGAACCGAGACCTGGATGAGCAGCGAGGGCGCTTGGAAGCATGCCCAGAGAATCGAGACC
TGGATTCTGAGACACCCCGGCTTCACCATCATGGCCGCCATCCTGGCCTACACCATCGGCACC
ACCCACTTCCAGAGAGCCCTGATCTTCATCCTGCTGACCGCCGTGGCCCCCAGCATGACCATG
AGATGCATCGGCATCAGCAACAGAGACTTCGTGGAGGGCGTGAGCGGCGGCAGCTGGGTGGA
CATCGTGCTGGAGCACGGCAGCTGTGTGACCACCATGGCCAAGAACAAGCCCACACTGGACT
TCGAGCTGATCAAGACCGAGGCCAAGCAGCCCGCCACCCTGAGAAAGTACTGTATCGAGGCC
AAGCTGACCAACACCACCACCGACAGCAGATGCCCCACCCAGGGCGAGCCCAGCCTCAATGA
GGAGCAGGACAAGAGATTCGTGTGTAAGCACAGCATGGTGGACAGAGGCTGGGGCAACGGCT
GTGGCCTGTTCGGCAAGGGCGGCATCGTGACCTGTGCCATGTTCACATGCAAGAAGAACATG
AAGGGCAAGGTGGTGCAGCCTGAGAACCTGGAGTACACCATCGTGATCACCCCTCACTCTGG
CGAGGAGCATGCCGTGGGCAACGACACCGGCAAGCACGGCAAGGAGATCAAGATCACCCCCC
AGAGCAGCATCACAGAGGCCGAGCTGACCGGCTACGGCACAGTGACCATGGAGTGTAGCCCT
AGAACCGGCCTGGATTTCAACGAGATGGTGCTGCTGCAAATGGAGAACAAGGCCTGGCTGGT
GCACAGACAATGGTTCCTGGATCTGCCTCTGCCCTGGCTGCCTGGCGCCGACACCCAGGGAAG
CAACTGGATTCAGAAGGAGACCCTGGTGACCTTCAAGAACCCCCACGCCAAGAAGCAGGACG
TGGTGGTGCTGGGCAGCCAGGAGGGCGCCATGCACACCGCCCTGACAGGCGCCACCGAGATC
CAGATGAGCAGCGGCAACCTGCTGTTCACCGGCCATTTGAAATGTAGACTGAGAATGGATAA
GCTGCAGCTGAAGGGCATGTCTTACAGCATGTGTACAGGCAAGTTCAAGGTGGTGAAGGAGA
TCGCCGAGACCCAGCACGGCACCATCGTGATCAGAGTGCAGTACGAGGGCGATGGCAGCCCC
TGTAAGATCCCCTTCGAGATCATGGATTTGGAGAAGAGACACGTGCTGGGCAGACTGATCACC
GTGAACCCCATCGTGACCGAGAAGGATAGCCCCGTGAACATCGAGGCCGAGCCCCCTTTCGG
CGACAGCTACATCATCATCGGCGTGGAGCCCGGCCAGCTGAAGCTGAACTGGTTCAAGAAGG
GCAGCAGCATCGGCCAGATGATCGAGACCACCATGAGAGGAGCCAAGCGGATGGCCATCCTG
GGCGACACCGCCTGGGACTTCGGCTCTCTGGGCGGCGTGTTCACCTCCATCGGCAAGGCCCTG
CACCAGGTGTTCGGCGCCATCTACGGCGCCGCCTTCTCCGGCGTGTCCTGGACCATGAAGATC
CTGATCGGCGTGATCATCACCTGGATCGGCATGAATTCCAGAAGCACCAGCCTGAGCGTGTCC
CTGGTGCTGGTCGGAGTGGTGACCCTGTACCTGGGCGTGATGGTGCAGGCCTGATGACTCGAG

Figure 14

DV3 opt prME (Thailande isolated from a French patient):

GGATCCGCCACCATGAAGTGTCTGCTGTACCTGGCCTTCCTGTTCATCGGCGTGAACTGCTTCC
ATCTGACCAGCAGAGACGGCGAGCCCAGAATGATCGTGGGCAAGAACGAGAGAGGCAAGAG
CCTGCTGTTCAAGACCGCCAGCGGCATCAACATGTGTACCCTGATCGCCATGGACCTGGGCGA
GATGTGTGACGACACCGTGACCTACAAGTGTCCCCACATCACCGAGGTGGAGCCCGAGGACA
TCGACTGTTGGTGTAACCTGACAAGCACCTGGGTGACCTACGGCACATGCAACCAGGCCGGC
GAGCACAGAAGGGACAAGAGAAGCGTGGCCCTGGCCCCCCACGTGGGCATGGGCCTGGATAC
CAGAACCCAGACCTGGATGAGCGCCGAGGGCGCTTGGAGACAGGTGGAGAAGGTGGAGACC
TGGGCCCTGAGACACCCCGGCTTCACCATCCTGGCCCTGTTCCTGGCCCATTACATCGGCACC
AGCCTGACCCAGAAGGTGGTGATCTTCATCCTGCTGATGCTGGTGACCCCCAGCATGACCATG
CGGTGTGTGGGCGTGGGCAACAGAGATTTCGTGGAGGGCCTGAGCGGCGCCACCTGGGTGGA
CGTGGTGCTGGAGCACGGCGGCTGTGTGACCACCATGGCCAAGAATAAGCCCACCCTGGATA
TCGAGCTGCAGAAGACCGAGGCCACCCAGCTGGCCACCCTGAGAAAGCTGTGCATCGAGGGC
AAGATCACCAACATCACCACCGACAGCCGGTGTCCTACCCAAGGCGAGGCCATCCTGCCCGA
GGAGCAGGACCAGAACTACGTGTGCAAGCACACATATGTGGATAGAGGCTGGGGCAACGGCT
GTGGCCTGTTCGGCAAGGGCAGCCTGGTGACCTGTGCCAAGTTCCAATGTCTGGAGTCTATCG
AGGGCAAGGTGGTGCAGCACGAGAACCTGAAGTACACCGTGATTATCACCGTGCACACCGGC
GACCAGCACCAGGTGGGCAACGAGACCCAGGGCGTGACCGCCGAGATCACCTCCCAGGCCAG
CACAGCCGAGGCCATCCTGCCCGAGTACGGCACCCTGGGCCTGGAGTGTTCCCCCAGGACCG
GCCTGGACTTCAATGAGATGATCCTGCTGACCATGAAGAACAAGGCTTGGATGGTGCACAGA
CAGTGGTTCTTCGACCTGCCCCTGCCCTGGACCAGCGGCGCCACCACCAAGACCCCCACATGG
AACAGAAAGGAGCTGCTGGTGACCTTCAAGAACGCCCACGCCAAGAAGCAGGAGGTGGTGGT
GCTGGGCAGCCAAGAGGGCGCCATGCACACCGCCCTGACCGGCGCCACCGAGATCCAGACCA
GCGGCGGCACATCTATCTTCGCCGGCCACTTGAAATGTAGACTGAAGATGGACAAGCTGAAG
CTGAAGGGCATGAGCTACGCCATGTGCCTGAACACCTTCGTGTTGAAAAAGGAGGTGAGCGA
GACCCAGCACGGCACCATCCTGATCAAGGTGGAGTACAAGGGCGAGGACGCCCCCTGTAAGA
TCCCTTTCAGCACCGAGGATGGCCAGGGCAAGGCCCACAACGGCAGACTGATCACCGCCAAC

CCCGTGGTGACCAAGAAGGAGGAGCCCGTGAACATCGAGGCCGAGCCCCCCTTCGGCGAGAG
CAACATCGTGATCGGCATCGGCGACAAGGCCCTGAAGATCAACTGGTACAGAAAGGGCAGCA
GCATCGGCAAGATGTTCGAGGCCACCGCCAGGGGCGCCAGAAGGATGGCTATCCTGGGCGAC
ACCGCCTGGGACTTCGGCAGCGTGGGCGGCGTGCTGAACAGCCTGGGCAAGATGGTGCACCA
GATCTTCGGCAGCGCCTACACAGCCCTGTTCTCCGGAGTGAGCTGGATCATGAAGATCGGAAT
CGGCGTGCTGCTGACCTGGATCGGACTGAATTCCAAAAACACCAGCATGAGCTTCAGCTGTAT
CGCCATCGGCATCATCACCCTGTACCTGGGAGTGGTGGTGCAGGCCTGATGACTCGAG

Figure 15

DV4 opt prME (Dengue hemorrhagic Birmanie, 60's):

GGATCCGCCACCATGAAGTGTCTGCTGTACCTGGCCTTCCTGTTCATCGGCGTGAACTGTTTCA
GCCTGAGCACCAGAGACGGCGAGCCCCTGATGATCGTGGCCAAGCACGAGAGAGGCAGACCC
CTGCTGTTCAAGACCACCGAGGGCATCAACAAGTGTACCCTGATTGCCATGGATCTGGGCGAG
ATGTGTGAGGACACCGTGACCTACAAGTGTCCCCTGCTGGTGAACACAGAGCCCGAGGACAT
CGACTGCTGGTGTAACCTGACCAGCACATGGGTGATGTACGGCACCTGTACCCAGAGCGGCG
AGAGGAGACGGGAGAAGAGATCCGTGGCCCTGACCCCTCACAGCGGCATGGGCTTGGAAACA
AGGGCTGAGACCTGGATGAGCAGCGAGGGCGCCTGGAAGCACGCCCAGAGAGTGGAGAGCT
GGATTCTGAGAAACCCTGGCTTCGCCCTGCTGGCCGGCTTCATGGCCTACATGATCGGCCAGA
CCGGCATCCAGAGGACCGTGTTCTTCGTGCTGATGATGCTGGTCGCCCCCAGCTACGGCATGA
GATGTGTGGGCGTGGGCAACCGGGACTTCGTGGAGGGCGTGAGCGGCGGCGCCTGGGTGGAC
CTGGTGCTGGAGCACGGCGGCTGTGTGACCACCATGGCCCAGGGCAAGCCTACACTGGACTTC
GAGCTGACCAAGACCACCGCCAAGGAGGTGGCCCTGCTGAGAACCTACTGTATCGAGGCCAG
CATCAGCAACATCACCACCGCCACCAGGTGTCCCACCCAAGGCGAGCCTTATTTGAAAGAAG
AGCAGGACCAGCAGTACATTTGTAGAAGACGTGGTGGACAGAGGCTGGGGCAACGGCTGT
GGCCTGTTCGGCAAGGGCGGCGTGGTGACCTGCGCCAAGTTCAGCTGTAGCGGCAAGATCAC
CGGCAACCTGGTGCAGATCGAGAACCTGGAGTACACCGTGGTGGTGACCGTGCACAACGGCG
ATACCCACGCCGTGGGCAACGATACCAGCAACCACGGCGTGACCGCCATGATCACCCCCAGA
AGCCCCAGCGTGGAGGTGAAGCTGCCCGACTACGGCGAGCTGACCCTGGACTGCGAGCCCAG
AAGCGGCATCGACTTCAACGAGATGATTTTGATGAAAATGAAAAAGAAGACCTGGCTGGTGC
ACAAGCAATGGTTCCTGGACCTGCCCCTGCCCTGGACCGCCGGAGCCGACACCAGCGAGGTG
CACTGGAATTACAAGGAGAGAATGGTGACCTTCAAGGTGCCCCACGCCAAGAGACAGGACGT
GACCGTGCTGGGCAGCCAGGAGGGCGCCATGCACAGCGCCCTGGCCGGCGCCACCGAGGTGG
ATAGCGGCGACGGCAACCATATGTTCGCCGGCCACTTGAAATGCAAAGTGAGGATGGAGAAG
CTGAGAATCAAGGGCATGTCCTACACAATGTGTAGCGGCAAGTTCAGCATCGATAAGGAGAT
GGCCGAGACCCAGCATGGCACCACCGTGGTGAAGGTGAAGTACGAGGGCGCCGGCGCTCCCT
GCAAGGTGCCCATTGAGATCAGAGACGTGAACAAGGAGAAGGTGGTGGGCAGAATCATCAGC
TCTACCCCCCTGGCCGAGAACACCAATAGCGTGACCAACATCGAGCTGGAGCCTCCCTTCGGA
GACAGCTACATCGTGATCGGCGTGGGCAACAGCGCCCTGACCCTGCACTGGTTCAGAAAGGG
CAGCTCTATCGGCAAGATGTTCGAGTCCACATATAGAGGCGCCAAGAGAATGGCCATCCTGG
GCGAGACCGCCTGGGATTTCGGCAGCGTGGGCGGCCTGTTCACCAGCCTGGGCAAGGCCGTG
CACCAGGTGTTCGGCTCCGTGTACACCACCATGTTCGGCGGCGTGAGCTGGATGATCCGGATT
CTGATCGGCTTCCTGGTGCTGTGGATCGGCACGAATTCCAGAAACACCAGCATGGCCATGACC
TGTATCGCCGTGGGCGGAATCACCCTGTTCCTGGGATTCACCGTGCAGGCCTGATGACTCGAG

Figure 16

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2010/051072

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/12 C07K14/18 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)
A61K C07K 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, CHEM ABS Data, WPI Data, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 03/102166 A2 (MAXYGEN INC) 11 December 2003 (2003-12-11) paragraphs [0080], [0108], [0116], [0216], [0232], [0236], [0249], [0291], [0294] paragraph [0307]; sequences 215-218 paragraphs [0332], [345], [533]	1-13, 16-18,21 14,15, 19,20
Y	WO 2007/038425 A2 (UNIV MINNESOTA [US]; FERGUSON DAVID M [US]; GOODELL JOHN [US]) 5 April 2007 (2007-04-05) page 20, line 18 - page 21, line 3	14,15, 19,20
X	WO 2004/076664 A2 (UNIV SOUTH FLORIDA) 10 September 2004 (2004-09-10) page 2, line 24 - line 29	18,21

-/--

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

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 June 2010	19/07/2010

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	Mandl, Birgit

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2010/051072

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STEIN DAVID A ET AL: "Nucleic acid-based inhibition of flavivirus infections." FRONTIERS IN BIOSCIENCE, vol. 13, 2008, pages 1385-1395, XP002589203 ISSN: 1093-4715 page 1388, right-hand column, last paragraph - page 1390, left-hand column, paragraph 1	18,21
X,P	WANG PEI-GANG ET AL: "Efficient assembly and secretion of recombinant subviral particles of the four dengue serotypes using native prM and E proteins." PLOS ONE, vol. 4, no. 12, 2009, page E8325, XP002588733 ISSN: 1932-6203 the whole document	1-21
A	KONISHI E ET AL: "Dengue type 2 virus subviral extracellular particles produced by a stably transfected mammalian cell line and their evaluation for a subunit vaccine" VACCINE, vol. 20, no. 7-8, 15 January 2002 (2002-01-15), pages 1058-1067, XP004332976 ISSN: 0264-410X cited in the application * abstract	1-21

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 18, 21(all partially)

Present claims 18 and 21 encompass compounds defined only by their desired function, contrary to the requirements of clarity of Article 6 PCT, because the result-to-be-achieved type of definition does not allow the scope of the claim to be ascertained. The fact that any compound could be screened does not overcome this objection, as the skilled person would not have knowledge beforehand as to whether it would fall within the scope claimed, except for the compounds disclosed in the description, namely not further described siRNAs. Undue experimentation would be required to screen compounds randomly. This non-compliance with the substantive provisions is to such an extent, that the search was performed taking into consideration the non-compliance in determining the extent of the search for claims 18 and 21. The search of claims 18 and 21 was consequently restricted to siRNAs.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.2), should the problems which led to the Article 17(2) declaration be overcome.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2010/051072

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

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

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

2. ☒ Claims Nos.: 18, 21(all partially)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

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

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

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

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

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

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

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

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2010/051072

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 03102166 A2	11-12-2003	AU 2003267943 A1	19-12-2003
		CA 2481479 A1	11-12-2003
		CN 1909922 A	07-02-2007
		EP 1572941 A2	14-09-2005
		NZ 535690 A	30-04-2009
WO 2007038425 A2	05-04-2007	NONE	
WO 2004076664 A2	10-09-2004	NONE	