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Procedencia PAPO TUTATIVA DE NUEVAS CONFERENCIAS

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FORMULARIO UNICO DE SOLICITUD DE PATENTE
PCT
ENTRADA EN FASE NACIONAL

(1) SOLICITUD DE

[X] Patente de Invención [ ] Patente de Modelo de Utilidad

[ ] Capitulo I [X] Capitulo II

(2) SOLICITANTE (71)

Nombre: FUNDAÇÃO OSWALDO CRUZ FIOCRUZ
Dirección: A BRAS L 365 -CASTELO MOURISCO- 5º ANDAR MANGUNHOS O E JANEIRO (BR)
Nacionalidad o Domicilio: BRASIL
L ga de Constitución: RIO DE JANEIRO
Teléfono: Fax:
E ma l:

IDENTIFICACIÓN
C C [ ] NIT [ ]
C E [ ] Otro [ ]
Cual: ____
Número: ____

(3) REPRESENTANTE O APODERADO

Nombre: LAURA MICHELSEN NIÑO
Dirección: CALLE 93B N 12 48 OF 204
Teléfono: 601 9660 Fax: 611 4209
E mail: turn@turn et com

IDENTIFICACIÓN
C C [X] NIT [ ]
C.E. [ ] Otro [ ]
Cual: ____
Número: 39 783,287 T P 67 897

(4) INVENTOR (ES) (72)

Nombre: BONALDO MIRNA C GALLER RICARDO FREIRE MARCOS DA SILVA GARRAT RICHARD C
Dirección: A BRASIL 4365- CASTELO MOURISCO-5º ANDAR MANGUNHOS RIO DE JANEIRO
Nacionalidad o Domicilio: RIO DE JANEIRO BRASIL

(5) PCT (86)

Solicitud Internacional No: PCT/BR02/00036 Fecha: MARZO 8 DE 2002
Publicación Internacional No: WO 02/072835 Fecha: SEPT 19 DE 2002

(6) Título (54): USO DE FLAVIVIRUS PARA LA EXPRESION EPITOPOS DE PROTEINA Y DESARROLLO DE NUEVOS VIRUS VIVOS ATENUADOS VACUNANTES PARA INMUNIZAR CONTRA FLAVIVIRUS Y OTROS AGENTES INFECCIOSOS

(7) Cla ificación Internacional (51): C12N 15/40 15/86 A61K 39/00 39/015

(8) Prioridad

SI [X] NO [ ]

(33) País de Origen	(32) Fecha	(31) Número de Solicitud
GRAN BRETAÑA	09/03/2001	0105877 5

(9) C mprobant d p go N 03 631 Fech 3/01/2003

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NIT : 800176089-

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Dependencia: 2020 DIVISION DE NUEVAS CREACIONES

RECIBO OFICIAL DE CAJA : 03 - 631
FECHA : ENERO 3 DE 2003

***** C O N S I G N A C I O N *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
TRIANA URIBE & NICHELSEN	CONSIGNACION	BANCO POPULAR	050-03113-6	9022171	03/01/2003	4,480,080.00

***** C O N C E P T O *****

CANT.	PENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-01 SOLICITUDES	1 TRAMITES DE SOL. DE PATENTE DE IN	400,000.00
		TOTAL : $	400,000.00

SON: CUATROCIENTOS MIL PESOS

RESPONSABLE : _____

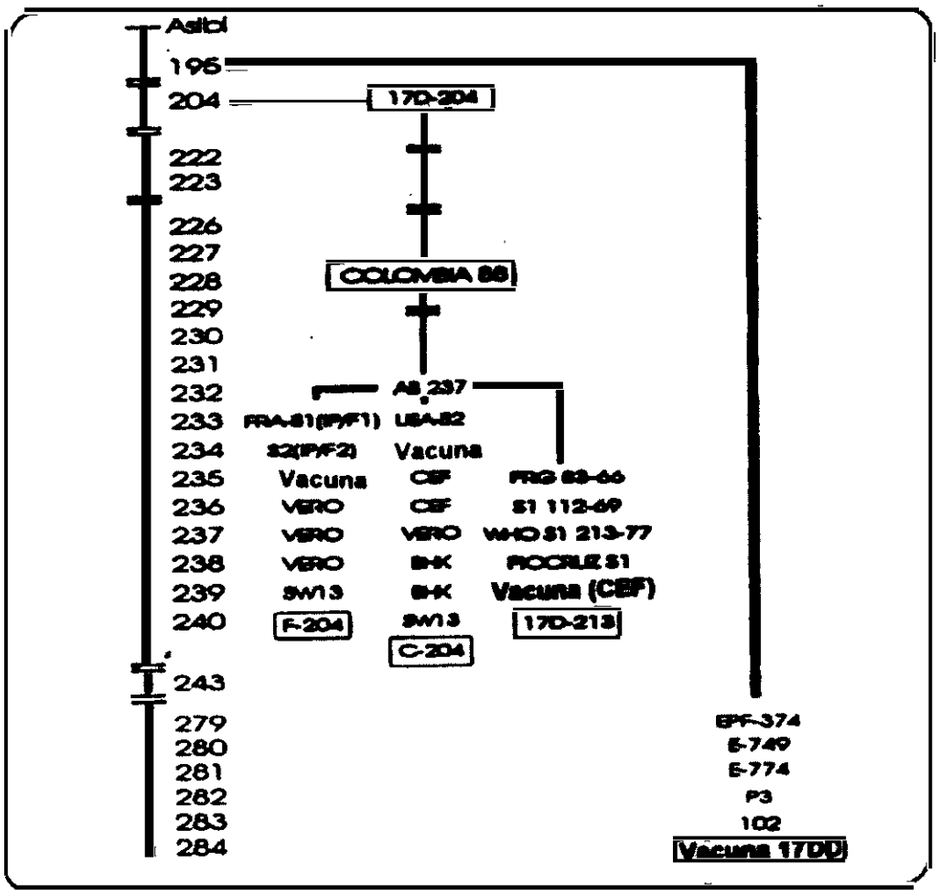
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Carrera 13 No. 27-00 Pisos 5, 7 y 10
Conmutador 382 0840
Fax 350 5220
E-mail: info@sic.gov.co
www.sic.gov.co
Bogotá, D.C., Colombia

(10) **ANEXOS**

- [X] Comprobante de pago de la tasa de representación de la solicitud.
- [X] Documento que acredite la existencia y representación legal cuando el solicitante sea persona jurídica.
- [X] Poderes, si fuere el caso.
- [ ] Documentos de cesión del inventor al solicitante o a su causante.
- [ ] Declaraciones.
- [X] Copia de la solicitud internacional. (Resumen, descripción, reivindicaciones, dibujos).
- [X] Traducción de la solicitud internacional al castellano. (Resumen, descripción, reivindicaciones, dibujos).
- [X] Copia del examen preliminar internacional efectuado por la Autoridad Internacional de Examen Preliminar (IPEA)
- [ ] Traduccion del examen preliminar internacional efectuado por la IPEA.
- [ ] De se el caso, copia del contrato de acceso.
- [ ] De ser el caso, documento que acredite la licencia o autorización de uso de conocimientos tradicionales de las comunidades indigenas.
- [ ] De se el caso, certificado de depósito del material biológico.
- [X] Arte final 12x12.
- [ ] De ser el caso, información sobre otras solicitudes de patente o títulos obtenidos en el extranjero por el titular o su causante, relacionadas parcial o totalmente con la invención de esta solicitud.
- [ ] De ser el caso, modificaciones efectuadas a la solicitud internacional.
- [ ] Otros.

(11) FIGURA CARACTERÍSTICA:



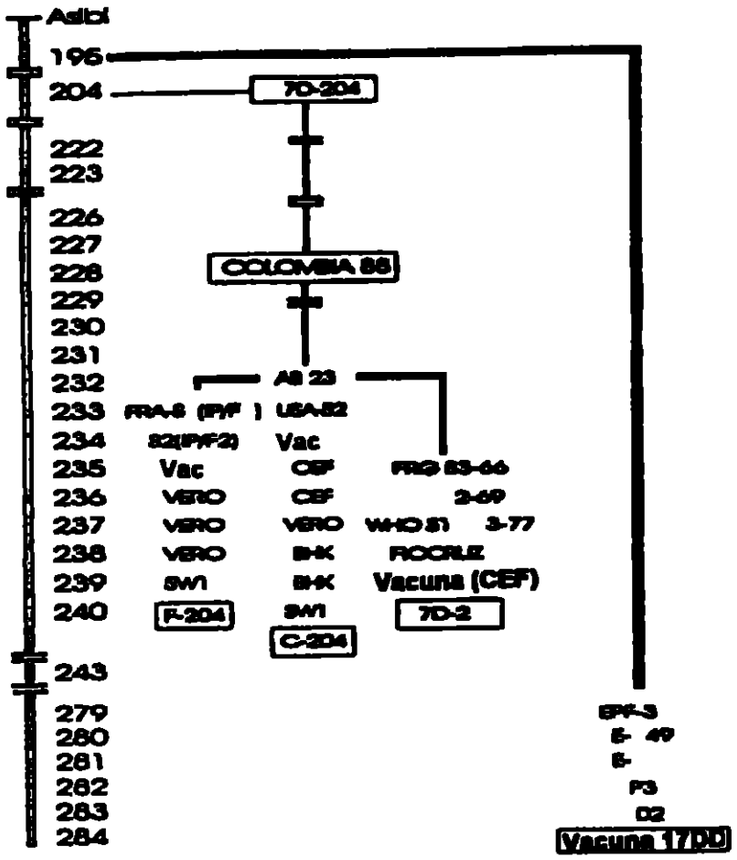
(12) Solicito la concesión de la patente

NOMBRE LAURA MICHELSEN NIÑO

FIRMA. _____

C.C. 39.783.287 T.P. 67.897

Instrucciones para la presentación del os anexos, ver reverso de esta pagina.


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REPUBLICA DE COLOMBIA

NOTARIA CUARENTA Y UNA

DEL CIRCULO DE SANTAFE DE BOGOTA D C

Carrera 12 No 93-26
Tels 621 4451/61/71/81/91 2186087 256 7730 Fax 218 6082

PRIMERA Copia de la Escritura Pública No 1785-

de fecha 13 de JULIO de 19 98

ACTO

PROTOCOLIZACION

OTORGANTES

LAURA MICHELSEN NIÑO

05 DIC 2002

AGUSTIN MORALES RIVEIRA
NOTARIO
Miembro del Colegio de Notarios de Colombia

EL KOLOR GRAFICAS LTDA 888

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AA 3125362

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ESCRITURA PUBLICA NUMERO (1785) MIL

SETECIENTOS OCHENTA Y CINCO

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(41) DEL CIRCULO DE SANTAFE DE BOGOTA

DISTRITO CAPITAL

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JULIO

DE MIL NOVECIENTOS NOVENTA Y OCHO (1998)

CLASE DE ACTO PROTOCOLIZACION

PERSONAS QUE INTERVIENEN EN EL ACTO LAURA MICHELSEN NI O

Dentro del Ci lo Not i l d l D t ito C pit l de S t fé

de Bogotá Dep t me to de C di m República d

Colombi e donde queda ubicada la Notaría Cu t y Un

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AGUSTIN MORALES RIVEIRA

Comp ecie on o mi Ute escrii LAURA MICHELSEN NINO

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ADRIAN ESTHER BAUTISTA
SECRETARIA
DECRE O 53 DE 1985
NOTARIA 41 DE SANTAFE DE BOGOTA

Mi t io de R l ione E t io

(HASTA AQUI LA MINUTA ESCRITA PRESENTADA POR EL(LA)(LOS) COMPARECIENTE(S)

ADVERTENCIAS

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AUXILIAR NOTARIA 41 DE SANTAFE DE BOGOTA D.C.

05 DIC 2002

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LA PRESENTE ESCRITURA PUBLICA SE EXTENDIO EN LAS HOJAS DE PAPEL NOTARIAL NUMEROS AA 3125362- 363

EN CONSTANCIA SE FIRMA COMO APARECE EN LA HOJA DE PAPEL NOTARIAL NUMERO AA 3129679

DERECHOS NOTARIALES $ 6 900 RECAUDO PARA LA SUPERINTENDENCIA DE NOTARIADO Y REGISTRO $1 710; y PARA EL FONDO NACIONAL DE NOTARIADO $1.710 RESOLUCION 00 7 DEL 15 DE ENERO DE 1998

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ADRIANA ESTHER DAUNSTA G
NOTARIA 41 DE SANTAFE DE BOGOTA D.C.
SECRET

05 DIC 2002

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TRADUCCION OFICIAL 286-98 DE UN DOCUMENTO ESCRITO EN IDIOMA INGLES

N de la T Se traducen las autenticaciones adjuntas a un poder escrito en idiomas Inglés y Español otorgado en la ciudad Rio de Janeiro Brasil el 23 de Abril de 1998 a favor de los doctores FERNANDO TRIANA LAURA MICHELSEN, JUAN CARLOS URIBE y JUAN PABLO TRIANA por Eloi de Souza Garcia en nombre y representación Fundaçao Oswaldo Cruz sociedad domiciliada en Avenida Brasil 4365 Castelo Mourisco 5 andar Mangunhos Rio de Janeiro

N del T La firma de Eloi de Souza Garcia fue autenticada ante André Luis Perira Pinto el 15 de Mayo de 1998

N del T La firma de André Luis Perira Pinto en el documento antes traducido fue legalizado ante el Consulado de Colombia en Rio de Janeiro el 18 de Junio de 1998 No 44/98 Pagado el impuesto de timbre y derechos consulares por US$100 oo La firma del Cónsul Francisco Jordan Peñaranda fue reconocida por el Ministerio de Relaciones Exteriores de Bogotá el 1o de Julio de 1998 mediante Sello No 250917 suscrito por el Jefe de Legalizaciones

ADRIANA E T EC ETAPA E O ESA DE LARA NOTARIA 41 DE SANTAFE DE BOGOTA LE oficial protocal

MINISTERIO DE RELACIONES EXTERIORES

ES TRADUCCION FIEL Y COMPLETA DEL DOCUMENTO ANTES DESCRITO QUE PARA IDENTIFICACION SE SELLA POR LA SUSCRITA TRADUCTORA OFICIAL ESTE 3 DE JULIO DE 1998

PILAR GAMBOA MORALES
TRADUCTORA E INTERPRETE OFICIAL
RESOLUCION No. 1937 DEL
MINISTERIO DE JUSTICIA

Pilar Gamboa Morales
Resolución 1937 de 1991
Ministerio de Justicia
C C 39 682 927 de Usaquén

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EFE DE LEGALIZACIONES S SAN FEDO D.C.

NO SE ASUME RESPONSABILIDAD DEL TEXTO

05 DIC 2002

A 9

TRIANA URIBE & MICHELSEN

POWER OF ATTORNEY

I (we), the undersigned ELOI DE SOUZA GARCIA
representation FUNDAÇÃO OSWALDO CRUZ
domiciled in Av. Brasil, 4365 Castelo Mourisco 59 andar Manguinhos / Rio de Janeiro
due compliance with the terms of paragraph 1st. of art. 1 543 of the Commercial Code do hereby appoint **FERNANDO TRIANA**, tpa. N 45.265 c.c. N 79 154 036 **LAURA MICHELSEN** tpa. N 67 897 .c No. 39 783.287 **JUAN CARLOS URIBE**, tpa. N 54.493 N 79 159 313 and **JUAN PABLO TRIANA**, tpa. 63 077 c.c. N 79.487.222 domiciled at Calle 93 B N 12-48 (204), Santafé de Bogotá, Colombia, the first as principal and the others as alternates, the third of the appointment, as our representatives for all industrial property matters with facility to receive notifications and to appoint attorney and out of Court. The first alternate shall replace the principal case of absence of temporal or definitive impediment. The same rule will apply with the second third alternate representatives as to replace the first or second one, or when the second third to replace the principal one. In such cases, the principal representative, hall state his tentative not to act as representatives through a declaration given before a Notary Public Colombia, or before Notary or nother Public Official or a Colombian Consul abroad and in case of impediment, the appropriate certificate shall office. If any of the appointees were to be definitely absent, the following alternate shall take his place. When the absence or impediment of the principal representative, or of the first or second third alternate in their turn cases, the representation can be taken again the sequence, by the principal the first or second or third alternated pending case, and the mere fact its exercise will office. The exercise of the representation by the first second or third alternates the turn shall end to the m

ADRIAN E LI SECRETARIA NOTARIA 41 DE SANTAFE DE BOGOTA DECRETO 5

In addition, we grant Power of Attorney favor of the above named attorneys-at law so that they may obtain the protection of my (our) industrial property and against the fair competition, for which purpose I (we) authorize the said attorneys to represent me ( ) before any authorities or person with without jurisdiction, peculiarly those administrative, administrative contentious and judicial matters concerning the following) Obtainment of patents models and designs the registration of marks and trade logans the deposit of commercial names and trade emblem the obtainment of the declaration and authorization of denomination of rigits renewal assignment, change from or domicil voluntary cancellation and the registration of licenses fuse of the above rights, b) Obtainment of plant variety protection) Obtainment of functioning licenses health registrations and other petitions before the sanitary or administrative authorities d) Obtainment of copyright registrations for any type of artistic, literary scientific, musical deco, films, video games choreography works, software or any other production in the artistic, scientific or literary fields capable of being reproduced expressed by any means known now or in the future Registration of agreements related with the execution transfer of intellectual property rights, Deposit of names of periodical publication and television programs Obtention of reduced postal tariff) Action of opposition cancellation nullity of agreement, writ of mandamus, granting of licenses and in general the penal and administrative rights relating to all those rights, actions and rights instituted by me ( ) against me ( ) And that all the above matters they may substitute this Power of Attorney compromise, conciliate, admit the facts of the case, desist, cancel receive, resign, and ratify acts done by representatives without Power of Attorney

Given and signed in April 23, 1998

RIO DE JANEIRO, BRAZIL

Signature

Dr. ELOI DE SOUZA GARCIA
Presidente da FIOCRUZ

Cra 31 N 12-48 (204) Bogotá Colombia
Tels (57 1) 611 4292 621 5810 621 7360 Fax (57 1) 611 4209
E-mail tmc@colomsat.net.co

FILAR GAMBOA MORALES

ESCRITÓRIO DE REPRESENTAÇAO NO RIO DE JANEIRO
SEÇÃO CONSULAR RJ

de Andre Luis Pereira Pinto

Adriana Vargas Mar

ANDRÉ LUÍS PEREIRA PINTO
3 SUBSTITUTO
11 CIRC R C PN e TAB

PODER

Y (osotros) baj su crito(s) ELOI DE SOUZA GARCIA
representac ón d FUNDACÃO OSWALDO CRUZ
dom ciliado( ) en Av. Brasil, 4365 Castelo Mourisco 50 andar- Manguinhos/Rio d Janei
por l presente mbro (nombramos) **FERNANDO TRIANA**, tpa N 45.265 .c N 79 154 036 **LAURA MICHELSEN** tpa. N 67 897 N 39 783 287 **JUAN CARLOS URIBE**, tpa. N 54 493 N 79 159 313 y **JUAN PABLO TRIANA** tp 63 077 .c N 79 487.222 domicil ados l C ll 93 B N 12-48 (204), Santafé d B gotá, Col mbia, en bedecim to lo dispuesto en l parágrafo 1ro d l Artic l 543 del Código de Comercio l pr mero com principal y los demás como plentes, en su ord como mi (stros) representantes en todos los asuntos de propiedad industrial co fac ltad para rec bi ot ficac o es y nombrar apoderados judici les o extrajudiciales. El primer represe ta te pl te reemplazará l pr pal en caso de ausen i mped m to temporal defi tivo d aquel La m m regl se pli ará nd l seg d y tercer represe tantes suplentes hayan d reemplazar l p m ro l segu d l pri pal En tales casos, l representante principal l pr mero, seg do plente, tercer pl te en caso, todos llos declararán l tención de no jercer l representac ó med te decl rac ó te t cad po Notari Públ co en C lombia, o pór Notario otro F nari Públ co, Cóns l d Colomb a, en l extranjero y i se tratare de impedimento, con el ert ficado respectivo S alguno d los nombrados faltare defi iti amente, el iguiente tomará su l gar Cuando cesare la sencia imped mento d l represe tante principal, de su primero segundo tercer pl te, su caso podrán jercer l representació en orden, uno otros según l caso, as m éndol por l sol hecho d ejerceri cesand ese mom to l ej rci i de l representació por l p mero seg nd terce supl te aso

Ad má co f ero (co ferimos) poder los bogados arrib nombrados, para btene l protección de m (ñ stra) prop edad d str l y contra l compete ia desl l yo fecto to zo(amos) los d chos poderados para q m (nos) representen te ualesq ra ent dades perso as, jerzan no j sd cc ó espe lmente ante las d l rd adm trativo, contenc oso-adm strat vo y jud l, en tod l concern ente lo g ientes asu tos) Obtenc ón d patentes de l nción, modelos y dib jos ndust l l reg stro d marcas y de lemas comerci l l d pós to de nombres comerci les y enseñas l bten ó d l decl rac ó y d l to zac ón de den minac on ge orígе renovación traspaso, camb de mbre domi li ca lac ó l ta y l registro d l ce las de de esos derechos b) Obte ón d l protecc ó para iedades vegetales c) Obte ón y tram tación de todo l rel ad co l cenc as de f nc m to, reg tros san tar os y cualq er tra petición ante las t dades sa tar as adm strat as d) Obten ó d l reg tro de derechos de autor para lq er t po d bra artíst ca, l terarıa, tíf ca, m cal deográf ca, nematográf ca, coreográficas, lq er otra producción en el campo arti t co ci tíf co o l terario capaz de ser reproducido o expresado po alq er medio conoc d ahora por conocer en l futuro Registro de contratos relac onados con la jecución transfere la e derechos telectuales Depósito de nombres de publ cac es per ód cas y de programas d telev ó Obtenc ó de tarifa postal reducida,) Las acc es de pos ó d cancelac ó d l dad de med das ca telares, de tutela, d concesió de l cen as y en ge eral los procesos les, pen les, adm trat vos o contenc oso-adm istrat vos relac ado co todos estos derechos acc nes y procesos q promovamos se nos promuev Y para q todos los to arr b determ ados puedan sust tuí ste m ndato transig concil adm t l hech d l proceso des t cancel rec b ren nciar y rat f ca los actos de agentes f sos

Dad y f rm d h y 23 DE ABRIL DE 1998

RIO DE JANEIRO, BRAZIL

F rm

Dr. ELOI DE SOUZA GARCIA
Pro OC

PILAR GAMBOA MORALES

CONSULADO GENERAL DE COLOMBIA

CONSULADO DE COLOMBIA
No 44/98.

El Suscrito Cónsul General de Colombia en Rio de Janeiro visto los documentos presentados por los interesados CERTIFICA. Que el Señor ANDRE LUIS PEREIRA PINTO quien como Escribiente Sustituto de la Notaría 11o de Rio de Janeiro Brasil autenticó la firma del signatario d l poder precedente ejercía en la fecha las funciones indicadas y su firma corresponde a la registrada en este Consulado

Que la Compañía FUNDAÇÃO OSWALDO CRUZ es una sociedad constituida de acuerdo con las leyes del Brasil y cumple su objetivo conforme a las mismas

Que el Señor ELÓY DE SOUZA GARCIA quien en su caracter de PRESIDENTE de la citada Compañía confiere el poder precedente y cuya firma fué autenticada ante la Notaría 11o de Rio de Janeiro Brasil es su representante legal en este país

El Cónsul no asume responsabilidad por el contenido de este documento

Rio de Janeiro 18 de junio de 1998

CONSULADO GENERAL DE COLOMBIA RIO DE JANEIRO

FRANCISCO JORDAN PEÑARANDA
Cónsul General de Colombia

PAGADO IMPUESTO DE TIMBRE
US$ 6,00 (SEIS DÓLARES)
PAGADO DERECHOS CONSULARES
US$ 100 00 (CIEN DÓLARES)

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05 DIC 2002

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AA 3129679

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HOJA NUMERO TRES

DE LA ESCRITURA PUBLICA NUMERO 1785

DEL 13 DE JULIO DE 1998

VIENE DE LA HOJA DE PAPEL NOTARIAL

NUMERO AA 3125363

L ig 13 JUL. 1998 p i o

i di (Art. 9 Dcto 2148/83)

LAURA MICHELSEN NIÑO

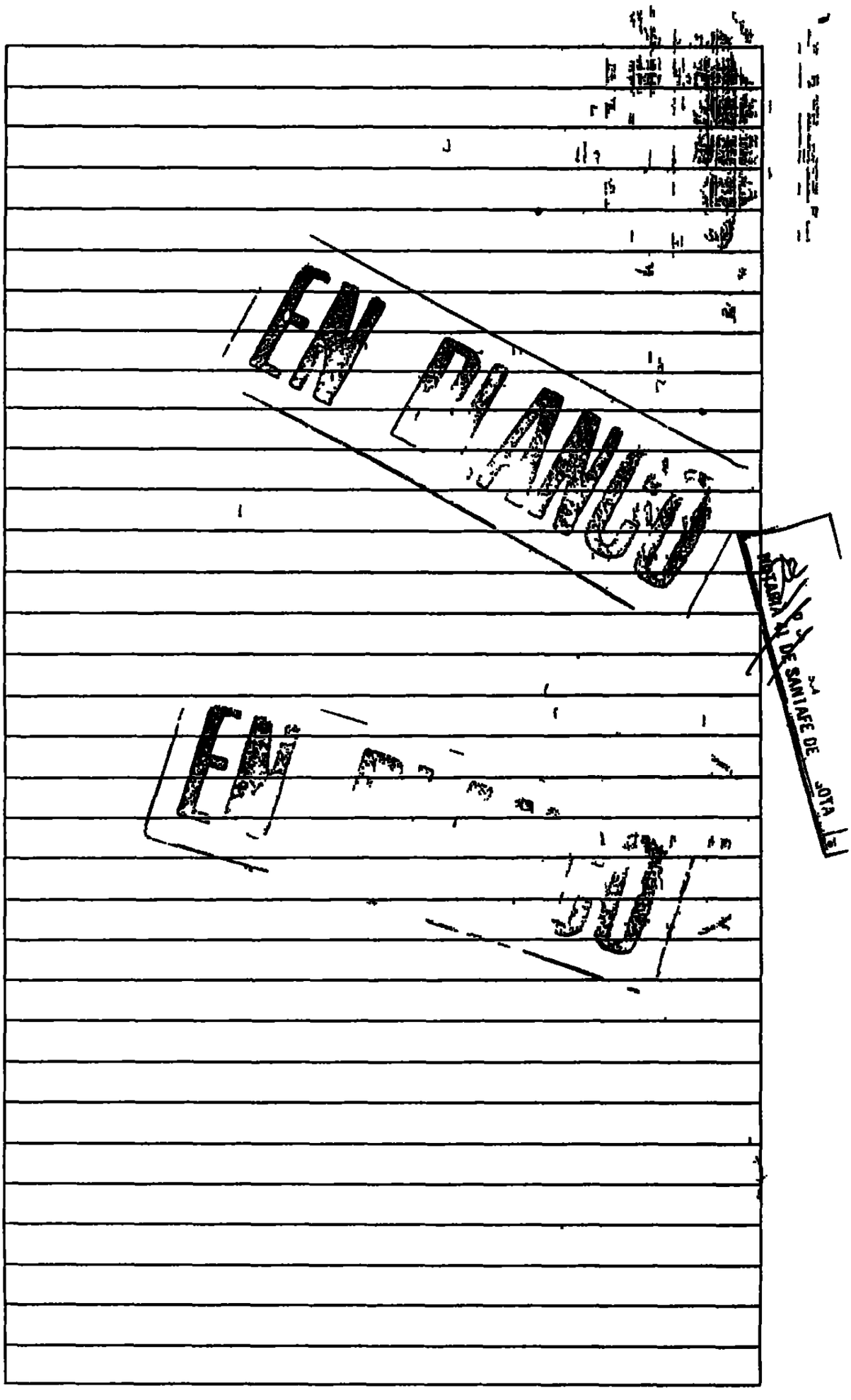
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EL NOTARIO

NOTARIA 41 DE SANTAFE DE BOGOTA

05 DIC 2002

ESTAPELNO EN COSTO LGUNO ARA EL USUARIO


NOTARIA 41 DE SANTAFE DE BOTA

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HOJA NUMERO - SIETE (07) (ULTIMA)

ES PRIMERA- (01) COPIA (FOTOCOPIA) TOMADA DE SU ORIGINAL CONFORME AL ARTICULO 79 DEL DECRETO 960 DE 1970

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DERECHOS RESOLUCION N 0037 DEL 15 DE ENERO DE 1998

SANTAFE DE BOGOTA D C JULIO 14 DE 1998

NOTARIA 41 DE SANTAFE DE BOGOTA DECRETO 960 DE 1970

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LA SECRETARIA

REPUBLICA DE COLOMBIA H B DECRETO 960 DE 1970 NOTARIA 41 DE SANTAFE DE BOGOTA

NOTARIA 41 DEL CIRCULO DE SANTAFE DE BOGOTA D C

05 JUL 2002


EN BLANCO
NOTARIA 41 DE SP
EN BLANCO

The Patent Office

INVESTOR IN PEOPLE

13

The Pate t Office
Concept House
Cardiff Road
Newport
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	Patents ADP number *(if you know it)*	8099152001
	If the applicant is a corporate body, give the country/state of its incorporation	Brazil
4	Title of the invention	USE OF FLAVIVIRUS FOR THE EXPRESSION OF PROTEIN EPITOPES AND DEVELOPMENT OF NEW LIVE ATTENUATED VACCINE VIRUS TO IMMUNIZE AGAINST FLAVIVIRUS AND OTHER INFECTIOUS AGENTS
5	Name of your agent *(if you have one)*	J A KEMP & CO
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USE OF FLAVIVIRUS FOR THE EXPRESSION OF PROTEIN EPITOPES AND DEVELOPMENT OF NEW LIVE ATTENUATED VACCINE VIRUS TO IMMUNIZE AGAINST FLAVIVIRUS AND OTHER INFECTIOUS AGENTS

The present invention relate to vaccine against infections caused by flavivirus M re particularly to the use of the YF vaccine virus (17D) to express at the level f ts envelope protein epitopes from other pathogens which will elicit specifi immune response to the parental pathogen. There are provided new plasmids which were deposited on December 21 2000 under the following accesion umber with the American Type Culture Collection (ATCC) 10801 University Blvd. Manassas, Va. 20110-2209 ( ) pYFE200/13 accession umber PTA 2854 ( ) pYF17D/8 accession number PTA 2855 (ii) pYFE200 accession umber PTA 2856 ( ) pYFE200/8 accession umber PTA 2857 and ( ) pYFE200/1 accession umber PTA 2858

BACKGROUND OF THE INVENTION

Flavivirus consists f 70 serologically cross-reactive, closely related human or veterinary pathogens causing many serious illnesses, which includes dengue fever Japanese encephalitis (JE) tick borne encephalitis (TBE) and yellow fever (YF) The Flaviviruses are spherical viruses with 40-60 nm in diameter with an cosahedral capsid which contains single positive-stranded RNA m lecule

YF virus the prototype virus f the family of the Flaviviruses with RNA genom f 10 862 nucleotides (nt) having 5 CAP structure and short 5 end nontranslated region (118 nt) and nonpolyadenylated ontranslated 3 end (511 nt) The first complete ucleotide sequence f flavivirus genome was determined on the genome of the YF 17D 204 vaccine strain virus by Rice et al (Rice C M Lenches E Eddy S R. Shin, S.J Sheets, R.L and Strauss, J H 1985 "Nucleotide equence of yellow fever virus implications for flavivirus gene expression and evolution Science 229 726-733)

The singl RNA also the viral messag and its translation in the infected cell results in the synthesis of polyprotein precursor f 3 411 amino acids which is cleaved by proteolyti processing to generate 10 virus-specifi polypeptides From the 5 terminus, the order of the encoded proteins s C prM/M E NS1 NS2A, NS2B NS3 NS4A NS4B and

NS5 The first 3 proteins constitute the structural proteins that is, form the virus together with the packaged RNA molecul and were named capsid (C 12-14kDa) membrane (M, and ts precursor prM, 18 22 kDa) and envelope (E,52 54 kDa) all being encoded in the first quarter of th genome The remainder of the genome codes for the nostructural proteins (NS) numbered in the order f synthesis from 1 through 5

Three large nonstructural proteins have highly conserved sequences among flaviruses, NS1 (38-41 kDa) NS3 (68 70 kDa) and NS5 (100-103 kDa) A rol in the replication of the negative strand RNA has been assigned to NS1 (Muylaert IR, Chambers TJ Galler R, Rice CM 1996 Mutagenesis of N linked glycosylation sites of YF virus NS1 effects on RNA accumulation and mouse neurovirulence *Virol gy* 222 159-168 Muylaert IR, Galler R, Rice CM 1997 Genetı analysis f Yellow Fever virus NS1 protein dentification of temperature-sensitive mutation which blocks RNA accumulation. *J Virol* 71 291 298 Lindenbach BD Rice CM 1999 Genetı interaction of flavivirus nonstructural proteins NS1 and NS4A as determinant f replicase function. *J Virol* 73 4611-4621 Lindenbach BD Rice CM 1997 trans-complementation of yellow fever virus NS1 reveals a role in early RNA replication. *J Virol* 71 9608 9617)

NS3 has been shown to be bifunctional with protease activity needed fo the processing of the polyprotein at sites the cellular proteases will not (Chambers TJ Weir RC Grakoui A, McCourt DW Bazan JF Fletterick RJ Rice CM 1990b Evidence that the N terminal domain f nonstructural protein NS3 from yellow fever virus is a serine protease responsibl for site-specific cleavages in the viral polyprotein. *Proc Natl.Acad.S USA* 87 8898-8902 Falgout B Pethel M, Zhang YM, Lai CJ 1991 Both nonstructural proteins NS2B and NS3 are required for the proteolyti processing of dengue virus nonstructural proteins *J Virol* 65 2467 2475 Yamshchiko VF Compans RW 1995 Formation of the flavivirus envelope rol f the viral NS2B-NS3 protease. *J Virol.* 69 1995 2003 Yamshchikov VF Trent, DW Compans RW 1997 Upregulation of signalase processing and induction of prM E secretion by the fl vivirus NS2B NS3 protease roles f protease components *J Virol* 71 4364-4371 Stocks CE Lobigs M 1998 Signal peptidase cleavage at the flavivirus C prM junction dependence on the viral NS2B 3 protease for efficient processing requires determinants in C the signal peptide and prM *J Virol* 72 2141 2149) It also contains ucleotide triphosphatase/helicase activities (Gorbaleny AE, Koonin EV Donchenko AP

Blinov VM 1989 Two related superfamil es f putative hel cases involved in replication, recombination repair and expression of DNA and RNA genomes *NuclA ids Res* 17 4713 4730 Wengler and Wengler 1993) being therefore also associated with viral RNA repl cation. NS5 the largest and most conserved viral protein, contains several sequence motifs believed to be common to viral RNA polymerases (Chambers TJ Hahn CS Galler R, CM Rice 1990a. Flavivirus genome organization, expression and evolution *Ann.Rev M crob l* 44 649-688 O Reilly EK, Kao CC 1998 Analysis f RNA-dependent RNA polymerase structure and function as guided by known polymerase structures and computer predictions f secondary structure *Virol gy* 252 287 303) and exhibits RNA dependent RNA polymerase activity (Steffens S Thiel HJ Behrens SE 1999 The RNA dependent RNA polymerases f different members of th family Flaviviridae exhibit similar properties in vitro *J Gen.Virol* 80 2583 2590) Finally a umber of cis and trans acting elements in flavivirus RNA replication have been dentified (Kromykh AA, Sedlak PL Westaway EG 2000 cis and trans-acting elements in flavivirus RNA replication. *J V rol* 74 3253 3263)

The 4 small proteins NS2A, NS2B NS4A and NS4B are poorly conserved in their amino acid sequences but not in their pattern f multiple hydrophobi stretches NS2A has been shown to be required for proper processing of NS1 (Falgout B Channock R, Lai CJ 1989 Proper processing of dengue virus nonstructural glycoprotein NS1 requires the N terminal hydrophobi signal sequence and the downstream nonstructural protein NS2A *J Virol* 63 1852 1860) whereas NS2B has been shown to associate with NS3 to constitute the active viral protease complex (Chambers TJ Nestorowicz A Amberg SM Rice CM 1993 Mutagenesis of the yellow fever virus nonstructural polyportein catalitically active NS3 proteinase domain and NS2B are required for cleavages at dibasi sites *J V rol* 65 6797 6807 Falgout B Pethel M, Zhang YM, Lai CJ 1991 Both nonstructural proteins NS2B and NS3 are required fo the proteolyti processing of dengue virus nonstructural proteins *J Virol* 65 2467 2475 Jan LR Yang CS Trent DW Falgout B Lai CJ 1995 Processing of Japane encephaliti virus non structural proteins NS2B NS3 complex and heterologous proteases *J Gen. V rol* 76 573 580) NS4A has been suggested to interact with NS1 integrating t into the cytoplasmi process of RNA replicati (Lindenbach and Rice, 1999) Since viral RNA synthesi takes place in th cytosol in associati with RER membranes t has been postulated

that these hydrophobic proteins would be embedded in membranes and through protein-protein interactions participate in viral replication complexes together with NS3 and NS5 (Rice CM 1996 Flaviviridae the viruses and their replication. In BN F elds, DM Knipe PM Howley (eds) Fields Virology 3rd ed, Raven Press USA, p 931 960)

Two strains of yellow fever virus (YF) isolated in 1927 gave rise to the vaccines to be used for human immunization. One the Asibi strain, was isolated from young african named Asibi by passage in Rhesus monkey (*Macaca mulatt*) and the other the French Viscerotropi Virus (FVV) from patient in Senegal

In 1935 the Asibi strain was adapted to growth in mouse embryonic tissue After 17 passages, the virus, named 17D was further cultivated until passage 58 in whole chicken embryoni tissue and thereafter until passage 114 in denervated chicken embryoni tissue only Theiler and Smith (Theiler M and Smith HH 1937 The effect of prolonged cultivation in vitro upon the pathogenicity of yellow fever virus. *J Exp Med.* 65 767 786) showed that, at this stage, there was marked reduction in viral viscero and neurotropism when inoculated intracerebrally in monkeys This virus was further subcultured until passages 227 and 229 and the resulting viruses, without human immune serum, were used to immunize 8 human volunteers with satisfactory results, as shown by the absence of adverse reactions and seroconversion to YF in 2 weeks (Theiler M, Smith HH 1937 The use of yellow fever virus modified by in vitro cultivation for human immunization. *J Exp M d* 65 787-800)

In the late 1930 s and early 1940 mass vaccination was conducted in Brazil and various countries in Africa. Fox, J.P et al (Fox, F P and Penna, H A. (1943) Behavior of 17D yellow fever virus in Rhesus monkeys Relation to substrain, dose and neural or extraneural inoculation. *Am. J Hyg* 38 52 172) described the preparation f the vaccine from 17D virus substrains These substrains differed in their passag history and they overlapped with regard to time of their use for inocula and/or vaccine production. The substitution of each on by the next was according to the experience gained during vaccine production, quality control and human vaccination in which the appearance of symptomatology led to the discontinuation of given strain.

As mentioned before, the YF virus Asibi strain was subcultured in embryonic mouse tissue and minced whole chicken embry with or without nervous tissue These passages yielded the parent 17D strain at passage level 180 17DD at passage 195 and th 17D-204 at

passag 204 17DD was further bcultured until passage 241 and underwent 43 additional passages in embryonated chicken ggs until the vaccine batch used for 17DD virus purificati (passag 284) The 17D-204 was further subcultured to produce Colombia 88 strain which, upon passag in embryonated chicken eggs gave rise to different vaccine seed lots currently in use in France (I Pasteur at passag 235) and in the United States (Connaught, at passage 234) Each of these 17D-204 strains was plaque purified in different cell lines the virus finally amplified in SW13 cells and used for cDNA cloning and sequence analyses These 17D 204 are named C-204 (Rice, C M. Lenches, E Eddy S R. Shin, S.J Sheets R.L and Strauss, J H (1985) "Nucleotide sequence of yellow fever virus implications for flavivirus gene expression and evolution Science 229 726-733) and F 204 (Despres, P Cahour A. Dupuy A Deubel V Bouloy M Digoutte J P Girard, M (1987) High genetu stability of the coding region for the structural proteins of yell w fever strain 17D J Gen Virol 68 2245 2247) respectively The 17D-213 strain was derived from 17D-204 when the primary seed lot (S1 112-69) from the Federal Republi f Germany (FRG 83-66) was used by the World Health Organization (WHO) to produce an avian leukosis virus-free 17D seed (S1 213/77) at passage 237

The 17D 213 at passage 239 was tested for monkey neurovirulence (R. S Marchevsky personal communication, see Duarte do Santo et al (Duarte dos Santos CN P st, PR, Carvalho R, Ferreira II Rice CM and Galler R. 1995 Complete nucleotide sequence of yellow fever virus vaccine strains 17DD and 17D 213 Virus Res 35 35-41) and was th subject of sequence analysis together with 17DD (at passage 284) and comparison to previously published nucleotide sequences of other YF virus strains (Duarte do Santo et al 1995 Asibi Hahn, C S Dalrympl J M Strauss, J H and Rice C.M (1987) "Comparison of the virulent Asibi strain of yellow fever virus with the 17D vaccine strain derived from t Proc Natl Acad. Sci USA 84 2029-2033 17D 204 strain, C-204 Rice C M Lenches E M Eddy S R. Shin, S J Sheets, R.L and Straus J H (1985) 'Nucleotide sequence of yellow fever virus implications for fl vivirus gene expression and evolution Science 229 726-733 F 204 Despres, P Cahour R. Dupuy A. Deubel, V Boul y M D goutte J P and Girard, M (1987) High genetic stability f the coding region for the structural proteins f yellow fever virus strain 17D J Gen. Virol 68 2245 2247) F gure 1 depicts the

passage history of the original YF Asibi strain and derivation of YF 17D vaccine strains

A total of 67 nucleotide differences corresponding to 31 amino acid changes were originally noted between the Asibi and 17D 204 genome sequences (see Hahn, C S et al 1987) The comparison between the nucleotide sequences of 17DD and 17D-213 substrains (see Duarte dos Santo et al, 1995) and the nucleotide sequence of 17D 204 substrain (see Rice et al 1985) showed that not all changes are common and thus not confirmed as being 17D-specific Therefore, the 17D substrain specific changes observed are very likely not related to attenuation but may reflect differences in behaviour of these strains in monkey neurovirulence tests Therefore 48 nucleotide sequence changes are likely associated with YF virus attenuation. These are scattered along the genome 26 are silent mutations and 22 led to amino acid substitutions The alterations noted in the E protein are important because it is the main target for humoral neutralizing response it is the protein where hemagglutination and neutralization epitopes are located, and it mediates cell receptor recognition and cell penetration, therefore targeting the virus to specific cells Importantly E protein accumulate the highest rate of nonconservative to conservative amino acid changes Altogether eleven nucleotide substitutions were observed in the E protein gene leading to 8 amino acid changes at positions 52 170 173 200 299 305 380 and 407 (respectively nucleotides 1127 1482 1491 1572 1870 1887 1965 and 2112 from the RNA 5 end) Among these four are nonconservative changes and presumably would have larger impact on protein structure and consequently function.

Alterations at amino acids 52 (G→R) and 200 (K→T) are located at the base of domain II in the 3 D structure proposed for the E protein of Flaviviruses (Rey F.A. Heinz F X Mandl C Kunz, C and Harrison, S C (1995) "The envelope glycoprotein from tick borne encephalitis virus at 2Å resolution *Nature* 375 291 298) which is conserved among Fl viviruses and contains cross-reactive epitopes as shown by Mandl C W et al (Mandl, M W Guirakhoo F Holzmann, H. Heinz, F X. and Kunz, C (1989) Antigeni structure of the flavivirus envelope E protein at the molecular level using tick borne encephalitis virus as a model *J Virol* 63 564-571) This domain II is highly crosslinked by disulphide bonds and undergoes low pH transition which is

related to exposing strictly conserved and hydrophobic stretch f amino acids which are supposed to be involved in the fusion of the viral envelope to the endosom membrane

Alterations at amino acids 170 and 173 in domain I f the E protein in the 3 D structure map very close to the position that neutralization ep tope was dentified for tick borne encephalitis (TBE) virus (see Mandl C W et al, 1989) A mutation at position 171 f TBE virus E protein was shown to affect the threshold f fusion activating conformational change f this protein and the 2 changes bserved for YF 17D virus may be related to same phenomenon It is conceivable that slower rate f fusion may delay th kineti f virus production and thereby lead to milder infection of the host. Further evidence for th importance of this area comes from the work f Ryman et al (Ryman KD Xi H, Ledger N Campbell GA and Barrett ADT 1997 Antigeni variants of yellow fever virus with altered neurovirulence phenotype in mice Virology 230 376-380) showing that t encodes an ep tope recognized by wild type-specifi monoclonal antibody and reversion at this site may have contributed to added neurovirulence in the mouse model f variant virus recovered from the 17D accine population.

Alterations at amino ac ds 299 305 380 and 407 are located in the domain III (see Rey F A et al 1995) This domain was suggested to be involved in viral attachment to cellular receptor and consequently being major determinant both of host range and cell tropism and of virulence/attenuation. The 4 amino acid changes reported for YF are located on the distal face of domain III Thi area has loop which s tight turn in tick borne encephalitis virus but contains 4 additional residues in all mosquito-borne strains Becaus viruses repl cate in their vectors this loop is likely to be host range determinant. This enlarged loop contains an Arginine-Glycine Asparti Acid (Arg Gly Asp) sequence in all 3 YF 17D vaccine strains Thi sequence motif is known to mediate umber f cell interactions including receptor binding and is absent not only in the parental virulent Asibi strain but also in other 22 strains f YF wild type virus (Lepiniec L Dalgarno L Huong VTQ Monath TP D goutte JP and Deubel V (1994) Geographic distribution and ev lution of yellow fever viruses based on direct sequencing f genomi DNA fragments *J Gen. Vrol* 75 417-423) Such fact

suggests that the mutation from Threonine (Thr) to Arginine (Arg) creating Arg-Gly Asp motif, is likely to be relevant for the attenuated phenotype of the YF 17D strain. Consistently Lobigs et al (Lobigs M, Usha R, Nesterowicz A, Marschall ID Weir RC and Dalgarno L 1990 Host cell selection of Murray Valley encephalitis virus variants altered at an RGD sequence in the envelope protein and in mouse neurovirulence *Virol gy* 176 587 595) identified Arg-Gly Asp sequence motif (at amino acid 390) which led to the loss f virulence f Murray Valley encephalitis virus for mice At least for YF however t is not the only determinant as shown by van der Most et al (van der Most RG Corver J Strauss JH 1999 Mutagenesis of the RGD motif in the yellow fever virus 17D envelope protein. *Virol gy* 265 83 95) It was suggested that the sequence in the RGD loop is critical for the conformation of E and minor changes in this region can have drastic effects on the stability of the protein. It is feasible, however that such changes in structure and/or stability can affect the spectrum of cells infected by influencing the overall E protein structure and although that particular area would not be directly involved in the receptor binding Since this loop is present in all mosquito-borne flaviviruses and most of them carry sequence that is very similar to the 17D-RGD motif t is conceivabl that mutations in this region in other flaviviruses will affect viral fitness

The importance of residue 305 was implied from the findings by Jennings et al (1994) who noted that the 17D virus recovered from human case of postvaccinal encephalitis had a E→K change at position 303 and was found to have increased neurovirulence for both mice and monkeys

It s noteworthy that the development of infectious cDNA for Japanes encephalitis (JE) virus made by Sumiyoshi H et al (Sumiyoshi H, Hoke CH and Trent DW 1992 Infectious Japanese encephalitis virus RNA can be synthesized from in vitro-ligated cDNA templates *J Virol* 66 5425 5431) allowed the dentification of mutation (Lys for Gl) at amino acid 136 f the E protein which resulted in the loss f neurovirulence for mice (see Sumiyoshi, H Tignor G.H and Shope R.E (1996) Characterization of highly attenuated Japanese encephalitis virus generated from molecularly cloned cDNA J Infect. Dis 171 1144-1151) This means that domain I is an important area which contains a critical determinant of JE virus virulence in contrast

to most f the data obtained from the analyses of virulence for several other fl viviruses for which t is suggested that domain III would be the primary site for virulence/attenuation determinants

For dengue type 2 virus Butrapet et al (2000) have suggested that attenuation determinants li outside the structural area, namely 5 UTR, NS1 and NS3 Littl known about the molecular basis of viscerotropism, the ability f wild type YF virus to replicate and damage extraneural tissue, specially hepati tissue or the mutations responsibl for the loss of thi trait in the 17D virus Further studies on 17D neurovirulence in the mouse model have uggested complex geneti basis being most likely multigeni involving the structural and nonstructural proteins as well as the 3 end nontranslated region, the latter would presumably restrict replication. Nevertheless such analyses f the E protein provides framework for understanding several aspects of flavivirus biol gy and suggests that t should be possibl to engineer viruses for the development f new live flavivirus vaccines

Being the major component of the virion surface, the envelope protein E pl y dominant role in eliciting neutralizing antibodies and the induction f a protecti response This has been conclusively demonstrated by active immunization of animals with defined subviral components and recombinant proteins and by passive protection experiments with E protein specifi monocl nal antibodies Linear epitopes have been mapped using synthetic peptides and are found in areas of the glycoprotein predicted to be hydrophili however the induction f neutralizing antibodies seems to be strongly dependent on the native conformation f E A umber f neutralizing sites have been inferred from studies with monoclonal antibody scape mutants and have been mapped onto the 3D structure Consistent with the suggested topology of the dimer on the virion surface most f these sites are located on the externally accessibl upper side of the subunit. The scattered distribution of these eutralization escape mutations over the entire subunit indicates that antibody binding to any of the structural domains can lead to virus neutralization.

The neutralization epitopes recognized by monocl nal antibodies are conformational ince E protein denaturation abolishes binding Moreover monoclonal antibodies will only react with syntheti peptides if they recognize an epitope which is

present on the denatured E protein. Since the dimeri subunit forms part f as yet undefined lattice on the virion surface t is likely that certain epitopes are composed of elements from different subunits

The mechanisms f neutralization by these antibodies remains speculative but relevant to the strategy of epitope insertion described below is the hypothesis that domain II may be involved in fusion f the viral envelope with the membrane f the endosome which occurs under acidi pH Fusion requires conformational changes that affect several neutralization epitopes, primarily within central domain I and domain II These changes are apparently associated with reorganization of the subunit interactions on the virio surface with trimer contacts being favored in the low pH form, in contrast to dimer contacts in the native form. Interference with these structural rearrangements by antibody binding represents one mechanism that may lead to virus neutralization (Monath and Heinz, 1996)

Low titer neutralizing activity and significant degree f passive protection in mice has been observed by passive immunizatio with monoclonal antibodies against prM This due to a certain degree of partial cleavage of prM to form M by the furin in the Golgi system and thereby prM remains associated with the virus particl being an additional target for antibodies

The NS1 protein, also known as the complement fixing antigen elicits an antibody response during the course of fla virus infection in man. It exists as cell associated and secreted forms and it has been shown that immunization of animals with purified NS1 or passive immunization of animals with monoclonal antibodies to t do elicit a protective immune response, the basis of which is still controversial
The primary immunological rol of nonstructural proteins, except for NS1 seems to be targets for cytotoxi T cells The specificity of T-cell responses to flaviviruses has been studied in human and mouse systems mainly with dengue and Japanese encephalitis serocomplex viruses In the course of dengue infection in man, both CD4+ CD8-- as well as the opposite CD4-- CD8+ T lymphocytes response have been detected and characterized. In bulk cultures of CD4+ lymphocytes as well as with CD4+ cell lones obtained from a single individual which had been infected with dengue, different

specifi cross-reactivity patterns with several other flaviviruses is observed. Similar bservations hold for CD8 cell from infected humans and mice

Antigeni determinants involved in cell mediated immunity have not yet been specifically localized in YF virus proteins as t has been for dengue and encephalitis virus such as MVE and JE Such cytotoxic T cell determinants are found in all 3 structural and in the nonstructural proteins as well, specially in NS3 Some of these epitopes have been mapped to their primary sequence on the respective protein L vingsto et al (L vingston PG Kurane I, Lai CJ Bray M, Ennis FA 1994 Recognition of envelope protein by dengue virus serotype-specifi human CD4+ CD8– cytotoxi cell clones *J Virol* 68 3283 3288) reported the identification f several HLA class II restricted CD4+ CTL lones from human donor capabl f mediating specifi lysis f virus-infected cells It has been suggested that CD4+ CTL may be important mediators of viral learance especially during reinfection with the same serotype f virus

Vaccination f humans with recombinant poxviruses expressing the structural proteins prM and E of Japanese encephalitis elicited CD4– CD8+ CTLs directed to the JE virus structural proteins although no specifi epitopes were identified (Konish E, Kurane I Mason PW Shope RE, Kanesa Thasan N Smucny JJ Hoke CH, Ennis FA 1998 Induction of Japanese-encephal ti virus-specifi ytotoxi T lymphocytes in humans by poxvirus-based JE vaccine candidates *Vaccine* 16 842 849)

More recently JE virus E protein epitope recognized by JE-specifi murine CD8+ CTLs has been reported. The epitope was found to correspond to amino ac ds 60-68 of the JE virus protein which are located in domain II (Takada K, Masaki H Konishi E, Takahashi M, Kurane I 2000 Definition f an epitope on Japanese encephaltis virus envelope protein recognized by JEV specifi murine CD8+ cytotoxi T lymphocites *Arch. Virol* 145 523 534) This epitope is located between strands and b of domain II including two amino acid residues from each and the remaining of the epitope encompassing the intervening short loop This area is exposed n the surface of the dimer

Functional T helper cell epitopes in the flavivirus E protein were dentified b measuring B-cell response after immunization with syntheti peptides (Roehrig JT

J hnson AJ Hunt AR 1994 T helper cell epitopes on the E glycoprotein f dengue 2 Jamaica virus *Virology* 198 31 38)

The capability to manipulate the genome f flaviviruses through infectious clone technology has opened new possibilities for vaccine development. This is so because virus can be recovered from complementary DNA by *in vitro* transcription and transfection of cultured cell with RNA, and these cDNAs corresponding to the complete viral genome allow introducing genetic modifications at any particular site f the viral genome The pioneer study of Racaniell and Baltimore (Racaniello VR and Baltimore D 1981 Cl ned poliovirus complementary DNA is infectious in mammalian cells *Sci nce* 214 916-919) first showed the feasibility to regenerate virus from cloned cDNA In th patent US 4 719 177 Racaniello and Baltimore described, in details the production of RNA viral DNA by reverse transcribing viral RNA and inserting the resulting cDNA molecule into recombinant DNA vector The process was particularly concerned to the production f poliovirus double-stranded complementary DNA (ds cDNA) They found out that the transfected full length poliovirus cDNA was tself infectious

In addition, with the development of *in itro* transcription systems (see Melton DA, Krieg PA, Rabagliati MR, Maniatis T Zinn K and Green MR 1984 Efficient in vitro synthesis of biol gically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter *Nucl Acids Res* 12 7035 7056) much higher efficiency in synthesis of full length viral RNA, as compared to cDNA transcription in the cell, became possible Furthermore, the development of improved transfection methodologies such as cationi liposomes and electroporation increased the efficiency of RNA transfection f cultured cells

The construction and cloning of stable full length dengue cDNA copy in strain of *Escherichia col* using the pBR322 plasmid vector was described by Lai, C.J et al (Lai CJ Zhao B Hori H and Bray M 1991 Infectious RNA transcribed from stably cloned full length cDNA f dengue type 4 virus *Proc Natl Acad. Sci USA* 88 5139-5143) They verified that RNA molecules produced by *vitro* transcription of the full length cloned DNA template were infectious, and progeny virus recovered from transfected cell was indistinguishable from the parental virus from which the cDNA

clone was derived. B t, as mentioned in th Patent Appl cation WO 93/06214 such an infectious DNA construct and RNA transcripts generated therefrom were pathogeni and that the attenuated dengue viruses generated thus far were genetically unstabl and had the potential to revert back to pathogeni form overtime To solve this problem, the Applicant proposed to construct cDNA sequences encoding the RNA transcripts to direct the production f chimeric dengue viruses incorporating mutations to recombinant DNA fragments generated therefrom A preferred embodiment introduces deletions in the 3 end noncoding region (Men R, Bray M, Clark D Chanock RM, Lai CJ 1996 Dengue type 4 virus mutants containing deletions in the 3 noncoding region f the RNA genome analysis of growth restriction in cell culture and altered viremi pattern and immunogenicity in rhesus monkeys *J Virol* 70 3930-3937 Lai CJ Bray M Men R, Cahour A, Chen W Kawano H, Tadano M, Hiramatsu K, Tokimatsu I Pletnev A, Arakai S Shameen G Rinaudo M 1998 Evaluation of molecular strategies to develop live dengue vaccine *Cl n. Diagn.Virol* 10 173 179 Whitehead SS Men RH Lai CJ Murphy BR, Reynolds MJ Perreault J Karron RA, Durbin AP 2000 A l ve attenuated dengue virus type 4 vaccine candidate with 30- ucleotide deletion in the 3 end UTR s attenuated and immunogenic in humans 19th Annual Meeting, American Society f Virology pp 125)

The construction f full length YF 17D cDNA template that can be transcribed *in vitro* to yield infectious YF virus RNA was first described by Rice et al (Rice CM, Grakoui A, Galler R and Chambers T 1989 Transcription of infectious yellow fever RNA from full length cDNA templates produced by *in vitro* ligation. *The N w B ologist* 1 285 296) Becaus of the instabil ty f full length YF cDNA clones and their toxi effects on *Escher hia l* they developed strategy in which full length templates for transcription were constructed by *vitro* ligation of appropriate restriction fragments Moreover they found that the YF virus reco ered from DNA was indistinguishabl from the parental virus by several criteria. The YF infecti us cDNA derived from the 17D-204 substrain Notwithstanding the YF virus generated from thi YF infecti us cDNA s rather attenuated, t cannot be used for human accination because of its residual neurovirulence, as determined by Marchevsky R.S et al (Marchevsky RS Mariano J Ferreira VS Almeida E, Cerqueira MJ Carvalho R, Pi surn JW Tra asso

da Rosa APA, Simões MC Santos CND Ferreira II, Muylaert IR, Mann GF Rice CM and Galler R 1995 Phenotypic analysis f yellow fever virus derived from complementary DNA. *Am. J Trop M d. Hyg* 52, 75 80) Although these results showed the virus was not ideally attenuated for YF 17D vaccine t was the first demonstration for flavivirus that it was possibl to develop from a few micrograms of DNA template whol seed lot under Good Manufacturing Practices (GMP) using current methodol gy for the production f YF vaccine

Galler and Freire (US patent application 09/058411) have approached the recovery of fully attenuated virus from YF cDNA by engineering a number of mutations into the original 17D-204 cDNA (Rice et al, 1989) based on th sequence f the 17DD substrain (Duarte dos Santo et al, 1995) This substrain has been used in Brazil for YF vaccine production since the late 1930 with excellent records of efficacy and safety Here, virus was recovered from the genetically modified cDNA template through the transfection of certified CEF cells under GMP (US patent application 09/423517) Altogether 3 transfection lots were derived which gave rise to two primary and three secondary seed lots by further passaging in CEF cells with all the relevant quality controls for human vaccine production using this cell system. Average titer of formulated virus was 6 7log10PFU/ml Analysis of viral genetic stability was carried out by serial passaging in CEF cultures and studying several parameters such as plaque size, mouse neurovirulence and ucleotide sequence determination with satisfactory results

Further nucleotide sequencing results on the chimeric 17D204/DD virus was btained after viral reisolation from viremic monkey sera. There were no genomic changes (Galler R, Freire MS and JaborAV unpublished) But YF 17D virus exists today as two main substrains used for vaccine production world wide, namely 17D-204 and 17DD and differences in their sequences have been noted (Galler R, Post PR, Duarte dos Santos CN and Ferreira II 1998 Geneti variability among yellow fever virus 17D substrains *Vac ne* 16 1024-1028)

espite the overall geneti stability of YF 17D virus major concern is the genetic stability of the foreign epitope since the virus does not need t and in fact ts replication has been somewhat restricted. Although viral genetic variability is

minimized by the use f seed lot system to produce production sized seeds at least 4 passages are necessary starting out from DNA (US 6 171 854)

The first aspect that has to be cons dered when using given flavivirus cDNA backbone fo the expression f heterologous proteins is whether one can indeed recover virus with the same phenotypi markers as riginally present in the virus population that gave rise to the cDNA library That s extrem ly applicabl to YF 17D virus given the well known safety and efficacy of YF 17D accine

In fact different technical approaches to constructing recombinant viruses based on flavivirus in particular YF 17D virus, are possibl and will vary according to the region f the genom selected for insertion and to the antigen to be expressed. One major approach has been the creation of chimeri viruses through th exchange f prM/M/E genes as first established for DEN-4 virus chimeras (Lai et al, 1998 US 5 494 671) The prM/M/E genes of dengue virus serotypes 1 2 and 3 were inserted into the dengue 4 infectious clone resulting in chimeri virus with reduced virulence for mice and monkeys (Lai et al, 1998) This all ws the removal of the major immunogens of the vector thereby reducing the criticism on previous immunity

The same type of construction was made for tick borne encephaliti (TBE) and Langat viruses (Pletnev AG Bray M, Huggins J Lai CJ 1992 Construction and characterizati f chimeri tick borne encephalitis/dengue type 4 viruses *Proc Natl Acad. Sc USA* 89 10532 10536 Pletnev AG Men R. 1998 Attenuation f Langat virus tick borne flavivirus by chimerization with mosquito-borne flavivirus dengue type 4 *Proc Natl Acad. Sci USA* 95 1746-1751) resulting in irus attenuated for mice

Chambers et al (Chambers TJ Nestorowicz A, Mason PW Rice CM 1999 Yellow fever/Japanese encefalitis chimeri viruses construction and biological properties *J Virol* 73 3095 3101) have described the first chimeri virus developed with the YF 17D cDNA from Rice et al (1989) by the exchang of the prM/M/E genes with cDNA derived from JE SA14-14 2 and Nakayama strains of JE virus The former corresponds to the live attenuated vaccine strain in use nowadays in China.

Guirakhoo et al (Guirakhoo F Zhang ZX, Chambers TJ Delagrave S Arroyo J Barrett ADT Monath TP 1999 Immunogenicity genetic stability and protective efficacy f recombinant, chimeric y llow fever J panese encephaliti virus

(Chimerivax JE) as live attenuated vaccine candidate against fever Japanese encephalitis *Virology* 257 363 372) Monath et al (Monath TP Soike K, Levenbook I, Zhang ZX, Arroyo J Delagrave S Myers G Barrett ADT Shope RE, Rattterree M, Chambers TJ Guirakhoo F 1999 Recombinant, chimeri live, attenuated vaccine (Chimerivax) incorporating the envelope genes of Japanese encephalitis (SA14-14-2) virus and the capsid and nonstructural genes of yell w fever (17D) virus is safe, immunogeni and protective in nonhuman primates *Vaccine* 17 1869-1882) and WO98/37911 have brought it closer to vaccine development. Here chimeri virus was recovered after transfection of certified FRhL cell with 5 additional passages of the virus to produce seed lots and experimental vaccine lot (5th passag) all under GMP in certified cell Virus yields in this cell system were not provided.

Chimeric virus retained nucleotide/amino acid sequences present in the original SA14-14-2 strain. This vaccine strain differs, in prM/M/E region, from the parental virus in 6 positions (E-107 E138 E176 E279 E315 E439) Mutations are stabl across multipl passages in cell culture (Vero) and mouse brain but not in FRhL cells Despite previous data on the genetic stabil ty f such virus, one of the 4 changes in the E protein related to viral attenuation had reverted during the passaging to produce the secondary seed.

In dose-response study neutralizing antibodies specifi for prM/M/E were elicited in all groups f monkeys with different doses even with as little as 100 PFUs and conferred full protection against IC challenge with wild type JE However the lower the chimeri virus dose the more residual histopathological changes were noted in the SNC after IC challenge with wild type JE virus

The first chimeri 17D/dengue virus developed (Guirakhoo F Weltzin R, Chambers TJ Zhang ZX, Soike K, Rattterree M, Arroyo J Georgakopoul s K, Catalan J Monath TP 2000 Recombinant chimeric yellow fever-dengue type 2 virus s immunogeni and protective in nonhuman primates. *J Virol.* 74 5477 5485) involved prM/M/E gene replacement (fusion at the signalase cleavage site) with den2 cDNA. All virus regeneration and passaging was done in Vero PM cells (a cell bank from Pasteur Merieux) allegedly certified for live vaccine virus production. Recombinant virus retained the original den2 prM/M/E sequences even after 18 serial passages in

Vero cells but some variation was noted in YF gene Phenotypi analysi f chimeri 17D/den2 virus showed t does not kill mice even at high doses (6 0 l g10 PFU) in contrast to YF 17D which kills nearly 100% at 3 0 log10PFU Antibody response and full protection was elicited by the 17D-DEN2 chimera in both YF immune and flavivirus-naive m nkey In dose response study even at the lowest dos (2 0 log10PFU) chimeri virus replicated sufficiently to induce protective neutralizing antibody response as no viremia was detected in these animals after challeng with a wild type dengue 2 virus

Although YF 17D virus known to be more genetically stabl than other vaccine viruses such as poliovirus, given the extremely low number f reports on adverse events following accination, few mutations have been detected occasionally when virus derived from humans were sequenced (Xi H Cass AR, Barrett ADT 1998 Yellow fever 17D vaccine virus isolated from healthy vaccinees accumulates few mutations *Virus Research* 55 93 99) Guirakhoo et al ha reported few changes in the YF mo ety f chimeri 17D/dengue 2 virus which had been passaged p to 18 times in cell ulture

Galler et al (in preparation) have also developed similar chimeri 17D-DEN 2 virus However the 17D backbone was genetically modified (US 6 171 854) These viruses were characterized at the genomi level by RT/PCR with YF/Den specifi primers and nucleotide sequencing over fusion areas and the wh l DEN2 mo eties The polyprotein expression/processing was monitored by SDS PAGE analysis of radiolabeled viral proteins immunoprecip tated with specifi antisera, including monoclonal antibodies Recognition f YF and DEN 2 proteins by hiperimmune antisera, and monoclonal antibodies was also accomplished by viral neutralization in plaque formation reduction tests and indirect immunofluorescence on infected cells The growth f recombinant viruses was examined in several cell substrates such as Vero LLC-MK2 C6/36 MRC5 and CEF Only YF virus grew in all f them to high titers but the himeri viruse failed to replicate in the accine-production certified cells (CEF and MRC5) similarly to DEN 2 virus Analysi f viral virulence was performed by intracerebral inoculation of mice (10^3 PFU) and the chimeri viruses turned out to be more attenuated in this system than the YF 17D virus W'th regard to the

immunogenicity studies in the mouse model indicate the chimeri virus does induce protective response against an otherwise lethal dose of mouse neurovirulent DEN 2 New Guinea C virus

One alternative to develop YFV 17D as a vector for heterologous antigens is the expression of particular epitopes in certain regions of the genome The feasibility f this approach was first demonstrated for poli virus (reviewed in Rose CSP Evans DJ 1990 P liovirus antigen chimeras *Trends B te hnol* 9 415-421) The solution of the three-dimensional structure of poliovirus allowed the mapping of type-specifi neutralization epitopes on defined surface regions f the viral particle (Hogl JM Chow M & Filman DJ (1985) Three-dimensional structure of poliovirus at 2 9 resolution. *S nce* 229 1358 1365) One f the surface loops f the VP1 protein was used for the insertion of type 3 epitope which was recognized by primate antisera to poliovirus type 3 showing that the chimera was not only viable but also that the inserted epitope was presented with the sam conformation as in the surface of the type 3 virus (Murray MG Kuhn RJ Arita M Kawamura N Nomoto A & Wimmer E (1988) Poliovirus type 1/type 3 antigenic hybrid virus constructed in vitro elicits type 1 and type 3 neutralizing antibodies in rabbits and monkeys *Proc.Natl.Acad.Sc USA* 85 3203 3207) However to argue strongly against t s the bservation that the sam site was used for the insertion of different epitopes f hepatitis A virus but the immunogenicity f the inserted peptides was very poor (Lemon SM, Barclay W Ferguson M, Murphy P Jing L Burke K, Wood D Katrak K, Sangar D Minor PD & Almond JW (1992) Immunogenicity and antigenicity of chimeric p cornaviruses which express hepatitis A virus (HAV) peptide sequences evidence for eutralization domain near the amino terminus of VP1 of HAV *Virology* 188 285 295)

Influenza viruses are also well studied from the structural view and 3D structures are availabl for both hemagglutin and neuraminidase viral proteins Li et al (Li S P loni V Isobe H, Zaghouani H, Guinea R, Moran T Bona C Palese P 1993 Chimeric influenza virus induces neutralizing antibodies and cytotoxi T cells against human immunodeficiency virus type 1 *J Virol* 67 6659-6666) have described insertion f HIV epitope into loop of antigeni site B f influenza virus and the generation f specifi B and T cell responses to the epitope

London et al (London SD Schmalj hn AL Dalrympl JM & Rice CM (1992) Infectious enveloped RNA virus antigeni chimeras *Proc Natl Acad.S USA* 89 207 211) devel ped chimeri Sindbis virus expressing singl well-defined neutralizing epitope of Rift Valley fever virus inserted by random mutagenesis Insertion sites, permissive for recovery of viruses with growth properties similar to the parental virus were found in one of the virion glycoproteins For these chimeras the epitope was expressed at the viri surface and stimulated partially protective immune response to RFV infection of mice

De Vries et al (de Vries AAF Glaser AL, Raasman MJB de Haan CAM Sarnataro GJG Rottier PJM 2000 Geneti manipulation f equine arteritis virus using full length cDNA clones separation of overlapping genes and expression f a foreign epitope *Virol gy* 270 84-97) have engineered equine arteriti virus also positive-stranded RNA virus with genom similar in size to YF 17D but with different replication strategy Inserti f a nonapeptide epitope recognized by an MHV specifi monoclonal antibody with functional O-glycosylation site resulted in recombinant virus expressing both properties of th epitope However growth f the virus was clearly reduced as compared to the parental virus although 3 serial passages did not result in the l ss of the insert

Bendahmane et al (Bendahmane M, Koo M, Karrer E, Beachy RN 1999 Display of ep topes on the suface f tobacco mosaic virus impact of charg and isoelectri point of the epitope on virus-host interactions *J M l Biol.* 290 9 20) have described the creation of chimeri tobacco mosai virus expressing defined epitopes of rabies and murine hepatiti virus Th foreign epitopes were expressed on the carboxi terminus f the viral coat protein, the stucture and biophysical properties of which is well established Although both insertions gave rise to viabl viruses the generation of lesions in plants infected by either virus differed. It was observed that the isoelectri point (pI) f the epitope affected the overall hybrid coat protein pI/charge and that was important for successful epitope display It was also noted the lack of tolerance to positively charged epitopes the surface f TMV

A number f other studies employing virus-like particles to express defined foreign protein ep topes ha been described as they seem to be abl to potentiate the

immunogenicity of foreign epitopes presented on their surface Heal et al (Heal KG Hill HR, Stockley PG Hollingdal MR, Taylor Robinson AW 2000 Expression and immunogenicity of liver stage malaria epitope presented as foreign peptide on the surface of RNA free MS2 bacteriophage capsids *Vaccine* 18, 251 258) have used the coat protein of bacteriophage MS2 to express foreign epitopes based on β hairpin loop at the N terminus of thi protein which forms the most radially distinct feature of the mature capsid. A chimeri capsid expressing *Plasmodium* liver-stage antigen epitope (LSA 1) stimulated in mice polarized Th 1 response similar to the human response to this antigen in nature

Of interest also the study by Sedlik et al (Sedlik C Sarraseca J Rueda P Leclerc C Casal JI 1995 Immunogenicity of poliovirus B and T cell epitopes presented by hybrid porcine parvovirus particles *J Gen. Virol.* 76 2361 2368) They have inserted poliovirus T and B cell epitopes into the single-stranded DNA genome of canine parvovirus Insertions were made at the N terminus of the viral VP2 on the particl structure. There was no antibody response to the epitope whereas T cell response could be observed *in vitro* for the expressed T cell epitope That suggested the N terminus is not an adequate site for the expression of humoral epitopes Rueda et al (Rueda P Hurtado A, Barri M, Torrecuadrada JLM, Kamstup S Leclerc C, Casal JI 1999 Minor displacements in the insertion site provoke major differences in the induction of antibody responses by chimeri parvovirus-like particles. *Virology* 263 89-99) have extended these studies with canine parvovirus and inserted a poliovirus humoral epitope into the 4 loop of the viral VP2 carboxi terminus on the particl structure. Only loop 2 allowed the generation of antibodies to the epitope but not poliovirus neutralizing antibodies That was accomplished by inserting the epitope at adjacent amino acids suggesting that minor displacements at the insertion site cause dramati changes in the accessibility of the epitope and the induction f antibody responses

Chimeri hepatitis B virus core parti les carrying hantavirus epitopes have been described by Ulrich et al (Ulrich R, Koletzki D Lachman S Lundkvist A, Zankl A, Kazaks A, Kurth A, Gelderblom HR, Borisova G Meisel H, Krueger DH 1999 New chimaeri hepatitis B virus core particles carrying hantavirus (serotype Puumala)

epitopes immunogenicity and protection against virus challenge *J B ot chnol* 73 141 153) Insertion of as much as 45 and 114 amino acids of hantavirus capsid protein resulted in protection of natural hosts to the virus

The formation of chimeric virus like particles with hamster polyoma virus major caps ed protein has been describe by Gedvilaite et al (Gedvilaite A, Froemmel C Sasnaukas K, Micheel B Oezel M Behrsing O Stanilius J Jandrig B Scherneck S Ulrich R 2000 Formation f immunogeni virus-like parti les by inserting epitopes into surfac-exposed regions of hamster polyomavirus major capsid protein. *Virology* 273 21 35) They have predicted from the 3D-structure 3 areas that would be surface exposed and expression f 5-amino acid hepatitis B virus capsid epitope resulted in the recognition by monoclonal antibody to the epitope

For Flaviviruses neutralizing epitopes to other viral agents could be inserted in regions of the viral envelope where one detects geneti variability by nucleotide sequencing Such an approach was first tested after changing 2 amino acids in the envelope protein gene f the YF infectious cDNA by the corresponding amino acid sequence f Murray Valley encephalitis virus (MVE) which was previously characterized by monoclonal antibody as neutralizing MVE epitope The chimera, however was not viabl suggesting that particular area f the E protein (amino acids 192 193 from the amino terminal) critical for YF virus viability (R.Weir and C Rice pers commun.)

In short, to obtain a Flavivirus based vaccine virus expressing foreign antigens for the development of new live accines using recombinant DNA techniques t is necessary cumulatively

(1) to design strategies that allow the introductio f foreign antigens without compromising the structure and function f the virus

(2) to assure that the infectious cDNA construct and RNA transcripts generated therefrom give rise to virus which not pathogeni does not have the potential to revert to pathogenic form, and that the foreign antigen sequence is genetically stabl once integrated into the viral genome

(3) the Flavivirus generated from cloned cDNA, in addition to being attenuated should retain its immunological properties and present the expressed foreign antigen such that it elicits the appropriate immune response

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a safe and effective Flavivirus vaccine virus obtained from cloned cDNA having phenotypic characteristics such as attenuation and immunogenicity and which should also stably express and elicit an immune response to foreign antigens

In one embodiment, the present invention relates to a method for the production of Flavivirus as a vector for heterologous antigens comprising the introduction and expression of foreign gene sequences into an insertion site at the level of the envelope protein of any Flavivirus, wherein the sites are structurally apart from areas known to interfere with the overall flavivirus E protein structure and comprising sites that lie on the external surface of the virus providing accessibility to antibody not disrupt or significantly destabilize the three-dimensional structure of the E protein and not interfere with the formation of the E protein network within the viral envelope

In another embodiment the present invention is related to a strategy that allows introducing foreign gene sequences into the *fg* loop of the envelope protein of YF 17D virus and other flaviviruses

Another embodiment of the present invention relates to a new version of YF infectious cDNA template that is 17DD like and which resulted of insertion of malarial gene sequences

In another embodiment of the present invention, there is provided new YF plasmids which have the complete sequence of the YF infectious cDNA and malarial gene sequences

Another embodiment of the present invention are Flavivirus as a vector for heterologous antigens wherein the Flavivirus is obtainable according to the method herein described.

In another embodiments there provided recombinant YF viruses which are regenerated from YF infectious cDNA and express different malarial epitopes

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 illustrates the passage history of the original YF Asibi strain and derivati f YF 17D accine strains

FIGURE 2 shows the sequence alignment f the soluble portions f the Envelope proteins from tick borne encephalitis virus (tbe) yellow fever virus (yf) japanese encephalitis virus (j) and Dengue virus type 2 (den2)

FIGURE 3 shows schemati representati f the CS protein of *Plasmodium sp*

FIGURE 4 displays the structure f the plasmid pYF17D/14

FIGURE 5 shows the structure f the plasmid pYFE200

FIGURE 6 shows the sequence alignment between tbe and yf, but with the introduction of an insertion sequence (highlighted in bold and underlined) between residues 199 and 200 f yf, located in the loop between β strands f and g As in figure 2 the alignment shown is that used for model building of the modified yf E protein and deliberate misalignments are shown shaded. Elements f secondary structure are shown as horizontal bars between the two sequences

FIGURE 7 sets forth two views of the modelled yf E protein including the SYVPSAEQI insertion sequence within the fg loop The domains are coloured individually domain I (red) domain II (yellow) and domain III (blue) In the upper panel the E protein dimer is seen perpendicular to the viral membrane and in the lower panel is viewed within the membrane plane perpendicular to the long axis of th dimer The insertion site (in cyan) lies cl se to the proximal interface between the two constituent monomers f the dimer and can be seen to be partially buried.

FIGURE 8 sets forth the superposition of ten model f the YF E protein including the insertio sequence GG(NANP)$_3$GG within the fg loop I each model the insertion sequence is shown in different color whil the remainder f the structure is shown in green. Th great di ersity in conformations for the loop whil essentially

preserving the rest f the structure, indicates that the large volume f space availabl to the insertion peptide

FIGURE 9 shows the molecular surface of the YF E protein dimer for one f the ten models of Figure 8 In the upper panel, the blue and red dots indicated on each monomer represent the entrance and exit to the insertion peptide In the middle panel the two-residue N (blue) and C- terminal (red) glycine spacers are shown, indicating their rol in lifting the $(NANP)_3$ sequence above the molecular surface In the lower panel the $(NANP)_3$ insertion is shown in green.

FIGURE 10 sets forth an indirect immunofluorescence assay using monoclonal antibody directed to $(NANP)_3$ repeat.

FIGURE 11 displays SDS PAGE gel f the 17D/8 virus obtained by immunoprecipitation f metaboli labeled viral proteins

FIGURE 12 illustrates the comparative plaque size analysis among YF 17D/8 virus, YF17D/14 and YF17D/G1/2-derived virus

FIGURE 13 shows viral growth curves in CEF (15a) and VERO cells (15b)

FIGURE 14 sets forth the size of virus plaques formed on Vero cell monolayers after serial propagation of the viruses in Vero and CEF cell cultures

FIGURE 15 shows the comparative growth curves of the different recombinant viruses in Vero cells

FIGURE 16 shows the plaque size analysis f the different recombinant YF viruses

DETAILED DESCRIPTION OF THE INVENTION

For many diseases the ideal vaccine is live attenuated derivative f the pathogen, which induces strong long lasting protective immmune responses to a variety of antigens on the pathogen without causing illness Development of such vaccine is often precluded by difficulties in propagating the pathogen, in attenuating t without loosing immunogenicity and ensuring the stability of the attenuated phenotype. One alternative is the use f known attenuated microorganisms for the expressi of any antigen f interest.

The abılity to genetically engıneer anımal vıruses has changed the understandıng f how these vıruses replıcate and allowed the constructıon f vectors to dırect the expreasıon of heterol g us proteıns ın dıfferent systems DNA vıruses such as SV40 Vaccınıa, adenovıruses and herpes have been used as vectors for number of proteıns More recently RNA vıruses, both posıtıve and negatıve stranded, (Palese P 1998 RNA vırus vectors where are we and where do we need to go? *Proc Natl Acad. Sc USA* 95 12750-2) have also become amenabl to genetıc manıpulatıon and are preferred vectors as they lack DNA phase rulıng ut ıntegratıon of foreıgn sequences ınto chromosomal DNA and do not appear to downmodulate the ımmune response as large DNA vıruses do (g vaccını and herpes)

Flavıvıruses have several characterıstı whıch are desırabl for vaccınes ın general and that has attracted the ınterest of several laboratorıes ın developıng t further to be used as vector for heterologous antıgens Partıcularly for YF17D vırus these characterıstıcs ınclude well-defined and efficıent productıon methodol gy strıct quality control ıncludıng monkey neurovırulence testıng, long lastıng ımmunıty cheapness sıngl dosıs estımated use ıs over 200 mıllıon doses wıth excellent records of safety (only 21 cases f post accınal encephalıtıs after seed lot system ımplementatıon ın 1945 wıth an ıncıdence ın very young ınfants (9 months) f 0 5-4/1000 and >9 months at 1/8 mıllıon)

The fact that th 3 D structure for the flavıvırus E protein vaılabl (Rey et al 1995) would support the approach first used for polıovırus by examınıng the 3D structure and selectıng sıtes for ınsertıon whıch are less lıkely to ınterfere wıth the overall E protein structure The major concern about ınsertıng epıtopes ınto flavıvıruses E protein, ın partıcular ınto the YF 17D E protein, relates to the fact that thıs protein ıs the maın target for humoral neutralızıng response, t ıs the protein where hemagglutınatıon and neutralızatıon epıtopes are, and t ıs the protein that medıates cell receptor recognıton and cell penetratıon, therefore targetıng the vırus to specıfi cells By ınsertıng new epıtope somewhere ın the E protein of gıven flavıvırus one or more of these propertıes could be changed unles the analy ıs f the 3D structure all ws the dentıfıcatıon of potentıal ınsertıon sıte structurally apart from areas known to medıate one o more of these processe

Regarding the T ck borne encephalitis virus E protein, two distinct crystal forms of ts soluble fragment were obtained by Rey et al (Rey et al 1995) In both, the E protein shows similar dimeric arrangement in which two monomers are related by molecular twofold axis which is crystallographic in on crystal form and non crystallographi in the other The repeated appearance of the same dimer in both cases suggests that this is not an artifact of crystallization but represents the true l gomeri arrangement f the E protein as inserted into the viral envelope at neutral pH. The dimer presents an elongated flattened structure with overall dimensions of approximately 150 55 30 Å. Its shortest dimension lies perpendicular to the viral membrane and the whol structure presents mild curvature yielding an external surface which is somewhat greater than the corresponding internal surface This curvature may aid in the closure f the spherical viral particle

Each monomer is composed of three domains domain I (the central domain) domain II (the dimerization domain) and domain III (the immunoglobulin-like recepto binding domain) all of which are dominated by β-sheet secondary structure Domain I s discontinuous being composed of three separate segments of the polypeptide chain, and is dominated by an up-and-down eight-stranded β barrel of complex topology Domain II is responsibl for the principal interface between the two monomers proximal to the two-fold axis and is formed by the two segments of the polypeptide chain which divide domain I It s an elongated domain, heavily crosslinked by disulphide bridges and composed principally of two structural components 1) five-stranded anti parallel β sheet onto one side of which pack the only two α helices of the structure and 2) a β sandwich made p of three-stranded β sheet packed against β hairpin. This β sandwich sub-domain includes the fusion peptide believed to be important for the fusogenic activity of the virus It nestles into cavity formed by the interdomain contacts between domains I and III f the opposite monomer and therefore forms part of second interface region between the two monomers which lies more distal to the twofold axi to that mentioned above Domain III is continuous and presents somewhat modified C type immunogl bulin (Ig) fold. Using the conventional nomenclature for such folds (Bork P Holm L Sander C 1994 The immunoglobulin fold, *J Mol Biol* 242 309-320) the C F and G strands of this domain face outwards

from the monomer and represent a region critical in the determination of host range and cell tropism and is probably therefore fundamental for cell attachment. The opposite face of the Ig like domain forms the interface with domain I, and together with regions from the β-sandwich sub-domain of the opposite monomer s important in forming the dimer interface distal to the twofold axis This interface is further protected by the carbohydrate moiety present on domain I

The β-strands from domain I are named A_0 to I_0, those from domain II named to I and those from domain III named A to G in all cases labeled consecutively from the N terminus (in domain III distorti f the typical C type Ig fold leads to the creation f additional strands A_x, C_x and D_x) With the exception f the two short α hel ces of domain II all connections between the β strands f given domain as well as the linkers which lead from one domain to another are either β turns or loops which vary greatly in length. In general terms all such loops are either buried within the structure (inaccessibl to solvent) or exposed o one or more of th internal external and lateral surfaces of the dimer

On exposure to low pH the network of E protein dimers on the viral surface must rearrange into trimers This must envolve larg alterations to the monomer monomer contacts and possibly also to the relative domain orientations within a given subunit, if not to internal reorganizati f the tertiary structure of the domains themselves In participating in both proximal and distal contacts, domain II is likely to suffer the greatest changes, consistent with the fact that the binding of monoclonal antibodies to this domain is strongly affected by the dimer to trimer transition (Heinz FX, Stiasny K, Puschnerauer G H lzmann H, Allison SL Mandl CW Kunz C 1994 Structural-Changes And Functional Control Of The T ck Borne Encephalitis-Virus Glycoprotein E By The Heterodimeri Association With Protein prM *Virology* 198 109 117)

For the design of insertions f epitop peptides into the E protein of a given Fl vivirus and the subsequent evaluation of their viability the in entors f the present inventi developed the following strategy Initially t was necessary to produce three-dimensional model for the E protein f a selected Fl vivirus The sequence f the yell w fever 17DD strain was used fo this purpose and ts alignment with that f tick

borne encephalitis (tbe) virus was generated initially with the program MULTALIGN (Barton GJ Sternberg MJE, 1987 A Strategy For The Rapid Multipl Alignment Of Protein Sequences Confidence Levels From Tertiary Structure Comparisons *J M l Bi l* **198** 327 337) and subsequently with reference to the 3D structure of the latter such that all insertions and deletions were restricted to stereochemically reasonable positions A final alignment, including sequences from japanese encephalitis (strain JaOArS982) and Dengue type 2 (strain PR 159) is shown in Figure 2 and s significantly different in several important respects from that given by Rey et al 1995 The most important differences which are relevant to the subsequent insertion design are now described

An insertion of two residues (122 and 123 in tbe) introduced in tbe leading to complete readjustment of the alignment up to the region of the glycosylation site between β-strands E_0 and F_0 On comparing the tbe and yellow fever (yf) sequences within the region 120 to 150 (tbe sequence numbering) the alteration leads to an increase in the number of sequence dentities from 2 to 8 A similar improvement observed for japanese encephalitis (je) and an increase from 1 to 10 identities occurs in the case of Dengue 2 (d2) Besides the clear improvement in sequence identity the new alignment accommodates the two residue insertion (122 and 123 in tbe) in surface loop (between β strands d and)

A deletion of ne residue prior to strand f in yf and d2 is closed and transferred to the large deletion between β strands f and g The deletion in this region of the alignment given in Figure 2 is thus 6 residues in length for both yf and d2 as t is in je, when compared to tbe.

The asparagine/asparti acid rich segment of yf (residues 269 to 272) becomes an insertion between β strands k and l f domain II

The sequence alignment was used to generate 10 models for the yf E protein dimer using satisfaction of spatial restraints derived from the tbe dimeri structure empl ying the program MODELLER (Sal A, Blundell, TL 1993 Comparative model building by satisfaction of spatial restraints *J M l Biol* **234** 779 815) For each model default coordinate randomizati in cartesian space f 4 Å was employed prior to model optimization using the Variable Target Function Method (Braun W Go N

1985 Calculation f protein conformations by proton proton distance constraints new efficient alg rithm *J M l Biol* **186** 611-626) and Simulated Annealing Del berate misalignment of residues surrounding insertions and deletions was used in order to relax the hom logy constraints of these residues, permitting the insertion/deletion whil simultaneously maintaining acceptabl stereochemistry

The model which presented the lowest pseudo-energy given by –ln(P) where P is the MODELLER molecular probability density function, also presented excellent overall stereochemistry yielding for exampl PROCHECK (Laskowski RA, Macarthur MW M s DS Thornton JM, 1993 Procheck A Program To Check Th Stereochemical Quality Of Protein Structures *Journal Of Appl d Crystall graphy* **26** 283 291) G-factor of –0 1 equivalent to crystal structure of better than 1 5 Å resolution. The model was also evaluated using the method of Eisenberg (E senberg D Luthy R, Bowie JU 1997 VERIFY3D Assessment f protein models with three-dimensional profiles *M thod Enzymol* **277** 396-404 Bowi JU Luthy R, Eisenberg D A, 1991 Method to Identify Protein Sequences that fold into Known 3 Dimensional Structure *Science* **253** 164-170 Luthy R, Bowi JU Eisenberg D 1992 Assessment Of Protein Models W th 3 Dimensional Profiles *Nature* **356** 83 85) presenting VERIFY_3D score f 348 cl se to the expected value of 361 for protein of 786 residues (in the dimer) and well above the acceptability threshold f 162 Furthermore the normality f the model was assessed by atomi contact analysis using the WHATIF overall quality score (Vriend G 1990 What If A Molecular Modeling And Drug Design Program *J M l Graphics* **8** 52 57 Vriend G Sander C 1993 Quality-Control Of Protein Models Directional Atomi Contact Analysis *J Appl Crystall gr* **26** 47-60) which gave value of –0 964 showing the model to be reliabl

The model for the yf E protein shows slightly reduced contact area between subunits compared with tbe (1,242Å per monomer compared with 1 503Å^2) partly due to the reduced size f the fg loop which makes intersubunit contacts via His208 in tbe There is subsequent reduction in interdomain hydrophobi contacts as detected by LIGPLOT (Wallace AC Lask wski RA, Thornton JM, 1995 Ligpl t A Program T Generate Schemati Diagrams f Protein Ligand Interactions *Pr t Eng* **8** 127 134) These are compensated by th appearance of two potential intersubunit salt bridges

(between E201 and R243 and between R263 and E235) which appear in several f the ten models and which are absent, due to amino acid substitutions, in tbe These salt bridges presumably aid in stabilizing the yf dimer The contact made between the fusion peptide of the cd loop in domain II and the cavity between domains I and III of the opposite subunit is essentially retained in the yf E protein

The model for the yf E protein together with the sequence alignment was used to select potential insertion sites for heterol gous B and T cell epitopes. In both cases such an insertion site should 1) not disrupt or significantly destabilize the three-dimensional structure of the E protein 2) not interfere with the formation of the E protein network within the viral envelope 3) lie on the external surface f the virus such that t is accessible to anti body Although this criterion may not be strictly obligatory for T-cell epitopes t remains appropriate as sites on the internal surface may interfer with viral assembly 4) The site should preferably present evidence that sequence length variation is permissibl from the differences bserved between different flaviviruses (the site should show natural variance) 5) In the case of sites which present sequence length variation, preferably yf should present a smaller loop in such cases

The first criterion limits insertion sites to loops and turns between elements of secondary structure The second and third eliminate sites on the internal and lateral surfaces of the dimer and those that are buried. Of the remaining possible insertion sites the following can be said. The loop between D and represents an interdomain connection and shows littl structural ariabil ty That between loops and d represents the fusion peptide, is partially buried and highly conserved. That between d and shows little structural variation and includes ½-cystine residue which is structurally important. That between E_0 and F includes the glycosylation site in tbe and is potential insertion site as t shows great structural ariability and s highly exposed. That between G_0 and H_0 may be partially involved in lateral contacts, is small β turn and shows littl structural variation. That between f and g presents all of the desirabl characteristics in that t is six residues shorter in yf, j and d2 compared with tbe and is externally exposed. That between k and l s potential site as t shows an asparagine rich insertion in yf which may accommodate asparagine-rich epitopi B-cell sequences as described below That between B and C_κ poss bl site but may form part of th

lateral surface That between D and E shows structural ariation and presents an asparagine-rich sequence in yf which may accommodate asparagine-rich epitopes From the above the most promissing insertion site is that between β-strands f and g which form part of the fi e-stranded anti parallel β sheet of domain II The large deletio of six residues in this loop in yf compared to tbe leaves space at the dimer interface for large insertion without creating steric hindrance Besides the *fg* loop another promising insertion s te is th E_0F_0 as t shows great structural ariability and is highly exposed Although not wishing to be bound by any particular theory t postulated that the presence f one or more glycine residues immediately flanking the inserted epitope is advantageous in introducing conformational flexibility to the epitope in ts subsequent presentation

One alternative of the present invention to develop fl vivirus in general as vector for heterologous antigens is the insertion and expression of particular antigens including epitopes into sites structurally apart from areas known to interfere with the overall flavivirus E protein structure, specially into the *fg* loop or the E_0F loop of given flavivirus E protein The foreign inserted antigen, including epitope, may vary widely dependent on the immunogenic properties desired in the antigen. For exampl the foreign inserted antigen may include antigens from protozo such as malaria, from virus such as yellow fever dengue Japanese encephalitis tick borne encephalitis, fungi infections and others Additionally the maximum lenght of the antigen/epitope will depend on the fact that t wo ld not compromise the structure and the function f the flavivirus envelope

More particularly one strategy described here s the insertion f malarial gene sequences into the *fg* loop f YF17D E protein. Whil comparatively short sequences having only a few amin acid residues may be inserted, t is also contemplated that longer antigens/epitopes may be inserted. The maximum lenght and the nature of the antigen/epitope will depend on the fact that t would not compromise the structure and the function f the y llow fever virus envelope

Malaria remains one f th most important vector borne human diseases The concept that vaccination may be useful tool to control the disease based mainly on

the fact that individuals continually exposed to infection by the parasitic protozoan eventually develop immunity to the disease

The life cycl of the malaria parasite is complex, the several stages in humans are morphologically and antigenically distinct, and immunity is stage specifi It is only now becoming possible to define the full pattern of parasite gene expression in each stage

In short, in the parasite life cycl sporozo tes are delivered by the bite of the infected mosquito find their way to the liver and invade hepatocytes Two proteins have been identified as implicated in the recognition and invasion of the hepatocytes, CS (circumsporozoite) protein (Frevert, U P Sinnis, C Cerami, W Shreffler B Takacs, and V Nussenzweig. 1993 Malaria circumsporozoite protein binds to heparan sulfate proteoglycans associated with the surface membrane of hepatocytes J Exp Med. 177 1287 1298) and Thrombospondin related anonymous protein or TRAP (Sultan, A A. V Thathy U Frevert, K. J Robson, A Crisanti V Nussenzweig, R. S Nussenzweig, and R. Ménard. 1997 TRAP is necessary for gliding motility and infectivity of *Plasmodium* sporozoites Cell 90 511 522) Antibodies to proteins on the parasite surface might conceivably neutralize sporozoites and prevent subsequent development of liver stages. In the hepatocyte the parasite differentiates and replicates asexually as schizont to produce enormous amounts of merozoites that will initiate the infection of red blood cell Antigens specifi for the liver stage have been identified (Call JM, Nardin EH, Clavij P Boudin C Stuber D Takacs B Nussenzweig RS & Cochrane AH 1992 Recognition of different domains of the Plasmodium falciparum CS protein by the sera of naturally infected individuals compared with those of sporozoite-immunized volunteers.J Immunol 49(8) 2695 701 Nardin, EH & Nussenzweig RS 1993 T cell responses to pre-erythrocyti stages f malaria. rol in protection and vaccine development against pre-erythrocyti stages.Annu Rev Immunol 1993 11 687 727)

It has been proposed that these antigens together with those from the sporozoites are in part processed by the host cell and presented o the surface together with class I MHC molecules Thi presentation can lead to the recognition by cytotoxic T

lymphocytes and killing f the infected cells o stimulation of the T cells to produce cytokines can ultimately lead to the death of the tracellular parasite

Merozo tes surviving the pre-erythrocyti stages initiate the asexual blood stage infection, which is responsibl for th d sease The paras te infects erythrocytes which do not express class I molecules and theref re cytotoxi T lymphocyte are not important. Antibody binding to the surface of the merozoite probably plays major rol in immunity to asexual blood stages P tentially these antibodies could neutralize parasites or lead to Fc-dependent mechanisms f parasite killing g macrophages Complete protection against sporozo te challeng bserved in irradiated *P be ghe* sporozoite-immunized mice and *P fal iparum* sporozoite-immunized humans results from immune responses to antigens expressed by the parasite at the preerythrocyti stages of ts life cycl (Nardin EH Nussenzweig RS 1993 T cell responses to pre-erythrocytic stages f malaria role in protection and vaccine development against pre-erythrocyti stages *Ann. Rev Immunol* 11 687)

Antibody CD4 and CD8 T cells have been impl cated in preerythrocyti immunity (Schofield L Villaquiran J Ferreira A, Schellekens H, Nussenzweig R, Nussenzweig V 1987 γ interferon, CD8 T cells and antibodies required for immunity to malaria sporozoites *Nature* 330 664-666 Rodrigue M L S Murata K, Rodriguez D Rodriguez JR, Bacik I Bennink JR, Yewdell JW Garcia Sastre A, Nussenzweig RS Esteban M Palese P Zaval F 1994 Influenza and vaccinia viruses expressing malaria CD8 T and B cell epitopes Compariso f their immunogenicity and capacity to induce protective response *J Immunol* 153 4636-4648)

There are 6 main preerythrocyti antigens in *P fal parum* parasites the circumsporozo te protein (CS) thrombospondin related adhesio protein (TRAP) l ver stage antigens 1 and 3 (LSA 1 and 3) *Pfs* 16 and sporozoite threonine and asparagine-rich protein A umber f ep topes dentified on th diferent plasmodial proteins are being expressed in different systems towards immunogenicity studies (Munesinghe DY Clavijo P Call MC Nardin EH, Nussenzweig RS 1991 Immunogenicity f multipl antigen peptides (MAP) containing T and B cell epitopes f the repeat region f the *P falciparum* circumsporozo te protein *Eur J Immunol* 21 3015 3020 Rodrigues et al 1994 Shi YA, Hasnain SE Sacci JB H lloway BP F j ka H, Kumar N W hlhueter

R, Hoffman SL Collins WE, Lal AA 1999 Immunogenicity and *in* vitro efficacy f recombinant multistage *Plasmodium falciparum* candidate vaccine *Proc. Natl Acad. Sc USA* **96** 1615 1620 Aidoo M, Lalvani A, Gilbert SC, H JT Daubersies P Hurt N Whittl HC Druilhe P Hill AVS 2000 Cytotoxic T lymphocyte ep topes for HLA B53 and other HLA types in th malaria vaccine candidate liver-stage antigen 3 *Infect Immun* **68** 227 232)

Figure 3 shows schemati representation of the CS protein f *Plasmodium* sp (Nardin Nussenzweig,1993) and the location of epitopes expressed by recombinant YF 17D viruses f the present invention.

The CS protein contains an immunodominant B epitope located in ts central area. This ep tope consists of tandem repeats f species-specific amino acid sequences In *P falciparum* this epitope, asparagine-alanine-asparagine-proline, (NANP) has been detected in all isolates and thus represents an ideal target for vaccine development. Initial clinical trials with syntheti or recombinant peptides administered with alum resulted in induction f rather modest levels of antibodies to sporozoites and only individuals with the highest levels of antibodies were protected against *P falciparum* with delayed onset f parasitemi in others (Hoffman SL, Nussenzweig V Sadoff JC Nussenzweig RS 1991 Progress toward malaria preerythrocyti accines *S nce* 252 520-521) Other studies in animals of passive transfer of monoclonal antibodies to the repeats of the CS protein also suggest that antibodies can provide protection against sporozoite infection. The problem that remains is to engineer vaccines that elicit levels of antibodies in humans with the appropriate specificity and affinity to destroy all of the sporozo tes before they enter the hepatocytes In this regard it is noteworthy that immunization of humans with a singl dose YF 17D virus suffices to induce detectable levels f neutralizing antibodies to YF even after 40 years of the primary immunization (Monath, 1999) and that lends further support for the applicant f the present invention to use 17D virus to express relevant pathogens epitopes especially the relevant malarial ep topes towards the development of new live viral vaccines capabl of protecting, being life-long against yellow fever and econd disease, for exampl malaria.

The liver stage of the parasite is also target for vaccine development because t offers additional antigens and in contrast to the short l ved existence f sporozoites in the

bloodstream f the mammal host, human malarias devel p in the l ver for several days The effector mechanisms against these intrahepatocyti forms are probably cytotoxi T cells that destroy the infected hepatocytes and γ-interferon that inhibits parasite development

Preerythrocyti immunity to *Plasmod um* s mediated in part by T lymphocytes acting against the liver stage parasite These T cells must recognize parasite-derived peptides o infected host cells in the context of major histocompatibility complex antigens T-cell mediated immunity appears to target several parasite antigens expressed during the sporozo te and liver stages of the infection. A number of such CTL epitopes, present on diferent proteins of th preerythrocytic stages, have been identified in humans l ving in malaria endemi areas and are restricted by variety of HLA class I molecul (Aidoo M, Udhayakumar V 2000 F ld studies f cytotoxi T lymphocytes in malaria infections implications fo malaria vaccine development. *Parasit l. Today* 16, 50-56)

Cytotoxi T cells mostly CD8 which require the class I antigen presentation pathway are primarily generated by intracellular microbial infections and have been most thoroughly investigated in viral infections Recombinant viruses expressing the desired foreign ep topes are therefore l gical pproach towards generating the cytotoxi T cell f the desired specificity

Miyahira et al (Miyahira Y Garcia Sastre A, Rodriguez D Rodriguez JR, Murata K, Tsuj M, Palese P Esteban M, Zavala F Nussenzweig RS 1998 Recombinant viruses expressing human malaria antigen can elicit potentially protective immune CD8 responses in mice *Proc. Natl Acad. Sc USA* 95 3954-3959) have studied in mouse model th immunogenicity f CTL epitope located on CS f *P fal iparum* The CTL epitope (DELDYENDIEKKICKMEKCS) was expressed in bicistroni neuraminidase gene f the influenza D strain. Recombinant vaccinia included the whol CS gene containing both humoral and CTL epitopes Immunizati f mice with either fl o vaccinia licited modest CS-specifi CD8 T cell response detected by interferon γ secretion of individual immune cells Priming f mice with the recombinant flu virus and boosting with the vaccinia recombinant resulted in striking enhancement f this response

A vaccinia virus expressing several *P f l iparum* antigens was developed and used in clinical trial Whil cellular immune responses were elicited in ver 90% f the individuals antibody responses were generally poor Of the 35 volunteers challenged, only one was completely protected, although there was significant delay on the onset of parasitemia (parasitemia (Ockenhouse CF Sun PF Lanar DE, Wellde BT Hall BT Kester K, Stoute JA, Magill A, Krzych U Farley L Wirtz RA, Sadoff JC Kaslow DC Kumar S Church LW Crutcher JM, Wizel B Hoffman S Lalvani A, Hill AV Tine JA, Guito KP de Taisne C Anders R, Ballou WR, et al 1998 Phase I/IIa safety immunogenicity and efficacy trial f NYVAC-Pf7 pox vectored, multiantigen, multistage vaccin candidate for Plasmodium falciparum malaria. J Infect Dis 177 1664- 1673)

Recombinant Sindbi virus expressing minigene encoding the CD8 T-cell epitope SYVPSAEQI of the CS protein f rodent malaria parasite *P yoel* when inoculated subcutaneously in mice induced a large epitope-specific CD8 T-cell response This immunization also protected mice against infection by sporozo tes (Tsuj M, Bergmann CC Takita Sonoda Y Murata K, Rodrigues EG Nussenzweig RS Zavala F 1998 Recombinant Sindbis virus expressing a cytotoxic T lymphocyte epitope f malaria parasite or f influenza virus elicit protection against the corresponding pathogen in mice *J Virol* 72 6907 6910)

It has also been shown that mice immunized by singl dosis f recombinant adenovirus expressing the CS protein of *P yoel* elicits a high degree f resistance to infectio mediated primarily by CD8 T cells (Rodrigues EG Zavala F Eichinger D W'lson JM, Tsuj M 1997 Single immunizing doses f recombinant adenovirus efficiently induces CD8 T cell mediated protective immunity against malaria. *J Immunol.* 158 1268 1274)

Because f the complexity f the parasite and ts life cycl there is consensus that highly effective malaria vaccine would require combination of key antigens and/or epitopes from different stages of the life cycl and that induction of both humoral and cellular immunity required for optimal efficacy Such vaccine would also circumvent the problems with host geneti restriction and antigeni variability in the case f singl antigen based vaccines So far however several attempts to attain

protective immmunity with different antigens preparations from several stages have not produced convincing results

The critical issues for the multivalent approach as with singl antigen are the dentification of antigens that will induce (partially) protective response in all or most of the target population, the delivery f these antigens in form that will stimulate the appropriate response and th delivery ystem must allow presentation f the antigens in form that stimulates the mmune system The development described here which utilizes fl vivirus for the expression f defined pathogen antigens/epitopes should address the issues of presentation to the target population. Regarding the YF 17D virus, t an extremely immunogenic virus inducing high antibody serocon ersion rates in vaccinees of different geneti background.

The appl cant of the present invention particularly explores the feasibility f using the YF 17D virus strain and substrains thereof not only as very effecti proven yellow fever vaccine but also as vector for protective antigens, particularly protective epitopes Thi will result in the development of vaccin simultaneously effective against yell w fever and other diseases which may occur in the same geographical areas such as malaria, dengue Japanes encephalitis, tick borne encephal tis fungi infecti ns etc

The main goal was to establish general approach to insert and expres singl defined antigens, including epitopes into sites structurally part from areas known to interfere with the overall flavivirus E protein structure, specially into the *fg* loop or the E_0F loop f the E protein f given fl vivirus such as y llow fever dengue, Japanese encephal tis tick borne encephal tis that can be used as new l ve vaccine inducing long lasting and protective immune response More parti ularly the present invention is related to general approach to expres singl defined ep tope on the *fg* loop of the E protein of YF17D virus

As used herein, the term Fl vivirus means wild virus attenuated virus and recombinant virus including chimeri virus

The geneti manipulation of the YF 17D genome was carried t by using the YF infectious cDNA as riginally developed by Rice et al (1989) which consists of two plasmids named pYF5 3 IV and pYFM5 2 Th YF genome was splited in two plasmids

due to the lack of stability f some virus sequences in the high copy number plasmid vector pBR322 However in the case of dengue 2 virus, full length cDNA was steady cloned in the same plasmid (Kinney RM, Butrapet S Chang GJ Tsuchiya KR, Roehrig JT Bhamarapravati N & Gubler DJ 1997 Construction of infectious cDNA clones for dengue 2 virus strain 16681 and ts attenuated vaccine derivative strain PDK 53 Virol gy 230 300-308) When cut by specifi restriction enzymes (ApaI or NsiI and AatII) specifi restriction fragments are generated which upon ligation reconstitute the complete YF genome in the cDNA form. Restriction f this cDNA form with XhoI linearizes the cDNA and allows *vitro* synthesis of capped RNA from the SP6 promoter sequence Such RNA when transfected into cultured cell gives rise to infecti us virus The phenotype of this virus has been tested in monkeys (Marchevsky RS Mariano J Ferreira VS Almeida E, Cerqueira MJ Carvalho R, Pissurno JW Travassos da Rosa APA, Simões MC Santos CND Ferreira II M ylaert IR, Mann GF Rice CM and Galler R. 1995 Phenotypic analysis f yellow fever virus derived from complementary DNA *Am J Trop Med. Hyg* 52, 75 80) and these studies indicated the need for genetic modification of the cDNA as to make the resulting virus more attenuated. This has been carried out by Galler and Freire (US 6 171 854) and resulted in new versions f pYF5 3 IV and pYFM5.2 named G1/2 and T3/27 respectively The physical map and sequences of both plasmids are shown elesewhere and these plasmids have been deposited at ATCC (ATCC # 97771 and ATCC # 97772)

Having the genome split into two plasmids is convenient for insertion cloning purposes but not deal for the recovery of the virus S in order to accomplish a higher specific infectivity of the transcripts the applicant developed a full length cDNA clone for the YF genome. It was identified in the literature plasmid pACNR1180 (Ruggli N Tratschin J D Mittelholzer C Hofmann MA. 1996 Nucleotide sequence of classical swine fever virus strain Alfort/187 and Transcription of infecti us RNA from stably cloned full length cDNA *J V rol* 70 3478 3487) which has replication origin named P15A that allows only limited replication of the plasmid reducing the number of plasmid DNA molecules per bacterial cell This plasmid system was used by the authors to stabilize the genom f the classical swine fever virus with a genome ize

cl se to that f YF virus This plasmid, pACNR1180 was provided by Dr JD Tratschin f Institute f Virology and Immunoprophylaxi Switzerland

Besides pACNR1180 ther vectors which provide the stabilization f the YF virus genome can be used to prepare the plasmids of the present invention. Specifi examples include plasmids which have repl cation origin that allows only limited replication of the plasmid reducing the umber f plasmid DNA molecules per bacterial cell vectors consisting f l w copy number plasmids such as pBeloBAC11 (Almazan F Gonzalez JM Penzes Z, Izeta A Calvo E Plana Duran J Enjuanes L 2000 Engineering the largest RNA virus genome as an nfectious bacterial artificial chromosom *ProcNatlAcadSc USA*97 5516-5521) Another possibil ty is the use f high copy umber plasmids such as pBR322

The new versi of pACNR1180 was named pACNR1180Nde/Sal It was obtained by removing most of the unique restriction site of pACNR1180 by digestion with NdeI/SalI, filling in the ends by treating with Klenow enzyme ligating and transforming *E. l* XL1 blue

To generate pYF17D/14 fragments NotI (13 059)-NsiI (11 158) and SalI (3,389)-XhoI (1951) from G1/2 plasmid (described in detail in US 6 171 854) were first ligated to NotI/XhoI-digested pACNR1180Nde/Sal The resulting plasmid was named NSK7 (map not shown) and used to clone the remaining of the YF cDNA contained in plasmid T3/27 (described in detail in US 6 171 854) by digestion with NsiI (11 158)-SalI (3 389) ligation of the appropriate fragments and transformation f the sam bacterium. Restriction enzyme mapping confirmed the expected physical structure f plasmid containing the complete YF cDNA. A total of 8 clones were analysed, virus was recovered from 5 ut of 6 tested and complete nucleotide sequence determination confirmed th expected YF sequence The plasmid contains 13449 base pairs and was named pYF17D/14 (F gure 4)

pYF17D/14 contains an amp illin resistance gen from po tion 13 196 to 545 and the p15A origin f replication (nts 763 to 1585) both derived from plasmid pACYC177 (Ruggl et al 1996) Nucleotides 12 385 to 12 818 correspond to the SP6 promoter The YF genom is transcribed in thi plasmid from the pposite strand as the complete genom spans ucleotide 12,817 to 1951 All insertions at the fg loop of the

yellow fever virus E protein are made at the EcoRV site of YFE200 plasmid and from there incorporated into pYF17D/14 by exchanging fragments NsiI/NotI Other representative sites are shown in Figure 4

Plasmid G1/2 contains the YF 5 terminal sequence (nt 1 2271) adjacent to the SP6 phage polymerase promoter and 3 terminal sequence (nt 8276-10862) adjacent to the XhoI site used fo production f run off transcripts To allow the insertion of foreign sequences into the fg loop as defined by the analysis of the 3D structure f the E protein we have created by in vitro mutagenesis a restriction site (EcoRV) at nucleotide 1568 The creation f this site led to two amino acid changes in the E protein at positions E-199 and E-200 (E→D T→I respectively).

It is important to emphasize that the creation of the restriction site of choice is dependent on the nucleotide sequence that makes up each loop and will vary according to the Flavivirus genome sequence to be used as vector Therefore, the restriction site used for on Flavivirus EcoRV site is specifi to the fg loop of yellow fever but (cortar?) may not be useful for insertion into the genome of other flavivirus Those skilled in the art will dentify suitable sites by using conventional nucleotide sequence analysis software for the design of other appropriate restriction sites.

It s noteworthy that amino acid 200 is a K in Asibi, T in all 17D viruses analyzed and I in E200 The fact that E200 is position that is altered in all 17D viruses would suggest that particular alterati important for the attenuation of 17D virus and alterations there might compromise that trait. However the mutation introduced for the creation of the insertion site does not lead to reversion to the original amino acid and both are very distinct in character Moreover t is likely that attenuation of 17D is multifactorial and not only related to the structural region as uggested the phenotype of chimeri 17D/JE-Nakayama in the mouse model f encephalitis (Chambers et al 1999) In addition no major alterations in the E protein structure was apparent when modelling these amino acids changes into the predicted 3D model Finally the YF17D/G1/2-derived virus grew in tissue culture to th same level and was as neurovirulent for mice as the parental 204/DD virus derived from G1/2/T3/27 cDNA.

In order to create the EcoRV site in the YF sequence we subcloned a NotI/ApaI fragment of G1/2 into the pAlter plasmid (Promega In) and performed the mutagenesis

as indicated by the manufacturer in the presence of mutagenizing synthetic ligonucleotide specifying the desired EcoRV site Mutants bearing the site were identified by restriction enzym analysi f plasmid DNA and the ApaI/NotI fragment contained in one f the clones was sequenced entirely to confirm the restriction site was the sole mutation in the YF sequence Such fragment was cloned back into G1/2 plasmid and the resulting plasmid was named pYFE200 the map f which is shown in F gure 5

This plasmid was derived from pYF5 3TV originally described by Rice et al, 1989 as modified by Galler and Freire (patent number US 6 171 854) and herein. It contains 6905 nucleotides and region 1 2271 corresponds to the 5 UTR, C prM/M and E genes This region fused through an EcoRI site at the E gene (2271) to another EcoRI site in the NS5 gene (position 8276) At position 1568 in the E gene we created the EcoRV site which is used for epitope insertion into th E protein fg loop This plasmid also consists of the NS5 gene from ucleotide 8276 to the last YF genome nucleotide (10 862) containing therefore part f the NS5 gene and the 3 UTR. Nucleotides 5022 to 5879 correspond to the ampicillin-resistance gen and 6086 to 6206 to the origin f replication, both derived from pBR322 plasmid Besides pBR322 other vectors known to specialists in the art may be used such as pBR325 BR327 pBR328 pUC7 pUC8 pUC9 pUC19 λ phage, M13 phage, etc The location f relevant restriction enzyme sites is shown in F gure 5

YFE200 plasmid has been deposited at ATCC under number PTA2856 pYFE200 was used to produce templates together with T3/27 which allowed the recovery of YF virus that resembles YF 5 2/DD virus (US 6 171 854) in growth properties in Vero and CEF cells plaque size, protein synthesis and neurovirulence for mice (data for E200 and the recombinants derived thereof are shown in the examples)

The template to be used for the regeneration of YF 17D virus is prepared by digesting the plasmid DNA (YFE200 and T3/27) with NsiI and SalI After digestion with XhoI to linearize the ligated DNA, the template was used for *in vitro* transcription Virus has been recovered after RNA transfection of cultured animal cells

The animal cell culture used herein may be any cell insofar as YF virus 17D strain can replicate Spe ifi examples include H 1 (deri ed from carcinoma f human

uterine cervix) CV 1 (derived from monkey kidney) BSC-1 (derived from monkey kidney),RK 13 (der ed from rabbit kidney) L929 (derived from mouse connective tissue) CE (chicken embry) cell CEF (chicken embryo fibroblast) SW 13 (derived from human adrenocortical carcinoma) BHK 21 (baby hammster kidney) Vero (african green monkey kidney) LLC MK2 (derived from Rhesus monkey kidney) etc

In preferred embodiment f the present invention, Vero cells are the preferred substrate in all production steps as the titers btained in different growth curves, as well as the geneti stabil ty gave better results Primary cultures of chicken embryo fibroblasts (CEF) may be second choice to be used as substrate in all production steps as these cells have been used for measles vaccine production for years with extensive experience in ts preparation and quality controls a number f Standard Operating Practices (SOP) s availabl and a patent application dealing with the production of YF vaccine in CEF cultures has been filled (EP 99915384 4)

Therefore the flavivirus system described here, more particularly the YF system, provides powerful methodology for the development of unlimited formulations of recombinant viruses expressing different epitopes It is anticipated that the appropriate formulation of several recombinant viruses should elicit the adequate immune response to cope with the different parasite stages

The following examples are illustrative of the invention and represent preferred embodiments Those skilled in the art may know or be abl to find using no more than routine experimentation, to employ other appropriate materials and techniques, uch as the above mentioned ectors, culture cells and transfection methods

Exampl 1 Structural analysis of th inserti of pecific protein epitopes

Ten models were produced for the inserti SYVPSAEQI in the fg loop regi using the alignment shown in Figure 6 in which the insertion is made between E199 and T200 The inserted residues will be refered to as 199A to 199I

The pseudo-energies f the best five models were comparable to those of the native yf model Their structures are variabl as ne skilled in the art would expect from an insertion f nine residues in length. The variation in structure f the loop leads to correlated variation in th neighbouring loop between β strands k and l The glutamine

sidechain f residue Gln199H (the eigth residue f the inserted peptide) in several of the best models shows conformation compatibl with the formation of hydrogen bo d via ts $N_{\varepsilon 2}$ to the carbonyl of Val244 f the opposite monomer in similar fashion to that made by the N of His208 in tbe One representative model had an overall G-factor of 0 07 equivalent to structure f < 1 0 Å resolution and has good stereochemistry in the region f the insertion. The total Verify_3D score for the segment from 199 to 200 (including the nine inserted res dues) is +3 69 (mean value of 0 34 per res due) indicating that the residue of the loop have been built into favourabl chemical environments By comparison the mean alue per residue for the equivalent loop in the crystal structure f tbe only 0 28 The verage contact area per monomer for this model 1 460 $Å^2$ comparable to that bserved in the crystal structure of tbe as anticipated by the introduction f the insertion close to the proximal interface (F gure 7)

During the constructio f the insertion te two bstitutions were made to the amino ac d sequence E199D and T200I The consequence of such substitutions was analyzed with reference to the model The substitution E199D is not expected to have serious consequences as t is conservative in nature, is bserved in tbe and may lead to salt bridg with K123 There also no significant change in the quality index for this residue as determined by WHATIF The substitution T200I appears acceptabl as the insertion leads to rotation f th T200 sidechain in many of the ten models resulting in t being directed towards a hydrophobi pocket close to W203 the aliphati region f R263 and L245 The substitution also retains the ramification on Cβ

Using an dentical approach to that described above the insertion DYENDIEKKI was introduced into the yf E protein. An dentical alignment to that shown in F gure 6 was used in thi case with the exception of the insertion sequence tself. Model were produced using an dentical protocol to that described above for the SYVPSAEQI insertion and were f similar quality Typically the models present PROCHECK G-factors of –0 1 (equivalent to crystal structures with resolutio between 1 0 and 1 5 Å) and have around 90% f the residue within the m st favourable regi ns f the Ramachandran plot Although the loop conformations btained show considerabl variation, several suggest the po sibl appearance f new inter-subunit interactions

which may affect dimer stability and/or influence the pH dependent dimer to trimer transition. Potential salt bridges suggested by the model include those between Glu199C Asp199E and/or Gl 199G (the third, fifth and seventh residues of the insertion respectively) with Arg243 (native yf umbering) of the opposite subunit as well as Lys199H with the carbonyl of Leu65 of the opposite subunit. The salt bridge seen in the native yf model between Arg263 f one monomer and Glu235 f the other is retained In none of the models did Lys199H form hydrogen bond equivalent to that made by His208 to the opposite subunit in tbe but potential hydrogen bond to the carbonyl f Leu65 is poss ble Lys199I may form salt bridge with Gl 199 f the sam subunit and such an interaction should be feasibl even after the glutami acid to asparti acid substitution. On dimerization each subunit loses an verage f 1 483 Å f accessibl surface area (based on one such model) comparabl to that of tbe, principally due to the reinsertion of a large loop between β-strands f and g

Of note is the fact that neither of the two loop insertions described lead to steric clashes between the subunits, they preserve good steochemistry as well as (in the case of the best models) yielding reasonable chemical environments for the amino acid sidechains of the insertion. This is achieved without an exaggerated increase in the contact area between monomers suggesting that there is indeed no spatial restriction to the proposed insertions

Compared with the loop observed experimentally in the crystal structure of the E protein from the tbe virus, the two insertions described above are three residues longer These additional residues are accommodated in most models by an additional protrusion of the structure on the external surface (although in one model short stretch of α helix is observed) Thi suggests that t may be possibl to accommodate even larger peptides within this insertio site

Ten models were generated for the insertion GG(NANP)$_3$GG and protocol dentical to that described in the previous two examples Different from the previous two examples however is the fact that this insertion is a B-cell epitope, is considerably larger includes a repeated tetrapeptide and rich in proline residues In this case in order that the B-cell epitope should be as exposed as possibl (in order to be antibody accessible) and given adequate conformational freedom, as well as in fac l tating its

insertion into the fg loop in the form of an Ω loop (Leszczynski JF Rose GD 1986 Loops In Globular Proteins A Novel Category Of Secondary Structure *Science* 234 849 855) two glycine residues (spacers) were added to both the N and C-terminus of the epitope, $(NANP)_3$ Due to the reduced size of the glycine sidechain and to its achiral Cα atom, greater region of Ramachandran space accessible to its mainchain dihedral angles φ and ψ increasing the chance of sucessful insertion which does not prejudize the local structure in the region of the insert. This approach produced models which presented excellent stereochemistry (PROCHECK G-factors of the order of −0 1 equivalent to crystal structures of resolution 1 0 to 1 5 Å) The loop insertion itself is also free of stereochemical strain. We surmise that this is the result of the N and C-terminal glycine spacers which serve to lift the loop free of the external surface of the protein. In several of the models one or more of these glycines adopt backbone conformations which would be prohibited for other amino acids The remainder are generally in extended (β) conformations These factors appear to emphasize the importance of their inclusion.

Those skilled in the art will know that reliable prediction of the structure of such loop is beyond current theoretical approaches There is therefore considerable spread in the resulting conformation in the ten models generated (Figure 8) This wide distribution of conformations (of approximately equal pseudo-energy) does not lead to significant alteration of the remainder of the structure, emphasizing that the loop has access to large volume of space as originally designed. This does not mean that the loop would necessarily be unstructured, merely that many possible structures are accessible to the loop increasing the probability that that which is immunologically relevant may be adopted, even within the context of its insertion into the yfE protein. Figure 9 illustrates better the volume considerations

The (NANP) sequence in the ten models has mean relative accessible surface area (compared to its unfolded structure) of 63 7% This compares with mean value of 27 4% for the structure overall demonstrating that the insertion has very large relative accessibility as intended. If the glycine spacers are eliminated this value for the $(NANP)_3$ sequence falls to 53 6% demonstrating that the spacers have role in increasing the exposure of the epitope Examination of the models shows that

ıncreasıng the length f th glycıne spacer beyond two resıdues would appear to brıng no addıtıonal advantage ın exposıng the epıtope but may represent an entropıc cost for the structure whıch could lead to ts destabılızatıon. Two glycınes appears the optımum to us

It ıs reasonable expectatıon that the envelope proteıns of japanese encephalıtıs vırus and Dengue 2 would accommodate the above descrıbed ınsertıons equally well as yellow fever vırus The alıgnment of F gure 2 shows that sımılar sıx resıdue deletıon present ın all three vıral envelope proteıns compared to tbe Models for the j and d2 E proteın, produced usıng sımılar protocol to that for yf frequently show β turn structure for the fg loop, stabılızed by maınchaın hydrogen bond. A sımılar β turn also observed ın yf f the alıgnment restraınts are relaxed around resıdues f the fg loop ıtself The models for j show potentıal ıntersubunıt salt brıdge between Lys201 wıth Glu243 (j numberıng) of th opposıte subunıt. Thıs glutamıc acıd ın yf ınteracts wıth Arg263 whıch has been substıtuted by valıne ın j Sımılar contacts to those of yf are also observed around the dıstal dımer ınterface sıte A representatıve model for the j E proteın has a PROCHECK G-factor of –0 1 89 9% of resıdues ın the most favourable regıons of the Ramachandran plot, good sterochemıstry ın the regıon of the fg loop (whıch adopts a type I β turn) a good WHATIF qualıty score for the fg loop (resıdues 203 to 212 yıeldıng and verage of 0 768) and burıes a mean accessıble surface area f 1 048 Å per subunıt on dımerızatıon. Sımılar results are obtaıned for d2 ın whıch the fg loop adopts eıther the type I or type II β turn conformatıon. From these data those skılled ın the art wıll be abl to apply the ınsertıon strategy descrıbed above for yf to other flavıvıruses such as j and d2

The sıte whıch comprıses the regıon f β strands f and g ıncludıng the fg loop whıch form part of the fıve-stranded antı parallel β-sheet of domaın II of the flavıvırus envelope proteın comprıses the regıon of amıno acıd 196 to 215 wıth reference to the tıck borne encephalıtıs vırus sequence descrıbed ın figure 2 More partıcularly the sıte ıs the loop area between β strands f and g whıch form part of the fıve-stranded antı parallel β-sheet of domaın II of the flavıvırus envelope proteın (amıno acıd 205 to 210 wıth reference to the tıck borne encephalıtıs vırus sequence descrıbed ın figure 2)

Additionally the site which comprises the region f E_0 and F strands including the E_0F_0 loop which form part f the eight stranded β barrel of domain I f the flavivirus envelope protein comprise th regio of amin ac d 138 to 166 with reference to the tick borne encephalitis virus sequence described in figure 2 More particularly the site the loop area between E_0 and F_0 strands which form part of the eight stranded β barrel f domain I (amino acid 146 to 160 with reference to the tick borne encephalit virus sequence described in figure 2)

Exampl 2

) Derivation f Plasmid pACNR1180Nde/S l

pACNR1180Nde/Sal the new version f plasmid pACNR1180 is obtained by removing most of the unique restriction sites of pACNR1180 by digestion with NdeI/SalI, filling in the ends by treating with Klenow enzyme ligating and transforming *E.col* XL1 blue Thi new vers f pACNR1180 was named pACNR1180Nde/Sal

b) Derivation of Plasmid pYF17D/14

T generate pYF17D/14 fragments NotI (13 059) NsiI (11 158) and SalI (3,389)-Xh I (1951) from G1/2 plasmid (described in detail in US 6 171 854) were first lig ted to N tI/XhoI-digested pACNR1180Nde/Sal The resulting plasmid was named NSK7 (map not shown) and used to clone the remaining of the YF cDNA contained in plasmid T3/27 (described in detail in US 6 171 854) by digestion with NsiI (11 158)-SalI (3 389) ligation f th appropriate fragments and transformation of the sam bacterium. The plasmid contains 13449 base pairs and was named pYF17D/14 (F gure 4)

pYF17D/14 contains an ampicilun resistance gene from position 13 196 to 545 and the p15A origin of repl cation (nts 763 to 1585) both derived from plasmid pACYC177 (Ruggli et al 1996) Nucleotides 12,385 to 12 818 correspond to the SP6 promoter The YF genome is transcribed in this plasmid from the oppo te strand as th complete genome spans ucleotide 12 817 to 1951 All insertions at the fg loop of 17D virus E protein are made at th EcoRV site of YFE200 plasmid and from there

ıncorporated ınto pYF17D/14 by exchangıng fragments NsıI/NotI Other representatıve sıtes are shown ın F gure 4

) Derıvatıo of Plasmıd YFE200

In order to create the EcoRV sıte ın th YF sequence we subcloned NotI/ApaI fragment f G1/2 ınto the pAlter plasmıd (Promega Inc) and performed the mutagenesı as ındıcated by the manufacturer ın the presence of mutagenızıng syntheti olıgonucleotıde specıfyıng the desıred EcoRV sıte Mutants bearıng the sıte were ıdentıfied by restrıctıon enzyme analysıs f plasmıd DNA and the ApaI/NotI fragment contaıned ın one of the clones was sequenced entırely to confirm the restrıctıon sıte was the sole mutatıon ın the YF sequence Such fragment was cloned back ınto G1/2 plasmıd and the resultıng plasmıd was named YFE200 the map of whıch ıs shown ın Fıgure 5

d) Preparatıon f Large mounts of plasm d DNA

T prepare plasmıds DNAs from bacterıa, glycerol stocks of the *E. col* harborıng each of the two YF plasmıds, pYFE200 and pYF17D/14 must be avaılable Lurı Broth-50% glycerol medı used ın the preparatıon f the stocks, whıch are stored at 70°C Frozen alıquots of the pDNA are also avaılabl

The bacterıa are grown ın 5 ml LB contaınıng ampıcıllın (50 µg/ml) for YFE200 and ampıcıllın (50 µg/ml) plus tetracyclın (15 µg/ml) overnıght at 37°C for NSK14-harborıng bacterıa. Thıs used to ınoculate 1 100 large volumes of LB (usually 100-200 ml) At OD_{600} of 0 8 chloramphenıcol ıs added to 250 µg/ml for the amplıfıcatı of the plasmıd DNA and ıncubated further overnıght. The plasmıd ıs extracted usıng the alkalıne lysıs method The final DNA precıpıtate ıs ressuspended ın TE (Trıs-EDTA buffer) and ces um chlorıde added untıl a refractıon ındex of 1 3890 ıs reached. The plasmıd DNA ıs banded by ultracentrıfugatıon for 24 hours The banded DNA ıs reco ered by puncturıng the tube extractıng wıth butanol and extensıve dialysıs

The yıelds are usually 1 mg f pDNA/l ter f culture for pYFE200 and 0 02 mg/lıter for pYF17D/14

pYFE200 was deposited on December 21 2000 under accesion umber PTA 2856 with the American Type Culture Collection (ATCC) 10801 University Bl d. Manassas Va. 20110-2209

Exampl 3

Preparation of DNA template

The template to be used for the regeneration of YF 17D virus is prepared by digesting the plasmid DNA (YFE200 and T3/27) with NsiI and SalI (Promega Inc) in the same buffer conditi ns as recomended by the manufacturer Ten µg f each plasmid are digested with both enzymes (the amount required is calculated in terms of the number f pmol hits present in each pDNA in order to achieve complete digestion in 2 hours) The digestion is checked by removing an aliquot (200 ng) and running t on 0 8% agarose/TAE gel When the digestion is complete the restriction enzymes are inactivated by heating

Linearization f the DNA resulting from the ligation f both NSiI/SalI-digested plasmids carried out by the use of XhoI, and is performed with buffer conditions according to the manufacturer (Promega) Th resulting product thereafter phenol chloroform extracted and ethanol precipitated The prec pitate is washed with 80% ethanol and resuspended in steril RNase-free Tris EDTA buffer A template aliquot is taken for agarose gel analysis together with commerc al markers for band sizing and quantitation The template is stored at 20 C until use for *tro* transcription.

Exampl 4

RNA transcriptio from cDNA template f th present in entio

Preparation of the DNA template from the full length pYF17D/14 clone for *tro* transcripti simpler as t requires less pDNA usually 1 2 µg which is digested with XhoI for linearization. Digestion, cleaning of th DNA and quality f the template are carried ut as described above Transcription is as described in US 6 171 854 RNA produced from XhoI linearized pYF17D/14 DNA templates were homogeneous and mostl full lenght in contrast to the two-plasmid system-derived RNA (data not shown)

Exampl 5

RNA transfection

Transfection of such RNA were carried out in similar manner as described in US 6 171 854 (Galler and Freire) Transfection of such RNA gave rise to virus which was similar to YF v5 2/DD in its plaque size growth in Vero and CEF cells, neutralization by hyperimmune sera to YF and protein synthesis

Example 6

C nstructio of th recombinant YF17D/8 virus expressing humoral B cell ep tope.

For the expression of the $(NANP)_3$ humoral epitope of *P falciparum* (see Table 1 below) two surrounding glycines were added on each side as to compensate for the likely formation of β turn in the ep tope given the presence f consecutive proline and asparagines and give the loop more flexibl structure Therefore the synthetic oligonucleotide insertion at the EcoRV site of pYFE200 plasmid which corresponds to the amino acid sequence depicted in Table 1 gives rise to plasmid pYFE200/8 This plasmid was deposited on December 21 2000 under accesion number PTA 2857 with the American Type Culture Collection (ATCC) 10801 University Blvd. Manassas Va. 20110-2209

Blunt-ended ligation f the synthetic oligonucleotide disrupts the EcoRV site and that was used to screen for plasmids bearing the insertion EcoRV⁻ plasmids were sequenced around the insertion region to verify the orientation of the insert. Plasmids with correct insert orientation which therefore kept the required open reading frame were used for further sequence encompassing the whol structural region contained in this plasmid, more specifically the area comprised between the NotI and NsiI sites The NotI/NsiI cDNA fragment f 1951 bp was ligated to the NsiI/MluI fragment of 1292 bp f the the T3 plasmid and the NotI/MluI backbone of 10,256 bp of the full lenght clone pYF17D/14 Resulting plasmids were first screened for size and thereafter for the productio of infectious transcripts by l pid mediated RNA transfection of cultured Vero cells as described (Galler and Freire US 6 171 854) The resulting virus

was named 17D/8 After transfection, YF 17D/8 had titer (measured by plaquing Vero cell monolayers) of about 4 0 log $_0$PFU/mL. After one-single passage in Vero cells viral stocks had titer of 6 1 log $_0$PFU/ml The presence f the insert in the viral genome was checked by sequencing the cDNA made to the virus present in the cell culture supernatant derived from the transfection.

T bl 1 Amino acid eq ence and pecificity f (NANP)$_3$ hum ral epitop fo insertion into YF E protein

Seq ence	Antigen epitope	*source*	Clo
EMD GGNANPNANPNANPGG IES	CSP B	*P fal iparum*	17D/8

Example 7

Epitope expressi by th YF 17D/8 recombinant virus

To investigate the expression of the 16-amino ac d ep tope in the E protein of 17D/8 virus t was performed an indirect immunofluorescence assay using monoclonal antibody directed to the (NANP)$_3$ repeat ((MAb 2A10 Za ala F Cochrane AH, Nardin EH, Nussenzweig RS Nussenzweig V 1983 Circumsporozoite proteins f malaria parasites contain single immunodominant region with two or more dentical epitopes *J Exp M d.* 157 1947 1957) and a mouse polyclonal hyperimmune serum to YF 17D (ATCC) The IFA was made using glutaraldehyde-fixed VERO cells infected for 48 h with YF17D/14 virus or with recombinant virus YF17D/8 carrying (NANP)$_3$ epitope at m (multiplicity f infectio) of 1 The samples were treated with twofold dilutions of YF Hiperimmune ascitu fluid (ATCC) and mouse IgG directed against the immunodominant B cell epitope NANP of P falciparum CS protein purified from 2A10 monoclonal antibody as described (Zavala et al, 1983 gift f Dr M Rodrigues, Escola Paulista de Medi ina) Positive cell were evidenced by the binding f FITC conjugated anti mouse IgG

Only Vero cell infected with 17D/8 virus were recognized by mab 2A10 whereas the YF hyperimmune serum detected the YF antigens in both cases (see Figure 10)

Exampl 8

Radi labeling, Radioimm precipitatio and P lyacrilamid gel electroph resis.

Additional evidence for correct plasmodial epitope expression on the surface of the 17D/8 virus was btained by immunoprecip tation of metaboli labeled viral proteins. VERO cells were infected at multiplicity f 1 PFU/cell After 24 h incubation, the cells were labeled with [^{35}S]methionine for 1 h and lysed under nondenaturing conditions as described previ usly (Post et al 1990) Cell extracts were immunoprecipitated by mouse policlonal hyperimmune ascitic fluid to YF (ATCC) two monoclonal antibodies against viral protein NS1 (Schlesinger et al, 1983) and *P falciparum* repeat-specifi monoclonal antibody (2A10) Immunoprecipitates were fractionated with protein A-agarose and analysed by SDS-PAGE (Laemmli, 1970) For fluorographic detection, gels were treated with sodium salicylate and autoradiographed (Chamberlain, 1979) The results are shown in Figure 11 Immunoprecipitation profiles are btained from protein extracts of mock infected Vero cell (lanes 1,2,3) 17D/14 (lanes 4 5 6) or 17D/8 (lanes 7 8 9) virus-infected monolayers These different extracts were immunoprecipitated with murine hyperimmune serum against yellow fever virus from ATCC (lanes 1 4 7), $(NANP)_3$ repeat specific monoclonal antibody or 2A10 (lanes 2 5 8) and with two monoclonal antibodies direcetd against NS1 (lanes 3 6 9) Molecular weight markers are shown on the left side of the figure. The gel position f some of yellow virus proteins are indicated on the right side

Both parental and recombinant viruses produced the same protein profiles when the proteins were precipitated by the mouse hyperimmune serum and 2 monoclonal antibodies to YF 17D NS1 protein (F gure 11 lanes lanes 4 6 7 and 9) However Mab 2 10 precipitated exclusively the E protein of the recombinant 17D/8 virus (lanes 5 and 8) consistent with the interpretation f correct expression and exposure on the surface f the epitope as predicted by the structural analysis (F gures 11 13)

Example 9

Viral neutralizatio ass y by pecifi era

A third set f experiments to show the correct E protein surface expressi of the $(NANP)_3$ epitope was to examine viral neutralization by specifi sera. We used plaque reduction neutralizati assay in Vero cell seeded at the density of 65 000/cm on 96-well microplates as described elsewhere (Stefano et al 1999) Neutralizing antisera included an in house standard rhesus monkey YF immune serum (L13) and mab 2A10 Plaque neutralization titers were calculated as the highest dilution f antibody reducing 50% f plaques of input virus estimated by plating mixture f virus serially diluted in fetal calf serum

As observed in tabl 2 the anti YF L13 serum neutralized both viruses with the same efficiency (approximately 1 3 500) M noclonal antibody 2A10 however only neutralized the recombinant 17D/8 virus with an extremely high titer (1 181 000) indicating the specificity of the neutralization.

Table 2 YF 17D virus eutralizatio by immun sera

erum	YF 17 D	YF 17D/8
L 13	1 3 715	1 3 388
2 A 10	< 1 79	1 181 970

In the case f the expression f the plasmodial $(NANP)_3$ epitope, the monoclonal antibody recognizes the linear sequence in itself as shown by the specific ty f the neutralization That suggests that the ep tope well exposed in the fg loop and ts recognition is not hindered by ts involvement in other viral epitope structures It is also the first demonstration that E protein linear epitope can be eutralizing fo a flavivirus

Fusion requires conformational changes that affect several eutralization ep tope primarily within central domain I and domain II These changes are apparently associated with reorganization of the bunit interactions on the vin surface with trimer co tacts being favored in the l w pH form, in contrast to dimer contacts in the

native form Interference with these structural rearrangements by antibody binding represents one mechanism that may lead to virus neutralization (Monath and Heinz, 1996) Insertion of the plasmodial epitope in a loop of domain II also led to specific viral neutralization providing further evidence for the importance of this area in viral infectivity

Exampl 10

YF 17D/8 Recombin t virus ttenuatio

In order to demonstrate that the recombinant 17D/8 virus does not exceed ts parent YF 17D virus in neuroin asiveness and neurovirulence were carried out mouse tests

In our analysis, groups of 16 3week-old Swiss mice were inoculated by the ic route with 3 0 log PFU of the 17DD vaccine virus, a cDNA-derived 17D LS3 virus and 17D/8 virus Table 3 represents the verage of two separate experiments It is evident that the 17D/8 virus consistently kills less animals than the two other 17D viruses 26 out of 32 for 17D/8 as opposed to 31 of 32 for th 17DD vaccine and LS3 viruses The averag survival tim for animals inoculated with 17D/8 virus was also considerably longer as compared to the values obtained for the other two 17D viruses (12 5 vs 10 1 or 10 6 respectively)

T ble 3 M use eurovirulence of YF17D viruses

virus	IC dos (PFU)	% m rtality (dead/tested)	Average urvival tim (days)
control		0 (0/32)	
17DD	10^3	96 9 (31/32)	10 6
17D-LS3	10^3	96 9 (31/32)	10 1
17D/8	10^3	81 3 (26/32)	12 5

Swiss 3 week-old mice

YF 17D viruses were also examined for their capability of invading the central nervous system after peripheral (intra peritoneal, ip) inoculation into 2 5 7 9-day ld Swiss mice As shown in Tabl 4 below the 17D/8 virus again behaved favourably as compared to the other 17D viruses used in being less neuroinvasive for 2 and 5-day old mice

Epitope insertion at this site may affect the threshold of fusion-activating conformational change f this protein and t is conceivable that slower rate f fusion may delay the extent f virus production and thereby lead to milder infection of the host resulting in the somewhat more attenuated phenotype of the recombinant virus in the mouse model and lower extent f replication in cultured cells

Table 4 M se N roinvasiveness f YF 17D Viruses

lrus	IP dose	2-d y % mortality (dead/ tested)	Average surv val time	5-day % mortal ty (dead/ tested)	Average surv val t me	7-day % mortality (dead/ tested)	Average surv val me	9-day % mortal ty (dead/ tested)	Average survival im
co trol		0 (0/12)		0 (0/16)		0 (0/7)		0 (0/16)	
17DD	10^3	75 (9/12)	11 1	87 5 (7/8)	11 7	0 (0/16)		0 (0/14)	
17D-LS3	10^3	78 5 (11/14)	11 5	40 (4/10)	15 5	21 4 (3/14)	17 3	5 5 (1/18)	18
17D/8	10^3	12 5 (1/8)	10 0	0 (0/19)		0 (0/17)		0 (0/16)	

Exampl 11

Viral yields f YF 17D/8 in cultured cells.

In Vero cells the YF 17D/8 virus produced tiny plaques (1 1±0 3 mm) when compared to virus YF5 2/DD (or YF 17D/14 virus 4.20±0 9 mm) and th small plaque 17D/G1/5 2-derived virus (1 89±1 05 mm) Figure 12 shows this data.

Viral growth curves were determined by infecting monolayers of VERO cells or primary cultures of chicken embry fibroblasts (CEF) at m f 0 1 and 0 02 or at m. f 0 1 0 02 and 0 002 respectively Cell were plated at density f 62,500 cell/cm and infected 24 h later Samples f medi were collected at 24h intervals postinfection. Viral yields were estimated by plaque titration on VERO cell

Based on singl step growth curves in Vero and CEF cells differences were also bserved between 17D/8 and the control virus 17D/14 In CEF culture which are certified for human accine production, both viruses produced peak titer at 48 hrs postinfecti (F gure 13A) However maximal yields of 17D/8 reached 5 10 whereas the parental virus 17D/14 without th insert reached 10 PFU/ml Thi difference of about 20 fold was kept until 96 hrs p In Vero cells all viruses replicated better than in CEF cells but again the 17D/14-derived virus grew to 25 fold higher titers than 17D/8 (Figure 13B)

Exampl 12

YF 17D ccm virus prod cti in certified cells

According to present manufacturing efficiency and the final viral titer per human dose t needs an initial virus titer f at least 6 0 1 g $_0$PFU/ml It btained titer of 6 1 log $_0$PFU/ml for 17D/8 in Vero cells For vaccine manufacture addition f stabilizer to the virus bulk reduces titer by 0 3 log $_0$PFU/ml, filling and freeze-drying process red ces another 0 6 log $_0$PFU/ml 0 6 1 g $_0$PFU/ml loss in therm stabil tv is rul yielding virus preparation ready for use as vaccine with final titer f 3 9 1 g $_0$PFU/ml This s the minimum dosis recommended by

the World Health Organization for YF vaccine in humans (WHO 1995 Requirements for Yellow Fever Vaccine)

Example 13

YF 17D/8 Recombinant virus genetic stability

Therefore, the following experiments were carried out in order to characterize the genetic stability of the new virus (YF 17D/8) using cell cultures to propagate the virus

With regard to insert stability

The integrity of the insert must be assessed by sequencing of the RT/PCR products made on RNA of culture supernatant virus The PCR product is sequenced directly to ensure all the amino acids are in place

It shown for YF 17DD/204 virus that to generate vaccine-production sized secondary seed lots at least 3 passages are necessary starting from the cloned cDNA plasmid (US 6 171 854) Although the oligonucleotides encoding the epitopes were designed with codons more often utilized in the viral genome to void potential translation problems as well as instability of the inserted sequence t is important to examine the maintenance of the insertion in the YF 17D virus genome

For this purpose 5 series of separate passages of YF17D/8 virus in Vero and CEF were performed with known MOI (0 1 1 0 PFU/cell) Then at selected (5th and 10th) passages the viral RNA was collected and subjected to nucleotide sequence determination (as RT PCR products) at and around the insertion site in the E protein.

The results of these analyses in CEF and Vero cells are shown in Tabl 5 The insert was present in both 5th and 10th passages suggesting its stability when the virus was passaged serially in Vero cells but not in CEF cells

T bl 5 Genet stability f YF 17D8 viru insert po erial passage in Vero nd CEF cells.

Fifth passag	insert	Tenth passage	insert
CEF			
17D/8 (5)	–	17D/8 (10a)	–
17D/8 (5 b)	–	17D/8 (10b)	–
Vero			
17D/8 (5)	+	17D/8 (10)	+
17D/8 (5 d)	+	17D/8 (10 d)	+
17D/8 (5)	+	17D/8 (10)	+
17D/8 (5 f)	+	17D/8 (10 f)	+
17D/8 (5 g)	+	17D/8 (10 g)	+

The letters and b represent two independent passage in CEF cells whereas c, d, e, f and g th five independent series of passages in Vero cells

Plaq size lysis

Aiming at the establishment f an *tro* marker for the recombinant virus we have studied the size of virus plaques formed on Vero cell monolayers One f the lineages from serial passages in CEF ( ) or Vero cells (b) for which sequence data on the insert regi were vailabl was also analysed for ts plaque size phenotype along the passaging process Figure 14 displays the results of such analysis It sh ws the plaque size of YF17D/8 is tiny compared to our larg plaque YF 17DD/204 virus and th two small plaque 17D G1/5 2 and 17D/E200 viruses All controls are viruses derived from loned DNA and have defined ucleotide sequence differences that are related to plaque size in Vero cells In addition the plaque size displ yed by both viruses is very homogeneous as expected from virus derived from loned cDNA.

One f each lineages f the serial passages of YF17D/8 virus in CEF ( ) and Vero (b) cell were also analysed for their plaque size phenotypes along the passaging process (5th and 10 passages samples 5p and 10p respectively) For the comparati plaque size analysi among viruses the larg plaque control 17D/14 virus the small plaque co trol, 17D/G1 2 virus and 17D/8 virus

representing second passage in Vero cells f the original virus recovered from RNA transfection, were used. Mean plaque diameters (mm) with the corresponding standards desviations were btained at least from 10 different plaques of each virus

In both cells the plaque size f serially passaged viruses tended to increase As in Vero cell passaging there was no insert loss based on the sequencing data and plaque size phenotype in YF 17D viruses is dependent on one or more amin acid changes (AV Jabor and R Galler unpublished) it is possibl that other sequence changes took place outside the insertion area sequenced W are presently analysing this possibility by sequencing the complete genome of YF 17D/8 virus but ts expected sequence is shown comparatively to other viruses in Tabl 6

45

T bl 6 Comp is n of YF fectiou pl m d cl seq ences

NT/gene	YT 5 2	17D/DD[b]	YF 5 2/DD	17D/8	17D/1	17D/13	NT⇒AA
1140/E	T	C	C	T	T	T	T⇒Val→C⇒Al
1436 1437/E	G A	A G	A G	G A	G A	G A	G A⇒D→A,G⇒S
1946/E	T	C	C	T	C	C	T⇒S→C⇒P
2219 2220/E	A C	G T	G T	AC	G T	G T	A C⇒T→G T⇒V
2356/E	T	T	C	T	T	T	
2602/NS1	T	T	C	T	T	T	
2677/NS1	C	C	T	C	C	C	
2681/NS1	G	G	A	G	G	G	G⇒A→A⇒T
8656/NS5	A	A	C	C	A	A	
8808/NS5	A	G	G	G	G	G	A⇒N→G⇒S
9605/NS5	G	A	A	A	A	A	A⇒D→G⇒N
10454	G	A	A	A	A	A	
10722	G	G	A	A	G	G	

Rice at al (1989) D arte d Santos t al 1995 Galler and Fre re, patent US 6 171 854

Exampl 14

Immunogenicity in mic

The results described abo suggested that the epitope is being presented in the correct conformation and s accessible to antibodies either in solubilized native E protein as well as on the virus surface. In this example t was looked further into the immunogenicity f the recombinant 17D/8 virus by examining ts capability f eliciting antibodies It was done by immunizing BALB/ mice separately with up to 3 doses (10^5 PFU/dose) by the intra peritoneal (IP) route given two weeks apart. Two weeks after the last dose blood was collected by intraorbital bleeding Sera f mice were pooled and used to neutralize the homologous virus in 50% end point plaque reductio neutralization tests Table 7 shows these results obtained from two independent experiments Intraperitoneal inoculation as the 3rd dose elicited an average antibody titer to the YF virus f 1 1 079 as measured by $PRNT_{50}$

T bl 7 Immunogenicity of YF 17D/8 virus in mice.

Immunizing virus	Intra Peritoneal	
	PRNT	Average PRNT
control	1 13 < 1 10	< 1 13
17D/E200	1 166 1 195	1 181
17D/8	1 741 1 417	1 1079

Reciprocal of th highest dilution f serum that resulted in 50% reduction of plaque umbers

These two different dil tions correspond to two set of independent experiments

Example 15

C tructio of others recombmant YF17D viruses expressing cytotoxı T cell epıtop

The recombinant vıruses are constructed as described ın Exampl 6 ın order to express cytotoxı T cell epıtope Th recovery f the vıruses from cDNA by transfection of Vero cell was carried out as ın Example 6 The resultıng vıruses YF 17D/1 and 17D/13 were further passaged twice ın Vero cells for the generation f workıng stocks The syntheti lıgonucleotide ınsertion at the EcoRV sıte of YFE200 plasmid whıch corresponds to the amino acid sequence dep cted ın T bl 8 below gıves rıse to plasmıds pYFE200/1 and pYFE200/13 These plasmıds were deposited on December 21 2000 under accesıon number PTA 2858 and PTA 2854 respectıvely wıth the Amerıcan Type Culture Collection (ATCC) 10801 Unıversıty Blvd. Manassas Va.

T bl 8 shows the predıcted charg and ısoelectrı poınts fo the epıtopes alone ıntegrated ınto the fg loop and ın the whol E protein context. As can be seen there ıs consıderabl varıation of the net charge and the pI ın each context, epıtope alone, ın the loop or ın whol E contexts Sınce the ınsertıo regıon ıs ınvolved ın the pH-dependent conformatınal transıtıon for fusıon f the envelope to endosom membrane t ıs possıble that thı vırus property could be ınfluenced to dıfferent extents by the sequence ın the epıtope

Prelımınary data suggest that 17D/13 vırus bearıng the *P yoel* CTL epıtope does ot dısplay the growth restrıctıons bserved for the other vıruses (17D/8 and 17D/1) ın both CEF and Vero cells (Fıgure 13) Tabl 8 shows the amino acid sequence and pecıfıcıty f selected epıtopes for ınsertıon ınto YF E protein.

T bl 8 Am id eq ence d p ifi ity f lected pitopes f erti i t YF E protein.

	YF fg loop	EMD DYENDIEKKI IES	EMD GGNANPNANPNANPGG IES	EMD SYVPSAEQI IES
A t g p tope		CSP CTL	CSP B	CSP CTL
S rc		P f l parum	P falciparum	P yoel
Cl		17D/1	17D/8	17D/13
Charge/P pt d pI		2 00/4 06	0 00/5 97	1 01/3 30
fg l p+ep tope charge/pI	2 00/3 69	4 99/3 83	4 00/3 49	3 00/3 58
YI E prote + p tope charge/pI	7 4/5 83	9 40/5 61	7 40/5 83	8 40/5 71

5 B B cell

CTL ytot I cell ep tope

47

Exampl 16

Viral yields f YF 17D/1 and 17D/13 in cultured cells

The growth characteristi s in Vero cell of YF 17D/1 and 17D/13 comparatively to other 17D virus control including te 17D/8 recombinant expressing the humoral epitope are analysed. Thi exampl is carried out following the protocol described in Exampl 11

Figure 15 shows the comparati growth curves of the 17D/14 17D/E200 and the malaria recombinant virus 17D/8 17D/1 and 17D/13 in Vero cells at mo of 0 1 pfu/ml It is evident that 17D/1 and 17D/8 viruses grow to lower titers and more slowly than our 17D/14 virus control On the other hand the 17D/E200 and 17D/13 viruses grew as efficiently as our control virus suggesting that the insertion of SYVPSAEQI epitope was not as deleterious in this aspect as the 2 others were The same type f growth profil in Vero cells was observed with different MOI (0 02) and in CEF cells with both MOIs (data not shown)

There are no significant differences in the verall charg and isoelectri point f the whol E protein containing these epitopes but differences can be bserved at the level f the fg loop (see Tabl 8) containing or not the above epitopes, the hypothesi that the charge f the epitopes may influence pH dependent fusion and thereby lead to modified growth properties cannot be ruled out at this stage

Example 17

YF 17D/1 and YF 17D/13 recombinant virus geneti stability

With regard to insert tability

As described in exampl 13 above for YF 17D/8 t was examined the stability of the insertio in the YF17D virus genome by nucleotide sequence analysis at the inserti site on th RNA f viruses subjected to serial passages For thi purpose each YF17D/1 and 17D/13 viruses in Vero were performed with known MOI (0 1 1 0 PFU/cell) Then at selected (5th and 10th) passages th viral RNA was collected and subjected to nucleotide sequence determination (as RT PCR products) at and around the insertion site in the E protein

The results f these analyses in CEF and Vero cells are shown in Table 9 The insert was present in both 5th and 10th passages suggesting its stability when the virus was passaged serially in Vero cells but ot in CEF cells Similarly to YF17D/8 the inserted epitopes are stabl throughout serial passaging of both viruses in Vero cells These results altogether suggest the tolearbility of this site to the insertion f wide variety of ep topes and endowing this system with an enormous potential for the creation f recombinant viruses expressing antigens of other pathogens in general

T ble 9 Geneti stability of YF 17D recombinant viruses inserts upon serial passage in Vero cells

F fth passage	insert	tenth passage	insert
17D/1 (5 h)	+	17D/1 (10 h)	ND
17D/13 (5)	+	17D/13 (10)	+

ND not determined

Plaqu ize analysis

As shown in example 13 above the 17D/8 recombinant virus displayed tiny plaque size phenotype as compared to our large plaque YF 17DD/204 (17D/14) virus and the two small plaque infectious cDNA-derived 17D G1/5 2 and 17D/E200 viruses The plaque size phenotype for the new 17D/1 and 17D/13 recombinant viruses was compared to the viruses previously characterized (Figure 15) All viruses represent second passage in Vero cells of the original virus recovered from RNA transfection.

As expected from ts growth profile in Vero cells (Figure 15) the 17D/13 virus displayed a plaque size similar to 17D/E200 and 17D/G1 5.2 still small if compared to 17DD/204 (17D/14) but larger than the tiny plaques induced by 17D/1 and 17D/8 viruses (F gure 16)

Exampl 18

YF 17D/1 and YF 17D/13 recombinant ıru ttenuation

Although mouse neurovirulence does not predict virulence or attenuation of YF viruses for humans, t was important to demonstrate that recombinant 17D/1 and 17D/13 viruses do not exceed ts parent YF 17D virus in mouse neurovirulence Th YF 17D vaccin virus display degree f neurotropism for mice by killing all age f mice after intracerebral inoculation and causes usually subclinical encephaliti in monkeys (Monath, 1999)

In th present analysis groups of 16 3 week-old Swiss mice were inoculated by the route with 3 0 1 g $_0$ PFU f the 17DD vaccine virus, 17D1 17D/8 17D/13 and 17D/E200 viruses The results shown in Tabl 10 are representative f two separate experiments

T bl 10 M use eurovirulence f YF17D viruses

virus	dose (PFU)	% mortality (dead/tested)	Average survival tim (days)
control		0 (1/32)	
17DD	10^3	96 9 (31/32)	9 6
17D/1	10^3	81 3 (26/32)	15 6
17D/8	10^3	71 9 (23/32)	11 7
17D/13	10^3	53 1 (17/32)	15 3
17D/E200	10^3	93 8 (30/32)	11 0

Swiss 3 week-old mice

It is evident that the 17D/8 and 17D/13 viruses consistently kill less animals than the ther 17D viruses 96 9% for 17DD and 81 3% for 17D/1 and 93 8% for 17D/E200 As bserved previously the verag survival tim for animals inoculated with 17D/8 virus was also considerably longer as compared to

th values btained for 17DD and 17D/E200 viruses (11 7 vs 9 6 or 11 0 respectively) The 17D/1 and 17D/13 viruses killed mice at a much slower pace with ASTs of 15 4 and 15 1 respectively but 17D/1 killed virtually all mice whereas 17D/13 was more attnuated and only killed 75%, similarly to 17D/8

Epitope insertion at this site may affect the threshold of fusion-activated conformational change of the E protein and t is conceivable that slower rate of fusion may delay the extent f virus production and thereby lead to milder infection of the host resulting in th somewhat more attenuated phenotype of the recombinant virus in the mouse model and lower extent of replication in cultured cells

The results shown here indicate that inserts f different sizes and charges are tolerated and they are likely to influence the viral properties above In no case however the recombinant virus became more neurovirulent fo mice than ts vaccine counterpart, further confirming the potential of using this site for the insertion f foreign epitopes and the development of new live attenuated 17D vaccines

LIST SEQUENCING

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II) Invention. Use of Flavivirus for the expression of protein epitopes and development of new live attenuated vaccine virus to immunize against flavivirus and other infectious agents

III) Number of Sequences 01 (on)

INFORMATION FOR SEQ ID NO 1

I) Sequence characteristics
L) length 10862 base pairs
Lb) type ucleı acid
Lc) strandedness smgl
I d) topology linear
Le) m lecule type cDNA
I f) original source ındı ıdual ısolate Yfi 5.2/DD
I.g) sequence description SEQ ID NO 1

1 AGTAAATCCT GTGTGCTAAT TGAGGTGCAT TGGTCTGCAA ATCGAGTTGC

51 TAGGCAATAA ACACATTTGG ATTAATTTTA ATCGTTCGTT GAGCGATTAG

101 CAGAGAACTG ACCAGAACAT GTCTGGTCGT AAAGCTCAGG GAAAAACCCT

151 GGGCGTCAAT ATGGTACGAC GAGGAGTTCG CTCCTTGTCA AACAAAATAA

201 AACAAAAAAC AAAACAAATT GGAAACAGAC CTGGACCTTC AAGAGGTGTT

251 CAAGGATTTA TCTTTTTCTT TTTGTTCAAC ATTTTGACTG GAAAAAAGAT

301 CACAGCCCAC CTAAAGAGGT TGTGGAAAAT GCTGGACCCA AGACAAGGCT

351 TGGCTGTTCT AAGGAAAGTC AAGAGAGTGG TGGCCAGTTT GATGAGAGGA

401 TTGTCCTCAA GGAAACGCCG TTCCCATGAT GTTCTGACTG TGCAATTCCT

451 AATTTTGGGA ATGCTGTTGA TGACGGGTGG AGTGACCTTG GTGCGGAAAA

501 ACAGATGGTT GCTCCTAAAT GTGACATCTG AGGACCTCGG GAAAACATTC

551 TCTGTGGGCA CAGGCAACTG CACAACAAAC ATTTTGGAAG CCAAGTACTG

601 GTGCCCAGAC TCAATGGAAT ACAACTGTCC CAATCTCAGT CCAAGAGAGG

651 AGCCAGATGA CATTGATTGC TGGTGCTATG GGGTGGAAAA CGTTAGAGTC

701 GCATATGGTA AGTGTGACTC AGCAGGCAGG TCTAGGAGGT CAAGAAGGGC

751 CATTGACTTG CCTACGCATG AAAACCATGG TTTGAAGACC CGGCAAGAAA

801 AATGGATGAC TGGAAGAATG GGTGAAAGGC AACTCCAAAA GATTGAGAGA

851 TGGTTCGTGA GGAACCCCTT TTTTGCAGTG ACGGCTCTGA CCATTGCCTA

901 CCTTGTGGGA AGCAACATGA CGCAACGAGT CGTGATTGCC CTACTGGTCT

951 TGGCTGTTGG TCCGGCCTAC TCAGCTCACT GCATTGGAAT TACTGACAGG

1001 GATTTCATTG AGGGGGTGCA TGGAGGAACT TGGGTTTCAG CTACCCTGGA

1051 GCAAGACAAG TGTGTCACTG TTATGGCCCC TGACAAGCCT TCATTGGACA

1101 TCTCACTAGA GACAGTAGCC ATTGATAGAC CTGCTGAGGC GAGGAAAGTG

1151 TGTTACAATG CAGTTCTCAC TCATGTGAAG ATTAATGACA AGTGCCCCAG

1201 CACTGGAGAG GCCCACCTAG CTGAAGAGAA CGAAGGGGAC AATGCGTGCA

1251 AGCGCACTTA TTCTGATAGA GGCTGGGGCA ATGGCTGTGG CCTATTTGGG

1301 AAAGGGAGCA TTGTGGCATG CGCCAAATTC ACTTGTGCCA AATCCATGAG

1351 TTTGTTTGAG GTTGATCAGA CCAAAATTCA GTATGTCATC AGAGCACAAT

1401 TGCATGTAGG GGCCAAGCAG GAAAATTGGA ATACCAGCAT TAAGACTCTC

1451 AAGTTTGATG CCCTGTCAGG CTCCCAGGAA GTCGAGTTCA TTGGGTATGG

1501 AAAAGCTACA CTGGAATGCC AGGTGCAAAC TGCGGTGGAC TTTGGTAACA

1551 GTTACATCGC TGAGATGGAA ACAGAGAGCT GGATAGTGGA CAGACAGTGG

1601 GCCCAGGACT TGACCCTGCC ATGGCAGAGT GGAAGTGGCG GGGTGTGGAG

1651 AGAGATGCAT CATCTTGTCG AATTTGAACC TCCGCATGCC GCCACTATCA

1701 GAGTACTGGC CCTGGGAAAC CAGGAAGGCT CCTTGAAAAC AGCTCTTACT

1751 GGCGCAATGA GGGTTACAAA GGACACAAAT GACAACAACC TTTACAAACT

1801 ACATGGTGGA CATGTTTCTT GCAGAGTGAA ATTGTCAGCT TTGACACTCA

1851 AGGGGACATC CTACAAAATA TGCACTGACA AAATGTTTTT TGTCAAGAAC

1901 CCAACTGACA CTGGCCATGG CACTGTTGTG ATGCAGGTGA AAGTGCCAAA

1951 AGGAGCCCCC TGCAGGATTC CAGTGATAGT AGCTGATGAT CTTACAGCGG

2001 CAATCAATAA AGGCATTTTG GTTACAGTTA ACCCCATCGC CTCAACCAAT

2051 GATGATGAAG TGCTGATTGA GGTGAACCCA CCTTTTGGAG ACAGCTACAT

2101 TATCGTTGGG AGAGGAGATT CACGTCTCAC TTACCAGTGG CACAAAGAGG

2151 GAAGCTCAAT AGGAAAGTTG TTCACTCAGA CCATGAAAGG CGTGGAACGC

2201 CTGGCCGTCA TGGGAGACGT CGCCTGGGAT TTCAGCTCCG CTGGAGGGTT

2251 CTTCACTTCG GTTGGGAAAG GAATTCATAC GGTGTTTGGC TCTGCCTTTC

2301 AGGGGCTATT TGGCGGCTTG AACTGGATAA CAAAGGTCAT CATGGGGGCG

2351 GTACTCATAT GGGTTGGCAT CAACACAAGA AACATGACAA TGTCCATGAG

2401 CATGATCTTG GTAGGAGTGA TCATGATGTT TTTGTCTCTA GGAGTTGGGG

2451 CGGATCAAGG ATGCGCCATC AACTTTGGCA AGAGAGAGCT CAAGTGCGGA

2501 GATGGTATCT TCATATTTAG AGACTCTGAT GACTGGCTGA ACAAGTACTC

2551 ATACTATCCA GAAGATCCTG TGAAGCTTGC ATCAATAGTG AAAGCCTCTT

2601 TCGAAGAAGG GAAGTGTGGC CTAAATTCAG TTGACTCCCT TGAGCATGAG

2651 ATGTGGAGAA GCAGGGCAGA TGAGATTAAT ACCATTTTTG AGGAAAACGA

2701 GGTGGACATT TCTGTTGTCG TGCAGGATCC AAAGAATGTT TACCAGAGAG

2751 GAACTCATCC ATTTTCCAGA ATTCGGGATG GTCTGCAGTA TGGTTGGAAG

2801 ACTTGGGGTA AGAACCTTGT GTTCTCCCCA GGGAGGAAGA ATGGAAGCTT

2851 CATCATAGAT GGAAAGTCCA GGAAAGAATG CCCGTTTTCA AACCGGGTCT

2901 GGAATTCTTT CCAGATAGAG GAGTTTGGGA CGGGAGTGTT CACCACACGC

2951 GTGTACATGG ACGCAGTCTT TGAATACACC ATAGACTGCG ATGGATCTAT

3001 CTTGGGTGCA GCGGTGAACG GAAAAAAGAG TGCCCATGGC TCTCCAACAT

3051 TTTGGATGGG AAGTCATGAA GTAAATGGGA CATGGATGAT CCACACCTTG

3101 GAGGCATTAG ATTACAAGGA GTGTGAGTGG CCACTGACAC ATACGATTGG

3151 AACATCAGTT GAAGAGAGTG AAATGTTCAT GCCGAGATCA ATCGGAGGCC

3201 CAGTTAGCTC TCACAATCAT ATCCCTGGAT ACAAGGTTCA GACGAACGGA

3251 CCTTGGATGC AGGTACCACT AGAAGTGAAG AGAGAAGCTT GCCCAGGGAC

3301 TAGCGTGATC ATTGATGGCA ACTGTGATGG ACGGGGAAAA TCAACCAGAT

3351 CCACCACGGA TAGCGGGAAA GTTATTCCTG AATGGTGTTG CCGCTCCTGC

3401 ACAATGCCGC CTGTGAGCTT CCATGGTAGT GATGGGTGTT GGTATCCCAT

3451 GGAAATTAGG CCAAGGAAAA CGCATGAAAG CCATCTGGTG CGCTCCTGGG

3501 TTACAGCTGG AGAAATACAT GCTGTCCCTT TTGGTTTGGT GAGCATGATG

3551 ATAGCAATGG AAGTGGTCCT AAGGAAAAGA CAGGGACCAA AGCAAATGTT

3601 GGTTGGAGGA GTAGTGCTCT TGGGAGCAAT GCTGGTCGGG CAAGTAACTC

3651 TCCTTGATTT GCTGAAACTC ACAGTGGCTG TGGGATTGCA TTTCCATGAG

3701 ATGAACAATG GAGGAGACGC CATGTATATG GCGTTGATTG CTGCCTTTTC

3751 AATCAGACCA GGGCTGCTCA TCGGCTTTGG GCTCAGGACC CTATGGAGCC

3801 CTCGGGAACG CCTTGTGCTG ACCCTAGGAG CAGCCATGGT GGAGATTGCC

3851 TTGGGTGGCG TGATGGGCGG CCTGTGGAAG TATCTAAATG CAGTTTCTCT

3901 CTGCATCCTG ACAATAAATG CTGTTGCTTC TAGGAAAGCA TCAAATACCA

3951 TCTTGCCCCT CATGGCTCTG TTGACACCTG TCACTATGGC TGAGGTGAGA

4001 CTTGCCGCAA TGTTCTTTTG TGCCGTGGTT ATCATAGGGG TCCTTCACCA

4051 GAATTTCAAG GACACCTCCA TGCAGAAGAC TATACCTCTG GTGGCCCTCA

4101 CACTCACATC TTACCTGGGC TTGACACAAC CTTTTTTGGG CCTGTGTGCA

4151 TTTCTGGCAA CCCGCATATT TGGGCGAAGG AGTATCCCAG TGAATGAGGC

4201 ACTCGCAGCA GCTGGTCTAG TGGGAGTGCT GGCAGGACTG GCTTTTCAGG

4251 AGATGGAGAA CTTCCTTGGT CCGATTGCAG TTGGAGGACT CCTGATGATG

4301 CTGGTTAGCG TGGCTGGGAG GGTGGATGGG CTAGAGCTCA AGAAGCTTGG

4351 TGAAGTTTCA TGGGAAGAGG AGGCGGAGAT CAGCGGGAGT TCCGCCCGCT

4401 ATGATGTGGC ACTCAGTGAA CAAGGGGAGT TCAAGCTGCT TTCTGAAGAG

4451 AAAGTGCCAT GGGACCAGGT TGTGATGACC TCGCTGGCCT TGGTTGGGGC

4501 TGCCCTCCAT CCATTTGCTC TTCTGCTGGT CCTTGCTGGG TGGCTGTTTC

4551 ATGTCAGGGG AGCTAGGAGA AGTGGGGATG TCTTGTGGGA TATTCCCACT

4601 CCTAAGATCA TCGAGGAATG TGAACATCTG GAGGATGGGA TTTATGGCAT

4651 ATTCCAGTCA ACCTTCTTGG GGGCCTCCCA GCGAGGAGTG GGAGTGGCAC

4701 AGGGAGGGGT GTTCCACACA ATGTGGCATG TCACAAGAGG AGCTTTCCTT

4751 GTCAGGAATG GCAAGAAGTT GATTCCATCT TGGGCTTCAG TAAAGGAAGA

4801 CCTTGTCGCC TATGGTGGCT CATGGAAGTT GGAAGGCAGA TGGGATGGAG

4851 AGGAAGAGGT CCAGTTGATC GCGGCTGTTC CAGGAAAGAA CGTGGTCAAC

4901 GTCCAGACAA AACCGAGCTT GTTCAAAGTG AGGAATGGGG GAGAAATCGG

4951 GGCTGTCGCT CTTGACTATC CGAGTGGCAC TTCAGGATCT CCTATTGTTA

5001 ACAGGAACGG AGAGGTGATT GGGCTGTACG GCAATGGCAT CCTTGTCGGT

5051 GACAACTCCT TCGTGTCCGC CATATCCCAG ACTGAGGTGA AGGAAGAAGG

5101 AAAGGAGGAG CTCCAAGAGA TCCCGACAAT GCTAAAGAAA GGAATGACAA

5151 CTGTCCTTGA TTTTCATCCT GGAGCTGGGA AGACAAGACG TTTCCTCCCA

5201 CAGATCTTGG CCGAGTGCGC ACGGAGACGC TTGCGCACTC TTGTGTTGGC

5251 CCCCACCAGG GTTGTTCTTT CTGAAATGAA GGAGGCTTTT CACGGCCTGG

5301 ACGTGAAATT CCACACACAG GCTTTTTCCG CTCACGGCAG CGGGAGAGAA

5351 GTCATTGATG CCATGTGCCA TGCCACCCTA ACTTACAGGA TGTTGGAACC

5401 AACTAGGGTT GTTAACTGGG AAGTGATCAT TATGGATGAA GCCCATTTTT

5451 TGGATCCAGC TAGCATAGCC GCTAGAGGTT GGGCAGCGCA CAGAGCTAGG

5501 GCAAATGAAA GTGCAACAAT CTTGATGACA GCCACACCGC CTGGGACTAG

5551 TGATGAATTT CCACATTCAA ATGGTGAAAT AGAAGATGTT CAAACGGACA

5601 TACCCAGTGA GCCCTGGAAC ACAGGGCATG ACTGGATCCT AGCTGACAAA

5651 AGGCCCACGG CATGGTTCCT TCCATCCATC AGAGCTGCAA ATGTCATGGC

5701 TGCCTCTTTG CGTAAGGCTG GAAAGAGTGT GGTGGTCCTG AACAGGAAAA

5751 CCTTTGAGAG AGAATACCCC ACGATAAAGC AGAAGAAACC TGACTTTATA

5801 TTGGCCACTG ACATAGCTGA AATGGGAGCC AACCTTTGCG TGGAGCGAGT

5851 GCTGGATTGC AGGACGGCTT TTAAGCCTGT GCTTGTGGAT GAAGGGAGGA

5901 AGGTGGCAAT AAAAGGGCCA CTTCGTATCT CCGCATCCTC TGCTGCTCAA

5951 AGGAGGGGGC GCATTGGGAG AAATCCCAAC AGAGATGGAG ACTCATACTA

6001 CTATTCTGAG CCTACAAGTG AAAATAATGC CCACCACGTC TGCTGGTTGG

6051 AGGCCTCAAT GCTCTTGGAC AACATGGAGG TGAGGGGTGG AATGGTCGCC

6101 CCACTCTATG GCGTTGAAGG AACTAAAACA CCAGTTTCCC CTGGTGAAAT

6151 GAGACTGAGG GATGACCAGA GGAAAGTCTT CAGAGAACTA GTGAGGAATT

6201 GTGACCTGCC CGTTTGGCTT TCGTGGCAAG TGGCCAAGGC TGGTTTGAAG

6251 ACGAATGATC GTAAGTGGTG TTTTGAAGGC CCTGAGGAAC ATGAGATCTT

6301 GAATGACAGC GGTGAAACAG TGAAGTGCAG GGCTCCTGGA GGAGCAAAGA

6351 AGCCTCTGCG CCCAAGGTGG TGTGATGAAA GGGTGTCATC TGACCAGAGT

6401 GCGCTGTCTG AATTTATTAA GTTTGCTGAA GGTAGGAGGG GAGCTGCTGA

6451 AGTGCTAGTT GTGCTGAGTG AACTCCCTGA TTTCCTGGCT AAAAAAGGTG

6501 GAGAGGCAAT GGATACCATC AGTGTGTTTC TCCACTCTGA GGAAGGCTCT

6551 AGGGCTTACC GCAATGCACT ATCAATGATG CCTGAGGCAA TGACAATAGT

6601 CATGCTGTTT ATACTGGCTG GACTACTGAC ATCGGGAATG GTCATCTTTT

6651 TCATGTCTCC CAAAGGCATC AGTAGAATGT CTATGGCGAT GGGCACAATG

6701 GCCGGCTGTG GATATCTCAT GTTCCTTGGA GGCGTCAAAC CCACTCACAT

6751 CTCCTATATC ATGCTCATAT TCTTTGTCCT GATGGTGGTT GTGATCCCCG

6801 AGCCAGGGCA ACAAAGGTCC ATCCAAGACA ACCAAGTGGC ATACCTCATT

6851 ATTGGCATCC TGACGCTGGT TTCAGCGGTG GCAGCCAACG AGCTAGGCAT

6901 GCTGGAGAAA ACCAAAGAGG ACCTCTTTGG GAAGAAGAAC TTAATTCCAT

6951 CTAGTGCTTC ACCCTGGAGT TGGCCGGATC TTGACCTGAA GCCAGGAGCT

7001 GCCTGGACAG TGTACGTTGG CATTGTTACA ATGCTCTCTC CAATGTTGCA

7051 CCACTGGATC AAAGTCGAAT ATGGCAACCT GTCTCTGTCT GGAATAGCCC

7101 AGTCAGCCTC AGTCCTTTCT TTCATGGACA AGGGGATACC ATTCATGAAG

7151 ATGAATATCT CGGTCATAAT GCTGCTGGTC AGTGGCTGGA ATTCAATAAC

7201 AGTGATGCCT CTGCTCTGTG GCATAGGGTG CGCCATGCTC CACTGGTCTC

7251 TCATTTTACC TGGAATCAAA GCGCAGCAGT CAAAGCTTGC ACAGAGAAGG

7301 GTGTTCCATG GCGTTGCCAA GAACCCTGTG GTTGATGGGA ATCCAACAGT

7351 TGACATTGAG GAAGCTCCTG AAATGCCTGC CCTTTATGAG AAGAAACTGG

7401 CTCTATATCT CCTTCTTGCT CTCAGCCTAG CTTCTGTTGC CATGTGCAGA

7451 ACGCCCTTTT CATTGGCTGA AGGCATTGTC CTAGCATCAG CTGCCTTAGG

7501 GCCGCTCATA GAGGGAAACA CCAGCCTTCT TTGGAATGGA CCCATGGCTG

7551 TCTCCATGAC AGGAGTCATG AGGGGGAATC ACTATGCTTT TGTGGGAGTC

7601 ATGTACAATC TATGGAAGAT GAAAACTGGA CGCCGGGGGA GCGCGAATGG

7651 AAAAACTTTG GGTGAAGTCT GGAAGAGGGA ACTGAATCTG TTGGACAAGC

7701 GACAGTTTGA GTTGTATAAA AGGACCGACA TTGTGGAGGT GGATCGTGAT

7751 ACGGCACGCA GGCATTTGGC CGAAGGGAAG GTGGACACCG GGGTGGCGGT

7801 CTCCAGGGGG ACCGCAAAGT TAAGGTGGTT CCATGAGCGT GGCTATGTCA

7851 AGCTGGAAGG TAGGGTGATT GACCTGGGGT GTGGCCGCGG AGGCTGGTGT

7901 TACTACGCTG CTGCGCAAAA GGAAGTGAGT GGGGTCAAAG GATTTACTCT

7951 TGGAAGAGAC GGCCATGAGA AACCCATGAA TGTGCAAAGT CTGGGATGGA

8001 ACATCATCAC CTTCAAGGAC AAAACTGATA TCCACCGCCT AGAACCAGTG

8051 AAATGTGACA CCCTTTTGTG TGACATTGGA GAGTCATCAT CGTCATCGGT

8101 CACAGAGGGG GAAAGGACCG TGAGAGTTCT TGATACTGTA GAAAAATGGC

8151 TGGCTTGTGG GGTTGACAAC TTCTGTGTGA AGGTGTTAGC TCCATACATG

8201 CCAGATGTTC TCGAGAAACT GGAATTGCTC CAAAGGAGGT TTGGCGGAAC

8251 AGTGATCAGG AACCCTCTCT CCAGGAATTC CACTCATGAA ATGTACTACG

8301 TGTCTGGAGC CCGCAGCAAT GTCACATTTA CTGTGAACCA AACATCCCGC

8351 CTCCTGATGA GGAGAATGAG GCGTCCAACT GGAAAAGTGA CCCTGGAGGC

8401 TGACGTCATC CTCCCAATTG GGACACGCAG TGTTGAGACA GACAAGGGAC

8451 CCCTGGACAA AGAGGCCATA GAAGAAAGGG TTGAGAGGAT AAAATCTGAG

8501 TACATGACCT CTTGGTTTTA TGACAATGAC AACCCCTACA GGACCTGGCA

8551 CTACTGTGGC TCCTATGTCA CAAAAACCTC AGGAAGTGCG GCGAGCATGG

8601 TAAATGGTGT TATTAAAATT CTGACATATC CATGGGACAG GATAGAGGAG

8651 GTCACCAGAA TGGCAATGAC TGACACAACC CCTTTTGGAC AGCAAAGAGT

8701 GTTTAAAGAA AAAGTTGACA CCAGAGCAAA GGATCCACCA GCGGGAACTA

8751 GGAAGATCAT GAAAGTTGTC AACAGGTGGC TGTTCCGCCA CCTGGCCAGA

8801 GAAAAGAGCC CCAGACTGTG CACAAAGGAA GAATTTATTG CAAAAGTCCG

8851 AAGTCATGCA GCCATTGGAG CTTACCTGGA AGAACAAGAA CAGTGGAAGA

8901 CTGCCAATGA GGCTGTCCAA GACCCAAAGT TCTGGGAACT GGTGGATGAA

8951 GAAAGGAAGC TGCACCAACA AGGCAGGTGT CGGACTTGTG TGTACAACAT

9001 GATGGGGAAA AGAGAGAAGA AGCTGTCAGA GTTTGGGAAA
GCAAAGGGAA

9051 GCCGTGCCAT ATGGTATATG TGGCTGGGAG CGCGGTATCT TGAGTTTGAG

9101 GCCCTGGGAT TCCTGAATGA GGACCATTGG GCTTCCAGGG AAAACTCAGG

9151 AGGAGGAGTG GAAGGCATTG GCTTACAATA CCTAGGATAT GTGATCAGAG

9201 ACCTGGCTGC AATGGATGGT GGTGGATTCT ACGCGGATGA CACCGCTGGA

9251 TGGGACACGC GCATCACAGA GGCAGACCTT GATGATGAAC AGGAGATCTT

9301 GAACTACATG AGCCCACATC ACAAAAAACT GGCACAAGCA GTGATGGAAA

9351 TGACATACAA GAACAAAGTG GTGAAAGTGT TGAGACCAGC CCCAGGAGGG

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9601 ATGTAACAGA CTGAAGAGGA TGGCGGTGAG TGGAGACGAC TGTGTGGTCC

9651 GGCCCATCGA TGACAGGTTC GGCCTGGCCC TGTCCCATCT CAACGCCATG

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9951 ACAAAAGGGA CATGAGGCTA CTGTCATTGG CTGTTTCCTC AGCTGTTCCC

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AAAATGGAGA

10151 GATGTCCCTT ATCTAACCAA GAGACAAGAC AAGCTGTGCG GATCACTGAT

10201 TGGAATGACC AATAGGGCCA CCTGGGCCTC CCACATCCAT TTAGTCATCC

10251 ATCGTATCCG AACGCTGATT GGACAGGAGA AATACACTGA CTACCTAACA

10301 GTCATGGACA GGTATTCTGT GGATGCTGAC CTGCAACTGG GTGAGCTTAT

10351 CTGAAACACC ATCTAACAGG AATAACCGGG ATACAAACCA CGGGTGGAGA

10401 ACCGGACTCC CCACAACCTG AAACCGGGAT ATAAACCACG GCTGGAGAAC

10451 CGGACTCCGC ACTTAAAATG AAACAGAAAC CGGGATAAAA ACTACGGATG

10501 GAGAACCGGA CTCCACACAT TGAGACAGAA GAAGTTGTCA GCCCAGAACC

10551 CCACACGAGT TTTGCCACTG CTAAGCTGTG AGGCAGTGCA GGCTGGGACA

10601 GCCGACCTCC AGGTTGCGAA AAACCTGGTT TCTGGGACCT CCCACCCCAG

10651 AGTAAAAAGA ACGGAGCCTC CGCTACCACC CTCCCACGTG GTGGTAGAAA

10701 GACGGGGTCT AGAGGTTAGA GAAGACCCTC CAGGGAACAA ATAGTGGGAC

10751 CATATTGACG CCAGGGAAAG ACCGGAGTGG TTCTCTGCTT TTCCTCCAGA

10801 GGTCTGTGAG CACAGTTTGC TCAAGAATAA GCAGACCTTT GGATGACAAA

10851 CACAAAACCA CT

CLAIMS

1 A method for the production of Flavivirus as vector for heterologous antigens comprising the introduction and expression of foreign gene sequences into insertion sites at the lev l of the envelope protein f any Flavivirus, wherein the sites are structurally apart from areas known to interfere with the overall fl vivirus E protein structure and comprising

( ) sites that l on the external surface f the virus providing accessibility to antibody

(ii) not disrupt or significantly destablize the three-dimensional structure of the E protein and

(iii) not interfere with the formation f the E protein network within the viral envelope

2 The method according to claim 1 wherein one site comprises the region f β-strands f and g including the fg loop which form part of the five-stranded anti parallel β sheet f domain II of the flavivirus envelope protein.

3 The method according to claim 2 wherein the site is the loop area between β strands f and g which form part of the five-stranded anti parallel β sheet of domain II of the flavivirus envelope protein.

4 The method according to claim 2 wherein the foreign sequence has been inserted in the region of amino acid 196 to 215 with reference to the tick born encephalitı virus sequence described in figure 2

5 The method according to claim 3 wherein the foreign sequence has been inserted in the region of amino acid 205 to 210 with reference to the tick borne encephalitis virus sequence described in figure 2

6 The method according to claim 1 wherein another site comprises the region of E_0 and F strands including the E_0F loop which form part of the eight stranded β barrel f domain I

7 The method according to claim 6 wherein the site s the loop area between E_0 and F strands which form part of the eight stranded β-barrel of domain I

8 The method according to claim 6 wherein the foreign sequence has been inserted in the region f amino acid 138 to 166 with reference to the tick borne encephalitis virus sequence described in figure 2

9 The method according to claim 7 wherein the foreign sequence has been nserted in the region of amino ac d 146 to 160 with reference to the tick borne encephal tis virus sequence described in figure 2

10 Th method according to claim 1 wherein th Flavivirus is selected from th group consisting of any Flavivirus including yellow fever virus tick borne encephalitis virus dengue virus and japanese encephalitis virus

11 The method according to claim 10 wherein the virus wild type attenuated or recombinant virus

12 The method according to claims 10 and 11 wherein the Flavivirus is a yellow fever virus

13 The method according to claim 12 wherein the virus is recombinant yellow fever virus

14 The method according to claim 13 wherein the yellow fever virus is the YF17D virus strain and substrains thereof

15 Th method according to claims 1 to 14 wherein the foreign epitope is malarial gene sequence

16 The method according to claim 15 wherein the malarial gene sequence the $(NANP)_3$ humoral epitope

17 The method according to claim 15 wherein the malarial gene sequence s the DYENDIEKKI cytotoxi T lymphocyte (CTL) epitope

18 The method according to claim 15 wherein the malarial gene sequence the SYVPSAEQI ytotoxi T lymphocytes (CTL) ep tope

19 The method according to claim 1 wherein one or m re glycine residues is inserted in the region immediately upstream and downstream f the foreign ep tope

20 A DNA construct consisting sentially f vector genetically stabl Fl vivirus genom and foreign gene sequences introduced in an insertion site according to any of laims 1 to 19

21 The DNA construct according to claim 20 wherein the Flavivirus is selected from the group consisting of any Flavivirus including yellow fever virus tick borne encephalitis virus, dengue virus and japanese encephalitis virus

22 The DNA construct according to claim 20 wherein the vector is selected from the group consisting of low copy number plasmids

23 The DNA construct according to claim 21 wherein the vector is selected from the group consisting of pACNR1180 and pBel BAC11

24 The DNA construct according to claim 20 wherein the vector is selected from the group consisting of high copy number plasmids

25 The DNA construct according to claim 20 wherein the genetically stable Flavivirus genome is derived from any YF 17D strain.

26 The DNA construct according to claim 25 wherein the genetically stable Flavivirus genome the YF genome bearing the complete sequence set forth in SEQ ID NO 1 or functionally equivalent sequences thereof

27 The DNA construct according to claim 20 wherein the foreign gene sequence is derived from malaria, yellow fever dengue, Japanese encephalitis, tick borne encephalitis and fungi infections

28 The DNA construct according to claim 27 wherein the foreign gene sequence is malarial gene sequence

29 The DNA construct according to claims 26 and 28 wherein the malarial gene sequence is the $(NANP)_3$ humoral epitope

30 The DNA construct according to claim 28 wherein the malarial gene sequence is the DYENDIEKKI cytotoxi T lymphocytes epitope

31 The DNA construct according to claim 28 wherein the malarial gene sequence is the SYVPSAEQI cytotoxi T lymphocytes epitope

32 The DNA construct according to claim 29 which is plasmid pYF17D/8

33 DNA construct having the structure of plasmid pYFE200

34 DNA construct having the structure of plasmid pYFE200/1

35 DNA construct having the structure of plasmid pYFE200/13

36 DNA construct having the structure of plasmid pYFE200/8

37 A Flavivirus as vector for heterol gous antigens comprising foreign gene sequences inserted at tes in the level f ts envelope protein, wherein the sites are structurally apart from areas known to interfere with the overall flavivirus E protein structure

38 The Flavivirus according to claim 37 wherein the foreign gene sequence introduced in the region f β-strands f and g including the fg loop which form part f th five-stranded anti parallel β sheet f d main II f the flavivirus envelope protein.

39 The Flavivirus according to claim 38 wherein the site is the loop area between β-strands f and g which form part of th five-stranded anti parallel β sheet f domain II of the fl vivirus envelope protein.

40 The Fl vivirus according to claim 38 wherein the foreign sequence has been inserted in the region f amino ac d 196 to 215 with reference to the tick borne encephal ti virus sequenc described in figure 2

41 The Flavivirus according to claim 39 wherein the foreign sequence has been inserted in the region of amino acid 205 to 210 with reference to the tick borne encephaliti virus sequence described in figure 2

42 The Flavivirus according to claim 37 wherein another site comprises the regi f E_0 and F strands including the E_0F loop which form part f the eight stranded β barrel f domain I

43 The Flavivirus according to laim 42 wherein the site is the loop area between E_0 and F_0 strands which form part f th eight stranded β barrel f domain I

44 The Flavivirus according to laim 42wherein the f reign sequence has been inserted in the regio f amino acid 138 to 166 with reference to the tick born encephal tis virus sequence described in figure 2

45 Th Flavivirus according to laim 43 wherei the foreign sequence has been inserted in the region of amino acid 146 to 160 with reference to the tick borne encephalitis virus seq ence de cribed in figure 2

46 The Fl vivirus according to claim 37 wherein the Flavivirus is selected from the group consisting of any Flavivirus including yellow fever virus tick borne encephalitis virus, dengue virus and japanese encephalitis virus

47 The Flavivirus according to claim 46 wherein the virus is a wild type, attenuated or recombinant virus

48 The Flavivirus according to claims 46 and 47 wherein the Flavivirus is yellow fever virus

49 The Flavivirus according to claim 48 wherein the virus is recombinant yellow fever virus

50 The Flavivirus according to claim 49 wherein the yellow fever virus is the YF17D virus strain and substrains thereof

51 The Flavivirus according to claims 37 to 50 wherein the foreign epitope is malarial gene sequence

52 A vaccine composition to immunize against flavivirus and other infectious agents consisting essentially of a virus according to claims 37 to 51

53 The vaccine composition according to claim 52 wherein the flavivirus is a yellow fever virus and the other infectious agent is the causative agent f malaria.

54 The vaccine composition according to claim 53 wherein the malarial gene sequence is the $(NANP)_3$ humoral epitope

55 The vaccine composition according to claim 53 wherein the malarial gene sequence is the DYENDIEKKI cytotoxi T lymphocytes epitope

56 The vaccine composition according to claim 53 wherein the malarial gene sequence is the SYVPSAEQI cytotoxi T lymphocytes epitope

57 The vaccine composition according to claims 37 to 51 comprising sufficient amount f the virus and pharmaceutically acceptable vehicl

58 A Flavivirus as a vector for heterologous antigens wherein the Fl vivirus is obtainable according to any of claims 1 to 19

ABSTRACT

USE OF FLAVIVIRUS FOR THE EXPRESSION OF PROTEIN EPITOPES AND DEVELOPMENT OF NEW LIVE ATTENUATED VACCINE VIRUS TO IMMUNIZE AGAINST FLAVIVIRUS AND OTHER INFECTIOUS AGENTS

The present invention relates to vaccine against infections caused by flavivirus. More particularly to the use of the YF vaccine virus (17D) to express at the level of its envelope protein epitopes from other pathogens which will elicit specific immune response to the parental pathogen.

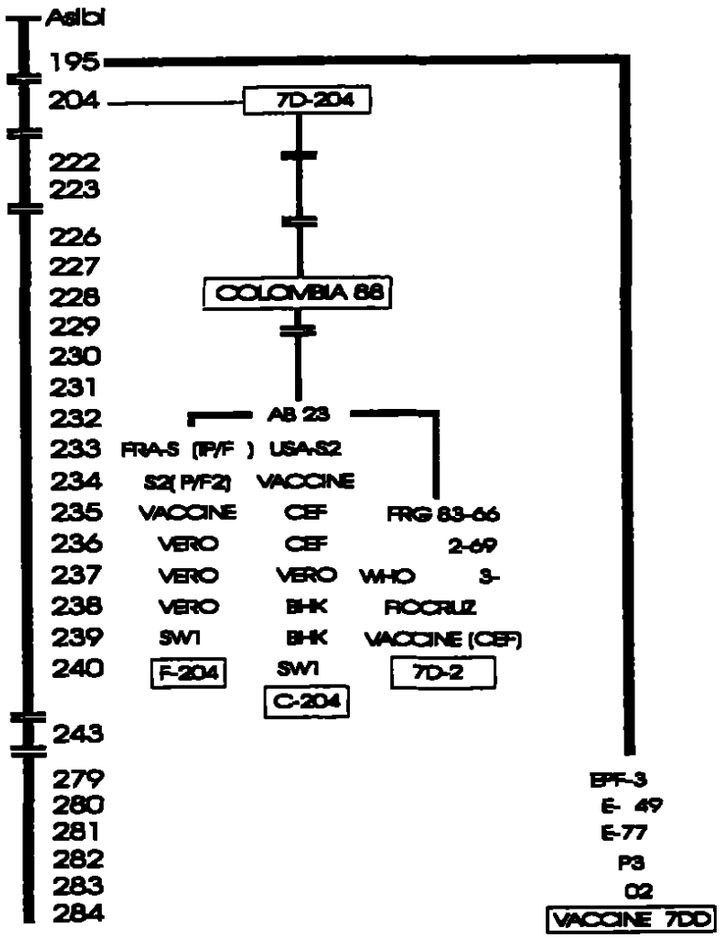
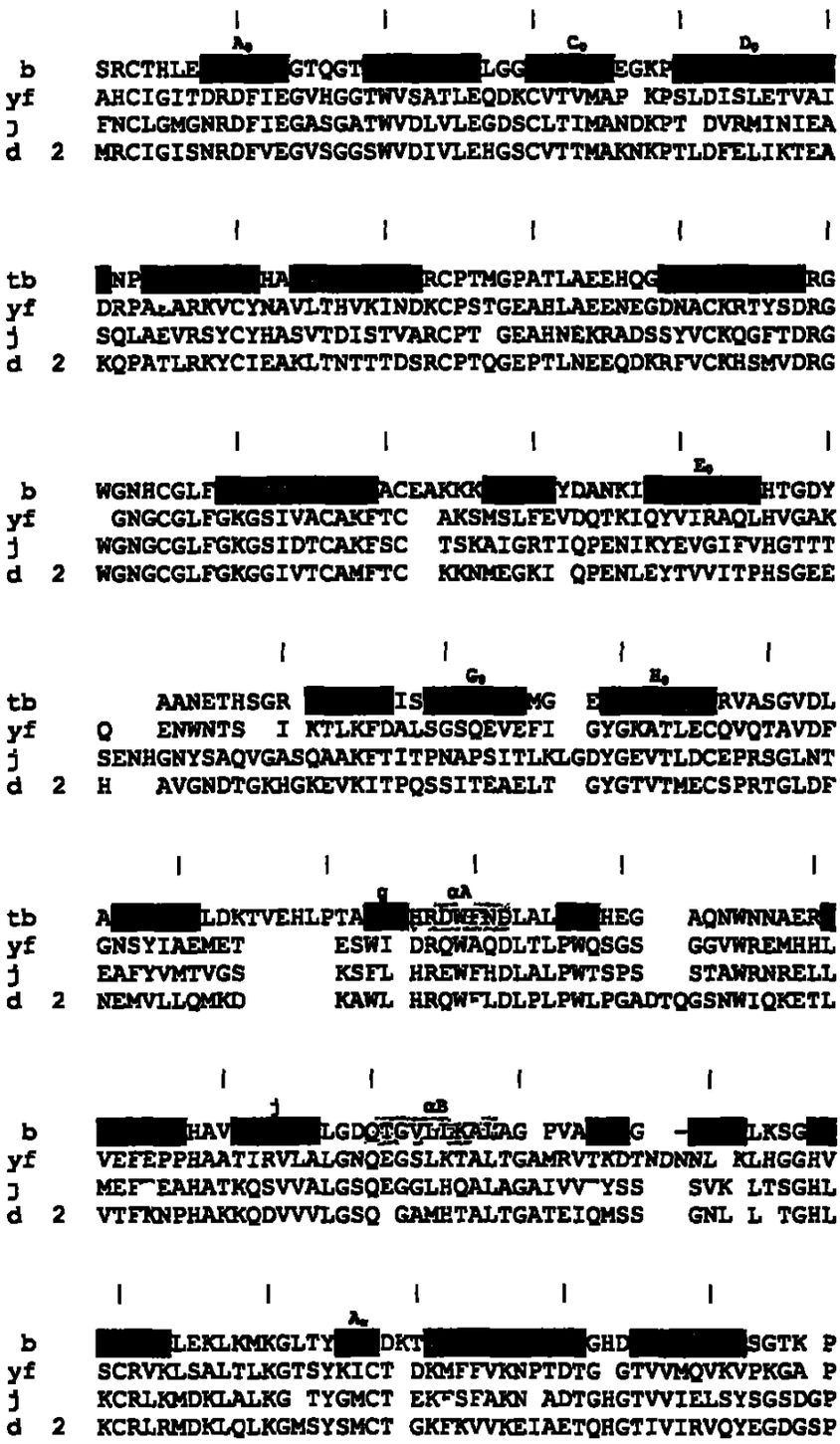

AsIbI
195
204
7D-204
222
223
226
227
228
COLOMBIA 88
229
230
231
232
AB 23
233
FRA-S (P/F) USA-S2
234
S2(P/F2) VACCINE
235
VACCINE CEF FRG 83-66
236
VERO CEF 2-69
237
VERO VERO WHO 3-
238
VERO BHK FIOCRUZ
239
SW1 BHK VACCINE (CEF)
240
F-204 SW1 7D-2
C-204
243
279
280
281
282
283
284
EPF-3
E- 49
E-77
P3
02
VACCINE 7DD

FIGURE 1


b SRCTHLE GTQGT LGG EGKP
yf AHCIGITDRDFIEGVHGGTWVSATLEQDKCVTVMAP KPSLDISLETVAI
j FNCLGMGNRDFIEGASGATWVDLVLEGDSCLTIMANDKPT DVRMINIEA
d 2 MRCIGISNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEA
tb NP HA RCPTMGPATLAEEHQG RG
yf DRPA ARKVCYNAVLTHVKINDKCPSTGEAHLAEENEGDNACKRTYSDRG
j SQLAEVRSYCYHASVTDISTVARCPT GEAHNEKRADSSYVCKQGFTDRG
d 2 KQPATLRKYCIEAKLTNTTTDSRCPTQGEPTLNEEQDKRFVCKHSMVDRG
b WGNHCGLF ACEAKKK YDANKI HTGDY
yf GNGCGLFGKGSIVACAKFTC AKSMSLFEVDQTKIQYVIRAQLHVGAK
j WGNGCGLFGKGSIDTCAKFSC TSKAIGRTIQPENIKYEVGIFVHGTTT
d 2 WGNGCGLFGKGGIVTCAMFTC KKNMEGKI QPENLEYTVVITPHSGEE
tb AANETHSGR IS MG E RVASGVDL
yf Q ENWNTS I KTLKFDALSGSQEVEFI GYGKATLECQVQTAVDF
j SENHGNYSAQVGASQAAKFTITPNAPSITLKLGDYGEVTLDCEPRSGLNT
d 2 H AVGNDTGKHGKEVKITPQSSITEAELT GYGTVTMECSPRTGLDF
tb A LDKTVEHLPTA HRDMFNP LAL HEG AQNWNNAER
yf GNSYIAEMET ESWI DRQWAQDLTLPWQSGS GGVWREMHHL
j EAFYVMTVGS KSFL HREWFHDLALPWTSPS STAWRNRELL
d 2 NEMVLLQMKD KAWL HRQWFLDLPLPWLPGADTQGSNWIQKETL
b HAV LGDQTGVLEKALAG PVA G LKSG
yf VEFEPPHAATIRVLALGNQEGSLKTALTGAMRVTKDTNDNNL KLHGGHV
j MEF EAHATKQSVVALGSQEGGLHQALAGAIVV YSS SVK LTSGHL
d 2 VTFKNPHAKKQDVVVLGSQ GAMETALTGATEIQMSS GNL L TGHL
b LEKLKMKGLTY DKT GHD SGTK P
yf SCRVKLSALTLKGTSYKICT DKMFFVKNPTDTG GTVVMQVKVPKGA P
j KCRLKMDKLALKG TYGMCT EK SFAKN ADTGHGTVVIELSYSGSDGP
d 2 KCRLRMDKLQLKGMSYSMCT GKFKVVKEIAETQHGTIVIRVQYEGDGSP

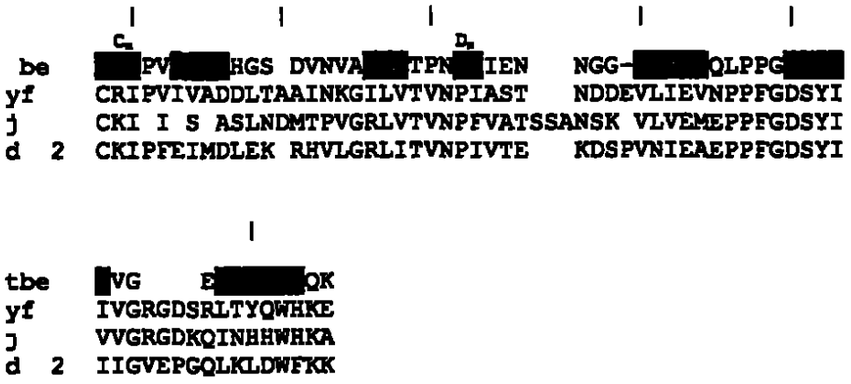
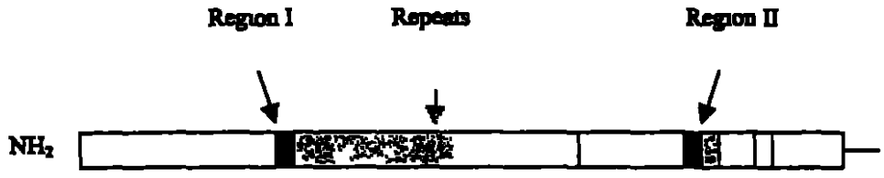

C
D
be [redacted]PV[redacted]HGS DVNVA[redacted]TPN[redacted]IEN NGG-[redacted]QLPPG[redacted]
yf CRIPVIVADDLTAAINKGILVTVNPIAST NDDEVLIEVNPPFGDSYI
j CKI I S ASLNDMTPVGRLVTVNPFVATSSANSK VLVEMEPPFGDSYI
d 2 CKIPFEIMDLEK RHVLGRLITVNPIVTE KDSPVNIEAEPPFGDSYI
tbe [redacted]VG E[redacted]QK
yf IVGRGDSRLTYQWHKE
j VVGRGDKQINHHWHKA
d 2 IIGVEPGQLKLDWFKK

FIGURE 2



Sp

P fal parum

repeat (DPNANPNVDPNANPNV)

CD8 CTL(DYENDIEKKI)

(NANP)

P yoelii

CD8+CTL(SYVPSAEQI)

FIGURE 3

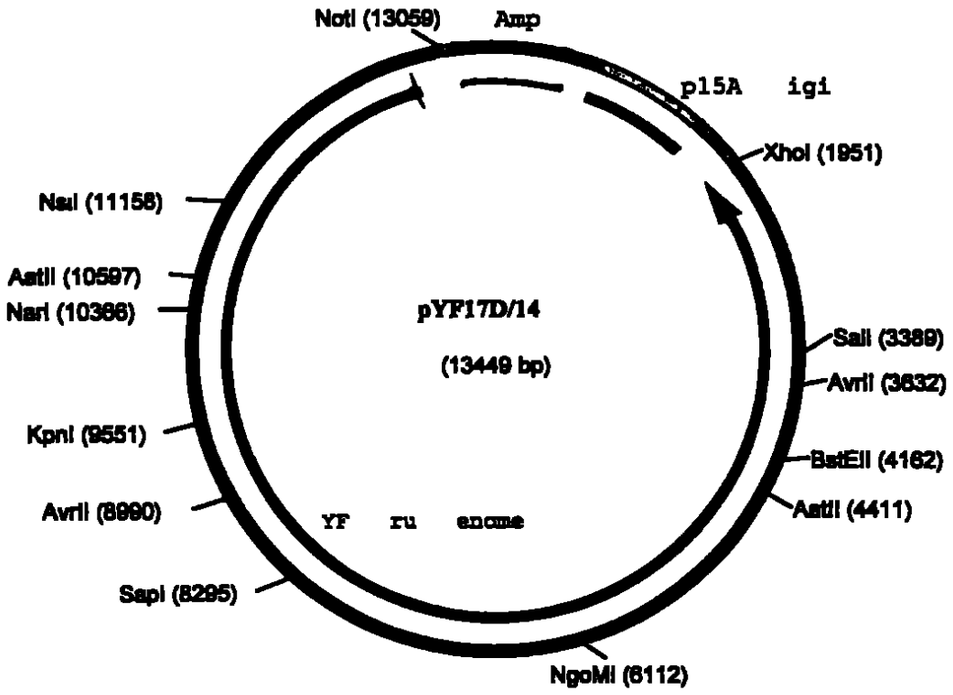

NotI (13059)
Amp
p15A
igi
XhoI (1951)
NsiI (11158)
AstII (10597)
NarI (10366)
pYF17D/14
(13449 bp)
SalI (3389)
AvrII (3632)
KpnI (9551)
BstEII (4162)
AvrII (8990)
AatII (4411)
YF ru enome
SapI (8295)
NgoMI (6112)

FIGURE 4

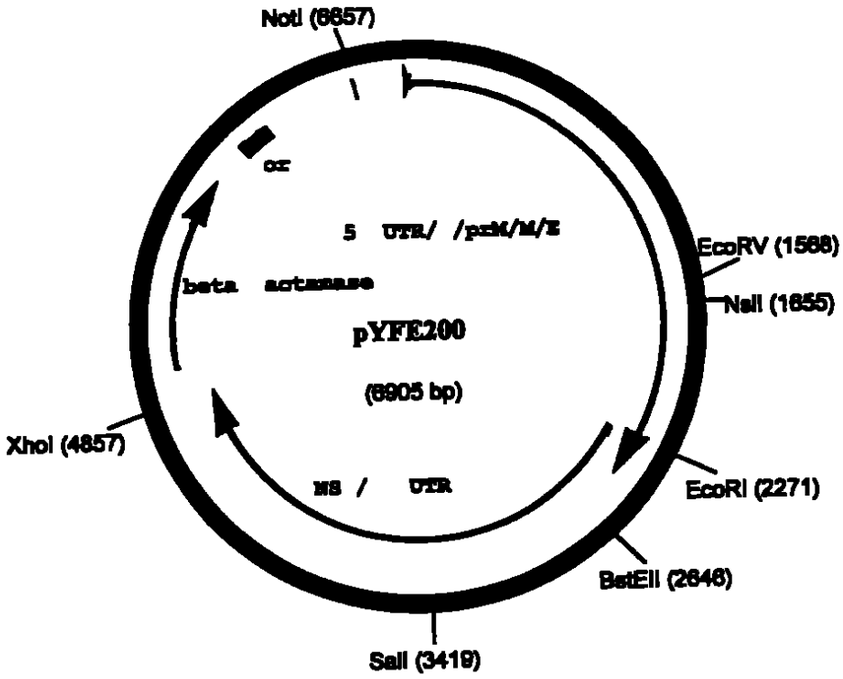

NotI (6857)
ori
5 UTR/ /pzM/M/Z
beta actamase
pYFE200
(6905 bp)
NS / UTR
EcoRV (1568)
NaII (1655)
XhoI (4857)
EcoRI (2271)
BstEII (2646)
SalI (3419)

FIGURE 5

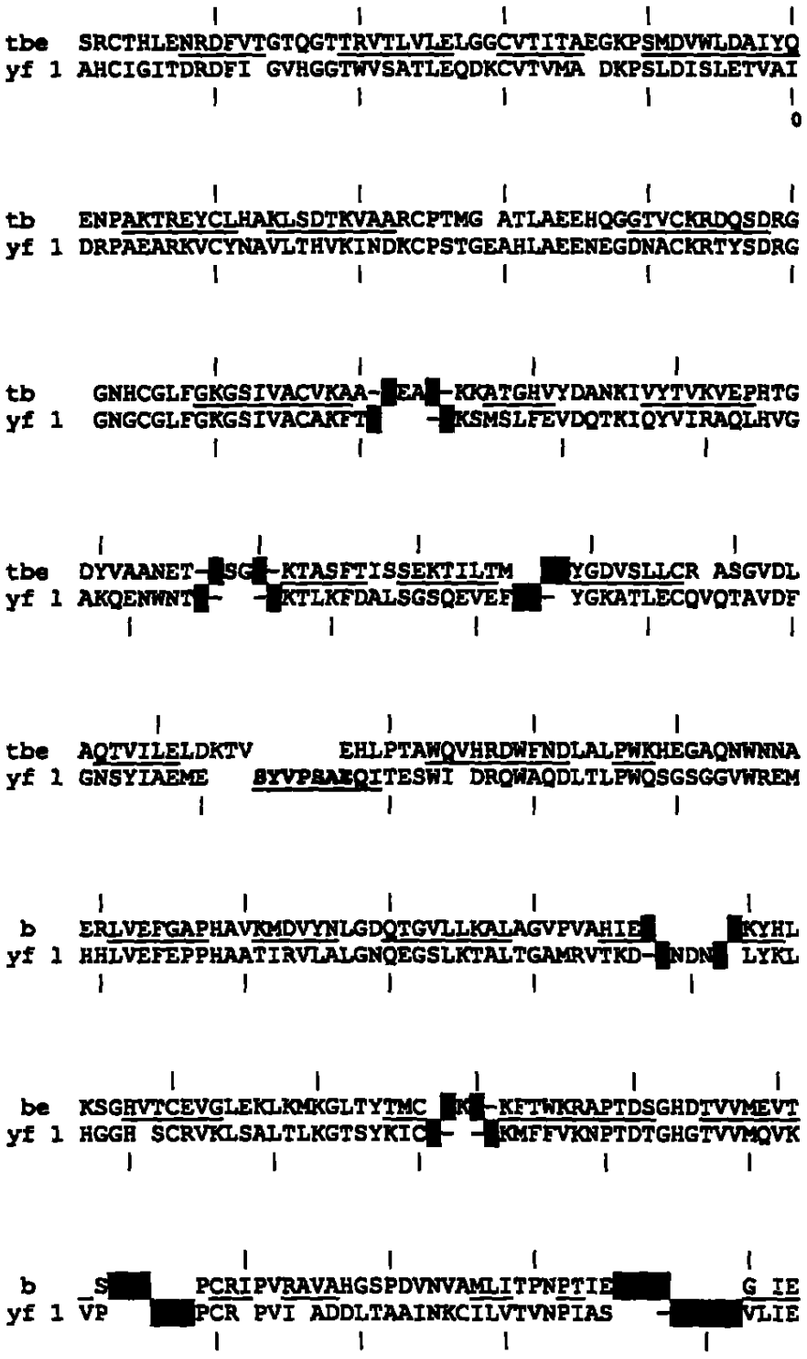

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yf 1 AHCIGITDRDFI GVHGGTWVSATLEQDKCVTVMA DKPSLDISLETVAI
tb ENPAKTREYCLHAKLSDTKVAARCPTMG ATLAEEHQGGTVCKRDQSDRG
yf 1 DRPAEARKVCYNAVLTHVKINDKCPSTGEAHLAEENEGDNACKRTYSDRG
tb GNHCGLFGKGSIVACVKAA- EA -KKATGHVYDANKIVYTVKVEPHTG
yf 1 GNGCGLFGKGSIVACAKFT -KSMSLFEVDQTKIQYVIRAQLHVG
tbe DYVAANET- SG -KTASFTISSEKTILTM YGDVSLLCR ASGVDL
yf 1 AKQENWNT- -KTLKFDALSGSQEVEF- YGKATLECQVQTAVDF
tbe AQTVILELDKTV EHLPTAWQVHRDWFNDLALPWKHEGAQNWNNA
yf 1 GNSYIAEME SYVPSAEQITESWI DRQWAQDLTLPWQSGSGGVWREM
b ERLVEFGAPHAVKMDVYNLGDQTGVLLKALAGVPVAHIE KYHL
yf 1 HHLVEFEPPHAATIRVLALGNQEGSLKTALTGAMRVTKD- NDN LYKL
be KSGHVTCEVGLEKLKMKGLTYTMC K -KFTWKRAPTDSGHDTVVMEVT
yf 1 HGGH SCRVKLSALTLKGTSYKIC- -KMFFVKNPTDTGHGTVVMQVK
b S PCRIPVRAVAHGSPDVNVAMLITPNPTIE G IE
yf 1 VP PCR PVI ADDLTAAINKCILVTVNPIAS -VLIE



FIGURE 6



FIGURE 7



FIGURE 8

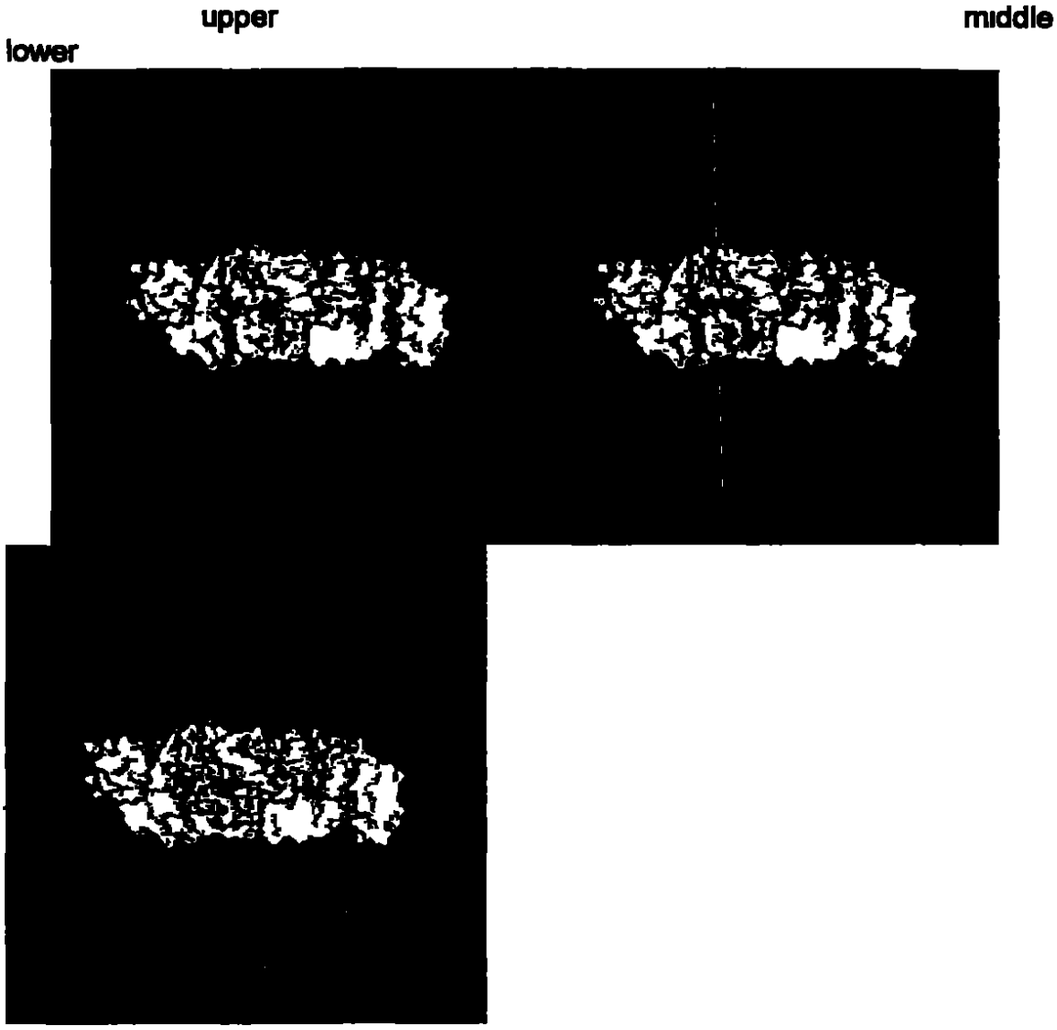


FIGURE 9

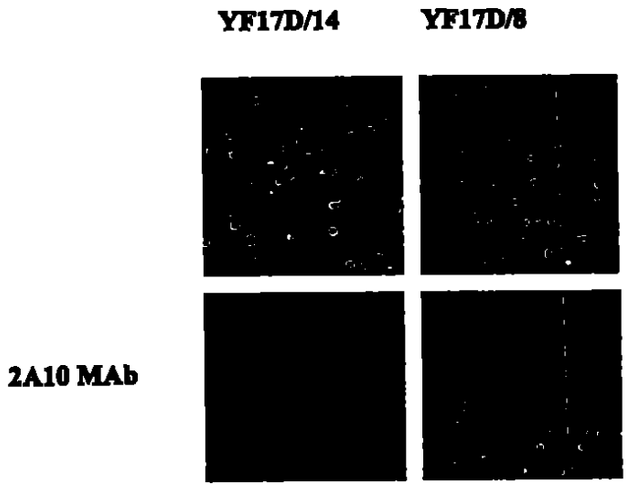


FIGURE 10

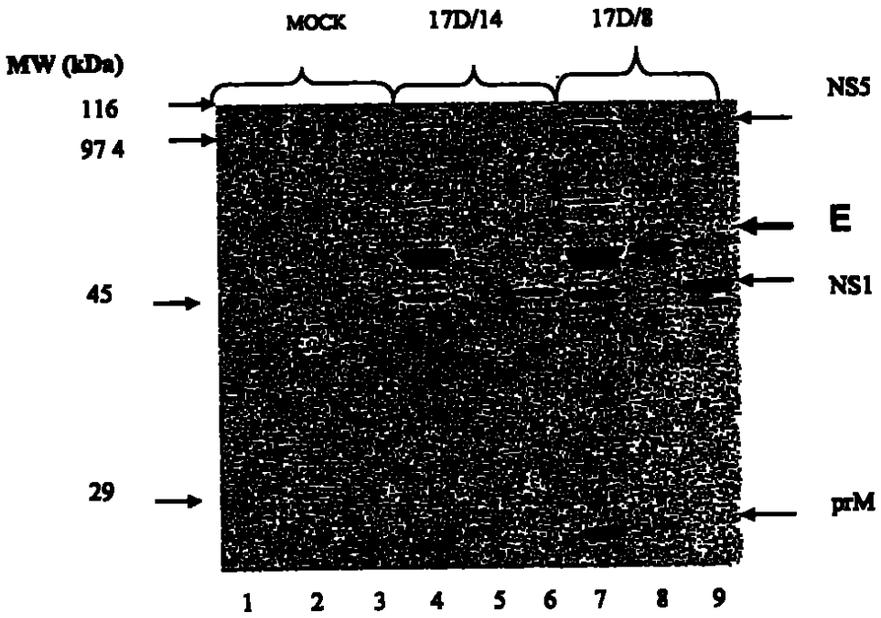


FIGURE 11

70

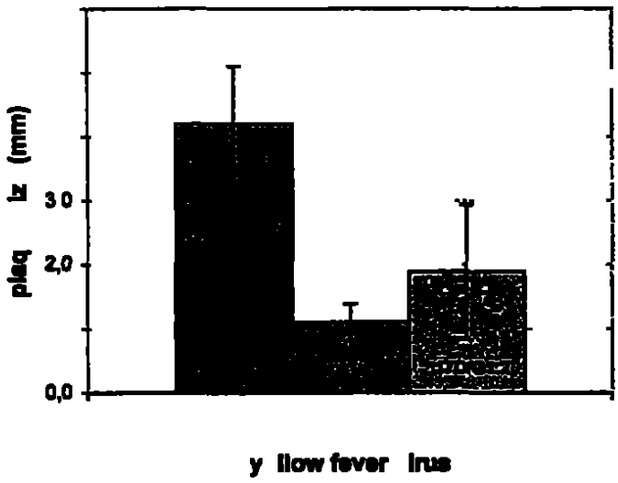


FIGURE 12

71

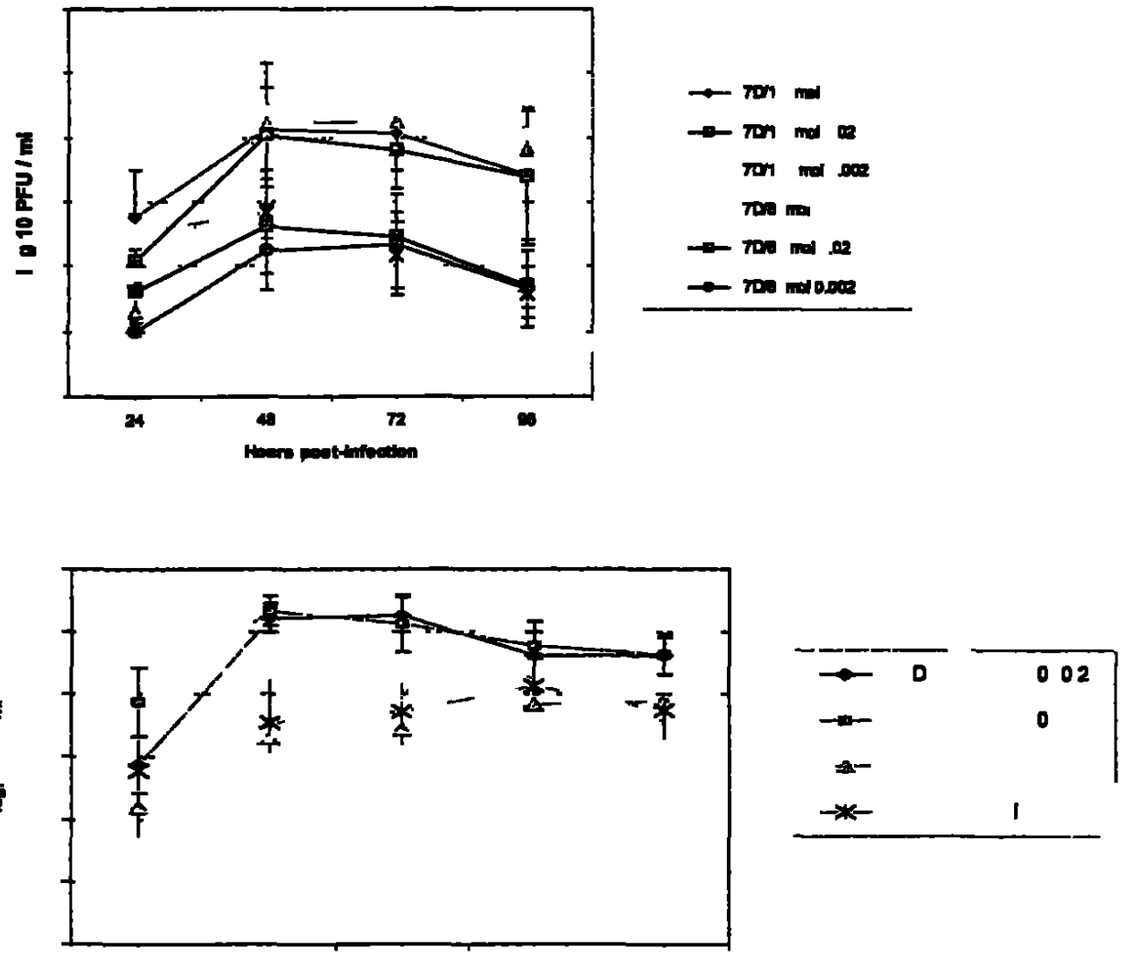

Log 10 PFU / ml
24
48
72
96
Hours post-infection
7D/1 mol
7D/1 mol .02
7D/1 mol .002
7D/8 mo
7D/8 mol .02
7D/8 mol 0.002
D
0 02
0
I

FIGURE 13

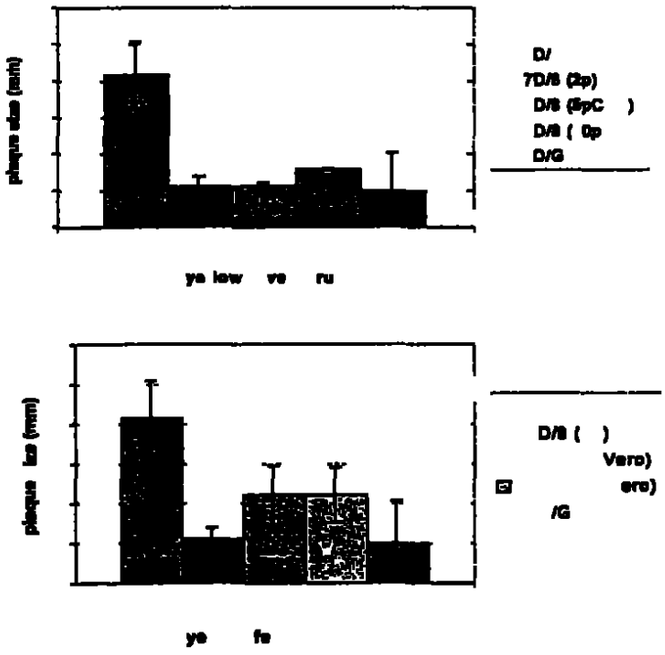

plaque size (mm)
D/
7D/8 (2p)
D/8 (SpC)
D/8 (0p
D/G
ye low ve ru
plaque size (mm)
D/8 ( )
Vero)
ero)
/G
ye fe

FIGURE 14

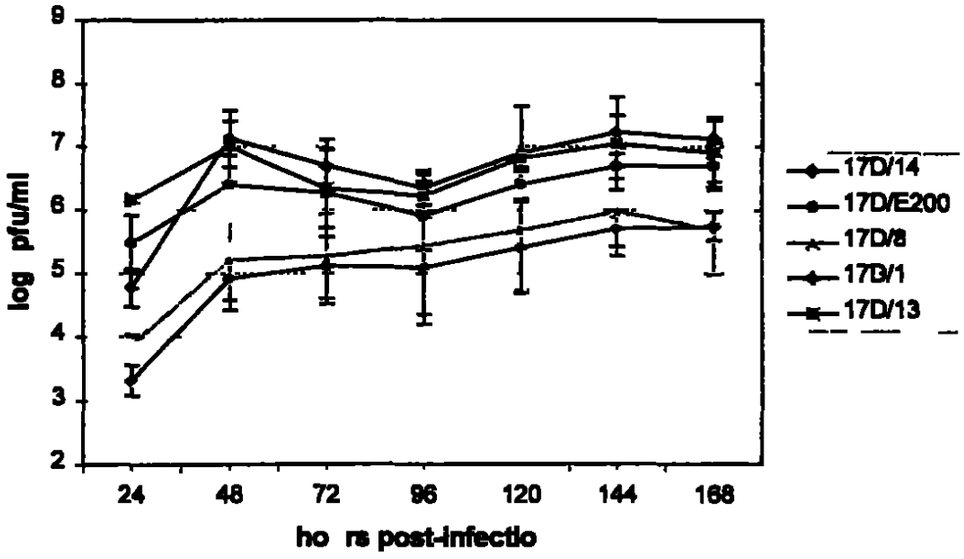

9
8
7
6
5
4
3
2
log pfu/ml
24
48
72
96
120
144
168
ho rs post-infectio
17D/14
17D/E200
17D/8
17D/1
17D/13

FIGURE 15

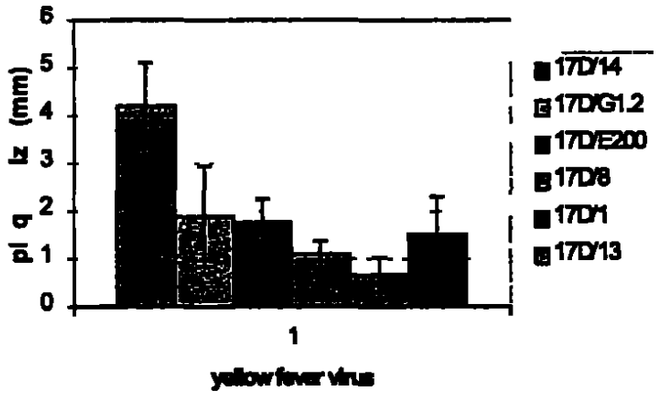

6
5
4
3
2
1
0
pl q lz (mm)
1
yellow fever virus
17D/14
17D/G1.2
17D/E200
17D/8
17D/1
17D/13

FIGURE 16

USO DE FLAVIVIRUS PARA LA EXPRESION EPITOPOS DE PROTEINA Y DESARROLLO DE NUEVOS VIRUS ATENUADOS VIVOS PARA VACUNA PARA INMUNIZAR CONTRA FLAVIVIRUS Y OTROS AGENTES INFECCIOSOS

L p nt ınven ión el n n un v un nt nf n u da p r Fl vıvı us Más p t ul rm nt c n l u o d l v us YF pa vacuna (17D) p p n u nıv l d s d ll pít p de p t ín p v n nt d t p tógen q e l c ta án un pu t nmun p f al p tóg n p ental S pr p n n n plá m d qu d p t n n d mb 21 2000 b ȷ l g nt núm o d d l Am n Typ Cultu C ll t n (ATCC) 10801 U v ty Blvd M n V 20110 2209 ( ) pYFE200/13 úm o d PTA 2854 ( ) pYFE17/8 úm d PTA 2855 (ı) pYFE200 núme de c PTA 2856 (v) pYFE200/8 núme o d c s PTA 2857 y (v) pYFE200/1 núme o de c PTA 2858

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ANTECEDENTES DE LA INVENCION

Lo Flav v rus cons ten de 70 patógenos humanos o veterına serolog cament de reacción cruzada cercanamente relac onados que or gınan muchas er as enf rmedades que ıncluyen f ebre de dengue encefal tı ȷ ponesa (JE) encefalitı producıda por garrapata (TBE) y f ebr amarılla (YF) Los Fl v virus son vırus fér cos con 40 60nm en diámetro con un cápsido co ahedral que cont ene una molécul de ARN de hılo pos tiva sencilla

El vırus YF es l vırus prototıpo de l famılı de lo Fl vivırus con un genoma ARN de 10 862 nucleótidos (nt) que tıen una tructur 5 CAP y una eg ón no t aducída de extremo corto 5 (118 nt) y un extremo 3 no traducido no poliadenılado (511 nt) La prımer secuenc a de nucleót dos compl ta de un genoma de Flavıv rus se determınó en l genoma del virus de la epa de la vacuna YF 17d 204 po Rıce t al (Rice C M ; Lenche E Eddy S R Shın S J Sheet R L y Str us J H 1985 Nucleot de ecuence f yellow fever vırus implications for Flavıvirus gene expres ón and evolution Science 229 726-733)

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El ARN nc ll tamb én l men aj v al y su tr duc ıón n l élul nf t d da como ultad l sínt s s de un prec rso d polipr teína de 3 411 mi á d qu div dıd p un p o am ento p t lít pa g ner 10 p lipépt d pe íf o d v u Del t rmin l 5 l den d l s p t ín c dif cada s C p M/M E NS1 NS2A NS2B NS3 NS4A NS4B y NS5 La p m 3 p t ín n t tuy n l p t ín t u tu l st f rman l vi u junt n l m lé ul d ARN emp d y n d nomin do áp d s (C 12 14kD) memb n (M y u p u p M 18 22 kD) y su env ltu (E 52 54 kDA) st nd tod s d f ad n l p im ua to del genom El st d l g n m d f p l p t ín n e t u tu l (NS) num das en l o d n de sínt s s d 1 ha ta 5

Tr s p teínas g nde no structu al s tien n u n i ltam nte n v d nt e lo Flaviviru NS1 (38 41 kD) NS3 (68 70kD) y NS5 (100 103 kD) Un p p l n l pli ión d l ARN d h lo n g tiv gn do NS1 (Muyla t IR Chambers TJ G ll R R Cm 1996 Mut g n i f N l nk d gly yl ti n t f

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YF v u NS1 ff t n RNA cumul ti n nd mou n u vi ul nc Vi logy 222 159 168 Muyl t IR G ll R R CM 1997 G net naly i f Y ll w F v v u NS1 p t in dentifi at n f temp tu n t v mut tion wh h blok RNA umul t n J vi ol 71 291 298 L nd nb ch BD Ri CM 1999 G n ti nt t n f Flav v us nonst u tu l protein NS1 y NS4A s d term n nt f r plı a funct n J Vi l 73 4611 4621 L nd nb h BD R c CM 1997 t ns mpl m nt t n f y ll w f v v u NS1 v l a l n rly RNA pl at n J Virol 71 9608 9817)

El NS3 h mo t do bifunci nal como un tiv d d d pr t n c p l p o sam ent d l p l p t ín n l s it donde la p te as celula es no lo rán (Chamb s TJ We r RC Gr kou A M Co rt DW B n JF Fl tt r k RJ R ce 1990b Ev d n n qu l dom nio N t rm nal de l proteína n t u tu l NS3 d l v u d l f b e ama ill n un in p t p n abl d l divi ne p cifi d iti n la p lip ot ína v l P N tl Ac d S i USA 87 8898 8902 F lg ut B P thel M Zh ng YM L i CJ 1991 Amb p t ín n

e t u tu l NS2B y NS3 s qui r n p l p am nt p t ul t c d l p t ína n e t u tu l d l vi u del d ngu n J Vi ol 65 2467 2475 Yam h hi v VF C mp ns RW 1995 Format n of th Fl v virus envel p ol f th vir l NS2B NS3 p ot a e J Vı l 69 1995 2003 Yamshıch cov VF T ent DW Compan RW 1997 Up gul t on f signal s p o ss ng and nduct n f p M E c eti n by th vi al NS2B NS3 p t e l s f pr t e component J Virol 71 4364 4371 St k CE L bıg M 1998 Sign l peptid e cl v ge t th Fl viv ru C prM junction d penden e on th vir l NS2B 3 prote for effi i nt proc ng r qu s d t rm nant n C the signal p ptide and prM J V l 72 2141 2149) It also contain nucle tidé t iph t /h li s a tiv ti (Go b l ny AE Koon n EV D n h nk AP Blin v VM 1989 Two el ted up famıl f put tiv h li e inv lv d n r pl t n omb n ti n p nd exp e i n f DNA nd RNA g n m Nucl Ac ds Re 17 4713 4730 W ngl nd W ngl 1993) t ndo p lo t nt también ciad c n pl ón d ARN vi l El NS5 l p ot ín vi l más g nd y má c n v d c nti ne v ios motivo de u n i qu s en son c mun s la ARN polimeras v le (Chambe s TJ H hn CS Galle R CM Rice 1990

Fl v v us gen me o g n t n xp sión and evolution Ann Rev M ob l 44 649 688 O R lly EK Kao CC 1998 Anál s of RNA dep nd nt RNA p lym as st uctu e nd fun t n gu d d by kn wn p lym s t uctu es nd mput pr dicti n f e unda y t u tur Vir l gy 252 287 303) nd xhib t RNA d p nd nt RNA p lyme t v ty (St ff n S Thi l HJ B hr ns S 1999 Th RNA d p nd nt RNA p lym of d ffe nt member f th fam ly Fl v v d xh bit m l r p op t in v t o J G n Vir l 80 2583 2590) F nally number ci and t an a t n lem nt nflav vi us RNA replication hav b e d nt fi d (K omykh AA S dl k PL W st way EG 2000 s nd t an t ng lem nt n Flavivı us RNA pl ti n J V l 74 3253 3263)

L c t p t ín p qu ñ NS2 2A NS2B NS4A y NS4B stán ns vad n u u n d am n á ido p n n u pat ón t am nt h d óf g El NS2A h mo t d qu qui l p e ami nto decuad d NS1 (F lg ut B Chann ck R L ı CJ 1989 P per proc s ng f d ngu v u n n t u tu l glyc p ot n NS1 qu e the N t rm n l hıd oph bı s gnal s quen nd th d wn t eam n nst uctu l p ot in NS2A J V ol 63

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1852 1860) M nt l NS2B h mo tr do qu s a l n NS3 p con t tui l omple] d p te v al t v (Chamb TJ N t wi A Amb g SM Ri CM 1993 Mut g n s f th yell w f ve n n t u tu l p lup t n t l t lly tiv NS3 p ten is d ma n nd NS2B qu d f l vag t d b si sit s J V l 65 6797 6807 F lg ut B P th l M Zh ng YM La CJ 1991 Both n n t u tu l p t in NS2B nd NS3 qu d f th p t lyt p ing f d ngu v u non tru tur l pr t ns J Virol 65 2467 2475 J n LR Y ng CS T nt DW F lg ut B L i CJ 1995 Pr ing f J p n e n ph l ti vi u n n t u tu l p t n NS2B NS3 mpl x nd h t l gu p ot s J G n V ol 76 573 580) El NS4 e ha ug id p ınte tu c n l NS1 nt g ánd l d nt d l p e c t pl smático de l pl ón d ARN (L nd nba h and R 1999) En a ón a que l ínte s del ARN v l t ne lug r n el cit ol n s o n l s m mb n RER s ha postulad qu sta p t ín h d óf ba st ían en membran y t vé de nt n I n p t ín I n p t ip n n l mpl] d pli ión v l junt n NS3 y NS5 (R CM 1996 Fl viv d th v as and th pli ati n I BN f ld DM Kn p PM Howl y (d) F lds Virology 3 d d R v n Pre USA p 931 960)

Dos s p d v u de fı b amarilla (YF) l d n 1997 d e n luga l v cunas pa a s r util ad p nmunı ión n human Un l s pa Asibi s i ló d un ɔ v n f i n d n min d A bi p l p d l m n Rh (M mul tt) y l tro el vi u V t pi f n é (FVV) d un pac ent en Sen gal

En 1935 la sep A ibi e adapto pa a c e n t ɔid emb ióni d atón D pué d 17 pa os l v u d nomın d 17D fu d c nalm nt cultiv d h t l p 58 n t ɔ d mb ión c c mpleto d pollo y lueg d t h t l p s 114 olam nt el teɔ do mb ón de poll de ne v do Th iler y Sm th (Th l M nd Smith HH 1937 Th ff t f p l nged ultiv t n v t up n th p th g n ity f y ll w f v vi u *J Exp M d* 65 767 786) mo t ó qu n t et p xi tía un du ón m d n l ví y n u t pi mo vi l u nd n ul nt b l ment n monos El vi us fu d má sub lt v d ha t l s p os 227 y 229 y l vı u sult nt n u o nmun hum n e util on p inmuni 8 humanos volunta os on e ult dos s tisf to s como se most ó p r l u enc de i n dv y e o nve ión YF n dos eman

(Th l M Smith HH 1937 Th u f y ll w fev vi u mod f d by in vit ultiv t on f r human mmun t n *J Exp Med* 65 787 800)

A f n l s d l s ñ 30 y om en s d l 40 s p duj una v cun c ón m iv en B sil y v ı p í e n f ı n JUL et l (f x FP y Pena HA (1943) B h v of 17D yell w f v v ru in Rh u monk ys R l t n t ub t n d s nd neu al xt n u al in ul t n *Am J Hyg* 38 52 172) d ib l pr p r c ón d l v n d ub pa de viru 17D E ta ub ep dif ían n su h to a de p o y ll s t l p b n n l ión l t emp de u u o par p du ión d nócul s y/o v un L ust tuc ón d ad un p l s gu nt fu de acu rdo a la expe i nci g n d du nt l p du ión de la vacuna el control d cal dad y la va unación de humanos en el cual la pa c ón d s ntom tologí conducía a la descont nuac ón d un p d da

C mo m n on nt i rm nte l p Asib del v u YF ub ult vo n t jid d tón mb óni y emb ón d p ll mpl t d m nu d n o n t ȷ d n v oso E tos paso p duj on la p padr 17D en el

n v l d p o 180 17DD en l p 195 y 17D 204 n el pa 204 El 17DD fu ubcult vad di on lmente ha t l p 241y uf ó 43 pa s d ci n l n hu v d p ll mb i nad h st l t nda de vacuna utilı ada p l pu fi ón d l vi u 17DD (p s 284) L 17D 204 fu ub ult v d ad i n lm nt p pr duci l p C l mb 88 l u l al p so en hu vo d p ll emb nad dio rıg n d fer nt s l t d s mill d v un habit alment n u o en F nc a (I P steu n el p 235) y en lo Est d Un do (C nn ght en l p s 234) C da una de st p 17D 204 fue pu ıficad en pl a n d f ent l g d célul s l v ru f n lm nte ampl f d en l élul SW13 y ut liz do p a anál si d l n ión y d u n a de cADN E t 17D 204 on d n min d C 204 (Ri C M L nch E Eddy S R Shin S J Sh t R L nd St u J H (1985) Nu l t d qu n f y ll w feve vı u impl cati n f Flav v u g n xp s n nd v luti n S nc 229 726 733) y F 204 (D p P C h u A Dupuy A D ub l V B ul y M Dıg utt J P Gi d M (1987) High g n t stabil ty f th oding r g n f th structural p ot ins of yellow f ver strain 17D J G n V l 68 2245 2247) esp ct vamente La sepa 17D 213 d v d l 17D 204 u nd util o el lot d

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sem ll p ima (Sl 12 69) p ov ni nte de la R public F d al d Al man (FRG 83 66) po la rg ni ción mund l d l lud (OMS) p p du l emill 17D l b d vi u d l l u si vi n (Sl 213/77) n l p 237

L 17D 213 en l pas 239 se ensayo pa n u v ul n i n mono (R S M h v ky p on l omm nic t n Du rt d Sant s t l (Du rt d S nto CN Po t PR C v lho R F r eir II R c CM nd G ll R 1995 Compl t nucl otide equ nce of y llow f v r v v in t in 17DD nd 17D 213 Vi R 35 35 41) y fu l bj to de nál i d u n i junt n l 17DD (n el p o 284) y c mp c ón on la se uen ia de nucleótid s previamente publ d d ot epas d vi us YF (Duarte dos Sant et al 1995 As bi Hahn C S D l ymple J M St uss J H nd Ri C M (1987) Comp on of the vi ulent A ib t n f yell w fev v u w th the 17D vacc n st in d iv d f m it P N tl Acad S i USA 84 2029 2033 17D 204 t n C 204 R c C M L n h E M Eddy S R Shin S J She ts R L and St u J H (1985) Nucl tide s quence f y llow fev v u impli t n f Flavıv u gene exp es i n nd

v lut n S n 229 726 733 F 204 D p P Cahou R Dupuy A Deubel V B ul y M D goutte J P nd G d M (1987) High genet c stabil ty f th d ng g n f th tructu l p t in of y ll w f v vi u t a n 17D J Gen V l 68 2245 2247) L f gu l d b l h st a d p d l ep Asib YF gin l y l de va ión de las ep de la v cun YF 17D

Un t tal d 67 dif ren i d nucleótido qu pond n 31 amb o de am noá ido se not on or g n lm nte ent e l s uenc genom ta Asib y 17D 204 (Ve Hahn C S et al 1987) La comparación ent e la cu n d nu leót d d la ub p 17DD y 17D 213 (v D t dos S nto t l 1995) y l ecu n ia d nucl ótid d l ub pa 17D 204 (v Ri et al 1985) mo t ó qu n t d l amb n mun s y í n nf mó mo sp íf 17D P l t nt l amb p íf d la ub p 17D ob v d s n muy pr b bl ment no relac onado on l tenu ión p ro pod í n efl ja dif en ias en el comportamiento de st p s n n y d neu ovilu encia n mon s P l tant l amb n l s u n d 48 nu l ótid n p bablem nt s d c n l tenu ón d l v u

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YF E t n p d junt n l genoma 26 mut n ilent s y 22 nduc n su tituci n d am n á do L alte i n not das en la p teín E on mp t nt n ón a que st l p in pal obj t v p l pu st d l n ut li ción humor l es dec t l p t ín d nde e lo li n los epítopo d h mo glut n c ón y n ut l ión y t m di en n m nt pt d élula y l p net ción d l élul p l tant punt nd l v u élula p fi D man mp t nt l p t ín E umul l má lt p p ón d ambi d amín á ido n n rv dor a on v dor En conjunto onc ust tu n de nucleót do ob e v ron n el de la p t ín E qu ndu amb d 8 am noácid en la p i n 52 170 173 200 299 305 380 y 407 (sp t v m nt nu leótid 1127 1482 1491 1572 1870 1887 1965 y 2112 del ext emo 5 d l RNA) Ent e t u t s n amb n con v do y p umibl m nt t nd í n un may imp t ob l t u tu d l p t ín y c nse uentemente l fun ón

L alter ion s n l aminoácidos 52 (G→R) y 200 (K→T) l l n n l b d l d min II n l t u tu 3 D pr pue t p a l p teína E del

88

Fl v v u (R y F A H in F X M ndl C Kun C y H n S C (1995) Th nv lop gly p t n f m t k b n n ph lit v u t 2Å s lution *N tur* 375 291 298) l u l e n rv ent e l s Flaviví u y nt n pit p d ón u da mo demu t p M ndl C W t l (M ndl M W Gui kh F H l m nn H H n F X y Kun C (1989) Antig ni t u tu of th Fl vivi u nvelope E p ot n t th m l ul l v l u ng ti k b n en ph lit v u as mod l *J V r l* 63 564 571) E te domin II e ltam nt r ti l d medi nt d nl c d d lfuro y f t n ón d baȝ pH el cu l e ta rela ionado con l po ión un tensión e t ctam nte cons rvad e h d óf b d am noá d qu up nen tán nv lu d n l fusión d l cub e t v r l d l m mb na d endos m

L alte ion s n l aminoácido 170 y 173 n el d m n I d l p t ín E n l m p d la tru tu 3D muy n l p ón n qu id nt fic un pít p d n ut l ón pa el v us de la encefaliti p du d p g pat (TBE) (v r Mandl C W et al 1989) Un mut ón n l p ón 171 de l p t ín E d l vir TBE mo t ó qu f taba l umbr l d l cambi

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nf rma n l d fus ón t v ón d st p t ín y 2 mb obs v d p el v u YF 17D p d ían e ta l n d n el m m fenóm no Es con eb ble que un p p ón má l nt d l fu ón pud t l nét ca de p du ión del vi us y de e ta man ra onduc una nf cción med a del huésp d Ev dencıa d ıonar pa a l mpo tanc a d ta ár a v ene del t ab ɔ d Rym n t l (Ryman KD Xi H L dge N Campb ll GA y B r t ADT 1997 Ant g n v i nt f y ll w f v v u with lt d n u vi ul nc ph n typ n mi e V l gy 230 376 380) Que mu t qu e t dif un pít p n id p r un nti u rp mo cl n l t p ilv tr y l r v r ión n t t o pud hab ntr bu d gr g rl neur virul n i n l mod lo de atón de un vi u v nt cup ad d l p bl ión d la v cun 17D

L alt r n d l amın á ido 299 305 y 407 l ali an n l d mini III (ve R y F A t l 1995) E t d min ugi ió p t inv lu do n l un ón vi l un c pt c lul y n u nt m nt un may d t rm n nt t nt d l p p c ón del huésp d como del t opismo d l élul y de la v ul n /at nu ón L cambi d los 4 aminoácidos

p t dos pa el YF s lo l a n sob la car d tal d l d m nio III E t á teni ci lo qu s un amb st ch n l vi us de l encefal tis p oduc da po g p t p nt n u t idu di i n l n t d l p p du d p mo qu to En ón qu l vi u pl c n n u v t e t i lo p ob blement un d t rmin nte de p op rc ón d l huésp d Est cl ag and do contien una ecu nc Argin n Gl c n á id pá t (Arg Gly Asp) en tod l s p s d l v un 3 YF 17D E t motiv d u n n do p m d n un núme o d nt r c nes d célul que ncluy n l un ón l rec pto y e ta usent n l n l p As b vi ulent p ent l s no tamb én n t 22 p d l v u tip lv t YF (L pin L Dalg n L Huong VTQ M nath TP Dig utt JP y D ub l V (1994) G ogr phi distr b tıon and evolution f y ll w f ve v use b ed on d ct sequ nc ng of g n m DNA f gment *J G n V r l* 75 417 423) T l f ct ugi l mut ón de t nina (Thr) a g n na (Arg) ea un mot vo Arg Gly Asp es probablemente l v nt p l fen t po t n do d la s pa YF 17 D C n st ntem nte L bıgs et al (Lobigs M Usha R Ne t w A Ma h ll ID W RC and D lg n L 1990 H t ll l ti n f Mu y Vall y n eph liti

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v u v nt lt r d t n RGD qu nc n the nv l p p t n nd n mou n u vi ul n *V r l gy* 176 587 595) d ntıf ó un mot v d u n i Arg Gly Asp (el am n á do 390) qu nduj un pé d d d l v ul nc d l vi u d l encef l tis Mu ay Vall y p ra t n Al m n p YF s n emba g n l determ nante om s mu t p v n de Mo t et al (van d M t RG Co v J Str uss JH 1999 Mut gén of th RGD m t f n th y ll w f v vi u 17D nv l p p ot n *V r l gy* 265 83 95) S ugi ió qu l c n n l l RGD s c ít c par la c nform ón de E y los camb os men re en e ta región pueden t ner fe tos drá t s en l tab l dad de l prot ína E f ct bl n mb go qu tal s mb s n l e t u tu y/ t b l d d pu d n f t l p t d la élul nf ct da p l nflu nc d l t u tu d l p t ín E t t l y unqu e á a pa t cul n st ía d tam nt inv lu d n l unión d pt r En ón qu t cl tá p nt en t d s l Fl viv u p du id p mo qu t y l may í d llo ll v n un u n i qu muy mil l motiv 17D RGD e nceb bl qu l mutac nes en st reg ón y ot os Flav v u f t n l titud v l

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L mp tan d l r s du 305 fu implic d d l h lla gos d J nn ngs t al (1994) qu nes not on que el v u 17D cup d d un d nc f l t p tv un l tuv un ambi E→K n l p i ión 303 y n nt ó qu t ní n u v lu n n em nt d t nt n ton mo n mon s

E n t bl qu l d ll d l cADN nf p l viru d l n f l ti j p n (JE) h h p Sum y h H t l (Sum yo hi H H ke CH nd t nt DW 1992 Inf ct s ȷ p n nc ph l t viru RNA n b ynth i d f m n v t l g ted DNA templ t *J V r l* 66 5425 5431) p rm tió l id ntif c ión d un mut ión (Ly p Glu) n l aminoá do 136 d l pr t ín E qu di mo sultad l pe did d l ur v ul n i p r l r tón (v Sumiy hi H Tign G H y Sh p R E (1996) Ch t iz ti n of highly tt nu t d ȷap nes en eph lit v rus gen rated f om mol ul y cl ned DNA J Infect Dis 171 1144 1151) Est sıgn f a qu el domin o I es un área impo tante que ont n un d t rmin nte rit co d l vi ul n d l vı u JE en cont ste con la mayo ía d lo dat bt n do d l náli d vi ulen ı pa v ios d los t o Flav vi u pa l s cu les se sugie e que el

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d min o III l itio primario p r l det rm n nt de v ul n i / t nu ción

P l v u t p 2 d l d ngu But pet t l (2000) h n ug d que l s det rm nant s d tenu c ón de l n po f r d l á structural a b r UTR 5 NS1 y NS3 Po s sab ce ca d la bas molécul d l vi t pi mo d i l ap cid d d l vi u YF t p lve t p r pli y d ñ l t jid xt n u l p ialm nt l t jid h pát l mut i n sp n abl s d la pé d d d t a ct í t c en l vi u 17D Estudio ad cionales en la n u v ul n 17D n l modelo d atón h n suge d un b e genét c compleja iendo más p obablement multigeníca ınvoluc ando las p oteín s e t uctu al s y no st t al sí mo tamb én l r gión no traduc d d l t m 3 l últ m p um blem nte st ingi í l epl c ón Sin mb g tal náli d l p ot ín E p p i n n un st u tu pa l entendimıent d v í p t d l bi l gí d l Fl viv ru y ug n qu d b po ble t ab j p ngenie í v u p l d ll de nu v v un d Fl v vi u v v s

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S nd l may omp n nte d l upe fici d l vi ón l p t ín E d ub e t jueg un p p l d m n nt n l t anti u p n ut li nte y l ndu ón d un spu st p ot to E to e ha m t d de man ncluyent m diante l nmun c ón ctiv d n m l n mp nent ubvi l s d finid y prot ín ecomb n nte y mediante experimentos de prot cc ón pa iva n anticu rpos monoclon les e pe íf c d l p teína E L s epítop s l neal s s h m p d ut l nd pépt d s intéti y se h n n ntr d n á s d l gli p ot ín p di h p hid fil c n emb g l ndu ión de nt u rp d n ut l ón p fu tem nt dep ndi nte d l nf rma ón n t v d E Un núm d ti d n tr li ón h n nf rid d tudi c n mut nt de c p d nt cu rpo mon lon le y e han map do sob e l e t uctu 3D Consistente con la top logí ug d d l dim obr la supe f cie d l vi ón la m y í de t t tán l li do b l l do upe r xt nam nte cc bl d la ubunid d L di t buc ón pa da de e tas mutacione de escape de neut al c ón sob e ubun dade completa ind can qu l un ón d l nt u rp a cu lqui d los domin

95

t u tu l puede conduc a l neut ali ón d l
v

Los epít p de neut l ación n ido po l
nt u p s mon clon les n c nformaci n l n ón
q l d n tu l ión d l p ot ín E ab l ó l
n ón Má un l s nt u rp mon l n l l
n n n péptid ntét i llo ono n
un pít p qu ste p nte n l p ot ín E
d n tu al d En ón qu l ubunid d d mé i
f rm pa te d un a un ind f n do n j d l
up f d l vi ión p bable qu t s pít p
tén mpu t d lem nt d dif nt subunid d

L m an smo d neut l ón por tos
nt u rp p rman n p ul ión p l v nt l
st t g d in ión d l pítopo d ita d l nte
h pót d qu l d mini II pu de e t involu d n
l fu ón d l ub t vi l n l membr n d l
nd m l u l u baj un pH á d L fu ón
q i amb d nf rma ión qu f t n l pítop
d n utrali ón v p n palm nte d nt d l
dom ni I y domin II nt les E t camb stán
p ntement ci d s con un rg ni ión d las

nt n ubun t i bre l up rf c d l
vı ión on nt t d t íme ndo f vo id s n
l f rm d pH b j en nt st c n l c nt t
dím n l f rma n tiv L nt f n i n t
pr d p s on s est uctu al p l unión d l
nt u p p e nt un mec n smo qu pu de conducı
l n ut l ıón del v us (M n th y He n 1996)

Act v d d n ut al nt d títul b ɔ y un g ad
signifı at v d p t ión p iv en at ne s ha
b v d m d nt l nmun a ión p iv c n nt u rp
mon l n l nt p M E t d b un i t g d
l v d n ón p i l d p M p f rma M m d nt l
fu n n l i t ma G lg y d ta forma l p M
perman e o d n l p rtícul d v ru qu un
bɔ t v ad i n l p l nti ue p

L pr teína NS1 tamb én c noc d mo l nt gén
de f ɔ ón d mplem nt l it un e puest du nt
nti rp d l d l inf cción d l Fl viviru n el
homb E t l mo f rma i d s y t d p
élul y h mo t d qu la inmun za ión d anim le
on NS1 pur ficad o nmun ac ón p siv de nimales n
nt ue p s monocl nal a ste el ta una respu ta

nmun p ot ct a la ba e d la cual s aun ont ove al El papel ınmunológ co p ma o de las p ot ín s n st uctu al s pt p a NS1 pa cen se l bj tiv p l élul it tóxi T L p fi d d d la pu t d l élul s T lo Fl v v u h tudi d n l homb y en l i tema d t n p n palm nt n l v us r ompl j d f lit ȷ p n En l u d l inf ión del d ngu n l h mb t nt l CD4 CD8 í mo tamb én l p t d l l nf t T opu st CD4 CD8+ s h d te tado y c ract r ad En ult vos de ma d l nf to CD4+ í m tambıén c n lon d célula CD4 bt n d d un nd v du s mpl qu se ha nfect d n d ngu ob v n p t n d e tıv d d u d p íf c d f nt n t Fl viv u Ob v i ne m l m nti n n p élul CD8+ d humano nfe t d y t n

L d t rm n nt ntig n involu ado n l nmun d d m d d p célul n h n l l d p íf m nt n l p t ín d l v u YF com lo ha d p a l s vı u del dengue y la ncefal tis t l c mo MVE y JE T l d t rm n nt d élula T t tó ca s u t n n t das la t e t uctu a y n p t ína

e t u tu l tamb én mo p lm nt en NS3 Algun d t s pit p s h n map d n u s uen p m b l p t v p ot ín Living t n t l (L ving t n PG Ku n I L CJ B y M Enni FA 1994 R gn t n f nv l p p t in by d ngu vi u e typ p fi human CD4 CD8 yt t i cell cl nes *J V rol* 68 3283 3288) ep ta n l dent fı ción de va os l ne CD4+ CTL HLA cl se II st ng d s d un don do hum n cap d m dia l sis pecific de las élul s nf ct d n v u Se ha ug do qu l CD4 CTL pu d n mp t nte m d d d l l mpi v l p c lm nt du nt l inf c ión n l m smo t p de v u

L va un ión d humanos n po tvi us mbin nt qu xp n l p tein tructu l p M y E de l en f l t ɔ p ne l tar n CD4 CD8+ CTL d ig d s la p ot ín est ucturale del vi u JE unqu n d nt fi n pitop p ífi (K n h E Ku n I M s n PW Sh p RE K n s Th n N Smu ny JJ H k CH Enn FA 1998 Indu tion f japan n ph lit v u p if c ytot x c T lymph cyt in hum n by p xvi u b s d JE v c n c ndid t *V c n* 16 842 849)

99

Má i ntem nt s h n reportado epítopos de p t ína E d v u JE econocidos por CD8+ CTLs de t p ífi s JE El pít po s encont ó qu c pondí a los am noá ido 60 68 d la pr teín d l v u JE que t ba locali do n el dom nio II (Tadaka K M ak H K n hi E Tak h shı M Ku ne I 2000 D f n ti n f n pit p n j p n se n ph lit vi u nv l pe pr t n r c gniz d by JEV pe fi mu in CD8+ yt t xi T lymph it *Ar h V r l* 145 523 534) E t epítopo sta local ado ntr l s hebras y b d l dom n o II que incluy dos e duos de amin ácidos d d un d l pítop s est nt s que c mp enden el cl t nterm d o E ta á a sta expuest s b e la up f c d l dime

L pit p d l élul yud nt T fun i n l d l p t ín E d l Flav vi us id ntifi on l med l pu t de l élul s B d pué de inmunı ción c n pépt d s ntéti o (R h ng JT J n on AJ Hunt AR 1994 T h lp ll pit p n th E gly op t n f d ngue 2 Jamaic v us *V r logy* 198 31 38)

L p c dad p man pul l g noma d l Flav v u t vés d t n l gí d cl n inf c o p l d s ll d va una E t e así p qu l s vi u se p d n cupe d l ADN omplem nt o med nt t n p ón in v t y t n f c ón de élul ult vad s on ARN y st cADN qu sp nden al g n ma v r l c mpl to p rm t n la nt oduc ón de mod f n g nét a n ualqui t p t ul d l g n ma vi l El tud pi n d R an ll y B lt m (R n ll VR y B ltimo D 1981 Cl n d p l vıru c mpl mentary DNA infe ti u in mammal n cells *Scienc* 214 916 919) mostr n prim r l f t b l d d de g n vi u de cADN cl n d En l p t nt t d un d n 4 719 177 Rac niello y Baltimor d s ib on en d talles l p odu ción d cADN vi al ARN m d nte la t an c pc ón eversa de ARN v al in t l molé ul d cADN sultante en el vecto de ADN mbınante El p es p t ularment t ní qu v n l p du ión d ADN complementa d doble h b d p li vi u (ds DNA) Ello av igu ron que l cADN del pol vi u d l ngitud mpl ta t n f tado er n m mo nf

Además de todos los sistemas de transcripción in vitro (véase Melton DA Krieg PA Rebagliati MR Maniatis T Zinn K and Green MR 1984 Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter *Nucl Acids Res* 12 7035 7056) un efecto mucho mayor en síntesis de ARN viral de longitud completa comparada con la transcripción de cADN en éllos hizo posible Adicionalmente todos los métodos de transfección mejorados tales como liposomas y la electroporación en momento en el transfección de ARN de élulas ultravioleta

La construcción y clonación de copias de cADN de ngud de longitud completa establecen una separación de Escherichia coli utilizando el vector plásmido pBR322 de bióspora La CJ tipificación (La CJ Zhao B Hori H y Bray M 1991 Infectious RNA transcripts from stably cloned full lnggth DNA of dengue type 4 virus *Proc Natl Acad Sci USA* 88 5139 5143) Ellos verificaron que los plásmidos purificados mediante los moldes de ADN clonados de longitud completa en infección y la progenia de

v u up r d d célul tr nsf t da
nd tıngu ble de l s vi u p ental s d l s u l s
habí de vad el cl n d cADN Per como s m n no n
l li tud de p t nte WO 93/06214 t l n t u ón d
ADN inf y t n t d ARN g n d d t
n p tóg n y que l v u d d ngu at nu do
g n d a í n g néti m nte nestabl y tení n l
p t n ı l d eve t n un f rm p tógen du nt l
t emp P olv t p oblema l s l cit nte
p pu c n t u cuenc a d ADN qu odif caban l
tran pt de ARN par d ig r l pr du ión d l
v us de dengue quimér co qu in rp ra las mut cion s
l f gm nt d ADN c mbin nt g n dos d t
Un mod lid d p f id indu l sup e ión n l gión
n dif nt d l xt mo 3 (Men R B ay M Cla k D
Ch n k RM L CJ 1996 D ngu typ 4 vi u mut nt
conta nıng delet ns n the 3 n n ding eg n f the
RNA g nom naly i of g wth t ti n n ll
ultu and lt ed v mia p tt n nd mmunog nicıty ın
he us monk ys *J Vır l* 70 3930 3937 Lai CJ Bray M
M n R C h u A Ch n W K w n H T d n M Hiramat u K
T k m tsu I Pl tn v A Ar k S Sham n G R n udo M
1998 Ev lu t n f mol ul t t gi t dev lop
l v d ngu v n *Cl n D ng V r l* 10 173 179

Wh t h d SS M n RH L CJ Murphy BR R yn lds MJ P r ault J K r n RA Durb n AP 2000 A L v tt nu t d d ngu vi us typ 4 v cin c ndid t with 30 nu l tid d l t n n Am c n i ty fo V l gy pp 125)

L n t u ón d un molde de cADN YF 17D d l ng tud complet que se puede transcribir en in vit o p r pr duci ARN de vi us YF nfeccioso se desc ib ó po R ce t l (Ric CM G ak ui A Gall r R and Chambe T 1989 T ns pt n f nf ti u y ll w f v RNA f m f ll length cDNA t mpl t produc d by n v tro lig tion *The N w B l g t* 1 285 296) En ón qu l n t b lid d d l s clones d cADN de YF de long tud mpl t y u fe t s tóxi *E her ch Col* ll d ll n un t t g en l ual los mold s de l ng tud omplet p l t nsc pción an con t u do m d nt lıg cıón n v t d f gm nto de e t ic ión p op ad Más aun ll se en ont a n qu lo v rus YF cup ad s d cADN e n ndıst nguibles de los v us p r nt l medi nte v o it i L cADN nf cı os de YF s de ivan de las subsepas 17D 204 N obstante los viru YF generados de este cADN nfeccioso de YF s l ont a ı at nu do pu de n s r p ra

v ón human porqu neur v lurenci re idu l om d term n p M ch vsky R S t l (M h vsky R S M n J F VS Alme d E C qu i MJ C v lh R Pi un JW T v s da R APA Simõ MC S nt s CDN F a II Muyl e t IR M nn GF R CM nd Gall R 1995 Ph n typi n ly s f y ll w f v vi us d iv d f m mplement y DNA *Am J Tr p M d Hyg* 52 75 80) Aunqu e to e ultados most n que l v u n d lm nt t nu do pa va una YF 17D ra l p im d mo t ón p un Flavıvi u d qu po bl de ollar d un s po m c g ni mo d moldes de ADN un lote de sem ll s c mpleta bajo buen s p á tic d elab c ón Good Manufactu ing P act ces (GMP) utili and m tod logí h bitu l p a la pr du ión d v un YF

G lle y F (oli tud d pat nte US 09/058411) h bí n p x mad l recupe ación d l vi us compl tament t nu d d l cADN d YF m d nt l p de ng nierí d un númer de mut ion n el cADN 17D 204 ig nal (R c et l 1989) b sados en la cu nci d l ubs p 17DD (Duart d Santos t l 1995) L ubs p había sıdo util z da en B sil p a l p ducc ón d v un YF d d fin l d l 30 c n x l nt

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g t de ef a y s gu id d Aquí el v u
up de un m ld d cADN g néti am nte modifi do
t vé d l t an f ción d élula CEF c rt fic das
b] GMP (li tud d p t nt US 09/423517) En njunto
3 l te d t n f ón s d iv on lo cuale d on
ıg n do l te d m lla p ima ios y t
ecund l pas d n lm nt a la células CEF
t dos l s cont oles de c lıdad relevantes para la
p oducc ón d v un d hum n utilizando este sistema
d élul El títul p m dio de los viru formulados fue
6 71 g10PFU/ml El anális de l e ţab lid d g nét
v l s ll v ab m di nt p o n er n ultivo
CEF y tud nd v i s p ám t s t les como el tamañ
d pl l n u vi ul n n t n s y l
d t rm n ón d e u n n nu l ótid n ult d s
t f t i

L s esultad s de la s cuencıación de nucleót d s
di n l b l viru 17D204/DD qu mé i s btuv
d spué del islami nto vi l d l u o d mon
v m c N hub ambi g nómi (G ll r R F i MS
y J b AV n publi d) P l vi us YF 17D exi t n
h y mo d sub ep ut l d p a l pr du ión d
v cun en el mund nter ab r l 17D 204 y l 17DD

y l d fer ncıas n us secuencias se han notado (Gall r R P t PR Du te d s Sant s CN y F r eira II 1998 G net c va ıabıl ty am ng yellow fever vi us 17D ubst a ns Va in 16 1024 1028)

A p sa d l t bil dad g néti t t l d l vi us YF 17D un may p cup ión es la stab l d d g nét d l pit p f án en ón qu l v ru n lo n c t y p l h ho d qu u pl c ón h d un p c t ngid Aunqu l v i b lid d g nét vi l s m nim po l o d lo stemas del lot d m ll pa p du mill s c n tamañ de pr ducc ón l m n u t p o son ne es ıo in iand del cDNA (US 6 171 854)

El p im sp t qu tení que e nside d u nd ut li b un columna de cADN de Flav vi u d d p a l e p e ión de p t ín h te ol ga e si un p di d he h e upe l v ru con lo mi mo ma d e f n t p co o gin lmente presentes n l p bl ıón d v u qu daban o gen la bibl otec de cADN E t t m dam nt pl abl a l v u YF 17D d da la b n c noc da gu dad y eficac a d la vacun YF 17D

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D h h dif nte p x ma n té n p n t u vi u mbin nt b do n un Fl vivi u n p ti ul l viru YF 17D son p ibl s y va ar n d u d n l gión d l g noma l i n d p l n ón al ntig no l se xp e do Un may aproxima ıón ha s d l creación de vi u quimé ıco a t vé d l nt cambio de p M/M/E como e stable ıó p ım o par l s qu me a d virus DEN 4 (La et al 1998 U 5 494 671) Los gene p M/M/E de l s ot po 1 2 y 3 d l v u del dengue n e t n d nt d tro lon inf c oso d l d ng d ndo como r ult do v ru qu mé co con v ul nci reducida p ra raton s y mon (L et al 1998) Est p rmıtió l remoc ón d l may í d inmun gen s d l vect r edu iend d t forma l ít c en la nmunid de pr v a

El mism t po d c nst ucc ón se hi p a nc f l t s p odu d p g ap ta (TBE) y viru l ng t (Pl tn v AG B y M Huggin J La CJ 1992 Con t u t on nd h r t ti n f him i ti k b n n ph lıt /dengu typ 4 viru s *Pr N tl Ac d S* USA 89 10532 10536 Pletnev AG Men R 1998 Att nu ti n f L ng t viru t ck b n Fl v vi u by

h m ti n w th mo quito b n Fl vivi u d ngu typ 4 *Pr N tl A d S ı* USA 95 1746 1751) resultando en v u t nu do pa tones

Chambe t l (Chamb s TJ Nesto ow cz A Mason PW Rı e CM 1999 Yellow f ver/j pan se enc falit s h m vı n t u t n nd biol g al p op t e *J V r l* 73 3095 3101) h n de it l p me v rus quimé d r ll d c n cADN YF 17D d R t l (1989) m d nt l int ambi d g n p /M/M/E n cADN d v d d p JESA14 14 2 y N k yama de viru JE L nt o c e pond la p d va una tenuad viv n u o t lm nt en Chin

Gui akh o t l (Guı kh F Zhang ZX Chamber TJ D l g av S Ar yo J B t ADT Mon th TP 1999 Immun g ni ty g n ti st bil ty nd p t tiv ff y f mb n nt him ic y ll w f v j pan nc phal ti vi u (Chime iv x J) a liv att nu t d v in nd d t ag ınst fev J pan e ncephal tis *V r l gy* 257 363 372) M n th t l (Mon th TP S ike K L v nb k I Chambe TJ Gu akhoo F 1999 R combinant ch me c l v att nuated vaccin (Ch m rivax) nco po atıng the envelope genes of Japanese encephal tis

(SA 14 14 2) vıru and the cap ıd and nonstructu al genes f y llow f ve (17D) v rus fe mmunog nı and p t tiv n n nhuman p m t s *V c ne* 17 1869 1882) y la WO98/37911 habí n ll g d má c ca al desa ollo de l v un Aquí l vi u qu mé co up n d spué d l t nsf ión n élula FRhL t fi da n 5 p d d n l d l v u p p du l l t d semill y el lot d v un exp iment l (quinta p d) tod s b j GMP n células e tif ad No e um n t ó l nd mient d l v u n l i t ma d élul

El vıru qu mé o retuvo l secu ncı d ucl ótid /am n á id pres nte en l s pa SA14 14 2 g n l E t pa v una difi l gión p M/M/E d un v u p nt l n 6 po i i n s (E 107 E138 E176 E279 E315 E439) L muta n s n tabl s t vés de tod l s p s d s n el ult vo de células (ve) y el er b d atón FRhL A p sa d lo dat p v s b e la e tab lıd d genét ca de tales vı us uno d los cuat o cambi n l p tein E el ion da con la tenu ción v l h bí n v t d du ante l p da pa p duci la emıll und

En un st dio d r p t do i nti uerpo neut l nt p cifi o p p M/M/E li t n n t d l g up s d monos on d f nte dosis un on un p c d 100 PFUs y c nfi ian p te ión compl t nt d mand IC n JE t p ilv t Sin mb go ent e más b ȝ l dos de viru qu mér a más cambios hıst pat lógico e iduales e notan en el SNC d spués d l demand IC on l v us JE tipo lv st e

El p m r vi u 17D/d ngu quimé ic d oll d (G r khoo F W lt n R Chamb r TJ Zh ng ZX Soike K R ttt M Ar y J G o g k p ul K C t i n J Mon th TP 2000 Re mb nant ch me yellow fev r d ngu typ 2 v us mmun gen and p te t ve n n nhum n p mat *J V r l* 74 5477 5485) nv lu mpl d g n p M/M/E (f ón n l s t d d visión d sign l) n un cADN d n2 T d l eg c ón d l vi u y l p fue he h n élul v o PM (un banco d célula de P t u M i u) p untament e tific d p p du ón d vi u d v un L vi u ombin nt tuv n l e u ncia d n2 p M/M/E o ginal un d pué d 18 p d n n élula v o p n to lguna va ión n los gen YF El nál s f not p co de los vi us 17D/den2 qu mérıco most ó que

e te o m tab l s ton un n d s lt (6 0 1 g10 PFU) nt st c n l YF 17D qu m tab c c del 100% n 3 0 log10 PFU La respue ta de anticu rp y la p t c ón mpl ta s lı tó n l quım 17D DEN2 t nt n m n nmun YF m n Fl viv u in u E un tudi d pu t l d má b j (2 0 1 g10PFU) l v u quimé pl d ufi nt m nt p nduci un pu t d nt u p n ut li nt p t t n r ón qu n e d t to vi mia n t ım l d sp é d l d m nd n l vir s 2 d l d gu d l t p lv tr

Aunq l v u YF 17D c noc do p r s má g ét m nt e t bl qu t vi u de v cun t le m l p l v u l núm t din iam nt b j d p t b v nt dv lu g d v un ión h n d t t d un p mut i n n lment cu ndo l v us d v d d hum n fu u n do (ISE H C ss AR B t ADT 1998 Y ll w f v 17D v in v us l t d f m h lthy v n umulat f w mutat n *V r Res r h* 55 93 99) Gui kh t al h n ep t d p cos cambıos en la porción YF d l vírus 2 17D/d ngu qu mé c el cual había d pa do hast 18 v ult v d élul

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G lle t l (n p p ón) tamb én h d ll d un v u 17D DEN 2 qu mé m l S n mb g la lumn d l 17D e genéti ament m dif c d (US 6 171 854) E t s v ru c act r n a un n vel g óm m dı nt RT/PCR n inı i d e YF/DEN e pe f s y s uen ıón de nucl ót dos sob á as d fus ón y po c on de DEN2 c mpl tas L xp ón/p ami nt d l p lip ot ín moni t p nál i SDS PAGE de la p ot ín v l di t quet d nmunop s ip t da c n nti ue pe f que n luy n nti u p s mon l n le El n m nt d l p t ín YF y DEN 2 m d nt nt u h p nmun y nt cue pos mon lon le tamb én s log o med nt la neutralızac ón viral e ensayos de ed c ó d f m ó d pl as ınmunoflu e n nd t b e élul nf tad El m nto d v u mb nant s xam no n v ri sub t to d élul t l m v LLC MK2 C6/36 MRC5 y CEF S l l v YF ó n t d ll g nd tít l p l v u qu mé no pli on n élul t fi d p p du ión d v un (CEF y MRC5) im larm nt a l s vi us de N 2 L análisı de la ul n v l s d s oll m d ante noculac ón

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nt b l d t ne (10 PFU) y l s viru qu mé i amb a n má at nu do en e te s st ma qu l v YF 17D C el c ón l nmun gen cıd d lo tud n l model d ton ndi on que l v u qu mé i no ndu un pu ta p t ct a cont a u lqu otr d s l t l d vi us DEN 2 d Nuev Guin neu v ulent de atón

Un altern tiv p d a ll YF 17D mo un v t p a ntígen s h te log s s l xp ón d pít p p t ul e en i ta regione del gen ma La f t b l d d n t pr xim ión fu p me d most d p p l vi u (v d n R s CSP Ev ns DJ 1990 p l vi u ntig n qu m s *Tre es b ote hn l* 9 415 421) L lución d l tru tur trimid n ional d l p l v u l p rmitió l map d l pít pos d n ut l c ón d tip p fi b gi n d up f d f n d s de la p tícul v ral (Hogle JM Chow M & Film n DJ (1985) La estructura trımidensional d l p li v u a un r soluc ón 2 9 *Scıence* 229 1358 1365) Un de l i l d sup f i de la p teín VP1 e utıl ó p a la nse ión del epítopo tipo 3 qu fu no ido po el ant sue o de pr mates al polıovirus t p 3 que mue t a qu la qu me a fue n s lo v ble ino

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qu tamb én l pít p n t d s p entó n l m sma nf rm ión qu l up f i d l v ru tip 3 (Mu y MG Kuhn RJ Ar ta M K wamur N Nomoto A &Wimme E (1988) v u híb do _ nt génic t p 1/t po 3 çonst uid _ vit li t nt u rpo n ut 1 nt s tipo1 y tip 3 n n j y mon *Pr c N tl Ac d S USA* 85 3203 3207) S n emba g pa gument r fue tem nt contra e to st l ob v c ón d qu el m mo sit o s ut li ó p l n ón d dif ente pít po del vı us d l h p tit A p l inmun gen id d de lo pépt do n rt do fue m y p bre (Lemon SM B r l y W Fergu on M Mu phy P J ng L Bu k K W d D K t ak K Sang D M PD & ALmond JW (1992) Immunogenıc ty nd ntg n ity f h m pi n vi u whi h xp h pat t A vi u (HAV) peptid sequences ev d nc f a n ut al at on domain ne r the am no terminus of VP1 of HAV *V rol gy* 188 285 295)

L v u d l nfluen también fue on bien tudi d d d l punt d vi t est u tu l y n u nt n d p nibl s e t u tu 3D t nt p p t ín v l s d hemat glut na y n u amınida a L t l (L S P l n V I ob H Zagh iani H Gu n R M n T Bon C Palese P 1993 Chim ric nfluen vi u

induce n ut l ıng nt b d nd cyt t x T cells ag n t human nmun d f n y v u type 1 *J V rol* 67 6659 6666) h n d sc it l ns rc ón del epítop HIV en un icl d un t B d influ n d l v rus d l influ n a y l g ne c ón d spu st d élul B y T e p ífi l pítop

London et al (L ndon SD Schm ljohn AL Dal ympl JM & R c CM (1992) Infe tıous envelop d RNA vırus ant g nı h m *Proc N tl A d Scı USA* 89 207 [redacted] D s ll n v u S ndb s quımér s que xp s n un pít p n ut l nt b n def nido s nc llo del vı u de la f b e R ft V ll y m di nt mut gén i l t i L ti d n ión p rm t n l up ión d vi u n p pi d d d mi nt simila l vı u p nt l n nt n en un lip p t ína d l v i n P t quim l pít p e xp ó n l sup f ci d l vi n y imuló un pu sta inmune p lm nt p t ct l nf c ión RFV del atón

De V i et al (d V ie AAF Gl s AL R m n MJB d Haan CAM S n tar GJG Rottie PJM 2000 G n t m n pul tı n f quin te ıt s vı us us ng full l nght DNA l ne pa t n of overlapping gene

d xp n f f ng ep t p Vi logy 270 84 97) h n t b ɔ d p ng ni í v u d te iti qu n tamb én un v us de ARN d h b p it va con un gen m im l n tam ñ l d l YF 17D pe n d f nt st at g a d pl ca ón L nse ión d l pit po non péptid c n cid p un nti u p mon clon l MHV p ifi n un s t o d O gli li ción fun i nal d o mo ult d un v u mb n nt que xpr sa amb p pi d d d l pit po S n mb go l c imi nto d l v u fu l am nt du ido c mp do n l v u p r nt l unqu t p d n ri no di r n mo sultad l p d d d l n e t

Bend hmane t al (Bend hmane M Koo M Ba r E Bea hy RN 1999 D splay f p tope n the sufac of t b m s v u imp t of ch ge nd s l t i poınt f the ep top on v rus ho t nte act ons J M l B l 290 9 20) h n d crito la e ón de vi u d l mos o d l t b quimé i qu xpr n pit p d f n d d v u d l h p tit n n ɔ y t n L pit p f án xp a n b e l t rmin l c b x d l pr t ína de e ub miento v ral l t u tu y l p p ed des biofísi s l s uale están b en establ c d s Aunqu ambas ínserci nes d n rigen a

v us v ables d g ne ación d l cc ne n plant nf tad s p ualqui de l v u dif id s Se b v qu l punt lé t (pI) del epít p f t b la p t ín d ub im nt d l híb d t t l pI/ g y qu mp t nt p mo t l pít p d m n to Tamb én s n to l f lt de tol an a l s pít p c g dos po it vam nt sob e la up f ie de TMV

Un núm ro de ot s estudio que empl a p rtícul s m l r vi u p r pr s pít pos d proteín f rán def n d n d to n ra ón qu llo p n r p de p t n a l inmunog ni d d d pít p fo áne s p e nt do sob e u upe fic Heal t l (H l KG Hill HR St kl y PG H llingfd l MR T yl R b n n A W 2000 Exp i n and nmunog n ty f liv t g mala i pit p p e nt d a f ngn p pt d n th u f f RNA f MS2 b t i ph g c ps d Vac ne 18 251 258) h n util ado la p ot ín d ncub im nt l b ct iofago MS2 p a exp s pít p f án b d n un l d ß h rp n n l N t rm n l d st p teín qu forma la a te í t má d lm nte d st nt d l áp do madu Un áp id qu méric qu xpre n pít p de ntíg no de t p n

l hig d d Pl mod (LSA 1) timulaba n t nes un espu t p l i d TH 1 milar a la e pu st d hum no al antígeno en la natu ale

Tamb én d nt és s l stud d Sedl k et al (Sedl k C S J Ru d P L cl c C C s l JI 1995 Inmun g n ity f p l virus B and T cell pit pe p nt d by hıb d p c n p rv vi u p ti le J Gen Vi l 76 2361 2368) Ell in t on epítop d élul T y B d p l uvi us n un g n ma d ADN d h b en ll d l p rv viru c nin L s in ci ne h ci ron en l N t rminal del VP2 v r l s bre la t u tu d l p tí ula N exist ó puesta del nt cuerp al epítop m ent as qu la respuest a las élul s T pudi n b rva in vit o para l pítop de la élul T p sad E o ugi ó que l N t rm nal n s el ıti de uad pa la exp es ón d lo epítop humo l Ru d t l (Ru d P Hu t d A B r M T u d d JML Kamstup S L cl c C C al JI 1999 M n d spl m nte n the in ti n it p ov ke m jo diff n n the nductıon of nt body resp nse by hım ıc pa vov us l ke part cles Vırology 263 89 99) h n ext nd d tos studio con parv vi u nin ns rtaron un epítop humoral de pol ovi us en los cuatr

l d l VP2 b x t rm n l v al sob e l est u tur d l p tí ul S l l l 2 p rmit ó l g n r ión d nti u rp l pít p pe no nt u rp n ut l nt d p li vi u E t l g al in t l pít p n am n á id ady nt que ug n d pl am nt m n n l it d n ión u amb s d amát co en l c s bilıdad del pít po y l ndu ón d l spuest s d l nti uerpo

L p tí ul d nú le d l virus B de la h p t t quimé que ll v pít pos d h nt virus an de c tos po Ul ch t l (Ulrich R Kol tzki D L chman S Lundkvist A Zankl A Ka ks A Kurth A G ld rblom HR B v G M is l H K u ge DH 1999 N w himae i h p t t B vi u pa t il y ng h nt v u (typ Puumal) pit p inmunog ni ity and pr te t on g n t v u h ll ng J Biot chn l 73 141 153) La n e ón de una ant dad t l c mo 45 y 114 am n ác dos d l p ot ín d l cáps d d h nt viru d mo lt d l p t ión d l s hué p d n tu l del vi u

L formac ón d l p tículas s m la e a vi us qu mé s n cáp id p n ip l d viru d prot ín d

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ham t n d it s po G dvilait t al (G dvil it A F oemm l C S sn uka K M chael B O sel M Beh ing O St n l u J Jand ng B Sch neck S Ul ch R 2000 F rmatıon of ınmunog n c v u lik pa ti l by n t ng ep topes int su fa expos d gions of hamste polyomavi u m jo apsid p tein V ol gy 273 21 35) Ell h n p d ho d l t uctu 3D 3 á e qu e t ían xpu st s la up f y la xpr sión d l pít p d áp d d 5 amin á id d l vi u d l h p t t B d n mo ult d l r n cim nt d l nt p mon cl nal l pít p

P lo Fl v vi us los pít po de n utr l ón ot os ag nte vi al pod ían se in ert do n eg n d l ub rt v al donde uno det ta l v r abil dad genéti a m d nt u n i ión d nucl ótidos T l p ox ma ón fu p m r n y d d pué d amb 2 am n á d s n l g n d l p t ín d ubi t d l cADN inf c i YF m di nt l p ndient u n i d amin á d s d l v u d l n f lit Mu y V ll y (MV) que fu p viam nt ca cte i ad po ant cuerpo mono lon l como un epítopo MVE neut alı ante La químe s n embargo no fue v able ug ıendo qu el á ea partıcula de la proteína E

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(Am noácid 192 193 del am n t rm n l) s c ti pa l v b l d d d l vi u YF (R We y C R p □ mún)

En um n p bt n un vi u d v un b s d en fl v virus qu xp e antíg no f án o p a l d ll d nuev s va un v va ut lizando técnicas de ADN r mbin nt n c sar o d man r cumul tıva

(1) D señ e t ategıa que p rmit n l ınt duc ión d ntíg n f án ın mp m t l e t u tu y la fun ón d l v rus

(2) A egu r qu l c nst uc ión d l cADN infe o y l t an ito d l ARN g n do d e t dan lugar a v rus qu no s n patógenos n t enen el p ten ial d verti e n la f rma patóg na y qu l u n i anfíg n f áne g nét am nt tabl una vez s ınteg d nt d l g noma vi l

(3) El Flav viru g ne do del cADN cl n d demá d s r tenuad d b t ner su p opiedades inm n lógi y p nt n ntíg no forán o expr do d t l form qu t licit l r p t nmune propi d

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RESUMEN DE LA INVENCIÓN

Es el obj to de l p s nt invención pr po ion un vi us p a v cuna de Flav v us segu o y fect vo bt n do d l cADN cl n do qu ti ne c a t ístic f n típi t le mo l tenu ión y la ınmunogen c dad y el cual también debe xp es tabl ment y lı it un e pu sta inmun a antígeno forán s

En un mod lid d l pre ent inv n ón e r l ci n n un mét d p l pr du ión de Fl viv ru como un v t p r ntígeno h te ol go que comp ende la ntr du ión y l e p e ión d s cu ncı s d g n fo án n un t d in c ón al n vel de l p teína de ubi t d u lqui fl vivi u n d nd l iti tán t u tu lm nt p t d d á n id p int f ri n l e t u tu d l p ot ín E d l Fl v v u t t l y qu mp nd

Sit o qu d c n an s b e la sup rfi ie xtern del v us p op cı nando cc ib lid d l nti u p n mp de tab l signif c t vam nt la st uctu t d mens onal de la proteína E y no interferir con la

fo m ón d l ed de p otein E d nt o de l ub e t vi l

En t mod l d d l p nt inv n ión e t l n d c n un t t gia que permite l nt oduc ón de s u n i de g n fo án n el ci lo fg d l pr tein d cub ta d l v u YF 17D y ot Fl v vir

Ot mod lid d d l p esente inv nción s r l n n un nu v v ón d un mold d cADN inf d YF qu e mil l 17DD y l u l d como r ult d l n ón d cu nci del g n d l m l i

En t mod l d d d l pr nt nv n ión p po n uno nu vo plá mid YF l s cu les tiene l ecuencia completa del cADN infeccioso de YF y la s u n i d l g n d l mal

Ot mod l dad d l p s nte inv n ión son l flav virus mo un ve t r p lo antigen s h te log s en d nd l fl v v u s bt nibl d a u d al mét do d to quí

En ot s modal d de propo ion n vi u YF mb n nt s qu e r gen n d un cADN d YF inf i o y xp san d fe ent s pítopos d mala ia

BREVE DESCRIPCIÓN DE LOS DIBUJOS

L fıgu 1 lu t a l hi to ı d pa o de la pa Asib YF igin l y l d iv ción de la sep s d l v un YF 17D

L f g r 2 mu stra l l neamıento de ecu nc a d l p c n lubles de la p oteín d ubi rta del vi u d l n f l ti p du d p l g apat (tb) el v us d la f b e amar lla (YF) l vi u d la n f l ti ȷ p ne (j) y l vi u d l d ngu t po 2 (d n2)

L figu 3 mue t una ep esentación quemáti d l pr t ín CS d Pl m di sp

L f gu 4 mue t a la t uctu del plá mido pYF 17D/14

La f g a 5 mu st l tructura d l plásmido pYFE200

L f gu 6 mu t el lin ami nt Y uen nt tb y YF p o n l nt du ión de un u n d ns ción (es ltada en letra gruesa y ub yada) nt lo e du s 199 y 200 d YF l cal ad s n el c lo nt e l s ß heb a f y g Como en la f gu a 2 el al n am nt m t do s quel ut l do pa a con t u un mod l d l p t ín YF modif ad y l de l n ami nt delib do en forma ombre d Lo l m nt d l t u tu s und i e mu t an omo b h ı nt l ent e 2 uen a

F gu 7 stable 2 vi ta de la p ot ína YF model d qu n luye la cuen i d ıns ción SYVPSAEQI d nt d l l fg L d min s tán l e d ndıvıdualment l dom nıo I rojo) el dom nio II (am ıll) y l d m n III (a ul) En l p nel upe io el díme de la prot ína E e ve p rpendicula a l m mbr n v r l y n l p n l infe i r v dentro d l pl n de la memb na perp nd ula al je l ngitudin l d l dím El it o de in e ón (en an) de c n c d l inte f p óxima nt e 2 monóm

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n t tuy nt d l dím y pu d n v mo p c lment nt d

L f gur 8 st bl e l superpo ción d 10 m del d p t ín E YF qu ncluye la se uencia d n e ón GG (NANP) GG d nt d l i l fg En d mod l l u n i d in ión e mu tr en un ol r d fe nt mientr qu el r sto d l e tru tur m str n v rde L gr n d ver id d n conf rma ion del cl m ent qu pr rv encialment el st d l t u tu ndic el g n v lum n d p i di pon bl p l péptid de in ón

L f gura 9 muestra la supe fic molecula del díme o de l p ot ín YF pa a un de l s 10 model d l f gu 8 En l p n l up o l punt s ul y j nd d b d un d l monómero p nta l nt d y lid d l pépt d d in ión En l p n l d l m d mu st n l e p d e N (ul) d d du y l gl in C t rmin l (ɔ) indi nd su p pel n l l v ntam nt d l s cu nci s (NANP) p nc ma de l sup fic mol ul En l p nel nf i l inse ión (NANP) se mue t a en ve de

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L f gur 10 t bl c un en yo d nmun flu n ind re ta utili ndo un ant cu rp mono lon l d g do a p ti ón (NANP)

L f gu 11 mue t un gel SDS PAGE d l vi u 17D/8 obt n do m d ante inmunoprec pitación de proteínas vi al s tiquetada met ból ca

L figu a 12 lu t a l nál sis de tamaño d pl c omp t v nt vi u YF 17D/8 vi us YF 17D/14 y de v do YF 17/G ½

L fıgu 13 mu st curva de crecimiento viral n CEF (15) y célul Ve (15b)

La fıgura 14 stablec el tamaño d la placas d v u f rmada n mon cap s de célula Ve d spué d p p g ón ı l del vi us n cultiv s Ver y CEF

L figu 15 mu t l u va de ecimi nt omp tiv d l v u mbin nt dif ent en élul s V o

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L figu 16 mu tr el nál si d tam ño de pl d l vi us YF ec mb nante d fer nt s

DESCRIPCIÓN DETALLADA DE LA INVENCIÓN

P mu h nf rm d d l v cuna de l un de vado t nuado vivo del patógeno el cual induc pue ta nmun p t t s de la g dur ión fu te un v iedad de ntígen s b el patógeno sin u a nf rm d d El d llo d tal v cun s menud p lu d p difi ult d n l p opag ión del p tóg no n l tenu ción de te in p rder l inmun g nic dad y n a gu l tab lid d d l f n tip t nu d Un lt n tiv el u d mi oo ganismo t nu d s on ido p a l e p e ión de cu lqu e ntígen d int é

L apa idad de maneja po ingen ía g néti am nt v us d nim les a cambi do el nt ndımi nt de ómo to vi u eplican y p rmiti l n t uc ón d v t s p dirigir l a xpr ión d p ot ín h t r log en dif entes sistema L s v rus ADN tal s c mo l SV40 la vacc nia los adenovi us y el h p h n ut lı do omo v tor p un núme o d

p ot in Má r c entemente lo virus ARN tanto d h b p t v mo n g t v (Pal P1998 RNA v u ve to whe e e we and wh e dow n ed to go) P Natl Ac d Sci USA 95 12750 2) se han hecho d cu do a l m n pul ción g nét c y n los vect re pr fe ído n r ón qu ll l f lta un f s de ADN qu recha la integ ción d secuen ias fo áne s n el ADN d l mo ma y n p n p submodula la r spu sta nmun omo l h n l s vi u ADN (por ejempl v ini y h rp)

L Fl viv u tien n v ia ct í ti qu on de abl s p a vacunas n g ne l y que han t ído l nt és d v s lab at io en de rollarl d i n lm nte par ut li do como un ve tor p r ntígen h te log Pa ti ularment p r l vi us YF 17D estas ca acterísticas incluyen una metodología d pr duc ón bi n d fin da y fici nte un c nt ol de al dad t to ncluyendo en ay de neu ovi ulenc en mon inmun dad de larg pl b j c to d i simpl l u timado t s bre l s dos i nto mill n d dos c n ex lent r gist os d gu id d (s lo 21 s s de encefalit p st v c n l d pué d l e t bl imi nt d l tema d lot s d semilla n 1945

on un inc d nci en nf nt s muy baj (9 me) d 0 5 4/1000 y >9 m se en 1/8 de millón)

El hech d que la st uctu 3 D d la prot ína E del Flav vi us est disp nibl (R y t l 1995) op t í l ap ximación u da prim am nte p a l pol ovı u al exam nar la t u tu a 3D y selec iona it os pa nse ción que nterf ri án probablemente menos on la e t u tu a de la proteína E tot l La may pre up ón c d l pítop s de ín ión d nt de l p t ín E d l Fl vivi us n p rti ul d nt d la p oteín YF 17D l i n n l h ho d qu st p t ín e l p nc pal objet vo p ra l r puest neut alı ante humoral es la proteína donde están los pít pos de hemaglut nac ón y neut alización y es l pr t ín que m d l n mi nto d pt d élul y de pen t ac ón d célula apuntando de est forma el v us a células esp cíficas Al inse ta un nu v pítop n ualqui pa t d la prot ína E de un Fl viv u d d un ma d t p opi d d s pu d ambia am n qu l t uctu a 3D p rmit l id nt fi ón itio d inser ión p t n ial st u tu lmente d st ntes de á eas conocidas para media un más de e tos pr ce os

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En la ón a l p teína E del viru d l n f l ti p du id po g p t 2 dif r nt f rma d c t le d su f gm nt s s lubles se obtuvi ron po R y t l (R y t l 1995) En ambos l p teína E mu t un xp i ón dimé i imil n l u l 2 monóme s stán rel cıonad s medı nte un eje de dos doblec s mol ul es el cu l es ist lográfico en una f rma de c tal y n c st log áfi o en l ot a L p n p tid del mi mo dím o en amb s s ug e que t n un art fa t de cri tali ción pero epr s nta un dispo ición oligoméri verd der d la p teína E gún s in e t d nt d la cubie t v l en un pH n ut El dímer pr senta una st uctur pl n d blong d n d m n n t t l d p x madam nt 150x55 30Å Su d men ón má t d ns p rpendicula l memb ana vi l y l t u tu mpl t pr nt un u v tu m d qu p du un up fi t n qu un p mayo qu l p ndi nt up fi e nt n E t u v tu pu d yud n l i d un p tí ul vi l esfé C d monóme o e ta mpu sto 3 dom nio el domin o I (el dom nio central) el dom nio II (el dominio de d m r c ón) y el dom nio III (l dominıo de unión al

re eptor será l nmunoglob na) todos los cual s e tán dom n d s po un t uctur secunda ia ß lámin El d min I d sc ntinuo tando ompu st de 3 s gmentos ep d d den d p lipéptido y e domın nt m di nt un ß b l d 8 h b qu sub n y b j n d top l gía complej El domin II es e pon able de la nte fas p ncıp l nt 2 monóme p óximos al ej d 2 d bl e y forma m diante d s s gmentos d l cad n de p lipéptid s que divıd en d min (I) E te s un dom ni longado fue tement eticulado med ante puent de d sulfu o y compuesto princ pal de 2 compon nte st u tu le 1) Un ß lám n ntip ralel d 5 h b ob un ti d l u l emp l 2 α hélic d l st u tu y 2) Un ß andwi h hecho ha ta d un ß lámin de 3 h bra emp cad s contra una ß h irpin E t ubdom nio de ß andwich ncluye el péptido de fu ión qu se c ee qu es importante pa a la actividad fusogénic del v us Este desc nsa en una av d d f rmad po l contactos de inte dom nio nt e los dominio 1 y 3 d l monómer opu st y por lo tanto forma parte de una egión de nt f se nt 2 monómer s que d sc nsan mas di t nt l j d 2 dobl e quel men i nad nt rm nt El domin 3 continu y p s nt un dobl d nmunogl bulin (Ig) tipo C un p o modifi d

Ut li ndo l nomen latura conv nci n l para t l s doble s (Bo k P Holk L Sander C 1994 Th mmun globulo n F l J Mol Bi l 242 309 320) la h b a C F y G d st dom ni s enfr nt n ha a fu r del m nóme y p e nt n un gión crítica n l det rmin ión d la p op ción del huésped y el t opismo de la célul y es p babl ment p lo tant fundam ntal p l un ón d l élul L puest d l domino mil Ig f rm l int f on l domin l y junt on l gi n d l ubdomini ß ndwi h d l monómer pu t mpo t nt n l formación d una int rf d dim d t nt d l ɔ de dos d bl c L int f t ad ion lment protegida por l po ión b h d t p nt n l d mini I

Las ß h bras del dominio 1 son denominada A_0 I quell s del d m n 2 d nom n da A a I y aqu lla del domin III domin das A G entonos los casos tiqu t d s on e ut vament d l N terminal (del domini III un dı t ón del IG C tip típi o nduc a l c ac ón d h b as dici n le A_α C y D) Con l excep ón d 2 α hél ces c rtas del dominio II todas las on xion s nt e l ß heb d un domino dado a í como

tamb én l s lig do es que conducen de de un dominio l ot o on ß d bl dos cıclo qu varían gr ndem nte en long tud En término gen l s todo l tales icl stán nt do d nt d l tru tur (in cce ibl l olv nt) xpu t b un o má de l sup fi i int n xt n y l t l d l dím r

Al exp ne e pH b jo l ed de dím ros de pr t in E d b disp n s n t ím r E t d b nv lucr r gr nd alt ra one l c nta tos monóm r y p blem nt tamb én l o i nt on r l tiv del dom n d nt o de una subunid d d da sino a la gan a ón nte n d la st uctu tercia a de l s dom ni mi mo Al p ti p t nto lo cont t s p óximo mo di t nt l domini II p bablement suf e los mayo cambio c n ist nte on l h ho d que la unión de los ant cuerpos monóclonales a este domin st fu rtem nte fect da po l t nsición dím o a dím (H in FX Sti ny K Pu hne uer G H l mann H All on SL MAndl CW Kuns C 1994 Structural Canges nd Functional Control Of The TickBorne Encephalit s Vi u Gly op tein E By The H te dime ic Associat n W th P ot in p M Vi ol gy 198 109 117)

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P l di ñ de ns rción d péptid s pitópico n la protein E de un Fl viviru dado y la subs cuente v lu ión d su viabilidad lo inventores de la p esente nv n ión d lla n la s gui nte estrat gia In ci lment fu nec ario producir un modelo t d m n on l p la pr t ín E de un flavivi u l cci n d L cu nci de la epa 17DD de la f ebr amar ll utili ó p r t propó ito y u alineami nto n qu ll d l vi u d l n ef liti p ducid p g p t (tb) g n ó ini i lmente n un pr g ama MULTALIGN (B t n G J St ngb rg MJE 1987 A Str t gy F Th R pid Multipl Aligm nt Of Protein Sequ nc C nf d n L v l F m T t ry Structu e Compa isons J M l Bi l 198 327 337) y ub uent ment con ef nci l t u tur 3D de la última todas aquellas ns c nes y upres ones se estring eron a posiciones te quimica ment r zonables Un aislamiento f nal qu in luy u nci de l encefalitis japon a (pa J OArS 982) y dengu tip 2 (pa PR 159) se muest en l figu a 2 y s s gnif tivament diferente en varios sp to mp t nt de quel dado po R y et al 1995

L s más ímpo t nt s dif rencias que son relevante al diseño de ınse c ón subs cuente se d scriben aho a

Un in ción d 2 idu (122 y 123 en tb) s int du n l tb nduci nd a un ajust completo del l n am nto h t l g ón del iti d h gl col ón nt l ß h b a E y F l mp a la u nci tb y d fi b e amar lla (YF) d nt o de l gión 120 150 (nume ción d s uenci tb) la ltera ión ondu e un n m nt n l núm r de ıdentid d s d s uenc s d 2 8 Un m ȷ imıla se bse va en la encefal tıs j p n (j) y un n m nto d 1 a 10 id nt dade u n l d l d ngu 2 (d2) Además d la clar meȷ n l id ntid d d la secuencia el nuevo lin ami nto c moda d s r sidu s d in rción (122 y 123 n tb) n un i l de up rfi ie (ntr las ß heb s d y e)

Un up ión de un e duo ntes de l heb f en YF y d2 ce ano y t n fer do a la gran supres ón nt l ß h b f y g La sup sión n esta egión del lin amient dad en la fıgu a 2 e así 6 esiduos d

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long tud tanto p ra YF como para d2 como lo es n ɔ uando e omp ra con tbe

El egm nt n p gin /á id párt o d l YF (idu 269 272) vuelv un in ción nt l ß heb k d l d minio II

El alin amı nt d c nci s utili ó p r g n 10 mod lo d l díme o de la prot ína E YF util and at sfacción d fija ione spaciale d v d s de la st uctu d mé ıca tbe que mplea el p g ama MODELLER (S li A Blund ll TL 1993 Comp tiv mod l building by tisfact on f p ti l t nt J Mol Biol 234 779 815) Para c da model empleó din ión al to a p r d fecto en el sp cio c rt ian de 4Å ntes de l opt mi ión del mod l utili nd l Mét do d Fun ión de Obj tiv Va iabl (B aun W G N 1985 C lcul tion f p tein onf rm ti n by p t n p t n di t nce con t int n w ffi nt lg rithm J Mol Biol 186 611 626) y Re c m nto Estımulado Los des lineamientos delibe ados de lo esıduos cundant d l s nse i nes y de las

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up e n util n n l f n de l j l
h m l gí d l fij ci ne d sto esiduo
p rmiti ndo l n ción/ up sión mu st s mant ní
imultán am nt l t r químic ptabl

El model qu p sentab la más baja seud en rgí
d d p ln (P) donde P e la fun ión de dens dad d
p bab lid d mol cul r MODELLER también pre entó
c lent t quími t tal p duci ndo po jempl
un PROCHECK (L k w ki RA M thu MW Moss DS
Tho nt n JM 1993 Pr ch k A P og am To Ch ck The
Ste e ch mi l Qu l ty Of P ote n St uctu s J u n l Of
Applıed C tallog phy 26 283 291) f ctor G de 0 1
qu val nte la est uctu a d c st l de una solu ıón
m j d 1 5 Å El model también fue valu do ut li nd
l mét d de Eis nberg (E nb g D Luthy R Bow e JU
1997 VERIFY3D Ass sment of p ote n Models w th three
d m n n l p f l M thod En ymol 277 396 404 B wie
JU Luthy R E nb g DA 1991 m t d t Id nt fy
P t n S qu n th t F l Int Kn wn 3 Dimen i n l
St uctu Sc nc 253 164 170 Luthy R Bowi JU
E nb g D 1992 As ment Of P ot n Models With 3
d m ns nal P f l s N tu 356 83 85) pr sent ndo un

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m rcado VERIFY_3D de 348 c rcano l v lor sp ad d 361 p a una p teín d 786 iduos (en el dím o) y b n po en ma d l umbr l d ept bil dad e 162 Adi n lm nt l n rm lid d d l mod l fu tim d p nál d nt t tómi utili nd un mar do d lid d tot lWHATIF (V i nd G 1990 Wh t If M l cul Mod ling And D ug DE íngn P ogram J M l G ph 8 52 57 V iend G S nde C 1993 Qu lity C nt ol Of P ot n M d l Dir cti nal Atomı C ntact Analysi Appl Cryst llogr 26 47 60) qued un val r de 0 964 most nd que el modelo es confiabl

El mod l p l p t ín E YF mu t un á de nta to l gerament educid nt l s ubunid d mpar da on tb (1 242Å p r monóm ro c mp r d c n 1 503Å) p i lm nt debido l tamañ du id del clo fg qu h c c ntact int ubunid d ví Hi 208 n tb N xist un r d cción sub ecu nte en lo ontacto h d f go del ınte d min c mo s dete to p LIGPLOT (W ll AC L w ko ky RA Tho nt n JM 1995 Ligpl t A P ogram To G n r te Schemat Di gram of Protein Ligand Int ract ons P ote n Eng 8 127 134) E tos comp nsan p l p r n i d 2 nte ubunid des d pu nt s d l

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(ntr E201 y R243 y nt R263 y E235) qu p c n n v i d l model n lo cu le están u entes debido la sust tu on d am n á ido n tbe E t pu nte d l p um blem nt ayudan a la tab li ión d l dím YF El nt to h ch ent l péptido de fus ón y l l d n l domin II n l avid d nt l d min s 1 y 3 de la ubunidad opuesta es sen ialmente t n da n l p teín YF E

El mod lo p r la p ot ín YF E junto on l l neam nt d l s secu nc a se util z p ra l n l ti d in e ón poten ial pa pít p het l g B y T n amb s t le t de nse ción deb n 1) no desordenar o desestabili a gn fic tivam nt la st u tu t idimen ion l d la pr t ín E 2) no nte f i con l f rmación d la d d l p t ína E dent d l cubi t vi l 3) D c n b l up rf ie xt na d l vi u d tal f rm qu t ibl l nti u rp Aunqu t rit i pu d n e t i tam nt blig torio p a l pít po de l célul T te p rman ce ap opi d en ón qu l s t s d l uperf ie nte na pu d n ınterfer con el ensamblaje viral 4) El sitio debe

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pr nt r pr f iblem nte v d n i d qu l v r ción d l l ngitud d l u n p rmi ible d l dif e ias ob erv da entre los Flavivırus dif rentes (es d l it debe d v i n a natural) 5)en l d l s s ti qu pr sentan variac ón de l longitud de ecu nc pref ıblem nte YF debe presentar un cicl má p qu ño n t le c s

El pr m c te io l m t l s s tio de ins rción a i l y ambi nt lem nt d estructura cunda i El gundo y te c o limina itios s b e la upe fici nterna y l te l del dímero y quellos qu están nt d De l tánt s sit os de in ión p bl s pu d d c r l sigui nte El cicl entr D y p s nt un nex ón d inte domin y mue t a pequeñ variab lid d tru tu l Aquel ent lo ci los e y d p e nt un péptido d fusión que esta p lm nt nt d y ltam nt c n rv do Aqu l nt d y m str p qu ñ varia ıón tructural ncluy un idu d ¼- i tin y s tru tur lment mp t nte Aqu l ent e E y F ncluye un sit o de gli li ión n tb y s un sit o d in ción pot ncial n l m did qu st mu t g n v iabil dad

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e tru tur l y e altamente expuesto Aquel entre G y H puede esta p cıalmente nvolucrado en contactos l t l s e un p queñ ß cambio y muestr p queñ v c ón t u tu l Aqu l nt e F y G p esenta t da la ca t íst as des abl p qu est es 6 e ídu más cort s n YF ȝ y d2 comparado con tbe y esta xte nam nte xpu st Aquel ntre K y L es un s tio p t n i l en ón a que te muestra un in c ón i n a pa agina en YF la cual podrí acomoda secuencia de células B ep tóp cas ícas en asparagina como se describe adelante Aquel entre B y C es un sit o posible p o puede formar p rte de l supe fic e lateral Aquell nt D_x y E muest v ación e tructur l y p es nta un u n ri n sp gin en YF l ual pued omoda pít po co en pa agina D lo nte ioŕes l sit o d in ión má p m i s qu l nt l ß h b f y g qu form n p t d l ß lámin ntipa l l d 5 heb a del dom n 2 La g an supresión de los 6 residuos n st cicl en YF c mpa ado con tbe deja spac a l int rf d l dím r p una m yor ns rc ón s n r r ımpedımento estér co Además de cıclo fg otros sitio de n e c ón p om so E F en a ón de qu este muest gran v rıab lidad estructural y esta altamene expuesto Aunque n de est lig do a ninguna t ría

p t ul p tul qu l p n i d uno ma
du d gl c na flanque nd nm diatam nt l epit po
n ert do s v nt jo o en la introducción de flexıbil dad
fo m n l l pit p n u p sent c ón ub ecu nte

Una lt n t v de la p ent inv n ón p
d s r oll fl v v us en gen al como un vecto pa
ntig n h t ol g s l in e ión y l xp e ón d
ntig n p t ul n luy ndo pit po n ti
t u tu lm nt d t nt s d á a con id p
nt f i n l t uctu d l p t ín E d
fl viv u t t l pe lm nt en l lo fg o n l
i lo E F d un p t ín d Flav vi u dad El
ntig n in t d f án qu n luy l pit p pu d
v i ampl am nt d p nd end d la p p dad
nmun gén d ad n l ntígen Po jempl l
ntig n n t d f án pu d incluí ntig n d
p t t l c mo mal d v u t l mo f eb
am ll d ngue en f liti jap n sa ncef l tis
p du id p g p t nf n fung y t
Adi ı n lm nt l máxim l ngitud del ntígen / pítop
d p nd á d l he h d que te n comp nde í l
est u tu de l fun ión de l cubi ta del Fl vivi u

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Má p t ul rm nt un t teg de it quí n l n ión d l s cu n i d l gen de la mala a d nt d l l fg d l p t ína YF 17DE M nt s qu pu d n in t u n i mp ativamente co t s que l t n n uno p r s du d am noác d también ont mpl qu pued n nserta antígen /epít p má l g La máx m long tud y la natu ale a d l ntíg no/ pít po dep nde á del hecho de que ste no c mp m t l t u tu d l fun ón d la cub t d l v r d l f br ama ill

L mal i p rm n como un d l má mp t nt nf rm d d hum n ig n d p r v t El nc pt d qu l vac n c ón pu d ut li mo h ami nt p nt l l nf rmed d t b s d p n ip lment n el h cho de que individu s nt nuam nt pu st s la infecc ón por el pa á to p to o ev ntu lm nt de ll n nmun dad l nfe m d d

El l d l p á it d l mal i mpl ɔ l s v t p en human s on mo f lógi a y ant gén cam nte dist nta y l etapa de nmun d d

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esp cif Sol ah a se ha hecho p s ble def nir l pat ón completo de l exp esión del gen d parás to en c da t p

En um n n l l d vid d l p ásito l s po o to qu s n sum ni t dos por l p cadur d l mo quit inf t d n u nt n l amin l hig d nv d n l h p t t S h n d nt fi d 2 p t ín mpl cad s n l c n im nto e inva ón de l h p t t s p t ín CS (i um p t) (F v t U P S nni C C ram W Shr ffler B T k c nd V Nu s n w g 1993 L p ot ín ci umsp o o t de la m la ı s une l s p teglı ales de sulfato d hep n s ad n l m mb n d l up fi i d l h p t t J Exp MED 177 1287 1298) y l p t ín nónim l i n d n t omb p ndin o TRAP (Sult n A A V Th thy U F v t K J R b on A C s nti V Nu sen we g R S Nu seen weig yR Ména d 1997 El TRAP s nece i p motilid d p d li amiento e nf ct vidad de l s poro it del plasmodio C ll 90 511 522) Lo anticue pos a las p oteína sob e la upe f i d l p rás to pod ían neut alı a c nsebiblemnete los esporo oitos y habita el desarrollo sub cuente de las tapas del hígado En el hepat cito el

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p rá t se difer nc y s replica a exualmente como un squi nt p a p du ntid de en rm d mero it qu n a n la inf cc ón en l s células sanguíneas J L ntíg n sp if c p a l t p d l híg d han ident fi do (C ll JM Na dın EH Clavijo P B ud n C Stub r D T ka B N s n w ig RS & C chr n AH 1992 R cogn tion of different doma ns f th pl mod um f l p um CS p t in by the e a of natu ally inf t d individu l mp d with thos of p it immun d v lunt J immun l 49(8) 2695 701 N din EH & Nu en we g RS 1993 T c ll rep nse t pr yth cyti st g s f mal ia r le in p ot ction and v c n d v l pm nt ga n t pre rythrcyt c tage Annu R v Immun l 1993 11 687 727)

Se ha propuest qu e tos antígen s junto con aqu ll de los esporo oitos sean en pa te proces do po l élul hué p d y p nt do obr l up f junt n l molé ul MHC l l E t pr ntación pu d ndu l n mi nto de l s l nfo to T it tóx s y m t l células infe t da o t mul ción d l s élul T p p du to in y fin lm nte conduc a l mue te del p ásit int celula

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L m t qu s b v v n l t p p
it iti n i n l inf ión d l t p nguín
xu l que e r sponsable de l nf rm dad s El
p á t inf ct l it ocit que no xpre n la
molé ul l l y p l t nt l linf t T
t tóx n n imp t nt L nticue p qu
un n l up f e d l me o to p obablem nt jueg n
un p pel imp tant n la inmunidad de las et pa
s nguín s xual Potenc alm nte estos nti uerpos
p d í n n ut lı l p ásit s c ndu me ni mos
F d p ndi nt d l m rte del pará ito por jemplo
ma óf g La pr t c ión c mpleta contr la demanda d
l p t ob erv da n at n s nmuni d s n
p it P b gu i di d s y human inmuni d
n p t P fal pa um die n omo ult d
spu st s inmun s a antígen s exp es dos por l p rásit
n l tap p e it cit as de su cicl d v d
(N d n EH Nu s n w g RS 1993 T cell pon t p
yth yt st ge f mal i ol in p ot ct n nd
v n d v l pm nt g in t p yth yti t g
Ann R v Immun l 11 687)

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L nt uerp la célula T CD4 y CD8 h n id
mpl d n la nmun d d p it cıt c (S h fi ld L
V ll qu n J F A s h ll ken H Nu nzw ig R
Nu en w g V 1987 Y nt f n CD8 T ll nd
nt b d qui d f immunity t m l i p it
N tu 330 664 666 R d igu M Li S Mur t K
R d gu D R d gu JR B k I B nnink JR Y dw ll
JW G S t A Nu en w ig RS Est b n M Pal P
Z v l F 1994 Influ n nd v ccin v us exp ss ng
mal CD8 T nd B c ll p top C mp n f th
mmun g n ity nd p c ty to indu e p ot tiv
p n J Immun l 153 4636 4648)

E i t n 6 ntig n s p e it citı os p nc p l s n
l p á it d P f lcip um l p t ín
un p t (CS) l p teín d dh ón
l i n d n t mb pond n (TRAP) l s ntíg n d
etapa de hígad 1 y 3 (LSA 1 y 3) los Pfs 16 y l
t n n d p t y l p t ín r c en spa gin
U núm o d pit p d ntif dos b e l dif nt
p oteínas d l pl smod están s ndo xp esad s en
d f ret s st mas h cıa los estribos d nmunogen d d
(Mun ngh DY Cl] P C ll MC NArd n EH

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Nu n w g RS 1991 Immun g n city of multipl ntig n peptid (MAP) c ntaıning T and B cell epitopes of the p t g n f th P f l ip um ci cusp o t p tein Eu J Immun l 21 3015 3020 Rod igues t l 1994 Sh YA H na n SE S ci JB Holloway BP Kuj k H Kuma N W hlhu t R H ffm n SL C llin WE L l AA 1999 Immun g n ity nd n vit o ffic y of mb n nt mult t g Pla modium F l ip um nd d t v n P N tl A d S USA 96 1615 1620 Aido M L lv nı A Gilb t SC Hu JT D ube s P Hu t N Wh ttl HC DRuilh P Hills AVS 2000 Cyt t xi T l m ph yt p t p f HLA B53 nd th HLA typ in the m l va ne candidate l ver stage antıgen 3 Infect Immum 68 227 232)

L f gu 3 mu t l p nt ón qu mátic d l p t ín CS d l pl m d p (N din Nu n w g 1993) y l l ali ción de l pítop xp d m di nte un vi us mbin nt YF 17D d l p ent nv n ón

L pr t ín CS nt n un pít p inmun domin nt B l c l do n su á e nt l E t epítop c ns te de t ndem p t d d uen d amin ác d esp ífıc

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d p En el P f l p um t pít p
p r gin l n n p gin p lin (NANP) h
d t t d n t d l ai l do y í r p nt un
bj t v d l p d oll d l v un L n yo
lín n l n pépt d s sıntét s o
mbin nt admin t d c n lumb d on c mo
ult d l ınduc ón d más mod sto n vele de
t cu rpos a spo ozo tos y s lo nd vıdual s c n lo
n v l má lt d ant cu rpo fu n p teg dos nt
P f lc p um on uf miento et a do d p sim en
t s H ffm n SL Nu sen weig V S doff JC Nuss n we g
RS 1991 P g s t w d mala p yth ocyt v ne
Sci n 252 520 521) Ot e tudí n nım l d
t f n ı p v d nt u p s mono l n l s l
pet dos d l p t ına CS tamb én sugie en que l
nt cu rp pu d n p op ion p tec ón nt
nf cc ón de spo zo t El problema qu sub iste s
t baj p ng í v unas que e c tan n v l s d
nt cu p n human n l p c fı d p piad y l
f n d d p d t ui tod lo spo oit nte d
qu ll nt n l s h p to t s A ste spe t s
n t bl que l nm n a ón d hum n s con na d
mpl d v u YF 17D s ufi nte p ındu
n v l d t t bl de nticu rpos d neut lı a ón al

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YF un d sp é d 40 ñ d l nmuni ión p m (M n th 1999) y que l p st sop t d nal l p nt ınv n ón p a l u d l viru 17D p xp s pít pos de patógenos rel vantes e pec lm nt l pít p de m l i l v nt h i l d r ll d nu v v un vi l v v pa d p teg nd d la ga vida c nt f b am rilla y un segund nfe m d d p ej mplo mal ria La tapa d l híg d n el parásito también es obj tıvo para l desa rollo de la v un p qu ta of ce antígenos ad i n le y n nt st l e i tenc a de vid c rt d lo p o itos n l t ent s nguín d l huésp d mamífe l s mal g s d l human s se de ll n en el híg d du nt v i di L m ni mos f t d t t form nt h p t ti n p bablem nt l élul T t tóxi s qu d st uy n lo h p t it inf t d y l ϒ nt fe n qu inh be l d llo d l p á t

L nmun d d p e trocítı a d l plasmodium m diad en p t p los linf t T que tú n c nt l p á t d lat p d el híg do Estas élul s T d ben e noc l pépt dos de ıv dos el pa á ito n l s élul d l hué p d inf ct d s n l nt xt d

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ntíg n s c mpl j s d h t mp t b l dad mayo L nmun dad m d d p élula T p re e como objet vo de v ntíg n s d p rá it xp es dos du ante la t p d espo it y d l híg d d la infe ión Un úme d tal s epítopos CTl presentes en d fe nte p t ín d l tap p e it cíti s h n d nt f d n human qu v v n n á endém c s de mal r y tán tr ngid un v i d d d molécul s l s l HLA (d o M Udh y kum r V 2000 Fi ld tudi of ytotox T lympho yt in mal ria nfe tions mpl t n f m la ia vaccin development P á tol T d y 16 50 56)

L élul s T it tóx ca p in ip lm nt CD8 qu n l s nd d p ent ón d l ntíg n cl s 1 n p im m nt g n d p nf i n m r b n nt lul y h n id má mpl t inv t g d s n nf n s v l L vi u mbin nt qu xp n l pit p f án d d n p l t nt un p o ma ón lóg c h l g n ión T t tóx d l p f d d se da

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Miy hi t l (M y h Y G ci S t A R d igu D R d igue JR Mu t K T uji M Pale P E t b n M Z b l F Nu en w g RS 1998 R mbin nt v p s ng human mal ntig n n li t p t nt ally p te t v immun CD8 resp nc n m ce P Natl Ac d Sc USA 95 3954 3959) han estud do n n m d l d tón l nmunogen idad de un pít p CTL l al d b CS de P f l pa um El pít po CTL (DELDYENDIEKKICKMEKCS) s xp ó n un g n d n u am n d b ci t ón d l p d nflu n D L v ni mb n nte n luy l g n CS mpl t qu nt ni t nt l pít p humo l s mo l CTL L nmun ión d t ne c n flu v ini li it n un pu t d élul T CD8 CS p if mod t d t t d p ón d int f ón yd l célul nmu ndiv du l El n c d d l t n s n l v flu ec mb nant y ayudand c n l v n mb nt die n mo ult d un m j pr ndent d est e pu[redacted]

Un vi de va n a qu xp s v r os ntig n d *P f lc parum* se desa llo y ut l ó n un nsay l n M ent as qu las respuestas inmunes celula es se

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l t n br el 90% d lp nt u p nd v du l las spu t fu n g n almente pobre De lo 35 v lunt r no ul d s lo uno fui compl tam nt p t g d unque hub un s gn fi tivo etr n l uf m nt d la p tem (p t mi (O k nhou CF Sun PF Lan DE W lld BT may BT K t K St ut JA M g ll A K y h U Fa ley L W rt RA S doff JC K l w DC Kum S Chu h LW Cr t h r JM Wı l B H ffm S L lv n A H ll AV T n JA G t KP d Ta n C And s R B ll u WR et al 1998 Ph I/II f ty mmun g nic ty and ffı cy tr l f NYVAC Pf7 p v to d multi nt g n multistage vac in candid t f pl m d um f lc p um mal i J Inf t D 177 1664 1673)

El v u S ndb c mbin nt qu xp un m nıg n qu c d f a l pit p d l célul T CD8 SYVPSAEQI d l p t ín CS d l p ás to d l m l ia d d *P y lı* u nd in ul ub után am nt n t n s ndujo una espuesta de célula T CD8+ espec f ca de epít po g ande Est nmun zac ón t n a un p ot g do nt la nf ón p espo to (T uji M B gm nn CC T kit S nod Y Mu ta K R d igues EG Nus n w g

RS Z v l F 1998 R c mb n nt Sindb v p s ıng yt t T lympho yt p t pe of a mal a p as t f nflu n v u l t pr te t n ga nst th p nd ng p th g n n m *J V r l* 72 6907 6910)

Tamb én h mo t d qu l t n nmuniz d p una d mpl d un ad nov u c mb n nt qu p l p t ín CS d *P y l* l it un lt g d d t l nf ón m dí d p m am nt p élul CD8 T (R d gu EG Z v la F E h nge D W l n JM Tju M 1997 L d s d ınmun ón impl d d nov u ec mb n nt nduj f nt m t nmu d d p te t m d d p élul T CD8 nt mal *J mm n l* 158 1268 1274)

En ón d l omplejıdad del pa á t y u c cl d v d x t un ns ns d qu un v un d m la lt ment f t v qu i í un mb n ón d tígen y/ epítop l ves d d fe entes tapa del l d l v d y qu l ndu ón t nt d nmun d d humo al com celular requıe e pa a la eficacıa óptıma T l v un tamb én vit í l s p bl m s n l

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t ón g nét d l hué p d y l v r ab lid d nt gén n l d v una b s da n ntíg no mpl H t h n mb g v i int nt p l g nmun d d p t t n d fe entes p epa cion s d ntíg n s d v ı s et p s no h n p duc do resultado onv n nt

L t ma ít o p l ap oximac ón mult v l nt mo un ntígen mple on l d nt f c ón d ntíg nos qu ndu rán n spu t (p lm nt) p t t n la may í o t d l p bl ón obȷ t v l um ni t d e to ntíg n s n una form que e t mula la respuesta má p pi da y l i t m d um n t debe permıt r la p s nt ión de l ntíg n s en una f ma qu t mule el si t ma nm n El d ollo d scr t aquí que ut l Fl v v u p l p ón d antíg no / pít p d p tóg n d f nido d b di ig r l t mas de p es nt ción l p bl c ón obȷ tiv C n rel ción l s vi u YF 17D n vi us t madam nt ınmun gén que indu e lt p p n d nv ión d nti uerpo n v una d d f r nt nt d nt g néti

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El s l c tante de l p sente inv n ón p rt cul rm nt s prob r la factib lid d d util a la ep de v rus YF 17Dy l subsepas de stos no olo como un v un muy fe tiv prob da contra f eb e ama ill s n tamb én mo un ve to pa ntíg nos p otecto es p t ul rm nte pit po p otecto es Estos saltaran el d s ll d un v un imultáneamente fect va cont a f b am rill y t enf rmed d s que pod í o u n l m ma á as ge g áfica t les como mala i d ngu n f l ti j p n ncefalıtis p oducid p g p t nf on fungo tc

L p nc p l meta fue establecer una p oximación g n r l p r nse t r y xpr sa antígen s mpl d fin d q n l y n epít p n l sit os d ár t u tu lm nte d t nt c n ido p int f i n l t u tu d l p t ín E d l Fl vivi u t t l p ífi m nt n l cl fg o l c cl E F de l p ot ín E de un Fl v vi us d do tal c mo fi b am ill d ngu nc f l ti j p ne n ef litis p odu d p g p t qu e pued util ar omo una

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nu v v un viv qu indu e un pu t inmune p t t d l g du ción Más pa ticularm nt la p ent ınvención esta lacionada con una aproximación gen ral pa a expres r un epítopo defín d del c clo fg de l pr t ín E del v u YF 17D

C m ut li qui l t rm n Flav vi u gn f v u lv st vı us atenu do y virus mb n nt que n luye v us quimérico

L m n pul ión g néti d l g noma YF 17D ll v b ut l nd l cADN d YF inf cci o gin lm nte d ll do p R ce t al (1989) qu con iste de d plásm do den minad pYF5 3 IV y pYFM5 2 El gen ma YF fu d v did n d plá m d d b d l f lta d t b l d d d lgun uen as d v us n el lto númer d op d l v cto plásmido pBR322 Sin emb go en l ca del vi u 2 d l d ngue cADN d l ngıtud mplet fue nstantem nte cl nado n el mismo plá mido (Kinn y RM But pet S Chang GJ T uchiy KR Ro hr g JT Bhama ap avati N & Gubler DJ 1997 C n t u tion f nfectiou cDNA clones for dengue 2

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v u t in 16681 nd it ttenuat d v ccine de iv te st a n PDK 53 Vi logy 230 300 308) Cuando se o t p l s n ma d tr ción p cif ca (Ap I N iI y AatII) l f gm nt d e t i ción sp if a son g n d l cu l l lig n tituy n l g n m YF mpl t n l f rma d cADN L e tri ión de e t f rm d cADN n Xh I l nea i a l cADN y perm t sínte s n v t d ARN c n ub e t d l uenci p mot SP6 T l ARN u ndo t nsf t n élul ltiv d d ig n vi inf ci s El fen tipo d t v h id n y d (M r h v ky RS M rian J F VS Alm da E C qui MJ C rvalh R P s u n JW T v so d R a APA Simões MC Santos CND F a II Muyl ert IR Mann GF R ce CM y Galle R 1995 Phenotypıc análıs s of yellow feve v us de ved f om omplementa y DNA *Am J Trop Med Hyg* 52 75 80) y e tos stud ndic ron la nece idad de mod fica ión genétıc d l cADN pa a elabo a el v us ultant má t nuado E to se ha llev do a cab po Gall y Fr i e (US 6 171 854) y dio como resultado nu v v n d pYF5 3 IV y pYF5 2 d nom nada G1/2 y T3/27 p ctiv mente El mapa físi o d la s cuencia de ambos plásmidos se muestra en otra parte y estos

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plásm d h n d p it do en ATCC (ATCC# 97771 y ATCC#97772)

Hab end l g nom dıvidido en do plásmıdos es onv nıente p p pó to d cl n ción d in c ón pe n s d l p la cup ión del v us Así con el f n d l g r un nfect v d d p cif a más lta d lo t n cr pt el s l c t nt d a llo un l n d cADN d l ngitud mpl t p a el gen m YF S d nt f n l plá mid de lit atu pACNR1180 (Ruggli n T ts h n J D Mittelh iz C H fmann MA 1996 L ecu nc d nucleótid d l s pa d l vi u de la fı b ama lla sw ne clá ica Alfo t/187 y la tr n r p ión de ARN nfeccioso de cADN longitud completa cl n d e t bl ment *J V rol* 70 3478 3487) qu t ne un g n d eplica ón d nom nado P15A qu permite ol epl c ón l mıt d d l plá mid redu iendo un num ro d molé ul de ADN d plásmıd po células bact rial Est i t ma d plá mid f util d p r los ut r p r st bil l g n ma d l v u d l fi br swin lási n un tamaño de g noma cer ano aqu l del vi u YF E te plásm do pACNR1180 p opo ono p el Doct

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JD Tr t h n d l In titut de Vi l gía Inmun p f l xi d Su

Ad má d l pACNR1180 ot s v cto qu p p i n n l tab li ción d l gen ma del vi us YF s pued n ut l pa a p epa los plásmidos de la p nt inv n ón Ejempl e p ífic n luyen plásm do qu t n un o ig n de eplic ión qu p rmit l pli ión límit d d l plá mido r du i nd l num ro d molé l de ADN de plásmido por célula de b t s d c r v t r s que con isten d bajo númer d p d plá mido t les omo el pB loBAC11 (Alma an F G n al JM P n Z I ta A C lv E Plan Du an J Enju n s L 2000 Engin ing the l g t RNA viru g nom s n infecti us b cterial a tif cial h mo m *ProcN tlA adS ıUSA* 97 5516 5521) Otr p b lid d el us de plá mid n lto núm d p s t le c mo el pBR322

L n eva v sión d l pACNR1180 se den minó pACNR1180de/S L Se btuvo al emov r l m yorí de los t d t cción únic d l pACNR1180 medi nte

dig t ón con Nd I/S lI ll n ndo n l ext mo al t t n l nz m Kl n w l g nd y t a formando *E C l* XL1 blu

P g n pYF17D/14 l s f gm nto N tI(13 059) N iI (11 158) y S lI (3 389) Xh I (1951) del plá m d G1/2 (descr to n det ll US 6 171 854) fu ron l g d p mer l pACNR1180Nde /SaI digerido NotI/XhoI El plá m d r sultante se denom n NSK7 (mapa no most ad) y se util zo para clona el rest del cADN YF c nt n d n el plásm d T3/27 (de c to en detalle en l US 6 171 854) m d nt dig st ón n N I (11 158) SalI (3 389) l lig ión de los fragmentos prop ad y l t n f rm ón d l m ma b cte El m pa d n ima de t ión nfirmó l t u tu físi sp d d un plá mid qu nt ní l cADN YF complet S anali on un tot l de 8 cl nes se cup ro virus de 6 nsay s y l d term na ón de ecu ncı d nucleótido complet nf rmó l cu n YF p d El plá m d ntí n 13449 pa e de b se y e d nom no pYF17D/14 (f gura 4)

El pYF17D/14 ont n un g n d r i t n i a l ampi lin p veni nt de l p ición 13 196 545 y l p15A g n d l epli ón (nt 763 1585) amb d v d d l plásm do pACYC177 (Ruggli t al 1996) L nu l ót do 12 385 12 818 o sp nden al p motor SP6 El g noma d l YF s t nsc ıb en est plásm do de heb a opuest en la medıda en que el genoma completo se xtiend sob e lo nucleótıd s 12 817 a 1951 Toda la ns ion s n l c lo fg d l p teına E del vi us d l fi b ama ll n h ch n el sitio EcoRV d l plá mid YFE200 y d alli in orpo ad d nt o d l pYF17D/14 m di nt int cambio de f agm nt N iI/N tI Ot tıo rep esentat vo s muestr n en la figura 4

L plásm dos G1/2 contien n l se uenc a t rm n l YF 5 (nt 1 2271) ady cent a p omoto de la p lime asa fag s SP6 y l cuen ı term nal 3 (nt 8276 10862) dy nt al sit Xh I p p ducción d t n c pto d d v P r p rm tir la in e ión de l c en i f n dent d l i lo fg omo d f nio m di nte l náli i d l tructu 3D d la p ot ına E hamo cr d po medio de mut génesis in vit o un siti de est cc ón (E oRV) en el nucleótido 1568 La creac ón de

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t ti nduj d amb n aminoá d n l p t ín E en l s p s cion s E 199 y E 200(E→D T→I r p t v mente)

E mp t nt nfat a que la re ción d l it o d tr ón de g nc d p nd d la se uen i d nu l ótido que h ce cada icl y va iedad de acu rdo con l u n ı d g n m d l Fl viv u l s util z da c mo vect r Po l t nt l sitio de re t i ción ut l ado p ra n itıo de Flav virus EcoRV es espec fic p el s t o fg de l f br ama lla pero (corta) pu d n se út l par ınserc ón n el genoma d ot os Fl v v ru Aqu ll exp rt s n l t cn ca dentif a n t d u d l util s p t lógic d análi is d u n d nu l ót d c nv n i nal p el dis ñ d t s it s d t i ón p pi do

E not bl que l amin ácid 200 s un K Asib T n t d l v us 17D n li do y I n E200 El hech E200 un pos ión qu s lt a n tod los vi u 17D uge í qu aquell alt a ión pa ticul e impo tant p a la at nuación del vırus 17D y las alteracion s

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p d í n mp met t t Sin emb g l mut ión
nt du d p l ión d l it d in ón n
nduce l eve ón del am noá ido o gin l y amb
s n muy dist nto en ca áct Más un es p obable que
la t nuac ón del 17D s a mult fact l y n s lo
l c n d n l gión t u tu l mo sug ió l
f not p d l 17D/JE Nak yama qu mé c en el mod l d
tón d n ef lit (Chambe s et al 1999) Ademá
n ngun lt ón p in ip l d l st uctu d l
p teín E ev dente cu ndo e modelan est s cambio de
aminoá id n l mod l 3D p di h Fin lm nt l
vi us d iv do d l YF17D/G1/2 e ó en ultivo de tejid
lo m smos n v les y fue tan neu ovi ulento pa a
t ne como el v rus 204/DD pa ental de vado del
G1/2T3/27 cADN

C n l f n d l it E oRV n la ecu nci
YF no t o ub lonamo n fr gm nto NotI/Ap I d G1/2 n
l plá m d pAIt (P m g In) y d llo l
mut gén mo l ndi l fab i nt d l pr n i
d ligun cl tid int tico mutag niz nte e p cifi ndo
el sit o EcoRV de eado Los mutantes que llevan el s tio
fu n d ntif do med nte nál sis de enzima de

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t ón d l ADN plá mid y l f agm nto ApaI/NotI nt nid n un d lo l n se u n i mpl tam nt p nf rm qu l iti d st cción fue l mut ión l n l u n i YF Al f gment t l nó n l plá mid G1/2 y el plá mido sult nte denom no pYF200 cuyo mapa e muestra en la figu 5

El plásm do d rivo del pYF5 3 IV o ginalmente d s it en Ric et l 1989 modificado p G 11 y F i (p tent num o US 6 171 854) in luido aquí E t nt n 6905 nu l ótid y la gi n 1 2271 qu p nd l 5 UTR C p M/M y l gen E Est gión fu i n t vé d l it o E RI n l g n E (2271) t it E RI en l gen NS5 (p sic ón 8276) E l p i ón 1568 n l g n E ó l iti Ec RV l u l ut li ó p in ión n l epit p d nt d l iclo fg d l p ot ína E Est plá mido tamb én cons te d un g n NS5 pue d l nucle tid 8276 hasta l ultimo nucleót d del g n ma YF (10 862) que c ntien po l t nt p t d l g n NS5 y 3 UTR Lo nu leótid 5022 5879 c p nd n l g n de r si t n ia l ampicilin y l 6086 al 6206 al rigen de l eplicac ón amb d v d del plásm do pBR322 además del pBR322 otros

v t s c noc do p l e p c l tas n l técn pu den utıl ar tales como el pBR325 BR327 pBR328 pUC7 pUC8 pUC9 pUC19 λ fago M13 fago etc La l l ac ón de l t s de en ma de st ón l v nt s mue t n l figu 5

El plásmid YFE200 se d pos to n l ATCC b j l núm PTA2856 El PYFE200 utili p a p odu i mold junt n el T3/27 que perm tió la cup ción del v ru YF que em j l v ru YFv5 2/DD (US 6 171 854) n p op ed des de rec miento n v o y células CEF tam ñ d pl ínt d p t ín y neu v lu nc p t nes (dat p E200 y mbin nt d iv d d t mo mu t n l s jempl s)

El mold l utili d p g n ón d l vı u YF 17D se p p a med nte digestión d l ADN del plásm d (YFE200 y T3/27) con NSiI y S lI D pués de d g t ón n Xh l p l ne a l ADN l g d l moldes se ut liza on pa a tran c p ión n v t o L vı us se han recuperado después de la t anscripción de ARN de la células animales cultivad s

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El cultiv d células an mal s util zada aquí puede se u lquie célul h ta qu la sepa 17D d l v rus YF s pueda epli Ej mplo e pecífic s ncluyen Hel (d vad d l c n m de ce vi uterino humano) CV 1 (d v d d l ñón d mon) BSC 1 (d v do del iñón d mon) RK 13 (de vad d ıñón de on ɔ) L929 (d v d d t jid on to de atón) célula CE (mb ón d poll) CEF (fib bl t de emb ión d p ll) SW 13 (d v d d r in ma ad n ti l human) BHK 21 (ñón d ham t b be) V (ñón d mon v d f i n) LLC MK2 (de ivad d l iñón d l mon Rh) t

En un m d lid d pr f id d l p nt nven ón l células V o on n subst to p fe ido en t d l t p d p du ión d los títul btenido n dif nt u v d ultiv í omo también l st b lid d g néti di m j e sultad Lo cultivo p m s d l f b obl st d emb ón d p llo (CEF) pu den se una segunda scog nc a a ser utilızada como ub t t n t das las t pa de p ducción n la med da

qu t célul s han ut li do p a p odu ción d v n de amp ón dur nt añ on expe ien a t ns v d u p ep ac ón y cont ol de c l dad un núm d p t ca de oper ión stánda St nd d Op t ng P ti (SOP) t di p nibl y un lic tud de patente que trabaja c n la p oducción de v cun d YF n ult v s CEF e h p entado (EP 99915384 4)

P l t nt l tema d Fl v v ru de t q í má p t ul rm nt l stem YF pr p n un m t d l gí potente p ra el des rroll de formulaciónes n l mit d d v u mbin nt qu exp n dif nt pít p S nt ip qu l f rmul ón p p ad de v rios vi us recombinantes debe el citar la pu t nmun p h c fr nte l d ferent tapa del pa ásito

Lo sigu nt s jemplos on lust ativos d la inv nción y repre ntan las modalidades prefe d s Aqu ll exp tos n l técn a podrían be se ap c s d en nt arl s utiliz ndo n da má que la

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xp m nt ción de rut na par empl r otro m terıale ap p ados y técnicas tales como los vecto es nt rm nt m n on d las élulas del cult v lo mét d s d t n f c ón

Ej mpl 1 Anál i t u tu l d l in ión d pit p d p t ín p íf

S p duj on 10 model s p la ns ión de SYVPSAEQI n l eg n d l icl fg util and el lin ami nt m t do n l figu a 6 en l cu l s h c l n ón nt l E199 y T200 L s s du s d ns ión e d nomin an como 199 a 199I

L eud n gí d lo 5 mejor mod lo fu n mp abl c n aquell del modelo nat vo YF Sus estructuras on v r ables como lo esperaría un expe t en l técn ca de una inse ción de nuev s esıduos de l ng tud La v riac ón en la e t uctura d l c lo c nduce a va ı ıón o elac on da en el ciclo vec no entre las ß heb as K e I L caden de glutamina late l del

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d Gln 199H (p jempl l oct vo r id o d l péptid n t d) n v os de los mej e mod l mu st una onf rmac ón c mpatible con la formación de un enl d h d og no p m d d su N al c bon lo d V 1244 d l monóm o pu t de man a im l aquel h h p l N d His208 en tb Un mod l p nt t v teni un f t g n l G d 0 07 qu v l nt un t u tur d meno 1 0 Å d l ón y t ní un bu n t quimi n l gión d l n e ión L n ta ión tot l Ve fy 3D p a l gm nt d d 199 200 (in luy nd l s nu v r idu in t do) 3 69 (un v l medi d 0 34 p e du) nd c ndo que l s esiduos n el la o han sido nst u d n ambi ntes químico f v abl s Po compa ción el valor medio po residuo pa a el la o quiv l nt n l tructur c i tal del tb lam nte de 0 28 El á e de contacto p omed o po m nóm pa te model es d 1 460 Å c mparable con l b v d n l t u tu c i t l d l tb mo nt cip d p l int odu ión de l ins ción ce c de la nt fac h dróf og s p óxima (Figura 7)

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Du nt l con t ucc ón d l sit o d in rción do u t tu i n fu on h h l u n i d m n á d E199D y T200I L n ecu n i d t l u t tu i n fu n li d n ef nci l mod l L u t tu ón E199D n sp ad p a qu t ng n u n i m u n v tiv n n tu l s bse v d en tb y pu d ll v un pu nt d l n K123 No h y un camb o ign f cativ en el índice de al dad pa este res duo como se determino po WATIF L u t tu ón T200 y p ec a pt ble a m d d que l n ión ll v un t ción d l ad n l te l T200 d l 100 mod l ult nd qu e t di gid h l bol ll hid ofóbico c no W203 l egión lifáti a d l R263 Y L245 La su t tu ión también t n l am f c ón b CB

Us ndo una en ñan dént ca l de ript nt rment l ns ión de DYENDIEKKI fue nt duc d n l p t in yf E Un l ne ón dént a la m t d n l f gu 6 fu u d n t n l x p ión d l u n i d in ión mi ma L mod l fu n pr du do nd pr tocolos idéntic al d scr to nt rm nt p l in ión SYVPSAEQI y fu n d

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l d d m l Típicament l s mod l p ent n un d l f t PROCHECK G 0 1 (equ v lent l t tu i t l n un lu ión ent e 1 0 y 1 5 Å) y t n n l d do 90% de l s duo dentr de la g n má f v abl s d l g áfi o Rama h nd n Aunqu l nf rma on s d l la o bten d mu t n v i ón n d abl mu h sug e n l p ibl p ición d un nu v int ci n s nt ub unid de qu pu d n fectar la st b lidad del dímer y/ l influencia del pH d pendiente del dím ro a l transición trímero Los p ent d l p t nc l ug d p r l s model s ncluyen aquell s ent e Glu199C Asp199E y7 Glu199G (el terce o qu nto y séptimo es duos de la ins rc ón sp tivam nt) c n Arg243 (num a ión nativa yf) de l ub unid d pu ta los mi m que l Lys 199H c n l b n l d l L u65 d l ub unid d opu sta El puent d l vi t n l model y yf n t v nt Arg263 d un m nóm o Glu235 d l t o es t n d En ningun de l m d l l Lys199H form un nl e de hid gen qu v l nt qu l h ch p el Hı 208 d l ub un d d pue t n tb pe un enl ce de hid ógeno potenc l b n l d Leu65 e po ble El Ly 199I pu den f rma un puente de sal con Glu199 en la m sma sub unıdad y tal nte c ón d be ía s r fa t ble aunque después de una

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u t tu ón á id glutámic á d párti En l
d m r a ón dad sub unidad p rd un p med o d 1 483
Å d á e d supe fic accesible (b sad n t l
m d l) mp bl n qu ll d l tb p n p lm nt
d b d l n c ón d un laz g nde ent e hilos ß f
y g

D n t l h ch qu ningun d l d
n i n d l d c t ll van a un l p
té ent l s ub unid de ellas p e van un
bu na ste eoquím l mismo que (en el aso de l s
m ɔ mod lo) un p du ión n bl d amb nt
químicos pa la cad na lat al de amin ác d s d la
ins c ón Esto se l g sin un inc ement exagerado en
l á d nt t ent los monóm o qu ugı en n
qu n h y un t i ión p ci l a l inse n
pu t

C mp d n l l b v do
p m nt lm nt n l t u tu i t l d l p t ín
E p tir d l v ru tb l d in ion de rit
nt iorm nt on tre es duos má la g Est
e duo ad ion les on comodados en la mayo ía de

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mod l p r un p n p tr ión di on l d l t u tu b l up fic ext n (unqu n un model un estı ami nt c t d la hél ce lf e b e v d) E t ugi qu pued s p sibl mod pépt d má l rg d nt d l iti d in ión

D e m del s fu n gene do para la ınse c ón GG (NANP) GG y un p otocolo déntico al descr to en los nt i d jempl A d f n i d l d jempl nt i in mb g ta l h ho de que ta in ón un epítop d élul B n d ablem nt má g nd e incluy un t t péptid p t d y i n du s d p l n En t 3 p h qu l pit p d élul B d b í t xpu t má p bl (p h ce que l nti u rpo se sibl) y d d l lib t d conform ci n l adecu da l m mo qu en f lit u n e ción d nt o del la o fg n l f rm d un l Ω (L ynsk JF Ro GD 1986 Lo p In Gl bul P te n A Nov l C tego y Of S c nd y St u tu *Sc ce* 234 849 855) dos es duos d gl in (p i do) fu on g g do tanto a l s t rm n l N y C d l epítop (NANP) Debido l tam ñ du id d l den lat al d glicina y a u

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át mo qu al Cα un reg ón mayor de sp c
R ma h nd n c ible u ángul s de caden
p n p l d éd al s φ y ψ increm ntando l
p tun d d d un nse ión x tosa que n
p jud li l t u tu l c l en l gión d l
in to E t nseñ n a p od modelo qu pr nt n
te quím xc l nt (fact es PROCHECK G d l d n
d 0 1 qu v l nte l t uctu as c tal de
resolución 1 0 a 1 5 Å) La inserción del lazo en si
mi ma t l b d pa t e quím c In st mo n
que t e el es lt do de lo e pa iadores de glic na
d t rmin l N y C qu s v n p l vant l l o
l b e d la supe f ie ext rna de l p oteína En v rio
d l mod l un má d t gli in d pt n l
nf rma ion d st u tu p incip l l qu p hibi í
n t amin á id El t n g n lm nt
nf rm ion n ext n ión xt ndid (ß) E t
fa to e d n énfas a la mpo tanc de su nclu ión

Aqu ll v ado n la técnica podrán sabe que
una pred cc ón c nfiable de la estructura de tal lazo
tán má llá de las nseñan a teóric s usuales Por
lo t nt xist ns d abl d t bución en l

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nf ma ión ult nt d l d e mod lo gene d (f gu 8) E t d t bu ión amplia de conform c nes (d aproxımadamente = a seudo ene gía) no llev a un lt ón gn f tiv d l st d la est uctu a nf t nd qu l l o tien ab ces a un gran volumen de esp c omo se d s ño o ginalmente Est no gnıf ca qu l la i n s am nte n t uctu m am nt n qu mu h p ible estructu s n c bl l l z nc ment ndo la p sib lid d en l u l l v nt nmun lóg ca ment y fue dopt d un d nt d l nt xt d u in ón d nt d la p t ín E yf L f gu 9 ilu t m ɔ c n de cı n s d volúmen

L cu nci (NANP) n l di z mod l s tıene una m d a l t v d á a d up fi i bl (mp d on u t u tu a n d r ll d) d 63 7% E t mp c n un v l medi d 27 4% p l t u tu gen l demo t and qu la nser ón t n un abilid d l tivament muy g nd omo h p pu t S lo p i d d gl cin on limin d s t v lo pa a l (NANP) cae a 53 6% demost ando que los espacıado es t enen un papel en incrementar la

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xpo ón d l epít pe El xamen d l s modelos mu str qu n m nt nd la l ng tud d l p i d r de gli in má llá d d e idu s pa ce í omo que n t n n ngun v nt ja dı on l l d sp ne l pit pop pu d p nta un t v n t óp o p a l t u tu l qu ll v í u d tabili a ión L s d s glicin s pa en er l ópt mo pa n t s

E un xp t t v onabl qu l p t ín d nv ltu d l vi u d n f l ti j p n y d ngu 2 mod í n l nteri e in ci n s des it g lm nt b n que l v ru de fi br ama ill L l n ón d l figu 2 mu st qu u8n el min ión d 6 du m l tá p nt n t d l t pr t ín d nv ltu v l mp do n l tb L mod l p l j y l p t ín d2 p du d u nd un pr t lo s milar pa aquel del yf frecu ntem nte muest a n e t uctu a d e pi a ß p a el la fg e tab l zad como un enlace de hidrogeno de cad na pr n ip l Un esp ß simil también e observa n yf i l t i n d l ne ón n r laj d s l d d d l re duos del l o fg mism L s mod l s pa a el je mu t an un puente de sal ınte subun dad

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pot nc al ent e Lys201 con Glu243 (nume ación ɔ) d l
ub un d d opuesta Est ác do glutám co n yf nte tú
n Arg263 qu h d u t tuid p v lin en je L
c nta to im la s aquell s d l yf tamb én s ob rv n
l d d d l it d nt f d dim r di t l Un
mod l p nt tiv p l p t ín E ɔ t ne un
f t PROCHECK G de 0 1 89 9% de s du s n l
g on s más f v bl s d l g áf o Ram hand n buen
st e quím ca n la egión d l l zo fg (que adopt el
t p l d l ß) una buen c l d d WHATIF d n t ón
p l l o fg (du 203 212 d p du ión y un
p m d d 0 768) y nti un ár d up f i
s ble m dia d 1 048 Å po sub unıd d b e l
dim i ión R ult d im l n bt nid p d2
n l l l l fg d pt s l tipo 1 o el tip 2 d
nf rm ión de l ß P e tos d to aqu ll
v d n l té n n p ce d pli l
st tegia d ın e c ón d script ante iorment pa a yf
n ot Fl v vi u t l s mo ɔ y l d2

El sıtio que mp nde la gión de hilos ß f y g
n l y l la fg que f rma pa t de las inco lam n s ß
nt pa lel am fic das d l d min 2 d l Fl vıv us d

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l p teína de env ltu Fl viviru que comp nd l g ón del amınoác do 196 a 215 con ef en ia a la u n d v u d n f l t t ck b ne de it n la f gu 2 Má pa t ularmente l sit es el á d l l nt l h lo ß f y g que forman pa te d las n lám n ß ntip l l am fi da del d m nio 2 d l p teína de nv ltu Flavivi u (amin ác d 205 210 n f n l u n i d vi u d n f liti t k b n d pt n la figur 2)

Adi n lm nt l t pu de mp nd l reg ón E y F d l h l E y F que ncluye l l o E F que f rma p t d l b il ß amif ad o tavo d l d m n o d l p ot ína de env ltu a Flav vırus que comp ende la g ón d am n á ıd s 138 a 166 on ef nc l u n d v u d n f lit ti k b n d pt n l f gu 2 Má p ticul rm nte el iti es el á ea d l o ntr lo h lo E y F que f rm n part d l b r il ß d 8 amifi n d domin 1 (am noá d 146 160 n f n l s u n d vi us d en f l ti t k b n d ipt n l f gu 2)

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Ejempl 2

) De v ión d l plá mido pACNR1180N de/Sal

El pACNR1180N d /S l l nu va ver ión del plásm do pACNR1180 bt n d emovi nd l may r p t d l t d t i ión úni del Pa n 1180 por dig st ón n Nd I/S lI ll n ndo n l xt emo po el t tam ento n nzima Kl n w lig nd y tra formand el *E l* XL1 ul E tá nu va ve ión de pACNR1180 fue llam d pACNR1180Nd /S l

b) D v ón d l plá mid Pyf17d/14

P g n l Pyf17d/14 f gmento d N tl (13 059) N l (11 158) y S lI (3 389) XhoI (1951) p tir d l plásm do G1/2 (desc ipt en detall en l pat nte estadoun d nse US 6 171 854) fue on ligados p m am nte l NotI/Xh I d ge do pACNR1180Nd /S l El plá mid e ult nt fu llamado NSK7 (el mapa no e mu t) y u do par cl nar el rest d cDNA d l YF nt n do n el plá m d T3/27 (d sc to en d talle en la US 6 171 854) p d g st ón con N Il (11 158) S lI (3 389) lig ión d lo f gm nt p opi do y

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tr n f ma ó d l b t i m ma El plásm d cont ne 13449 p d ba e y fu llamad pYF17D/14 (figu a 4)

El pYF17D/14 nti n un gen d si t n a l amp l n p t d l p i ón 13 196 545 y l p15A d ig n d pl ión (nt 763 1585) amb d iv d s d l plá m d pACYC177 (Ruggli t l 1996) L nu l ót d 12 385 12 818 o e pond n l promot SP6 El g n ma YF t s to en st plá m do d de l h lo pu t m d d qu l g n m mpl t b l nu l ót do12 817 a 1951 T d s la ins ci nes d l l o fg d l p t in E d l v u 17D son hech s n el sit EcoRV d l plá mido YFE200 y desd allí s in orp d nt pYF17D14 p nt cambi d l fr gm ntos N il/N tI Ot it p nt tiv mu t n n l f gu 4

) D v c ón del plásm do YFE200

P p d a Ec RV en la ecu ncia YF nosot s ubcl nam un f gmento NotI/ApaI del G1/2 n el plá m d pAlte (P omega Inc) y llevamos a cabo la muta géne mo nd ó po l fabric nte n la p es ncia

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d l ligun l ótid intét d mut geni ón
p f nd l it de ad E oRV Los mutant s
ll v b n qu l sit o fu n id nt fic dos po l
ál d n m d t ón d l DNA d l plá m d y
l f gm nt Ap I/N tI ont n d n un de lo cl ne fu
u nc d c mpl tam nt p nfirma l s t o de
e t ón l mut ón ún d l u n ia YF
T l f gm nt s fu n l n d s d nuev en l plásmido
G1/2 y l plá mid sult nt fu llamado YFE200 cuy
map mu t n l figu 5

d) P p r ión d c ntid des gr ndes de DNA de plá m d

Pa a p epa ar los DNA de plasmados a pa tir de la ba te c ntıdade d glicer l del *E coli* que lleva d un d l s plásmıdos YF pYFE200 y pYF17D/14 stán d p nibl El gli l l 50% Lu i B th n m dio e u d n l p p ión d l ntidad d p rt da qu lma n d 70 C Alí u t s ng l d d l pDNA también tán d pon bl

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L b t i n en 5 ml LB qu c ntien n mp lin (50µg/ml) pa l YFE200 y amp cilin (50µg/ml) más t t lin (15µg/ml) du nt l n h a 37 C p l b t que ll v l NSK14 E t s u d p n ul 1 100 d v lúm n s g nd s d LB (u u lmente 100 200ml) En OD de 0 8 s ag eg cl nfen c l a 250 µg/ml p ra la amplificac ón del DN del plásm do y ncubo d onalm nte dur nte la noche El plá m do s xtraído l mét do lı is lc lın El p p t do del DNA f nal es esuspendido en TE (Tris EDTA) y cl u o d c sio s g gado hasta que el índice d f ón d 1 3890 l an ad El DNA del plásm do e b nde d p ult ent ifug ón du nte 24 hor El DNA b nd do upe d po m d o d pin h l tub ext lo n but nol y dıáli is t nsiv

Lo p od t on u al 1 mg d pDNA/litro d c lt vo par l pYFE200 y 0 002 mg/lıtr p ra l pYF17D/14

El pYFE200 fue dep sitado en diciembre 21 2000 b ɔ l núm d c PTA 2856 on l Am i an Typ

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Cultu C ll t n (ATCC) 10801 Unıve sity Buleva Man V 20110 2209

Ej mpl 3

P p ión d plantill s d DNA

L pl nt ll l r u d p l eg n ción d l v u YF 17D s p p d p l d gest ón del DNA d l plásm do (YFE200 y T3/27) n NsiL y SalI (p omeg In) en l s mısm s condic ne d amo tıgu ión c mo om nd p l fab ant 10µg d cada plásm do on dıgerıdos con tanto enzımas (la cantidad requerida es lculad n térm n s del núm o de golpes de pool pr s nte en cad pDNA pa p de logra la digest ón ompl t n d h) La dig t ón verifi d movi nd un ali u t (200ng) y ié d l ob 0 8% d g /TAE d gel Cuando l dig stión mpl t l n im d t i ión n in tiv d p l

L lin i ión del DNA esultante d l l g ción de ambos NS I/S lI plásmidos d ge dos se ll va cabo

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p l o XhoI ll v d b c n nd i n d amo t gu ión d u d n l fab c nte (p m g) El p du t ult nt xt id n f nol l rof rmo y p ip t d n t n l El p cip t d s l vado c n 80% d t n l y e u p ndid n RN e libr tér l c n am tigu ón d T i EDTA Un lí u t de plantill t mada del anál s del gel de ga osa junto on los ma cado e come cıales pa a el tamaño de bandas y la u nt f c ón El t mplad e lmacen d 20 C hast qu us n l t n ip ón n v t

Ejemplo 4

Tr nsc pción d RNA p rtır de la plantılla DNA d la p es nt nv nción

L p p ón del la plant ll d DNA desde l l ng tud mpl t del cl n pYF17D/14 p la t n ip ón n v t má mpl y qu m n pDNA u u lm nt 1 2 µg qu dig rid n Xh I p u l n ón L d g tión limpi d l DNA y l lid d d l pl nt ll n ll vad a cab omo s d s b ó nt r rment L t ns ipc ón como s

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d s ib ó n US 6 171 854 El RNA p odu id del Xh I l n a z do d l pYF17D/14 de la plant lla d DNA de e t fue n h mogén s y l m yo í n long tud ompl t en nt t c n l s do sistemas de plá m d d v d d RNA (d t n mo t d s)

Ejempl 5

Transfección de RNA

L t an fe ón d l RNA fue llevada a cab de un man a s milar como se desc ib ó en la US 6 171 854 (G ll yF) L t nfecc ón d l t l RNA dio lugar al v u l u l m l r l YFiv5 2/DD en su tam ño de pl id n élul V y CEF n ut li ión p hip inmun YF y int d p t in

Ej mpl 6

C n t u c ón d l vi u YF17D/8 mbin nt qu e p un ep top de élul B humo al

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P l exp ón d l epitop humo l (NANP) d l *P f l p rum* (v tabl 1 más ab jo) d s gli in qu d n fue n ag g das s b e c da lad de modo qu s mp p un formación s mıl de una el ce ß n l pit p d d l pr n i d p lin c n ecut v y p gi na d do que el l zo tenía una st uctu má fl x bl P lo tanto la ınse ción de oligunócleotido ntét n l s t o EcoRV del plásm do pYFE200 qu p nd l ecu nc d aminoác d lustrad en la t bl 1 d lug l plá mid pYFE200/8 Este plásm do fue d p t d n di emb e 21 2000 b j l núme o de cc s PTA 2857 on l Am r c n Type Cultu e Collet on (ATCC) 10801 Un v ty Bul var M n sa Va 20110 2209

L lig ción t rm nad Blunt del ol gun cle tid s ntétic r mpe l iti E RV y t fue usad p s l c n r plá mid qu ll v n l n rción Lo plá m do EcoRV fu ron cuenc do lr d do de l g ón d n ión p v ifi l i nt ión del n t L plá mid n la ientación d in t ect fu n mantenid s nt nc c n el ma d l tu ab t fu n u ad p a l se uenc a dic onal que ll va la g ón e tructu al complet conten da en

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t plásm d má espe íf am nt l á omp ndid nt l t s NotI y N I El f gm nt d cDNA N tI/N ıI d 1951 bp fue l g do c n l f gment N I/MluI d 1292 bp d l plá m d T3 y l t u tu p n p l d l N tI/MluI d 10256 bp del cl n d l ngitud mpl ta pYF17D/14 L plá mid s ult nt fue n p m tam d en cu nt tamañ y lu go pa l p du ión d t a it s nf ccı sos po t nsf c ón d RNA m di d po líp do d la célul s vero c lt vad mo d s rib ó (Galler y Frei e U 6 171 854) El v us ult nt fue llamada 17D/8 Lu go de l t n f ón l YF 17D/8 tenía un t tulo (m dio po pl b mon c p de élulas ve) de d 4 0 l g PFU/ml De pué d un pas je ún o l mater l d p tid d élul v t ní un titulo de 6 1 l g PFU/ml L p nc d l n to n l g n ma vi l fu v fi d p u n i ión d l DNA h ho l vi u p nte n l b n d nte de ult vo de élul d iv do d l t n f c ón

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T bl 1 u n i d am noá id y p ifi d d d l pít po hum l (NANP) p l n rción d l p t ín E d l YF

Secuenc a	Ep topoantí geno	Fuente	Clón
EMD GGNANPNANPGG IES	CSP B	*P f l ıp rum*	17D/8

Ej mpl 7

E p ón pít p p r l vi u e mbin nte YF 17D/8

P nv t g l xpr ión del pítop de 16 amınoá do en la p oteína E del vi us 17D/8 fu llev do b un en ay d nmun flu n a indir ta us ndo un nt u p mono l n l dirig do l p t ión (NANP) ((MAb 2A10 Z b l F C ch an AH N dín EH Nu n w ig RS Nu nw g V 1983 Ci un poro oit p teins of mal ı pa sıtes cont ın a s ngle immunodomin nt egion w th two o m r dent al epítop s *J Exp Med* 157

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1947 1957) y un u hip inmun policlon l d r tón l YF 17D (ATCC) El IFA fu h h u nd célul V fij n glut ld do nfect d du nt 48 ho con YF17D/14 n un v u ombinante YF17D/8 que lleva el ep t p (NANP) n la pa te (multiplic dad de infección) d l L s mue t s fue on tratadas con dos diluciones de l fluıdo YF h pe nmune scético (ATCC) y l IgG de tón d r gido nt a el epít po de élul B inmunodom n nte NANP d l P f lcıparum de l proteína CS pu if ad parti del anticuerp monoclon l 2A10 como d ib ó (Z v la t l 1983 g ft of D M R d gu E l P ul t d M di in) L s élul p s tiva fuer n vid n d s p el leg d de IgG nt tón njug d on FITC

S lam nt l élul V infe t d con el vi u 17D/8 fu on n d p mab 2A10 mi ntr qu l r h p r nmun YF d tect loa ntígeno YF en ambos (v f gu 10)

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Ejempl 8

R d m ión d inmun p ipit c ne y l t fo s s d gel de poliacrilamida

Ev denc adicional pa a la expresión de epítopo pl modi l t b l up fi i d l v u 17D/8 fu bt n d p inmunop e pit ión d las p oteín v l metabóli ma c d La célul Ve fu on inf t d n un multipli d d d 1 PFU/célula D spué d 24 h d in ub ión l élul fu n ma d n [S] m ti n n du nt un hor y li das b j cond c ne n d natu al da como d sc ibió p viam nt (p st et al 1990) Los extractos de células f o nmun p e p t d p l fluid s ético nmun p li l n l d tón l YF (ATCC) d nti u rp mon cl n l nt l p t ín vi l NS1 (S hle inge t al 1983) y el ant uerpo monoclonal específ co d l *P f lc p rum* (2A10) Lo inmunop cipit do fu on f c n d c n ga a d p teín A y anali ado p SDS PAGE (L emmli 1970) P a la det cción fluorog áfica los geles fueron tratados con salicilato d sod o y ut diografiados (Chambe lain 1979) Los

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ult d mue t an en la figu a 11 Lo p fil d nmun p ip t ión n bt n do a p t d ext a to de p t ína d célul Ve nfe t d d mo k (p ta 1 2 3) 17D/14 (p ta 4 5 6) 17D/8 (pist s 7 8 9) d mon cap nfe t d del vi us Estos ext actos d f nt fu n inmun p pit d s on un sue h p ınmune d mu n cont a l vi u d la fieb ama ll p t d ATCC (p t 1 4 7) l nti u p m n l n l p if p t do (NANP) o 2A10 (p ta 2 5 8) y n d nt u rp mon l n les d igido ont l NS1 (p t 3 6 9) L m r d r s d p mol cul r n mo t d n l l d qu d d l figu L p ón d l g l de lguna de las protein s d vi u am llos s n nd cad en l l do d r ch

Tant v us pa ntal mo ec mbinant s p duJ on los m smos pe f les de p tein en donde l s p t ín fu n p ipit das p r l suer hip rinmune de tón y d s nti u rpos mono l nal a l prot ína YF 17D NS1 (figu 11 p t 4 6 7 y 9) Sin emba g el Mab 2 10 p p t xclu vam nte l p ot ína E del vi u 17D/8 mb n nt (pi t 5 y 8) con i tente con l nt p t c ón d la xp e ón correcta y exposición

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b l sup rf ci d l epítopos como pr dij p r l
náli t u tu l (figu 11 13)

Ej mpl 9

Ensay d n ut al a ón v al po ser esp c f co

Un t njunt d exp m nt p mo t l
e p ón d up f e d p teín E c t d l
(NANP) pít p fu xamin l n ut l ión v l p
á pec fic N tr u amo n n y d
n ut l c ón d r du ón d pl n élul v
emb da un d n id d d 65 000/cm br m c opl
d 96 po s como s des rib ó en lguna parte (Stefan et
al 1999) El antis ra neutr li nte incluyo un su ro
inmun d YF d l mico esu e tánd r en asa (L 13) y l
m b 2A10 L s título de n ut lı ción de placa fue n
l ul d n l dilu ión má lt del nticu po
du nd 50% d l s pla d l v us d ent ad
e t m d po l pl tin d d una m cla de v us d luido
lm nt n u o d c vo f t l

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C mo ob v en l tabl 2 l u nti YF L13 n ut l ó amb vi u n l misma fi ien ia (p m dam nt 1 3 500) El anticu rp mon cl n l 2A10 n mb go l n ut l z l vi u opil nt 17D/8 n un titul xt em dament alto (1 181 000) nd cando l sp if dad d l neut ali ión

T bl 2 n ut l ón del v ru YF 17D por erá nmun

Suero	YF 17D	YF 17D/8
L 13	1 3 715	1 3 388
2 A 10	<1 79	1 181 970

En l d l xpr ión d l epítop pl m di l (NANP) l anticuerp monoclonal conoce la se uenc a l ne l en s misma como se muest a po la p fidad d l n ut li ión E t ug que l pit po st bi n pu t n l l fg y u e ono imi nt n t nd d p u env lv mi nto n t t u tu d p t p vi l Tamb én es la p im a demo t ón d qu

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un pit p lin l d p t in E pu d n ut l do p un Fl v viru

L fu ón qu ambi nf rma n l qu f ct n v epítop s d n ut li ción p inc palm nt dentr del d m n c nt l I y el domıni II Est cambı stán apa ent mente as ciados con una g n ón d l s nte cc nes de ubunidad en la up rf d v n n t ime s n nta to iend f v r d n l f rma pH b ja en cont st c n nt t d dím n l f rma n tiv L int f nci t gl t u tu l p l l gad d nt u p p nt un m n smo qu pu d ll v l n ut al a ón d l v u (M nath y H inz 1996) La nser ón d l pit p pl mod l en el l d l domini II t mb én ll v l n ut li ión vi l pe íf um ni t nd v d nc d c n l d l imp rtan i d st á l nfe t vid d vi l

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Ej mplo 10

At nu c ón d l v u omb n nte YF 17D/8

P a pode demostra que el virus ecombinante 17D/8 n x de su p ri nte vi us YF 17D n n inv bid d y n uropir len ia fueron llev dos b n y b t n

En nu t náli l g up d 16 t n tip d tr man d n d fu r n in ul do p r l ut n 3 0 log PFU d viru d v una 17DD un DNA d vi u d v d d 17D LS3 y virus 17D/8 L tabla 3 p e nt el p omed de do expe ımento p d E vid nt qu l vi us 17D/8 n t ntem nt m tó m n nimal qu l t d v u 17D 26 d 32 p 17D/8 n p si ón 31 de 32 p l v cun 17DD y l vı us LS3 El tiempo d up viv n i p m di p l n mal in ulad c n 17D/8 fu n también c nsiderablement más l g mp d n l v l obt nido pa lo t viru 17D (12 5 v us 10 1 10 6 spectivament)

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Tabla 3 N u viluren ia en l tón de los vi u YF17D

V ru	IC		
	Do (PFU)	% mortalidad (muerte/evalua do)	Promedio de tiempo de upervivencia (día)
C nt l		0 (0/32)	
17DD	10^3	96 9 (31/32)	10 6
17D LS3	10^3	96 9 (31/32)	10 1
17D/8	10^3	81 3 (26/32)	12 5

R t n ui d t man d n id

En l s v rus YF 17D fue n tamb én examin d n u p d d d nv ón d l tema ne v o nt l d pué d l n ul ión p if r l (nt p it n l ip) n t n ui o d 2 5 7 y 9 dí d n cid Como mu t en l tabla 4 abaj el vi us 17D/8 nu vam nt s mp t f v abl ment en comp ación con l ot s v s 17 usad en que ra meno neur nva v en los t n de 2 y 5 dí

L ns ón d pit p en ste tio pu de afe ta el umb l de l fus ón de la actividad conformacional en

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l camb d st p t ín y c nc bibl qu un r t
m n d fus ón pued et da la extens ón de la
p odu c ón del v us y por lo tanto llevar a un
nf cc ón más su ve n l huésped resultando en un
f n t p lg más tenu d del ví u r combinante n el
model d atón y un meno xtensión de replicac ón en
élula d ultıvo

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Tabl 4 N u o nv ivid d en tón d l v u YF 17D

V ru	IP								
	D	Día 2 % m t lidad (n mu rt /n p bado)	Prom d d t empo d sup rviven cia	Día 5 % mo t lidad (n mue te/n p b d)	P om d d t empo d up rviv n ci	Dí 7 % mortalid d (n mue t /n p b do)	P medi d t emp d up rviv n ci	Día 9 % mo tal d d (n muert /n p ob do)	P omedio d t empo d sup rviven c
cont l		0 (0/12)		0 (0/16)		0 (0/7)		0 (0/7)	
17 DD	10^3	75 (9/12)	11 1	87 5 (7/8)	11 7	0 (0/16)		0 (0/14)	
17D LS3	10^3	78 5 (11/14)	11 5	40 (4/10)	15 5	21 4 (3/14)	17 3	5 5(1/18)	18
17D/8	10^3	12 5 (1/8)	10 0	0 (0/19		0 (0/17)		0 (0/16)	

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Ejempl 11

P oducc ón vi al de YF 17D/8 en células cultıvadas

En élul v o el vi u YF 17D/8 produjo pl ca pequeñas (1 1± 0 3 mm) cuando s compa o con el virus YF5 2/DD (o YF 17D/14 4 20±0 9 mm) y l pla p queñ del v us de v do 17D/G1/5 2 (1 89±1 05 mm) La figu 12 mu t t d t

L s urv d r mi nto v l fu n d t rmin d p mono apas inf tad s d célul v ro o cultivo p ma s d f b obl st de embrion s de pollo (CEF) at m d 1 y 2 o t m i de 0 1 0 002 y 0 002 espect vamente Las células fueron plateadas a una d n ıdad de 62 500 células /cm e infe tad 24 ho d pué L s muest s del medio fu ron recolectadas a nt v l d 24 ho s lueg d l inf ción L p duc i n viral s fue on e timad p r titulación d pl s bre élul ve

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Con ba en la tap ún a d cu va de e miento en ve y célula CEF la d f n ia fu on tamb én b v d s nt l 17D/8 y l vi us de cont ol 17D/14

En l ult v CEF que s n tifi d pa p du ón d v un hum n amb vi u p duj n un titul 48 h d spué d l inf c ón (f gur 13A) S n emb rg l pr du n máxım s d l 17D/8 lc n r n 5 10 m nt qu l vı us pa ntal 17D/14 n l n ert l n ó 10 PFU/ml Est dif en d e d 20 v c f m nt nid ha t 96 h r d p és d l inf ón En l Célul v t do l vi u pli on m jor que n l célul s CEF p ro nu vam nt l vır d iv do 17D/14 r ó h t 25 v d tít l s may s qu l 17D/8 (f gu 13B)

Ejemplo 12

P odu ón d v us d va un YF 17D en élul tif d

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De a ue do con l presente la eficien ia de f b ón y l titul vı al p r dos s humana se n ce t un título de vı us in cial de po lo men s 6 0 l g PFU/ml S bt n d un título de 6 1 l g PFU/ml p 17 D/8 n élul V P l f bri ción d l v un l d ón d stabilı ado es en l ma a d vi us du l titul d n 0 3 l g PFU/ml n un p c d ll n d y d p ng l ón du t 0 6 l g Pfu/ml un 0 3 l g PFU/ml e pie de n l t rmo tabil d d l qu un regl ll v ndo n p p ión d vi u l st par su us omo v una n un t tul d f n l d 3 9 l g PFU/ml E ta s l d sis mín m c m nd d p la O g ni ón Mund al de l S lud p v cun YF en humano (WHO 1995 Requi em nt fo Yell w Feve Va cin)

Ej mpl 13

E t b l dad genética del v rus recombinante YF 17D/8

P l tant l s guientes xpe imentos fueron llevados a cabo para poder caracterizar la estabil dad

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g néti d l nu v vi u (YF 17D/8) u ndo ult v d élul p p p g l v u

C n p ct l tabilid d d n e to

L int g d d d l n e t d be e l g d uen ndo l produ to RT/PCR h ch b RNA d l v us b n d nt d l ult v El p duct PCR es s uen ad d e tam nte pa egu tod l ami oá ido n lugar

S mu t p l vi u YF 17DD/204 qu l g n l t s d s m ll ecunda s tam das d m ns nad pa a gener r producción de A unas por l m nos t es p ɔ on n c p ni a d sd el plásm do cl nad d cDNA (US 6 171 854) Aunque los l gonucl ót d s que cod fic n l s epít pos fu n d ñ d n don má f u nt ment ut l ado en l g n ma vi l p vit l p blemas d t adu ión p t n l l mi mo qu l n tabil d d d l u n in t d imp t nt xamin r el mant nimi nto d l n ción n l g n m d vi u YF 17D

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P t p pósit 5 se es d p saj s s par dos d v ru YF 17D/8 en V y CEF fu on ll v d a bo n l MOI con d (0 1 1 0 PFU/células) Lu g n p je l n do (quint y dé m) l RNA vi l fu le t d y m t d d t rm n ón d u nc d nu l ót d (mo pr du t RT PCR) n y lr d d r d l t d n ión en l p t in E

L s sult dos de tos nál is n célula CEF y V s mu t n en l tabl 5 El nse t fu p es ntado t nt n l pas j 5 y 10 sugi iendo u est bilid d u ndo l v u a p d se lm nt p r célul V p o n n cél la CEF

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T bl 5 E tabilidad genétic d l virus YF 17D8 n t do lu go d un pas je rial n células Ve o y CEF

Quinto P aj	Insert	Décimo p aj	Inserto
CEF			
17D/8 (5 a)		17D/8 (10a)	
17D/8 (5 b)		17D/8 (10b)	
V			
17D/8 (5)	+	17D/8 (10)	
17D/8 (5 d)	+	17D/8 (10 d)	+
17D/8 (5 e)	+	17D/8 (10)	+
17D/8 (5 f)	+	17D/8 (10 f)	+
17D/8 (5 g)		17D/8 (10 g)	+

L l t y b p es ntan do pasaje ind p ndi nt n l célul CEF mientras c d f y g l in o i nd pendi nt d p s j s n célula - VERO

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Anál i d Tamaño de plac

C n l obj t d e tabl ce un ma c do in vit p el v u ombin nt hemos e tudi do l tamaño de l pl d vi u f rmada b l s mon cap s de élula VERO Un d l l naj s d los pas jes se ial s n CEF ( ) élul V o(b) p l ual s t bàn d sp nible l d t s de e u ncia en la r giones de n e to también fue on nalizad s en cuanto al fenotipo d l tamaño de pl ca junto con el pr ces de pas do La f gu 14 mu t l r ult d de t l náli Se mu t qu l tam ñ d pl c d l YF17 D/8 e muy p qu ño mp do n nue t a gran placa del v rus YF 17DD/204 y do pl c más p qu ñ s de vi us 17D G1/5 2 y 17D/E200 Tod s los cont ol s on d rivados d v ru p rtir de cDNA clonado y han ido dif ren i d cu ncı d nucleót dos qu on r l on d con l tamañ d placa en célula Vero

En adi ión al tamañ de pl most do pa amb v u t muy homogén o y s esp a qu l vi u d v d d l DNA lon do

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Uno d c da lin ami nto d los pasajes e ale del vı u YF17D/8 en célula CEF (a) y Ve o (b) fue on tamb én nal ad s en uant a su f notipos de tamañ d pl ca junto con el p ceso d p saj (5 y 10 p j mu t 5p y 10p r pe tivament) Par l análi s dę tamaño d placa omparativo nt vi us l nt l d pl c g ande del virus 17D/14 l c nt l d pl a p queña 17D/G 1 2 y un virus 17D/8 pre nt el segundo pas j n células Ve o y el vırus or ginal recupe do t avé de la tran fección RNA fu ron usados L s dıám tro m 4d os d placa (mm)con las desviacione estánd r co espondientes fueron obtenida en p r lo m nos a part de 10 plac s dıfe nte de cada vi us

En amb élul el tamañ d pl d lo vi u p do s r alment tendie n inc ementar Como n el p aj d célula Vero no habí p rdida d in erto con b s n los d t de ecu nc a y l f notipo d tamañ de pl c n l v u YF 17D d pendiente d un o má ambi d am n á d (A V Jab r y R G ll n public d s) s p ibl que t os amb s d secuencia tuviera luga po fue del área de inserción secuenciada Estamos

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t lm nt n li ndo t po ibilid d por medio d
u n i l g noma mpl to del virus YF 17D/8 p ro u
ecuenc a spe da se mue tra compa ativamente con ot os
vi us en la tabla 6

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T bl 6 c mp ión d l u n d l n d plá m do f YF

NT/g n	YF v5 2	17D/DD[b]	F v5 2/DD[c]	17D/8	17D/1	17D/13	NT→AA
1140/E	T	C	C	C	T	T	T→V 1→C→A1
1436 1437/E	G A	A G	A G	A G	G A	G A	G A→D→A G→S
1946/E	T	C	C	C	C	C	T→S→C→P
2219 2220/E	A C	G T	G T	G T	G T	G T	A C→T→G T→V
2356/E	T	T	C	C	T	T	
2602/NS1	T	T	C	C	T	T	
2677/NS1	C	C	T	T	C	C	
2681/NS1	G	G	A	A	G	G	G→A→A→T
8656/NS5	A	A	C	C	A	A	
8808/NS5	A	G	G	G	G	G	A→N→G→S
9605/NS5	G	A	A	A	A	A	A→D→G→B
10454	G	A	A	A	A	A	
10722	G	G	A	A	G	G	

R t l (1989) 9[b] Du t d s S nt t l 1995 GT 11 y F pat nt

st doun d ns 6 171 854

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Ejempl 14

Inmun genic dad en rat ne

L r sultad s des it s nte iorment ugirie n qu l pít p p ent d n l nform ón c r ct y a s bl l nticu rp s a n l p t ína n t v E olub li d lo mi mo qu l up rf ci d l v u En te ejemplo e miró adicionalment a la nmug n cid d del virus recombinante 17D/8 examinando su p d d p t nt u rp E t hi p medi d nmut ton BALB/C s pa adament con hast 3 dos s (10 PFU/d is) po vía intrape itoneal (IP) dadas n d m n d sp i Do emanas d spué l última d d s ng fu l t d p s ng ad int b t l El u o d atón fu inmer o y u do p r n ut l l vi u homól g n l n y d n ut l ón p du ón de pl d punto fin l l 50% La tabla 7 mu st a sto esultados obtenıdos a p rt r dè d xperimentos independient s La noculac ón int aperitoneal como la 3 cortó un promedio de títul d anticu rp del virus YF de 1 1 079 según s m dió po PRNT

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Tabl 7 Inm n g n cid d d l viru YF 17D/8 n at n

Inmun ació con v u	Intra Pe itoneal	
	PRNT	P omedio PRNT
Cont l	1 13 < 10 −	<1 13
17D/E200	1 166 1 195	1 181
17D/8	1 741 1 417	1 1079

El íp c d la d lución má lt del u qu ult n l 50% d du ión n l núm d pl s

Est d d luc nes o esp nden 2 conjunt de p m nto ind p nd nt

Ej mpl 15

C n t uc ón d otros virus recombınante YF17D qu p n un pit p d élul cit tóx T

L v us e mbin nte n onst uid mo d bió n l ɔ mpl 6 p p d r expre r un pit p d élul c t tóx T L cup r ón d l

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v u d l DNA p t n f ción d l s élul s V r fu llev d c b m l jempl 6 L v u e ult nt YF 17D/1 y 17D/13 fu n p d di n lm nt d v n élul s d V p la gen ra ón de pa tid d t b ɔ L in ón de lig nucl ótido ntético n l iti E RV d l plá m d YFE200 que c r p nde l cu n d min ác do most d n l t bl 8 más b ɔ d o lug a l s plásm do pYFE200/1 y pYFE200/13 E tos plá mid fu n dep t d l 21 d di mb e d l 2000 baɔ l núm d a PTA 2858 y PTA 2854 p tivam nt n l Am n Typ C ltu C ll t n (ATCC) 10801 Univ rsity Blvd Man a V

L t bl 8 mu t l g p dich y l s punt lé t p l pit p l int g do n l l z fg y en l ont xto d l protein E compl t C mo pued ve e h y una v riación consider ble en la c rga net y l pI en cad contexto el epítope solo en el la y en los ont to de l pr teína E completa Y qu la g ón d n e ón st inv luc da n l t nsi ón nf rma on l d p nd nt de pH po fu ón de l nv ltu n l m mb n ndo ma p sible qu l

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pr p dad s de ste virus an influenciadas diferentes
g d p l s cuenc a en el epítope

L d t p el mina es ug n qu el viru 17D/13
qu ll v l pít pe y *lii* CTL n mu st t i i n s
d m nt b v da pa l t vi u (17D/8
y17D/1) t nt n élul CEF y V (figu 13) L
t bl 8 mu t l s u n d amin á id y l
sp cıfi d d de l s epítop lec n do p a la
n ón en l p teín YF E

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Tabl 8 S u n d aminoá idos y pe fid d de pit p l nad p l in ión d nt d l p t ín YF E

	La o YF fg l	EMD DYENDIEKK I IES	EMD GGNANPNAP NANPGG IES	EMD SYVAPSAEQ I
Epit p d ntig n		CSP CTL	CSP B	CSP CTL
Fu nt		P f l ip ru m	P fal ip u m	P yo lii
Cl n		17D/1	17D/8	17D/13
C g /pépt do pI		2 00/4 06	0 00/5 97	1 01/3 30
La o fg+ g de pit p /pI	2 00/3 96	4 99/3 83	4 00/3 49	3 00/3 58
P oteína YF E+ g d pit p /pI	7 45/5 83	9 40/5 61	7 40/5 83	8 40/5 71

B élul B

CTL pit p d élul c t tóxica T

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Ej mpl 16

P du n v l d YF 17D/1 y 17D/13 n élul ult v d

L t í ti s d mi nt n élul V d YF 17D/1 y 17D/13 mp t vament t s v u d t l 17D in luy l omb n nte t 17D/8 qu xp el epit pe humo al s n nal ad s E t jemplo s llev do de cuerdo al protocolo descrit en el ej mplo 11

L f gu a 15 mu st a l s u v s de e mient mp tiv del 17D/14 17D/E200 y l virus ec mbin nte d mal 17D/8 17D/1 y 17D/13 en célul a una moi d 1 pfu/ml E v dent que l s v ru 17D/1 y 17D/8 c cen a títulos más lent s y má lentamente que nuestro vi u de ntr l 17D/14 P t o lado l s v us 17D/E200 y 17D/13 c e ie n tan ef cient mente c mo nu t v ru d nt l ug iend qu l in rc ón d l pit p SYVPSAEQI n t n imp t nt n te p t n l t a d s l fu r n El mi mo t p d p fil d r m nt n élul V fue bse v d un dife nte

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mo (0 02) y n élul CEF n amb moi (l s d t n
h n mo t ado)

N h y dif nc s gnif cativ n l g
g n l y l punt i lé t n la p ot ín mpl t E
qu nti n t pít p p l dif r n i pu d n
b v d n el n v l d l l o fg (ver tabl 8) qu
nt n n l pít po nt i e l h pót si d
qu l g de l s pít p pu d nflu n i l fu ión
d p ndi nt pH y p l tant llev modifi la
p p d d s d e mi nto no pu de e reglamentada en
t t p

Ej mpl 17

E t b l d d g nét del v us e omb nant YF
17D/1 y YF 17D/13

C n r pe t l t bilid d d l in t

Como d cr bió n l j mplo 3 ant rior p l
YF 17D/8 e xaminó l e tab lıd d d la nser ión en l
g m del v us YF17D p r l nál i d u nc d
nu l ót do n el sıtio de nse c ón sobre l RNA d lo

v u s m tid p ɔ i l P t p pó t
d vi u 17D/13 n V fu n ll v d s b n mo
n d (01 1 0 PFU/célul) Ent es en p ɔ
l i n d (5 y 10) l RNA vi l fue e l t do y
m t d l d t rm n c ón de e uen ia de nu l ótid
(om p du t RT PCR) en y l ded del sit o d
ns ón n l p t ín E

Los e ult do d e tos anál sis n CEF y célul s
V r n mo t ad s n l tabl 9 El n t t b
p es nt t nt n l s pa ɔ 5 y 10 ugi i nd u
tabil d d u ndo l vi u fue p do ialment n
célul s V ro p o no n célul s CEF S milarment al YF
17D/8 l pit po inse t do son e tabl s t vés de
t d l p ɔ i l d amb v ru n élul V
E t ult do t dos junt sug n l tol ab lid d
p ra la ns r ón d un ampl varied d d epitop s y
on ll va st s stema con un potenc al enorme p ra l
e ón de v us e mb nant que exp e n antíg no de
t p tóg n n g n l

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T bl 9 E t bil d d g nét c de l in ertos de v u mb n ntes YF 17D sob e pas je se ial en célul s V

Qu nt p j	Ins to	Décimo p s j	In t
17D/1 (5 h)		17D/1 (10 h)	ND
17D/13 (5)	+	17D/13 (10 i)	+

Nd n d t rmin d

Anális d tam ñ de pla

Com se mu str en el ejempl 13 nte i el vi u e mb nante 17D/8 mostró un tamaño de placa pequeño en f n tip c mp d on nu tr pl a grand YF 17DD/204 v u (17D/14) y l dos pl c p qu ñ s ınf cios d l v u d v d d cDNA 17D G1/5 2 y 17D/E200 Lo f n tıp d tam ño d pl a p l nu vo vi us mbin nt 17D/1 y 17D/13 fu on omp r do lo vi u p v am nte t ri do (f gu 15) t do l vi u p ent n l s gund p aje en células Ve del vi u g n l e up do de las tran fecciones RNA

C m p rab d su perf l d c imi nto n élulas V r (f gu a 15) l vi us 17D/13 most ó un tam ñ

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d pl mil l 17D/E200 y 17D/G1 5 2 t d ví p qu ñ mp n l 17D/204 (17D/14) p má g nd qu l pl muy pequ ñ t indu d p l v u 17D/1 y 17D/8 (f gu 16)

Ej mpl 18

Atenu c ón del virus r combin nte YF 17D/1 y YF 17D/13

Aunqu l n u vi ul nc d l tón n pr d l v ul n t nu ón p de l s v u YF p human fu mpo t nt demo t a qu l s viru mb n nt 17D/ 1 y 17 D/13 no exceden a su pa ı nte l v ru d YF 17D n l n u virul nc en atón La v un d l YF 17D mu t un grad de n urotrop smo par l t n po med o d mat t das las ed d s d t n d pué d l no ula ón ntr er b l y u u u lm nt n f l t ub lín n l m (M n th 1999)

En l p nt nál i grup de 16 t n u de 3 emana de nac dos fue n in cul dos p l

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vi n 3 0 1 g PFU del v u d vacuna 17DD 17D1 17D/8 17D/13 y 17D/E200 L s esult dos mostrados en l t bl 19 n ep entat v de dos expe m nto p d

T bl 10 Neu vi ul nci n tón d el vi us YF 17D

V rus	D s s (PFU)	% mortalıdad (mue te/ens y do)	Tiempo promedıo de sup rv venc (dıas)
C ntrol		0 (1/32)	
17DD	10^3	96 9 (31/32)	9 6
17D/1	10^3	81 3 (26/32)	15 6
17D/8	10^3	71 9 (23/32)	11 7
17D/13	10^3	53 1 (17/32)	15 3
17D/E200	10^3	93 8 (30/32)	11 0

R t n u d t s man d n idos

E vid nt qu l s vi u 17D/8 y 17D/13 ns tentem nt mat n menos an mal s que los otros vir 17D 96 9% p r 17DD y 81 3% p ra 17D/1 y 93 8% p ra 17D/E200 mo ob v pr viam nt el ti mp de upe v v n p m di p nımales ınocul d c n l

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vi us 17D/8 fu también nsiderablemente ma l rg n omp ón n l v lo e bt nid p l s vi u 17DD y 17D/E200 (11 7 ont 9 6 11 0 sp t vament) L v us 17D/1 y 17D/13 matar n ratones un v lo d d mas l nt con AST de 15 4 y 15 1 respect vam nt pe l 17D/1 m tó v tu lm nt tod lo t ne mi ntra q l 17D/13 fue mas atenuad y s lo mató l 75% m l rm nt l 17D/8

La n ón d l epít p n st ti pu de f t l umb l d l camb onforma ion l ct v d p fu ón d l p t ín E y e c n eb bl qu un vel c dad m lenta d fus ón pu de etarda la xt ns ón de la p du ción d v u y po lo tanto ll va a un infec ión ma u v d l hué p d ultando n l fen tip lg m t nu do del v u mbin nt n l mod l d tón y d m n s xt nsión d plic ción n élula d cult vo L ultad mo t do quí indi n qu l ns t d d f nt tam ñ y g n toler d y llo mil m nt tán d pu t influ n i l p pi d d v l nt i En n ngún n mbargo l vi u ombinante s volv ó más ne ov r lento para los raton s que su c nt aparte de

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va un nfi m nd di i n lm nt l p tenci l d us t t p l ns r ión de los epítopo ext ñ s y l des llo de nuev s vacunas vivas atenuadas 17D

Ej mpl 19

At nu ón del vi us combinante YF 17D/13 n mı s

L n ur v rul n i del ratón n habí id c nsid ad mo un ma do confiabl p a l at nuación d l v ru YF p humano ha t qu c entem nt u nd M n th t l (2002) e tudi l tenu ión d l v u d n f l t ɔ p ne quımé YF 17D qu habí p pu t S n emb rg l defini ión fin l p l t nu ión d l s vi us YF deb v nir d lo n y d n u vi ulen d m pt d nt n ci nalment m nd d p l O g n z ión Mundi l d l Salud p l a ct a ión d la nu va s milla YF y l v ru v un (WHO 1998) Aquí el examen patológı o u nt tat vo d l r bro y del t ɔid d médula espin l um n t un mét d sens tivo pa distingui s pa d l m mo v u c n d f en lgunas en n u vi ulenci

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(L v nb k t l 1987 M h vsky t al 1995 WHO 1998)

Hem amin d l t nu ión d l vı u YF 17D/13 l m t nu d n nue tr d mod l d tón en l en ayo d n u ov ulenci n l m c c nt (WHO 1998) pa a estable e su s gur dad p eclín ca Aquí 20 mı s u bt n d p rtir d l colon d l fund ón O w ld C u que p ab n ent 2 7 y 6 4 kg ndo 14 h mbr y 6 m ho fue n d st bu d gu lm nt n 2 g upo de 10 Un g upo d 10 mic e b ó un n ul c ón ún c d l YF 17DD d sem ll s cund d l lot d vir (B oM nguinhos Fi ru) n l lóbul f nt l d l b y un 2 g up b ó l v us p mental YF 17D/13 Cada 0 25 ml del ın cul do qu nt ní 6 53 l g PFU/ml d l vi u 17DD 6 62 log PFU/ml de 17D/13 Lo mic fu on bs rv d du nt 30 dí R g tros de l ob rv i n clíni fu n bten d u nd l gui nt ign s g do 1= ubi t v t n m g d 2= vo d t n lt n t vo mov m nt l nt g d 3= mov mi nt t mbl os tembl movim nt s des ordin do d b l dad d l híg do grado 4= inhab lidad pa pararse

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p ál t mb l m rt Un mi o qu mu r r b l
m 4 d sd l dí d mu t h t l dí 30

Los n veles de v rem a fue on medido en los dí
2 4 y 6 lueg de la nocula ión po las pl cas n muest
d élul Ve d l sue o de m co La suero onv rsión
fu m d d p l p enc de los anticuerp
n t l nt n l día 31 En ste dí l animales
fu n ut ni d y un n p i mpl t fu ll v d
b S xamin n l b y l u d pin l
y ma mo nd có (WHO 1998) Cin n v l d
b y 6 n v l s d c d un d los gr ndami nt
lumb y v c l fu n xamin do L niv l d
b ncluí n Bl que I l st atum c rpus en l n v l
d l qu sm ópt c bl que II l t lamus en l n vel d
l cu rpos mamila ios bloque III en mesencefalon en el
n vel d l c lic lo upe r bl que IV l ponse y el
b l en l n v l d l ol v s upe re el bloque
5 l médul bl ng d n l niv l m d o d l oliv s
nf (WHO 1998) L ma ci n numé i s fu on
d d d hemi ión d l cu d y d l
tr ctu s n c da hem ección del cerebro Las
l ıone fue on mar adas de acuerdo con el sıguıente

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t ma de g du ión 1 (mínimo) 1 3 p queño
nf lt ad s nflamato o fo l un p neu n
pu d n h b amb d p dido 2 (mode do)
nf lt dos inflamat s focales más extens vos camb s
n u n l p did qu fe ta n mas de una ter e
p t d l n on s 3 (v o) ambio ne ron l
p d d d l 33 l 90% d la n u nas n un
nf lt ón nflamato ia d fu a o foc l moder d IV
(g ve) má d l 90% d l n u on fu n ambi d
p d d n un nfilt ión inflam t ri va i bl p
f nt ment v r

T d l v lu n neurop tológica clínicas
h t lóg fu n h h p un inv st gado únic y
xp ım ntad qu n t b g l ódig d
tratam nto

T m ca n s p d fu n calcul das pa a
d mico Solam nt ár di c im n tori olam nt
á a objetiv y á as disc im natoria má objet v
(L v nb k t l 1987) El á ea bjetivo es la
u t nc neg a donde todos los vi us 17D epl c n
m nt qu l á s d m n to ia in luy n l

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nu l u ud t l p lídu gl b l putam n l nu l u t lámi m di l y nt i l nucleu t lámi l t l l s g ndam ent v cal s y lumba y lam nt l v u n u v lu ntos induc perdid n u n l gn f t va Un ma c d neu v ulen fin l t d d p r l ombin ión d l mar n amb á (ma c mb n d)

V m

L t bl 11 mu t l d t s b vi mi gi t d p l m o in ulado n cad vi u En l nf c ón xpe ment l d l mi s us p r l ut nt e bral en los v us 17DD yl 7D/13 la v em de su ro de mıco difiere ent e los viru en solamente cinc an mal s fu ron vi émicos en el día dado (2 4 6) de pués d la no ulac ón con este último mientra que la vi emi indu id ant o n 8 de 10 n mal L v emi fu má p v l nt n mb g upo en l dí cu to despué d l inf ón u d 5 de 10 mi mo t r n vi u cul to io m dibl L micos que ecibi ron l vi us 17D/13 también p ent ron m no viremia n lo días (5) c mp do con el 17DD (9) En adición al pico má alt d v r m p r l vi 17D/13 fu de 1 44 l g PFU/ml

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m nt qu p 17DD fue c de 10 v c may (2 42 l g PFU/ml) S n emb go ambo vi u e tán bi n p d b ɔ d l s lím te tablec d p WHO(1998)

L def ni ión pue t d lante p r l WHO (1998) d v s t p m del vi us 17D lim ta l nt d d de v u ulat ı po d b ɔ d 500 t n LD /0 03 ml p t d (10 d 10) n u y may igual a 100 LD /0 03 ml n 1 d 10 u d m co n 1 10 de dilu ión A es p t l v emi má lt ob rv da fue d 2 42 l g PFU/ml p l mi 810 in culado n l virus 17DD E t p nd 1 64 LD /0 03ml p l tanto bi n p deb ɔ d l límıt s t ble do Adic nalment el ng de títulos ob rv dos aquí e s mıla aqu l bs v d p l m o e u n ul d n l 17 D/JE SA 14 14 2 17D d n2 quimé i y l 17D 204 (Mon th t l 2000 2002 Gu akh et al 2000)

Marcación clín ca

La tabla 11 mu str l ma on clínic nd vıdu l s de pué d 30 dí d p d d

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b v ón E t gi t s l p omedio d lo v l
d d s n c d dí du ante e te pe do S mu tr n l
t bl 11 qu olament 2 mico (6U y 46) inoculad s on
el vı us 17D/13 most on cu lesquie sign s clín cos n
c mpa ac ón n 5 mi s n ul d n l v us 17DD
(114 240 303 810 y 031) El hech d qu va os
n mal most a n vi em a y tod s fue on ue
vert dos sp cíf camente a YF n l s nsay s d
n utr l c ón d edu ción d pl a (t bl 11) confirma
qu l n males fu n en fe t nf tado p l
vi u p tiv in ul do De l s m c in ul d
n vi u 17DD l mi s 114 810 y 240 t ní n l
v em a má lt s pero t davi un s marca ne mín mas
(0 07 0 014 y 0 64 p t vament) Pa l 17D/13 el
mi 253 n mo t ó n ngún ign líni o y t d ví t ní
l vi emi má lt n l g upo (tabl 11)

L g t o lín os st d d en la tabl 12
junt n l gi t h tológi El gi t
lín p medio p l vi u d v un YF 17DD fu d
0 11 m ent as que pa a el 17D/13 fue d 0 17 Un
eg st lg más lt pa este último s debido un mic
único que p e ento algunos s gnos de encefalit s (el mıco

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6U) E t mi p rti l r n mo tró v rem y m tró un u nv ión l YF y n p nt lt n h t lóg n l stema n v so cent al comp t ble c n l u d clínic (t bla 12) Últimamente los g t s p amb v u n mp abl s n u nto l egi t lín o ob rv d p r ot o grupo p t v u 17D (M ch vi ky t l 1995 M n th t l 2000 2002) sug nd l at nu ón d l v rus YF 17D/13

Reg tro h tológico

T d s l s 20 m os su in ulad s i c n los v us 17DD y 17D/13 de oll on les ones hist lóg s (t bl 12) n l i t m n v nt l (CNS) P r t d l an male n hub norm lidades n cu lqui d l s ó g n t n u l qu pud e ug i d ñ fun ión d b l n ead N nguno de los an male d ll ningun l ión h stológic n l híg do r ñ n d l n r ón pulmones Como e pr pu p L v nbo k t l (1987) l á bj tiv n l CNS d l m o e u pa div so vi u de vacun es la ustanc a neg a En este e tud o la sustancia neg a p e ntad n l s r gist h tológ cos más alt s p

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l mi in l do i c n ambo vi us C n base en los valo nd v du les mo trados en la tabl 12 el vıru 17DD tenia un regist o p omedio en esta área de 1 75 y t fu 1 40 p l vi u 17D/13 En 5 ns yos d n u vi ul n i c mpl t p a l s vi us d l l te d m ll 17DD 102/84 l r gi tr d l ár bjet v p med o fue de 1 49 (RS M rch vsky y R G lle n p ep ión)

Ent l á di imınat i en l CNS el put men el globos palidus y el nucleus caudatu fu n l á e má af tad s p r l gist s d l ón n d nd n n a po n ima de 2 n ning no d l v ru El mi 6U in ul d n l vi us 17D/13 y qu p ntó l ma ción clini má alt (1 00) ntr lo 20 nimal s mo t ó l t e g tr má b j n l á d min t i (0 33 tabl 12) El á d c min t p m di p l v rus 17DD fu de 0 78 y 0 53 pa el vi u 17D/13 val res que son erc nos uno l t y pa el val p omedio obse v do p 102/84 a t avés d ot os nsay s de neurovirulencia completa (0 67 RS Ma hev ky y R G lle en p pa ción)

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El gr d d n u virul n i d un viru d do l p m di d l á s objet vo/dis imın to i mbin d d t d s los mic s Pa a l vi u 17DD ste g st mb n d fue de 1 21 mientras qu p ra l 17D/13 fue de 0 96 L s val res pa a l s gistros de n vırulencia combinados n 5 ensayos completos con el v u 102/84 v nt 0 96 y 1 37 n un p m d o d 1 07 p a el v us YF 17D 204 l á a obj t v di min t y c mbin d fu n d 1 63 0 71 y 1 17 p tivam nt (Mon th et l 2002)

L b v ón d ign líni s mínimo d inf ón ningun lt r n d óg n xt aneu le g t h stológ CNS de nimale in ulado d nt d l ng e p ad y la vi emia limit da ugi un f n tip t nu d p l YF 17D/13 d vi u mb nant El he ho de que tod s l s nimal s mont ron un re puest de nt uerpo YF antineutral z nte l vi u 17D/13 ug qu también n p mat s n human l v u ombin nt h mant nido u inmun g n c dad pe a de l in e ción d epítope en u ub ta de p teín E

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L ultad most do aquí ind an que l
n t de d f nt s tamañ s y c gas son t le dos y
qu ll p s blem nt tán d pu st nfluen l
p pi d d vi l t l mo un b j fu ón d
v ltu v l d pendi nt d pH En ningún c in
mb g l v c mbin nt n y d v lv n
má n u vil ul nt p t nes qu su nt p t d
vacuna Lo en yo de uno de t le v us combinant s
(17D/13) p l n u v ul n i de m c tamb én ugi ó
qu l n ón n l l fg d l p t ín E d l 17D
no omprom tí f n t po t nu do má llá d
nf rma l u pot n ial de est sitio pa l
n ión d pít p xt ñ y el de oll d nu v s
v n 17D viva t nu d

T bl 11 P ám t o g t d p l n y d u v ul c d mı d l v u YF 17 D

Viru	Mıco	V remıa			Reg tro Clín co	Regı to Hıstológi Combinado	Sero conver ón	
		2d	4to	6t			P	P t
17DD	114	<<0 6	1 83	<0 6	0 07	1 10	<447	95471
	116	0 6	<0 6	<0 6	0	0 96	<447	30124
	159	<0 6	<0 6	1 08	0	0 35	<447	41210
	162	1 20	1 20	<0 6	0	1 51	<447	35872
	249	1 68	<0 6	<0 6	0 64	1 39	<447	141947
	303	<0 6	<0 6	<0 6	0 17	1 44	<174	46773
	810	0 9	2 42	<0 6	0 14	1 38	<174	72028
	934	<0 6	<0 6	<0 6	0	1 32	<100	100000

	4U	<0 6	1 20	<0 6	0	1 45	<100	100000
	031	<0 6	0 9	<0 6	0 10	1 26	100	16999
17D/13	178	<0 6	0 6	<0 6	0	0 79	<447	50646
	253	<0 6	1 44	<0 6	0	0 61	<174	3770
	423	<0 6	0 6	<0 6	0	0 69	<174	32393
	520	<0 6	0 9	<0 6	0	1 09	<174	13375
	540	<0 6	<0 6	<0 6	0	0 73	<174	8511
	558	<0 6	0 9	<0 6	0	1 28	<174	646
	4A	<0 6	<0 6	<0 6	0	1 33	<100	40179
	6U	<0 6	<0 6	<0 6	1 00	0 67	<100	10448
	46	<0 6	<0 6	<0 6	0 67	1 42	<100	7154
	T23	<0 6	<0 6	<0 6	0	1 02	<100	1136

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T bl 12 N u v l n i d lo v u YF 17D p l m u

V ru	Mico	Reg tro Clín co	Area D criminatorias	Area Objet vo	Reg tro Comb nado
	114	0 07	0 7	1 5	1 10
	116	0	0 42	1 5	0 96
	159	0	0 21	0 5	0 35
	162	0	1 03	2	1 51
17DD	240	0 64	0 78	2	1 39
	303	0 17	0 88	2	1 44
	810	0 14	0 76	2	1 38
	934	0	0 64	2	1 32
	4U	0	0 91	2	1 45
	031	0 10	0 52	2	1 26
PROMEDIO		0 112	0 78	1 75	1 216
	178	0	0 59	1	0 79
	253	0	0 23	1	0 61
	423	0	0 38	1	0 69
	520	0	0 69	1 5	1 09

	540	0	0 47	1	0 73
17D/13	558	0	0 57	2	1 28
	4A	0	0 67	2	1 33
	6U	1 00	0 33	1	0 67
	46	0 67	0 84	2	1 42
	T23	0	0 54	1 5	1 02
PROMEDIO		0 167	0 53	1 40	0 963

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LISTADO DE SECUENCIAS

I) S l it nt Fund ção O w ld Cru

I b) D ón Av n d B il 4365 C t l M u i l 05 M nguinh 21045 Rí d J n i RJ

II) Inv nc ón us d l Flavivirus pa l p ón d pítop d p teín y des oll d nuevos vı d va na v vo t nu d s p r nmuni c nt a l Fl v v us y tr s g nt nfec ios

III) Núme o de secuenc as 0 1 (uno)

1) INFORMACION PARA LA SEQ ID NO 1

I) Ca te i t s d l secuencı

I) l ng tud 10862 pa b s

I b) t p á d nu l

I) am fi ion s ún

I d) t p l gí lın l

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I) t po d molécul cDNA

I f) fu nt o gin l sl do indiv du l

Yf1v5 2/DD

I g) d c ipc ón d l s uen ia SEQ ID NO 1

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```
 1 AGTAAATCCT GTGTGCTAAT TGAGGTGCAT TGGTCTGCAA ATCGAGTTGC
51 TAGGCAATAA ACACATTTGG ATTAATTTTA ATCGTTCGTT GAGCGATTAG
101 CAGAGAACTG ACCAGAACAT GTCTGGTCGT AAAGCTCAGG GAAAAACCCT
151 GGGCGTCAAT ATGGTACGAC GAGGAGTTCG CTCCTTGTCA AACAAAATAA
201 AACAAAAAAC AAAACAAATT GGAAACAGAC CTGGACCTTC AAGAGGTGTT
251 CAAGGATTTA TCTTTTTCTT TTTGTTCAAC ATTTTGACTG GAAAAAAGAT
301 CACAGCCCAC CTAAAGAGGT TGTGGAAAAT GCTGGACCCA AGACAAGGCT
351 TGGCTGTTCT AAGGAAAGTC AAGAGAGTGG TGGCCAGTTT GATGAGAGGA
401 TTGTCCTCAA GGAAACGCCG TTCCCATGAT GTTCTGACTG TGCAATTCCT
451 AATTTTGGGA ATGCTGTTGA TGACGGGTGG AGTGACCTTG GTGCGGAAAA
501 ACAGATGGTT GCTCCTAAAT GTGACATCTG AGGACCTCGG GAAAACATTC
551 TCTGTGGGCA CAGGCAACTG CACAACAAAC ATTTTGGAAG CCAAGTACTG
601 GTGCCCAGAC TCAATGGAAT ACAACTGTCC CAATCTCAGT CCAAGAGAGG
651 AGCCAGATGA CATTGATTGC TGGTGCTATG GGGTGGAAAA CGTTAGAGTC
701 GCATATGGTA AGTGTGACTC AGCAGGCAGG TCTAGGAGGT CAAGAAGGGC
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751 CATTGACTTG CCTACGCATG AAAACCATGG TTTGAAGACC CGGCAAGAAA

801 AATGGATGAC TGGAAGAATG GGTGAAAGGC AACTCCAAAA GATTGAGAGA

851 TGGTTCGTGA GGAACCCCTT TTTTGCAGTG ACGGCTCTGA CCATTGCCTA

901 CCTTGTGGGA AGCAACATGA CGCAACGAGT CGTGATTGCC CTACTGGTCT

951 TGGCTGTTGG TCCGGCCTAC TCAGCTCACT GCATTGGAAT TACTGACAGG

1001 GATTTCATTG AGGGGGTGCA TGGAGGAACT TGGGTTTCAG CTACCCTGGA

1051 GCAAGACAAG TGTGTCACTG TTATGGCCCC TGACAAGCCT TCATTGGACA

1101 TCTCACTAGA GACAGTAGCC ATTGATAGAC CTGCTGAGGC GAGGAAAGTG

1151 TGTTACAATG CAGTTCTCAC TCATGTGAAG ATTAATGACA AGTGCCCCAG

1201 CACTGGAGAG GCCCACCTAG CTGAAGAGAA CGAAGGGGAC AATGCGTGCA

1251 AGCGCACTTA TTCTGATAGA GGCTGGGGCA ATGGCTGTGG CCTATTTGGG

1301 AAAGGGAGCA TTGTGGCATG CGCCAAATTC ACTTGTGCCA AATCCATGAG

1351 TTTGTTTGAG GTTGATCAGA CCAAAATTCA GTATGTCATC AGAGCACAAT

1401 TGCATGTAGG GGCCAAGCAG GAAAATTGGA ATACCAGCAT TAAGACTCTC

1451 AAGTTTGATG CCCTGTCAGG CTCCCAGGAA GTCGAGTTCA TTGGGTATGG

1501 AAAAGCTACA CTGGAATGCC AGGTGCAAAC TGCGGTGGAC TTTGGTAACA

1551 GTTACATCGC TGAGATGGAA ACAGAGAGCT GGATAGTGGA CAGACAGTGG

1601 GCCCAGGACT TGACCCTGCC ATGGCAGAGT GGAAGTGGCG GGGTGTGGAG

1651 AGAGATGCAT CATCTTGTCG AATTTGAACC TCCGCATGCC GCCACTATCA

1701 GAGTACTGGC CCTGGGAAAC CAGGAAGGCT CCTTGAAAAC AGCTCTTACT

1751 GGCGCAATGA GGGTTACAAA GGACACAAAT GACAACAACC TTTACAAACT

1801 ACATGGTGGA CATGTTTCTT GCAGAGTGAA ATTGTCAGCT TTGACACTCA

1851 AGGGGACATC CTACAAAATA TGCACTGACA AAATGTTTTT TGTCAAGAAC

1901 CCAACTGACA CTGGCCATGG CACTGTTGTG ATGCAGGTGA AAGTGCCAAA

1951 AGGAGCCCCC TGCAGGATTC CAGTGATAGT AGCTGATGAT CTTACAGCGG

2001 CAATCAATAA AGGCATTTTG GTTACAGTTA ACCCCATCGC CTCAACCAAT

2051 GATGATGAAG TGCTGATTGA GGTGAACCCA CCTTTTGGAG ACAGCTACAT

2101 TATCGTTGGG AGAGGAGATT CACGTCTCAC TTACCAGTGG CACAAAGAGG

2151 GAAGCTCAAT AGGAAAGTTG TTCACTCAGA CCATGAAAGG CGTGGAACGC

2201 CTGGCCGTCA TGGGAGACGT CGCCTGGGAT TTCAGCTCCG CTGGAGGGTT

2251 CTTCACTTCG GTTGGGAAAG GAATTCATAC GGTGTTTGGC TCTGCCTTTC

2301 AGGGGCTATT TGGCGGCTTG AACTGGATAA CAAAGGTCAT CATGGGGGCG

2351 GTACTCATAT GGGTTGGCAT CAACACAAGA AACATGACAA TGTCCATGAG

2401 CATGATCTTG GTAGGAGTGA TCATGATGTT TTTGTCTCTA GGAGTTGGGG

2451 CGGATCAAGG ATGCGCCATC AACTTTGGCA AGAGAGAGCT CAAGTGCGGA

2501 GATGGTATCT TCATATTTAG AGACTCTGAT GACTGGCTGA ACAAGTACTC

2551 ATACTATCCA GAAGATCCTG TGAAGCTTGC ATCAATAGTG AAAGCCTCTT

2601 TCGAAGAAGG GAAGTGTGGC CTAAATTCAG TTGACTCCCT TGAGCATGAG

2651 ATGTGGAGAA GCAGGGCAGA TGAGATTAAT ACCATTTTTG AGGAAAACGA

2701 GGTGGACATT TCTGTTGTCG TGCAGGATCC AAAGAATGTT TACCAGAGAG

2751 GAACTCATCC ATTTTCCAGA ATTCGGGATG GTCTGCAGTA TGGTTGGAAG

2801 ACTTGGGGTA AGAACCTTGT GTTCTCCCCA GGGAGGAAGA ATGGAAGCTT

2851 CATCATAGAT GGAAAGTCCA GGAAAGAATG CCCGTTTTCA AACCGGGTCT

2901 GGAATTCTTT CCAGATAGAG GAGTTTGGGA CGGGAGTGTT CACCACACGC

2951 GTGTACATGG ACGCAGTCTT TGAATACACC ATAGACTGCG ATGGATCTAT

3001 CTTGGGTGCA GCGGTGAACG GAAAAAAGAG TGCCCATGGC TCTCCAACAT

3051 TTTGGATGGG AAGTCATGAA GTAAATGGGA CATGGATGAT CCACACCTTG

3101 GAGGCATTAG ATTACAAGGA GTGTGAGTGG CCACTGACAC ATACGATTGG

3151 AACATCAGTT GAAGAGAGTG AAATGTTCAT GCCGAGATCA ATCGGAGGCC

3201 CAGTTAGCTC TCACAATCAT ATCCCTGGAT ACAAGGTTCA GACGAACGGA

3251 CCTTGGATGC AGGTACCACT AGAAGTGAAG AGAGAAGCTT GCCCAGGGAC

3301 TAGCGTGATC ATTGATGGCA ACTGTGATGG ACGGGGAAAA TCAACCAGAT

3351 CCACCACGGA TAGCGGGAAA GTTATTCCTG AATGGTGTTG CCGCTCCTGC

3401 ACAATGCCGC CTGTGAGCTT CCATGGTAGT GATGGGTGTT GGTATCCCAT

3451 GGAAATTAGG CCAAGGAAAA CGCATGAAAG CCATCTGGTG CGCTCCTGGG

3501 TTACAGCTGG AGAAATACAT GCTGTCCCTT TTGGTTTGGT GAGCATGATG

3551 ATAGCAATGG AAGTGGTCCT AAGGAAAAGA CAGGGACCAA AGCAAATGTT

3601 GGTTGGAGGA GTAGTGCTCT TGGGAGCAAT GCTGGTCGGG CAAGTAACTC

3651 TCCTTGATTT GCTGAAACTC ACAGTGGCTG TGGGATTGCA TTTCCATGAG

3701 ATGAACAATG GAGGAGACGC CATGTATATG GCGTTGATTG CTGCCTTTTC

3751 AATCAGACCA GGGCTGCTCA TCGGCTTTGG GCTCAGGACC CTATGGAGCC

243

3801 CTCGGGAACG CCTTGTGCTG ACCCTAGGAG CAGCCATGGT GGAGATTGCC

3851 TTGGGTGGCG TGATGGGCGG CCTGTGGAAG TATCTAAATG CAGTTTCTCT

3901 CTGCATCCTG ACAATAAATG CTGTTGCTTC TAGGAAAGCA TCAAATACCA

3951 TCTTGCCCCT CATGGCTCTG TTGACACCTG TCACTATGGC TGAGGTGAGA

4001 CTTGCCGCAA TGTTCTTTTG TGCCGTGGTT ATCATAGGGG TCCTTCACCA

4051 GAATTTCAAG GACACCTCCA TGCAGAAGAC TATACCTCTG GTGGCCCTCA

4101 CACTCACATC TTACCTGGGC TTGACACAAC CTTTTTTGGG CCTGTGTGCA

4151 TTTCTGGCAA CCCGCATATT TGGGCGAAGG AGTATCCCAG TGAATGAGGC

4201 ACTCGCAGCA GCTGGTCTAG TGGGAGTGCT GGCAGGACTG GCTTTTCAGG

4251 AGATGGAGAA CTTCCTTGGT CCGATTGCAG TTGGAGGACT CCTGATGATG

4301 CTGGTTAGCG TGGCTGGGAG GGTGGATGGG CTAGAGCTCA AGAAGCTTGG

4351 TGAAGTTTCA TGGGAAGAGG AGGCGGAGAT CAGCGGGAGT TCCGCCCGCT

4401 ATGATGTGGC ACTCAGTGAA CAAGGGGAGT TCAAGCTGCT TTCTGAAGAG

4451 AAAGTGCCAT GGGACCAGGT TGTGATGACC TCGCTGGCCT TGGTTGGGGC

4501 TGCCCTCCAT CCATTTGCTC TTCTGCTGGT CCTTGCTGGG TGGCTGTTTC

4551 ATGTCAGGGG AGCTAGGAGA AGTGGGGATG TCTTGTGGGA TATTCCCACT

4601 CCTAAGATCA TCGAGGAATG TGAACATCTG GAGGATGGGA TTTATGGCAT

4651 ATTCCAGTCA ACCTTCTTGG GGGCCTCCCA GCGAGGAGTG GGAGTGGCAC

4701 AGGGAGGGGT GTTCCACACA ATGTGGCATG TCACAAGAGG AGCTTTCCTT

4751 GTCAGGAATG GCAAGAAGTT GATTCCATCT TGGGCTTCAG TAAAGGAAGA

4801 CCTTGTCGCC TATGGTGGCT CATGGAAGTT GGAAGGCAGA TGGGATGGAG

4851 AGGAAGAGGT CCAGTTGATC GCGGCTGTTC CAGGAAAGAA CGTGGTCAAC

4901 GTCCAGACAA AACCGAGCTT GTTCAAAGTG AGGAATGGGG GAGAAATCGG

4951 GGCTGTCGCT CTTGACTATC CGAGTGGCAC TTCAGGATCT CCTATTGTTA

5001 ACAGGAACGG AGAGGTGATT GGGCTGTACG GCAATGGCAT CCTTGTCGGT

5051 GACAACTCCT TCGTGTCCGC CATATCCCAG ACTGAGGTGA AGGAAGAAGG

5101 AAAGGAGGAG CTCCAAGAGA TCCCGACAAT GCTAAAGAAA GGAATGACAA

5151 CTGTCCTTGA TTTTCATCCT GGAGCTGGGA AGACAAGACG TTTCCTCCCA

5201 CAGATCTTGG CCGAGTGCGC ACGGAGACGC TTGCGCACTC TTGTGTTGGC

5251 CCCCACCAGG GTTGTTCTTT CTGAAATGAA GGAGGCTTTT CACGGCCTGG

244

5301 ACGTGAAATT CCACACACAG GCTTTTTCCG CTCACGGCAG CGGGAGAGAA

5351 GTCATTGATG CCATGTGCCA TGCCACCCTA ACTTACAGGA TGTTGGAACC

5401 AACTAGGGTT GTTAACTGGG AAGTGATCAT TATGGATGAA GCCCATTTTT

5451 TGGATCCAGC TAGCATAGCC GCTAGAGGTT GGGCAGCGCA CAGAGCTAGG

5501 GCAAATGAAA GTGCAACAAT CTTGATGACA GCCACACCGC CTGGGACTAG

5551 TGATGAATTT CCACATTCAA ATGGTGAAAT AGAAGATGTT CAAACGGACA

5601 TACCCAGTGA GCCCTGGAAC ACAGGGCATG ACTGGATCCT AGCTGACAAA

5651 AGGCCCACGG CATGGTTCCT TCCATCCATC AGAGCTGCAA ATGTCATGGC

5701 TGCCTCTTTG CGTAAGGCTG GAAAGAGTGT GGTGGTCCTG AACAGGAAAA

5751 CCTTTGAGAG AGAATACCCC ACGATAAAGC AGAAGAAACC TGACTTTATA

5801 TTGGCCACTG ACATAGCTGA AATGGGAGCC AACCTTTGCG TGGAGCGAGT

5851 GCTGGATTGC AGGACGGCTT TTAAGCCTGT GCTTGTGGAT GAAGGGAGGA

5901 AGGTGGCAAT AAAAGGGCCA CTTCGTATCT CCGCATCCTC TGCTGCTCAA

5951 AGGAGGGGGC GCATTGGGAG AAATCCCAAC AGAGATGGAG ACTCATACTA

6001 CTATTCTGAG CCTACAAGTG AAAATAATGC CCACCACGTC TGCTGGTTGG

6051 AGGCCTCAAT GCTCTTGGAC AACATGGAGG TGAGGGGTGG AATGGTCGCC

6101 CCACTCTATG GCGTTGAAGG AACTAAAACA CCAGTTTCCC CTGGTGAAAT

6151 GAGACTGAGG GATGACCAGA GGAAAGTCTT CAGAGAACTA GTGAGGAATT

6201 GTGACCTGCC CGTTTGGCTT TCGTGGCAAG TGGCCAAGGC TGGTTTGAAG

6251 ACGAATGATC GTAAGTGGTG TTTTGAAGGC CCTGAGGAAC ATGAGATCTT

6301 GAATGACAGC GGTGAAACAG TGAAGTGCAG GGCTCCTGGA GGAGCAAAGA

6351 AGCCTCTGCG CCCAAGGTGG TGTGATGAAA GGGTGTCATC TGACCAGAGT

6401 GCGCTGTCTG AATTTATTAA GTTTGCTGAA GGTAGGAGGG GAGCTGCTGA

6451 AGTGCTAGTT GTGCTGAGTG AACTCCCTGA TTTCCTGGCT AAAAAAGGTG

6501 GAGAGGCAAT GGATACCATC AGTGTGTTTC TCCACTCTGA GGAAGGCTCT

6551 AGGGCTTACC GCAATGCACT ATCAATGATG CCTGAGGCAA TGACAATAGT

6601 CATGCTGTTT ATACTGGCTG GACTACTGAC ATCGGGAATG GTCATCTTTT

6651 TCATGTCTCC CAAAGGCATC AGTAGAATGT CTATGGCGAT GGGCACAATG

6701 GCCGGCTGTG GATATCTCAT GTTCCTTGGA GGCGTCAAAC CCACTCACAT

6751 CTCCTATATC ATGCTCATAT TCTTTGTCCT GATGGTGGTT GTGATCCCCG

6801 AGCCAGGGCA ACAAAGGTCC ATCCAAGACA ACCAAGTGGC ATACCTCATT

245

6851 ATTGGCATCC TGACGCTGGT TTCAGCGGTG GCAGCCAACG AGCTAGGCAT

6901 GCTGGAGAAA ACCAAAGAGG ACCTCTTTGG GAAGAAGAAC TTAATTCCAT

6951 CTAGTGCTTC ACCCTGGAGT TGGCCGGATC TTGACCTGAA GCCAGGAGCT

7001 GCCTGGACAG TGTACGTTGG CATTGTTACA ATGCTCTCTC CAATGTTGCA

7051 CCACTGGATC AAAGTCGAAT ATGGCAACCT GTCTCTGTCT GGAATAGCCC

7101 AGTCAGCCTC AGTCCTTTCT TTCATGGACA AGGGGATACC ATTCATGAAG

7151 ATGAATATCT CGGTCATAAT GCTGCTGGTC AGTGGCTGGA ATTCAATAAC

7201 AGTGATGCCT CTGCTCTGTG GCATAGGGTG CGCCATGCTC CACTGGTCTC

7251 TCATTTTACC TGGAATCAAA GCGCAGCAGT CAAAGCTTGC ACAGAGAAGG

7301 GTGTTCCATG GCGTTGCCAA GAACCCTGTG GTTGATGGGA ATCCAACAGT

7351 TGACATTGAG GAAGCTCCTG AAATGCCTGC CCTTTATGAG AAGAAACTGG

7401 CTCTATATCT CCTTCTTGCT CTCAGCCTAG CTTCTGTTGC CATGTGCAGA

7451 ACGCCCTTTT CATTGGCTGA AGGCATTGTC CTAGCATCAG CTGCCTTAGG

7501 GCCGCTCATA GAGGGAAACA CCAGCCTTCT TTGGAATGGA CCCATGGCTG

7551 TCTCCATGAC AGGAGTCATG AGGGGGAATC ACTATGCTTT TGTGGGAGTC

7601 ATGTACAATC TATGGAAGAT GAAAACTGGA CGCCGGGGGA GCGCGAATGG

7651 AAAAACTTTG GGTGAAGTCT GGAAGAGGGA ACTGAATCTG TTGGACAAGC

7701 GACAGTTTGA GTTGTATAAA AGGACCGACA TTGTGGAGGT GGATCGTGAT

7751 ACGGCACGCA GGCATTTGGC CGAAGGGAAG GTGGACACCG GGGTGGCGGT

7801 CTCCAGGGGG ACCGCAAAGT TAAGGTGGTT CCATGAGCGT GGCTATGTCA

7851 AGCTGGAAGG TAGGGTGATT GACCTGGGGT GTGGCCGCGG AGGCTGGTGT

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7951 TGGAAGAGAC GGCCATGAGA AACCCATGAA TGTGCAAAGT CTGGGATGGA

8001 ACATCATCAC CTTCAAGGAC AAAACTGATA TCCACCGCCT AGAACCAGTG

8051 AAATGTGACA CCCTTTTGTG TGACATTGGA GAGTCATCAT CGTCATCGGT

8101 CACAGAGGGG GAAAGGACCG TGAGAGTTCT TGATACTGTA GAAAAATGGC

8151 TGGCTTGTGG GGTTGACAAC TTCTGTGTGA AGGTGTTAGC TCCATACATG

8201 CCAGATGTTC TCGAGAAACT GGAATTGCTC CAAAGGAGGT TTGGCGGAAC

8251 AGTGATCAGG AACCCTCTCT CCAGGAATTC CACTCATGAA ATGTACTACG

8301 TGTCTGGAGC CCGCAGCAAT GTCACATTTA CTGTGAACCA AACATCCCGC

246

8351 CTCCTGATGA GGAGAATGAG GCGTCCAACT GGAAAAGTGA CCCTGGAGGC

8401 TGACGTCATC CTCCCAATTG GGACACGCAG TGTTGAGACA GACAAGGGAC

8451 CCCTGGACAA AGAGGCCATA GAAGAAAGGG TTGAGAGGAT AAAATCTGAG

8501 TACATGACCT CTTGGTTTTA TGACAATGAC AACCCCTACA GGACCTGGCA

8551 CTACTGTGGC TCCTATGTCA CAAAAACCTC AGGAAGTGCG GCGAGCATGG

8601 TAAATGGTGT TATTAAAATT CTGACATATC CATGGGACAG GATAGAGGAG

8651 GTCACCAGAA TGGCAATGAC TGACACAACC CCTTTTGGAC AGCAAAGAGT

8701 GTTTAAAGAA AAAGTTGACA CCAGAGCAAA GGATCCACCA GCGGGAACTA

8751 GGAAGATCAT GAAAGTTGTC AACAGGTGGC TGTTCCGCCA CCTGGCCAGA

8801 GAAAAGAGCC CCAGACTGTG CACAAAGGAA GAATTTATTG CAAAAGTCCG

8851 AAGTCATGCA GCCATTGGAG CTTACCTGGA AGAACAAGAA CAGTGGAAGA

8901 CTGCCAATGA GGCTGTCCAA GACCCAAAGT TCTGGGAACT GGTGGATGAA

8951 GAAAGGAAGC TGCACCAACA AGGCAGGTGT CGGACTTGTG TGTACAACAT

9001 GATGGGGAAA AGAGAGAAGA AGCTGTCAGA GTTTGGGAAA GCAAAGGGAA

9051 GCCGTGCCAT ATGGTATATG TGGCTGGGAG CGCGGTATCT TGAGTTTGAG

9101 GCCCTGGGAT TCCTGAATGA GGACCATTGG GCTTCCAGGG AAAACTCAGG

9151 AGGAGGAGTG GAAGGCATTG GCTTACAATA CCTAGGATAT GTGATCAGAG

9201 ACCTGGCTGC AATGGATGGT GGTGGATTCT ACGCGGATGA CACCGCTGGA

9251 TGGGACACGC GCATCACAGA GGCAGACCTT GATGATGAAC AGGAGATCTT

9301 GAACTACATG AGCCCACATC ACAAAAAACT GGCACAAGCA GTGATGGAAA

9351 TGACATACAA GAACAAAGTG GTGAAAGTGT TGAGACCAGC CCCAGGAGGG

9401 AAAGCCTACA TGGATGTCAT AAGTCGACGA GACCAGAGAG GATCCGGCA

9451 GGTAGTGACT TATGCTCTGA ACACCATCAC CAACTTGAAA GTCCAATTGA

9501 TCAGAATGGC AGAAGCAGAG ATGGTGATAC ATCACCAACA TGTTCAAGAT

9551 TGTGATGAAT CAGTTCTGAC CAGGCTGGAG GCATGGCTCA CTGAGCACGG

9601 ATGTAACAGA CTGAAGAGGA TGGCGGTGAG TGGAGACGAC TGTGTGGTCC

9651 GGCCCATCGA TGACAGGTTC GGCCTGGCCC TGTCCCATCT CAACGCCATG

9701 TCCAAGGTTA GAAAGGACAT ATCTGAATGG CAGCCATCAA AAGGGTGGAA

9751 TGATTGGGAG AATGTGCCCT TCTGTTCCCA CCACTTCCAT GAACTACAGC

9801 TGAAGGATGG CAGGAGGATT GTGGTGCCTT GCCGAGAACA GGACGAGCTC

9851 ATTGGGAGAG GAAGGGTGTC TCCAGGAAAC GGCTGGATGA TCAAGGAAAC

247

9901 AGCTTCCCTC AGCAAAGCCT ATGCCAACAT GTGGTCACTG ATGTATTTTC

9951 ACAAAAGGGA CATGAGGCTA CTGTCATTGG CTGTTTCCTC AGCTGTTCCC

10001 ACCTCATGGG TTCCACAAGG ACGCACAACA TGGTCGATTC ATGGGAAAGG

10051 GGAGTGGATG ACCACGGAAG ACATGCTTGA GGTGTGGAAC AGAGTATGGA

10101 TAACCAACAA CCCACACATG CAGGACAAGA CAATGGTGAA AAAATGGAGA

10151 GATGTCCCTT ATCTAACCAA GAGACAAGAC AAGCTGTGCG GATCACTGAT

10201 TGGAATGACC AATAGGGCCA CCTGGGCCTC CCACATCCAT TTAGTCATCC

10251 ATCGTATCCG AACGCTGATT GGACAGGAGA AATACACTGA CTACCTAACA

10301 GTCATGGACA GGTATTCTGT GGATGCTGAC CTGCAACTGG GTGAGCTTAT

10351 CTGAAACACC ATCTAACAGG AATAACCGGG ATACAAACCA CGGGTGGAGA

10401 ACCGGACTCC CCACAACCTG AAACCGGGAT ATAAACCACG GCTGGAGAAC

10451 CGGACTCCGC ACTTAAAATG AAACAGAAAC CGGGATAAAA ACTACGGATG

10501 GAGAACCGGA CTCCACACAT TGAGACAGAA GAAGTTGTCA GCCCAGAACC

10551 CCACACGAGT TTTGCCACTG CTAAGCTGTG AGGCAGTGCA GGCTGGGACA

10601 GCCGACCTCC AGGTTGCGAA AAACCTGGTT TCTGGGACCT CCCACCCCAG

10651 AGTAAAAAGA ACGGAGCCTC CGCTACCACC CTCCCACGTG GTGGTAGAAA

10701 GACGGGGTCT AGAGGTTAGA GAAGACCCTC CAGGGAACAA ATAGTGGGAC

10751 CATATTGACG CCAGGGAAAG ACCGGAGTGG TTCTCTGCTT TTCCTCCAGA

10801 GGTCTGTGAG CACAGTTTGC TCAAGAATAA GCAGACCTTT GGATGACAAA

10851 CACAAAACCA CT

248

REIVINDICACIONES

1 Un Mét do pa l p du ción de Flaviv us como un v t pa a ntígen s het ólog s qu comprend l ınt duc ón y xp e ión de e uen i d genes f áne dent d s ti d n ión n l niv l de l p t ín d nv ltu de u lqu Flav vı us en dond lo it tán p t t u tu alm nt d l s ár a c n cida p nt f on la st uctu de la p t ín E d l Fl v virus gene al y omprende

(ı) Sitio que des an an sobre l sup fi i externa del virus suministrando accesibil dad al ant cuerpo

(ii) No hay roturas o desestabili ación ignif cat v d l t uctu a t idim nsi nal de l p t ín E

( )N h y nte fe nc n l forma ión d l d de l p teína d nt d l env ltu vi al

2 El mét do d cue do n l ivindic ción 1 en dond un sıtio omp nd la egıón de hilos ß f y g qu ncluye el lazo fg que forma parte de la lámına ß

249

nt p l l de 5 ram f c on d domin o II de l pr t ín d nvoltu d l Fl vivi u

3 El mét do de u do n la ivindi ción 2 n dond l iti l á d l ent lo hil ß f y g qu f rman p t de la lám na ß nt pa alela de 5 mif cac ne del d min II de la p ot ína de nvoltu a d l Fl v v ru

4 El mét d de ue do n l re vindıcación 2 n dond l u nc extr ñ h ido in rtad en l gión d l amin ác d s 196 a 215 con efe en i l e uen d v u de ncef l tis tick bo n des it n la f gu a 2

5 El mét do d ue d n l r ivind cac ón 2 en d nd l e u nc xt ñ h id in t d n l gión d l aminoácid s 205 210 n fe nci a l u n d v u d en f l ti tick bo n d sc it en l figu 2

6 El mét d d u d n l iv ndi ción 1 n d nde t o it comprend la eg ón de los hilos E y F

250

qu n luy l laz E F que form p t d l t v d l ba l ß de 8 amıf ac on s del d m nio I

7 El mét d d a u d on la eivindic ción 6 n d nd l t s l á d l o ntre l hil E F qu f rma p t d l b l ß d 8 amifi n d l d m ni I

8 El mét d d u d con l vindic ción 6 n d nd la se uen a xt ñ ha id in ert da en la g ón d l s am noá id 138 a 166 con efe nc a la se u n ia de vi u d enc falitıs tick bo n desc ta en l f gu 2

9 El mét d d u d on l e vindic ón 7 n d nd la s uen ia xt ña h do ns rt d n l gión d l am n á dos 146 160 n f en l u n d vi u d ncef litı t k b n d s it n l f gu 2

257

10 El mét d d u d on la eiv nd c ón 1 n d nd el Fl viv us le n p rti d l g up qu n t de ualqu Fl viv u qu n luye l v u d f eb e amarılla l vı us d encefal tis t ck borne el v ru d l d ngu y el vi us d ncef litis japones

11 El mét do de cu do c n l e vindıca ıón 10 n d nd l v u un v u ombin nt o tenu do del t p n tu al

12 El métod d u d n l ivindic ón 10 y 11 n d nde l Flav vi u es un vi u de f ebre amar lla

13 El mét d d u d n l vindı ción 12 n d nd l v ru un viru d f bre amar ll r mb n nt

14 El mét d d u d n l iv ndi ión 13 n d nd l vi u d fı bre amarill s l p d vi u YF 17D y l s ubsep s d l m smo

252

15 El mét d d u d c n l r ivind c ón 1 14 n d nd l pit p xt ñ un cu n d l g n mal l

16 El mét d d u d n l ivindi ión 15 n d nd la se uen a de gen mala ial es el epítope humo al (NANP)

17 El mét do de cu rd con l r vind c ón 15 n d nd l uen ia de gen mal i l e el DYENDIEKKI del pítop de l nfoc tos T citotóxıcos (CTL)

18 El mét d d cu rd c n l r vind cac ón 15 n d nd l u n i d g n mal ri l l SYVPSAEQI del pit p d linf t T citotó i (CTL)

19 El mét d de cu d on la iv ndic ión 1 en dond un más es duo de glic na on ins rt d n l eg ón ınmed atamente aguas ar ba y aguas abajo del pit p xt ño

253

20 Un on trucción d DNA qu c n t s nc lm nt de un v cto un g nom d Fl v vi u st bl g nét ament y cuen i d g n xt ñ int odu id n un s t o de nserción d uerd c n u lqu d l v nd ne 1 19

21 La nst ucc ón d DNA d a u rdo n l r ivınd c c ón 20 n donde l Fl vivirus s l ccion a p tı d l g up que c ns st de ualquie Flav vi u n luy nd l v u d f b ama ill l v u de nc f l t t k b ne l vir d l d ngu y l viru de en f lit ɔ p n

22 L n t uc ón d DNA d u d on l vind ión 20 n d nd l v ct e l i n do p tı d l g up qu c n t de plásmid s d núm d b ɔ c p

23 L c n t uc ón d DNA de acue do n l e vınd ión 20 en d nde l v cto s sel cc onado a p tı d l g upo de pACNR1180 y Bel BAC11

254

24 L const uc ón de DNA de cuerdo on la iv nd c ón 20 n d nd l vect l c na pa t d l g up qu nsi te de plá mid s d núme d lt p

25 L on t ucc ón de DNA de acue do con la e v ndi c ón 20 n dond l gen ma de Fl viv us t bl g nét am nt d r v d ualqui r p YF 17D

26 L n t u ión d DNA d u d n l v nd ón 25 n dond el g noma de Fl viv u t bl g néticam nt s l gen ma YF que ll va la u n mpl t stabl id en l SEQ ID NO 1 o n q v l nt func on lm nt d l misma

27 L onst uc ón de DNA de cu rdo on l v nd c ón 20 n d nd lo cu nci d g n xt añ d v d d l m l i fi b ama illa d ngu n f l t s j p n n f l ti ti k bo n nf n s fung le

255

28 L con t uc ón de DNA d a ue do on l e v nd c ón 27en d nde la secuenc a de gen ext año es na se uenc d gen mala l

29 L on t u ión de DNA d ue do on la vind ón 26 y 28 n d nd l cuenci d l g n m l l s l pít pe humo l (NANP)

30 La construcción de DNA de acuerdo con la iv nd c ón 28 n d nd la s u n d g n mal i l l DYENDIEKKI d l epítop d linf cit s T citotóx (CTL)

31 L n t u ión d DNA d u d n l iv nd ión 28 n d nd la u n i d g n m l i l l SYVPAEQI d l pít p de l nf ito T totóxic (CTL)

32 L onst ucc ón d DNA d ue d c n l e v nd ac ón 29 que s el plásmid pYF17D/8

256

33 L n t u ión d DNA que ti n l t u tu d l l plásm d pYFE200

34 L n t u ón d DNA qu t en la st uctu a d l l plá m d pYFE200/1

35 La on t u ión d DNA qu t ne l e t uctu a d l l plá m d pYFE200/13

36 L n t u ión d DNA qu ti n l t u tu d l l plásmid pYFE200/8

37 Un Fl v vi us mo un ve to p r ntíg n h t ól g s qu omp nd u nci d g n xt ñ n t d n l t n l n v l d u p ot ín d nvoltu en d nd l s tios están e t u tu lm nt p t d l áre n ida p r int rf rir c n l t u tu de la p ot ína E del Fl viv u g ne al

38 El Fl v v us d a u d on la r ivind ca ón 37 n d nd l u n i d g n ext año e int du d n l gión de h lo ß f y g que ncluy el laz fg qu f ma p t d l lámin ß ntip lela de 5 am f on d dominio II d la prot ína d envoltu d l Fl v vı us

257

39 El Fl v v us de acuerdo con la eıv nd cacıón 38 n d nd el s t s l á e de la o nt lo h lo ß d f y g qu f rma p t d l lámin ß nt pa lel d 5 am fi i n d d min o II d l pr t ína d nv ltu d l Fl v vi u

40 El Fl viv u d cu d c n l re vind ca ón 38 n d nde l s uen i xt ñ ha sid in t da n l g ón d l am n ácid 196 215 c n efe encia a l cu n d v u d n f l ti t k b n de ít en l f gu 2

41 El Fl v vi u d ue do n l vind c ón 39 n d nde l u n i xt ñ h id n t d n l g ón d l am n á ido 205 210 n fe nci l v u de encefal tıs tıck bo ne d sc ita en la fıgura 2

42 El Fl v v u de cue do con l vindicac ón 37 n d nde otr ti omp nd l eg ón d lo h l E F ncluyend l zo E F que forma pa t d l b rr l ß d 8 am ficac ones del dom nio I

258

43 El Flaviv rus de cu rd con l re vind cación 42 n donde el s tıo es el área de lazo ent e los h los E F qu f rm p t d l b il ß d 8 am f c ne del d m n o I

44 El Flav vi us de cuerdo con la reivindica ión 38 n d nd l u n xt ñ h d n t d n l g ón d lo am noá d 138 215 con ref renci l n i d vi u d n f l ti ti k b ne d c it n l figu 2

45 El Fl v v u d u do on la r vind a ón 43 en donde l se u n xt añ ha id ins tad n l eg ón d l amınoácidos 146 a 160 con efe encia a la cu nc d vi u de n fal tis t ck bo n des ta n l f gu 2

46 El Flav vi u d a u do n l r ivindi ción 37 en donde l Fl viv rus se selecc ona a pa tir del g upo qu c n st de ualquie Flav virus que n luye el v u d f b ama ll l vi u de nc f l ti t ck

259

b ne l v us d l d ngu y el vi us d encefaliti
ȝ pon sa

47 El Fl v v u de cu do n l r ivind ción
46 n d nd l vi us un vi u mbinant o t nu do
d l t p s lv ȝ n tu l

48 El Fl v v u d u d n l vind ón
46 y 47 n d nd l Fl vivi us un viru de fieb
ama ıll

49 El Fl vivi u d u d n l r ivindi ción
48 n d nd l vi u un vi u d fieb ama ill
mb n nt

50 El Flav vi us de acue d con la r vind cac ón
49 n dond el vi u de fi br ama ılla l p d
v us YF 17D y las sub sepas del m smo

260

51 El Fl v viru d u d n l
v nd n 37 50 n d nde l pítop xt ñ
un ecu nc d l g n m l l

52 Un mp i ón d v un p r inmuni ont
Fl viv u y t g nt nf io qu nsi t
sen ialment d n virus de acuerdo con las
v ndi i n 37 51

53 L mp i ión d v un de cu d n l
v ndi ción 52 n d nde l Fl viv rus s un viru d
f b e ama ll y l s t o ag nte inf ci n
g nte c u tiv s d mal i

54 L omp ión d va un d acu do n l
v nd ón 53 n d nd l cu nci d g n mal i l
l pít p humo al (NANP)

55 L compo i ón d v cuna de acue do con l
v ndic ón 15 en dond la cuen ia de gen mala l
l DYENDIEKKI d l pít p d l nf it T t tóxi

261

56 L mp i ión d v cun d acue d n la v nd ón 53 n d nd la se uen ia de g n mal i l l SYVPSAEQI d l pit pe de linf cit s T citotóxico

57 L mp ón d va un de u d n l ıv nd n 37 51 que mp end un ntid d uf c ent d l viru y un vehículo farmacéutica mente ept bl

58 Un Fl viv u como un v cto p ntig n h t ól g n d nd l Fl vivi u obt nido d cu do n u lqu d l v ndi i n l 19

RESUMEN

L p ent inven ón se el ciona c n un v un
nt a nf c n us das p Flaviv us Má
p t cul rmente con el us d vı us para vacunas YF (17D)
p a e p n l n v l d su cub e t L pít p d
l p t ín d t p tóg n l u l eli t n n
pu t inmun p ífi l p tóg n p ent l

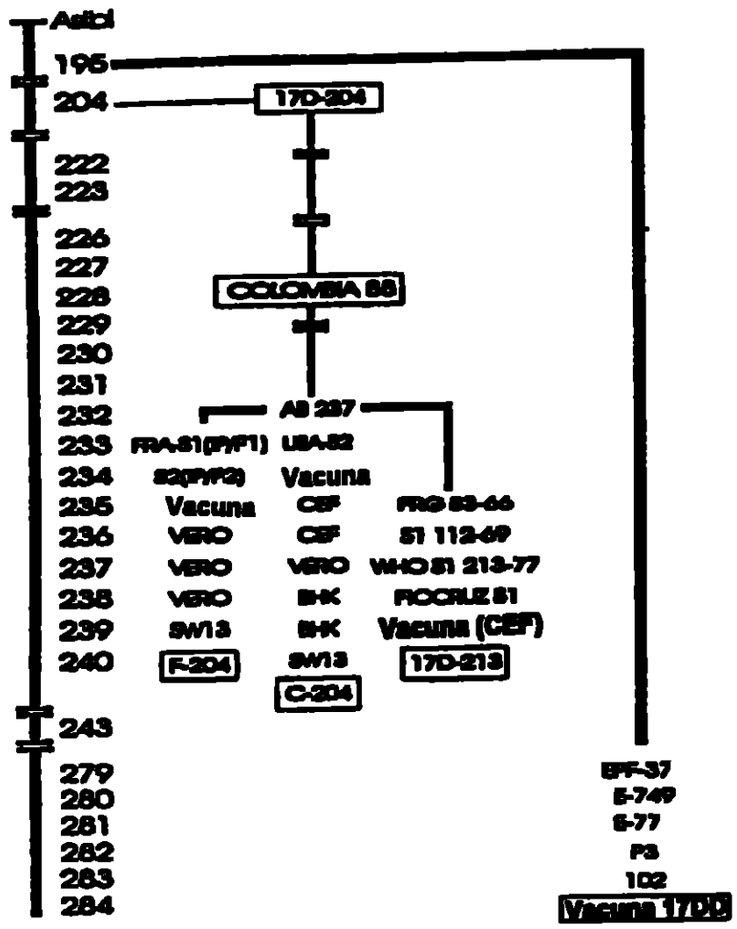

Asilci
195
204
170-204
222
223
226
227
228
COLOMBIA 88
229
230
231
232
AS 287
233
FRA-81(F/F1)
USA-82
234
82(F/F2)
Vacuna
235
Vacuna
CEF
FRG 83-66
236
VERO
CEF
81 112-69
237
VERO
VERO
WHO 81 213-77
238
VERO
BHK
RIOCRUZ 81
239
SW13
BHK
Vacuna (CEF)
240
F-204
SW13
170-213
C-204
243
279
280
281
282
283
284
EFF-37
E-749
E-77
P3
102
Vacuna 17DD

Figura 1

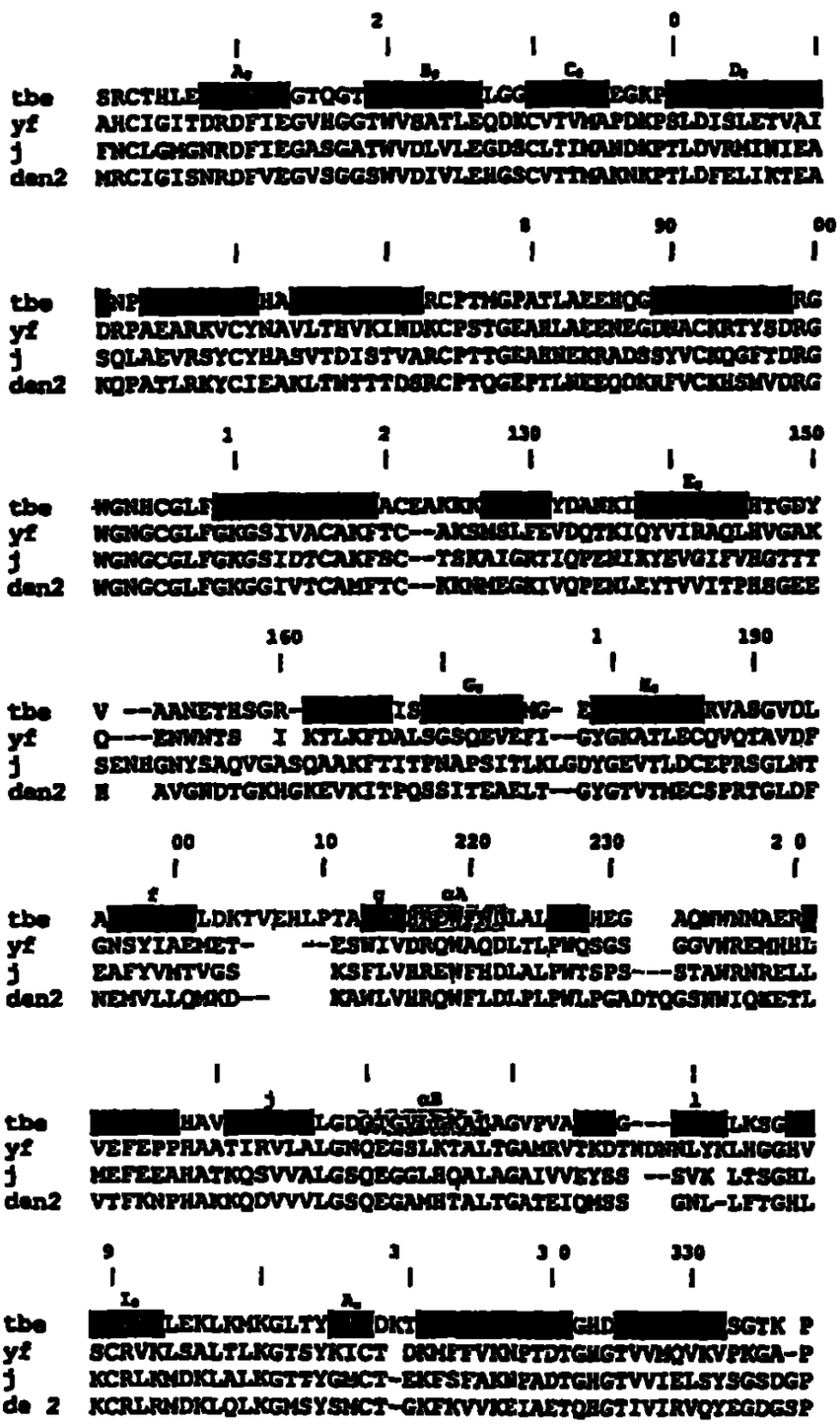
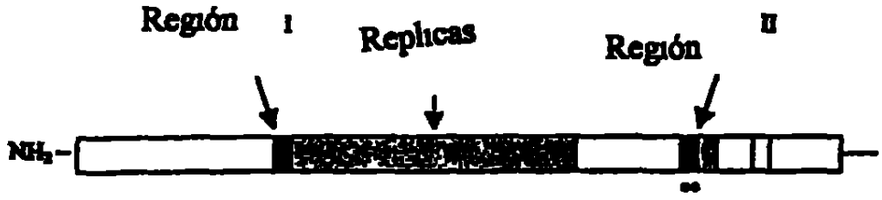

tbe
yf
j
den2



Figura 2



Especies

P.falciparum

réplicas(*DPNANPNVDPNANPNV*)$_2$ *CD8$^+$CTL(DYENDIEKKI)*

(NANP)$_2$

P.yoelii

CD8$^+$CTL(SYVPSAEQI)

Figura 3

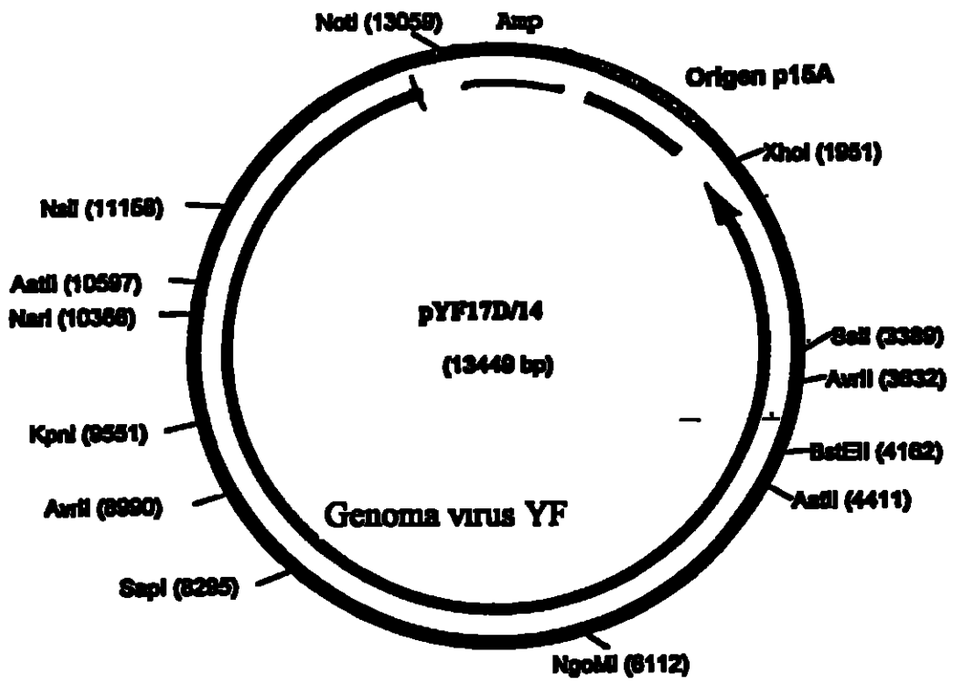

NotI (13058)
Amp
Origen p15A
XhoI (1951)
NaII (11158)
AatII (10597)
NarI (10368)
pYF17D/14
(13449 bp)
SalI (3389)
AvrII (3632)
KpnI (9551)
BstEII (4162)
AatII (4411)
AvrII (8990)
Genoma virus YF
SapI (8285)
NgoMI (8112)

Figura 4

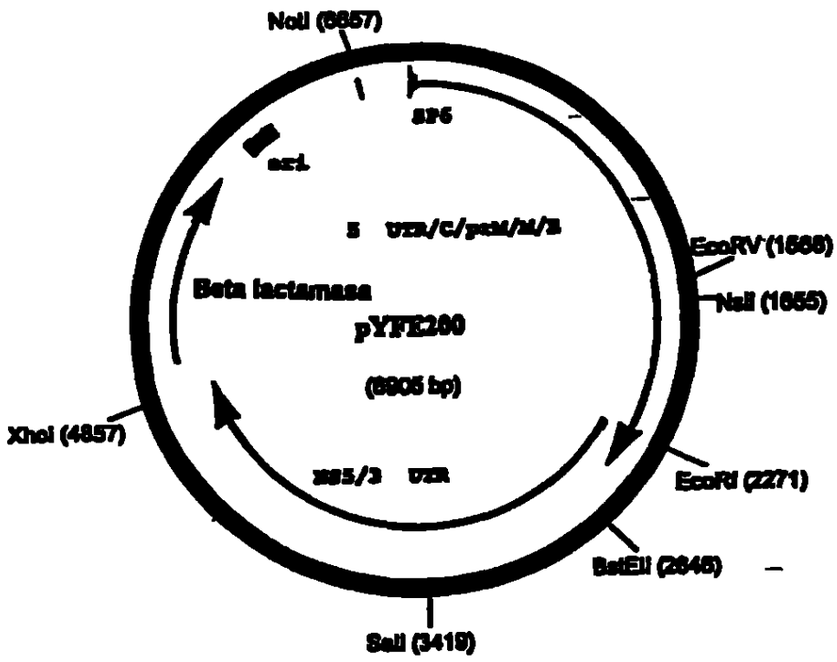
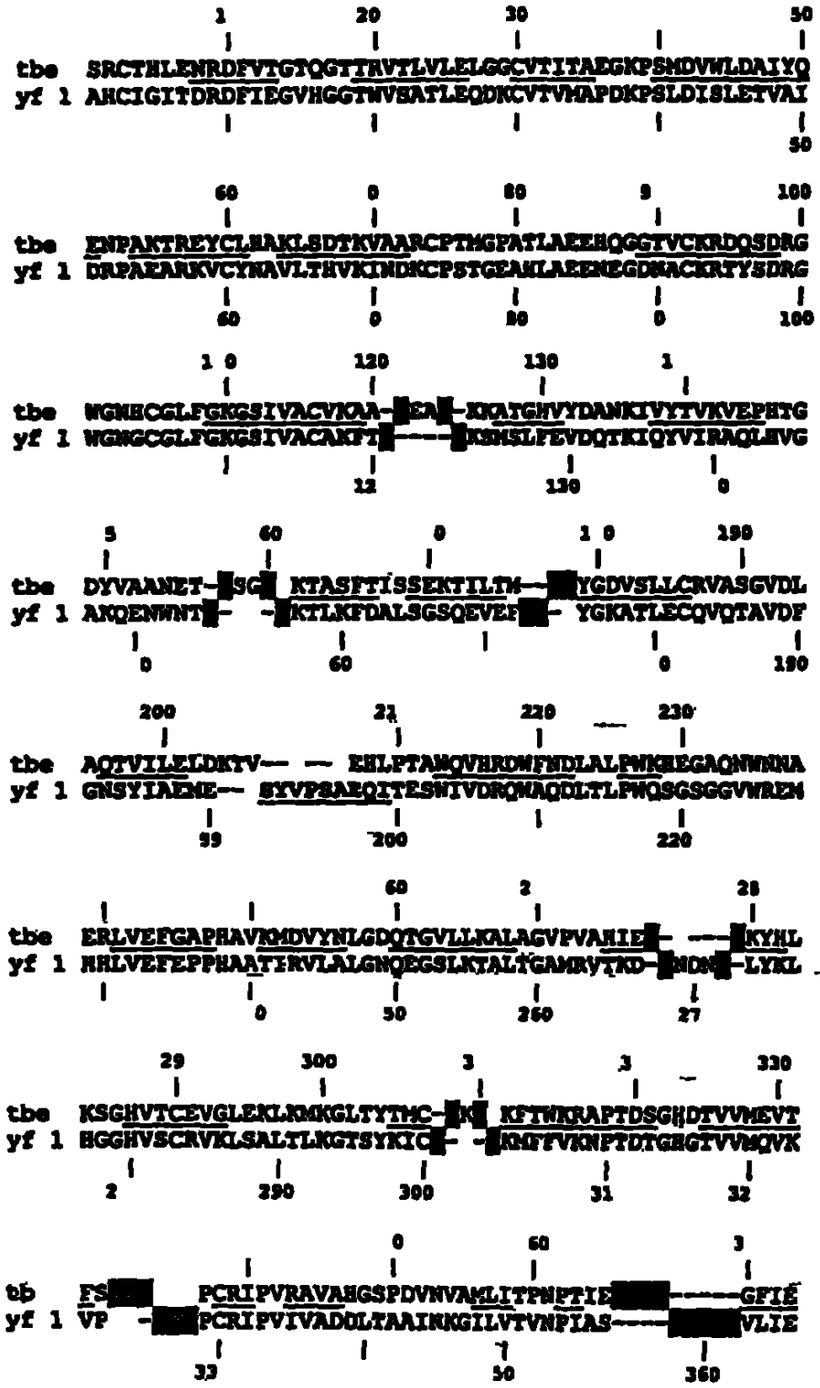

NotI (6857)
SP6
ori
5 UTR/C/pcM/M/3
Beta lactamase
pYFE200
(6905 bp)
EcoRV (1668)
NsiI (1655)
XhoI (4857)
385/3 UTR
EcoRI (2271)
BstEII (2648)
SalI (3419)

Figura 5


tbe SRCTHLENRDFVTGTQGTTRVTLVLELGGCVTITAEGKPSMDVWLDAIYQ
yf 1 AHCIGITDRDFIEGVHGGTWVSATLEQDKCVTVMAPDKPSLDISLETVAI
tbe ENPAKTREYCLEAKLSDTKVAARCPTMGPATLAEEHQGGTVCKRDQSDRG
yf 1 DRPAEARKVCYNAVLTHVKINDKCPSTGEAILAEENEGDNACKRTYSDRG
tbe WGNHCGLFGKGSIVACVKAA- EA -KKATGHVYDANKIVYTVKVEPHTG
yf 1 WGNGCGLFGKGSIVACAKFT ---- KSNSLFEVDQTKIQYVIRAQLEVG
tbe DYVAANET- SG KTASFTISSEKTILTM- YGDVSLLCKVASGVDL
yf 1 AKQENWNT - KTLKFDALSGSQEVEF - YGKATLECQVQTAVDF
tbe AQTVILELDKTV- - EHLPTAWQVHRDWFNDLALPWKHEGAQNWNNA
yf 1 GNSYIAEME- SYVPSAEQITESNIVDRQWAQDLTLPWQSGSGGVWREM
tbe ERLVEFGAPHAVKMDVYNLGDQTGVLLKALAGVPVAHIE - - KYHL
yf 1 RHLVEFEPPHAATIRVLALGNQEGSLKTALTGAMRVTKD - NDN - LYKL
tbe KSGHVTCEVGLEKLKMKGLTYTMC- K KFTWKRAPTDSGHDTVVMEVT
yf 1 EGGHVSCRVKLSALTLKGTSYKIC - KMFTVKNPTDTGHGTVVMQVK
tb FS PCRIPVRAVAHGSPDVNVAMLITPNPTIE ---- GFIE
yf 1 VP - PCRIPVIVADDLTAAIKKGILVTVNPIAS ---- VLIE


3
tb MQLPPGDNIIYV
LSHQWFQK
yf 1 VNPPFGDSYIIV-RGDS-LTYQWKKE

Figura 6

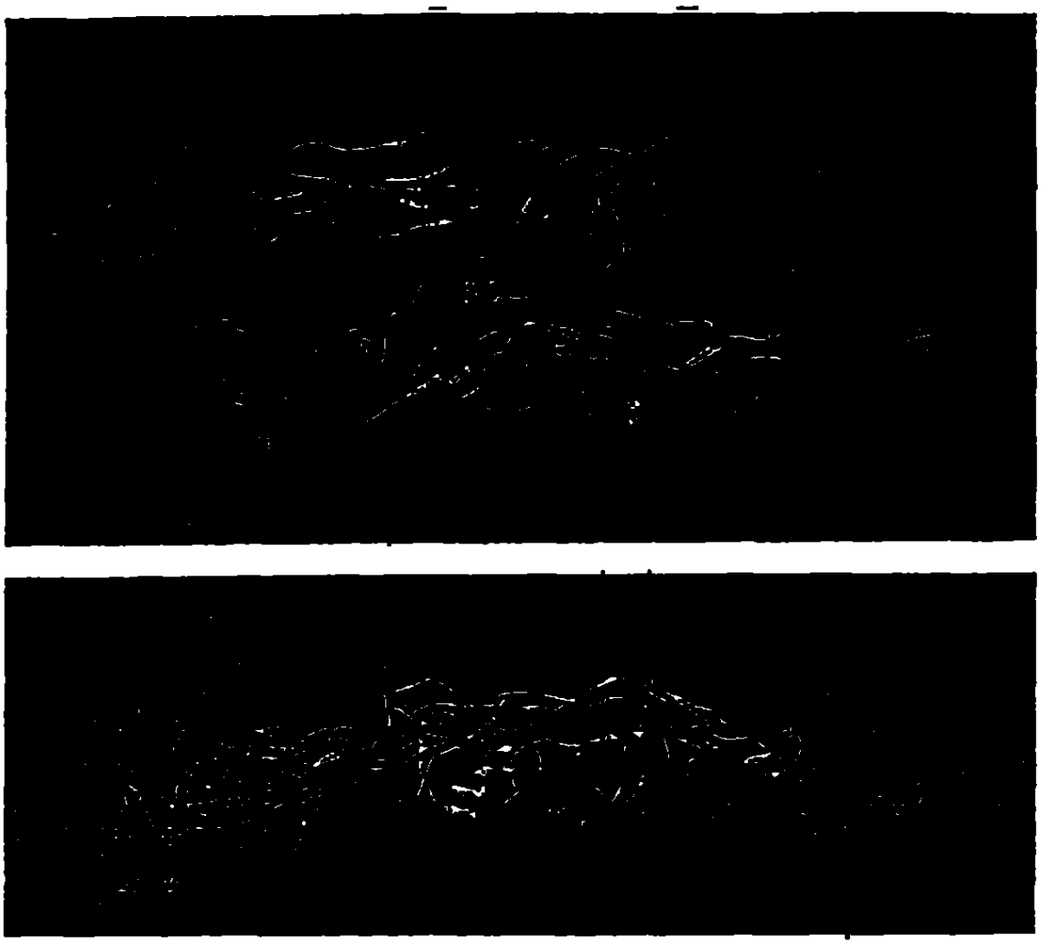


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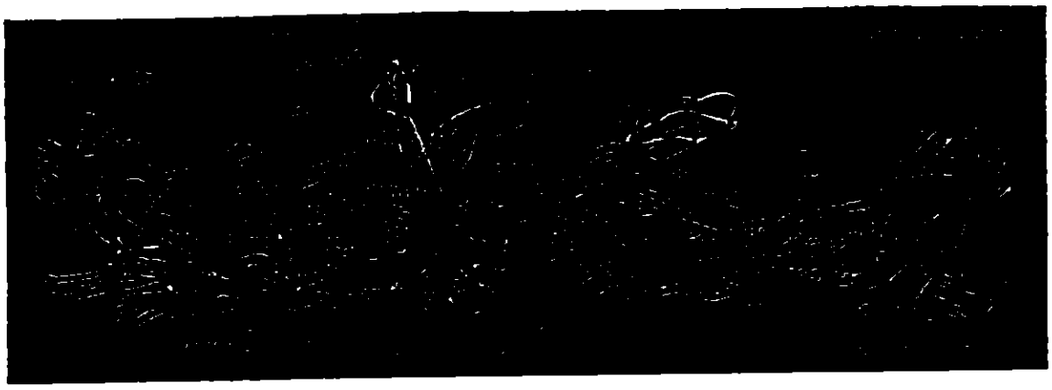


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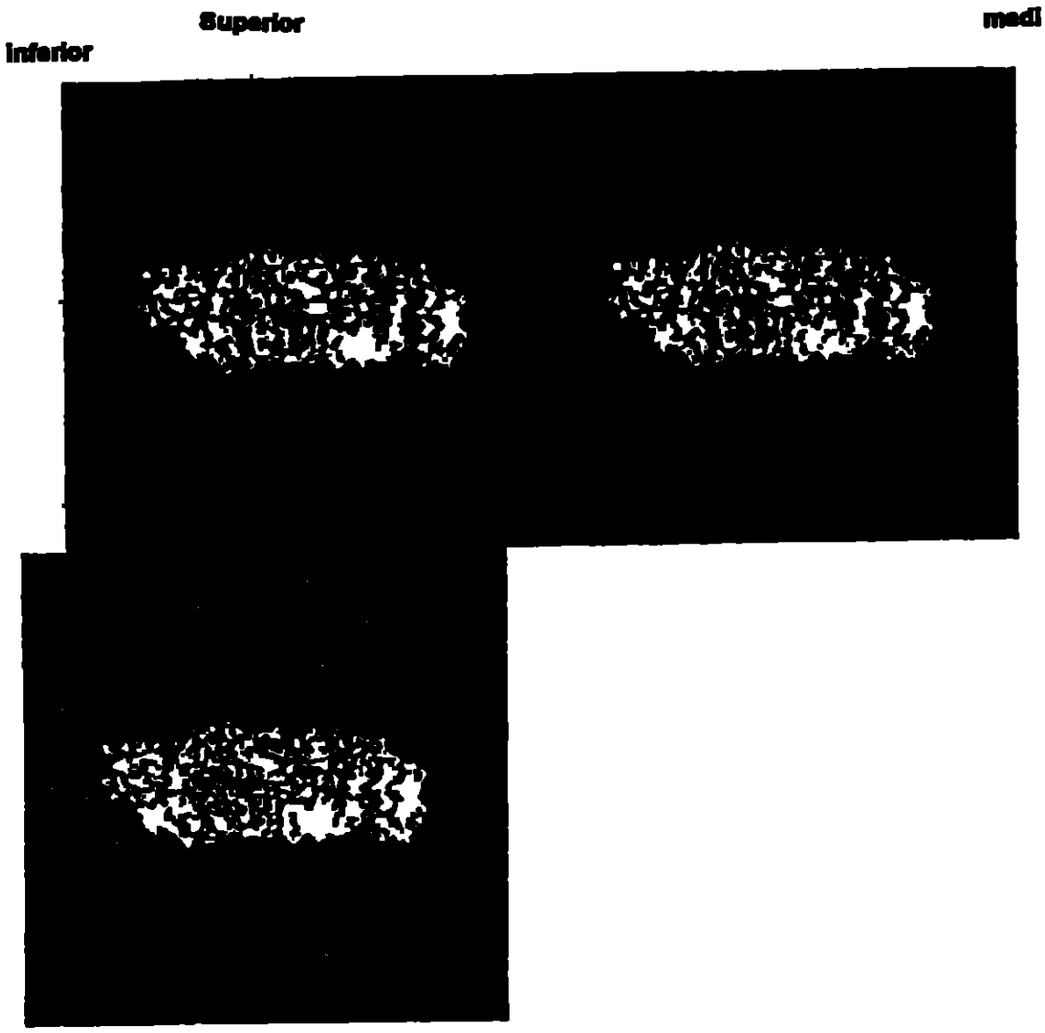


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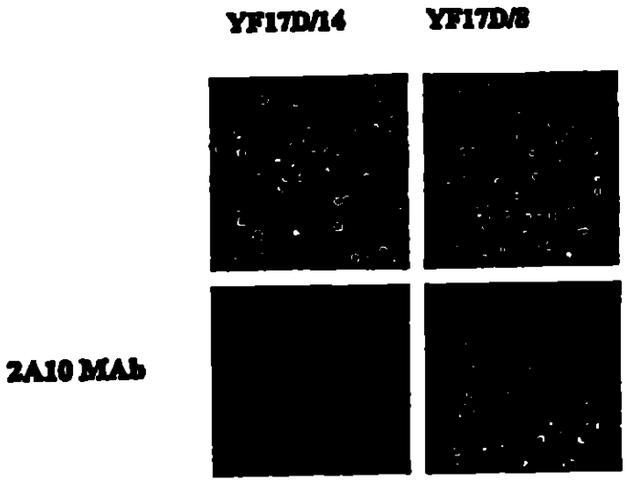


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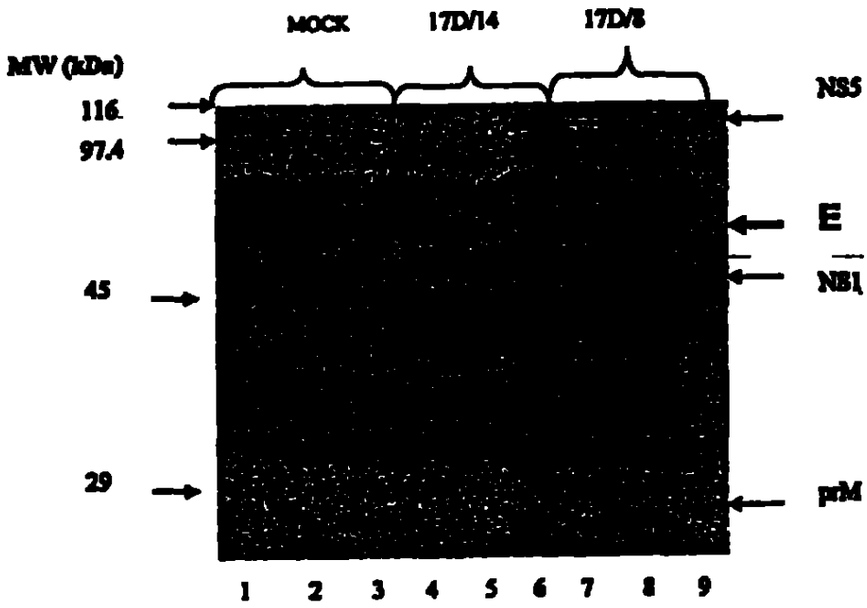


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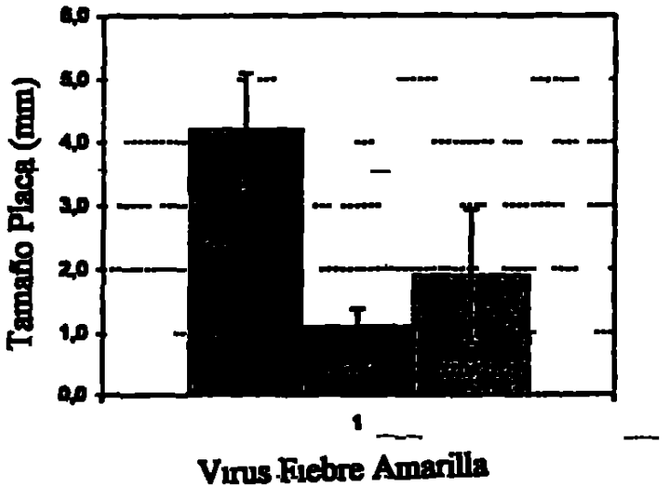
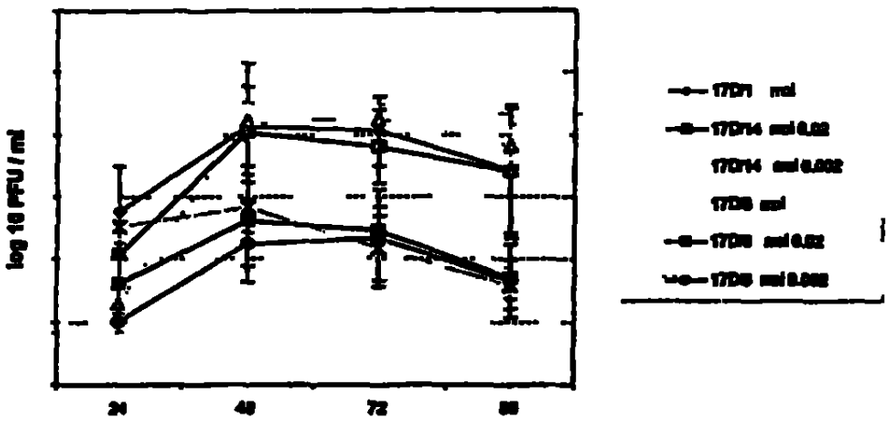
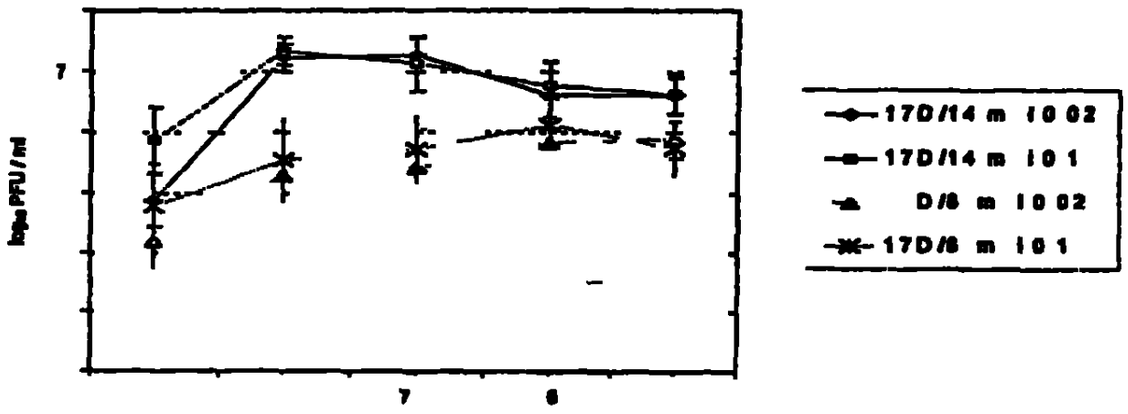


Figura 12

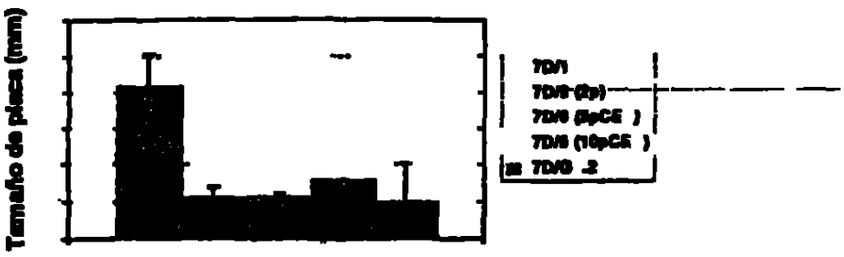


Horas Pos-infección

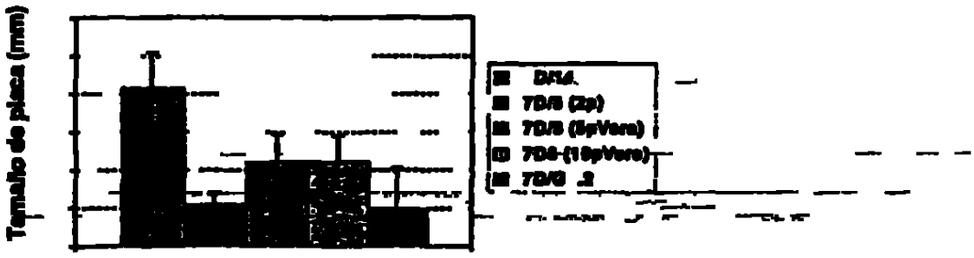


Horas Pos-infección

Figura 13



Virus Fiebre Amarilla



Virus Fiebre Amarilla

Figura 14

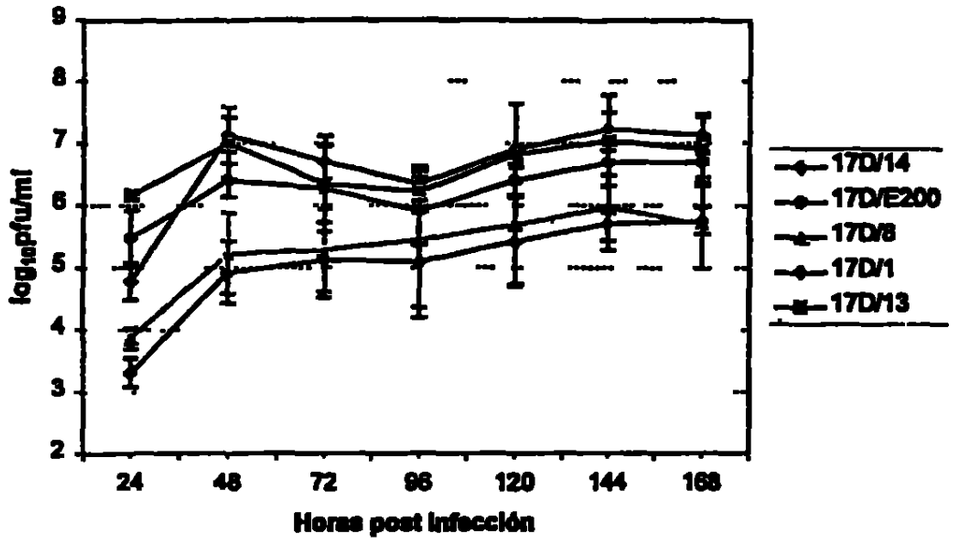
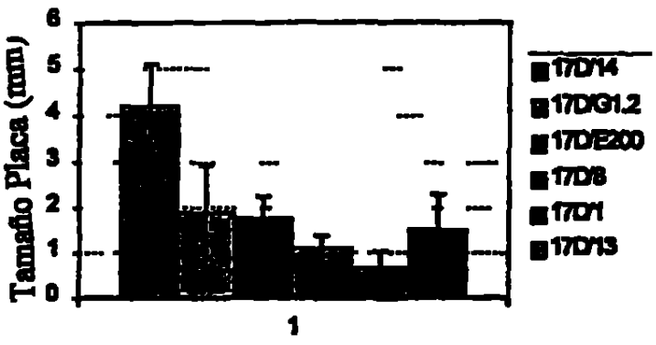

9
8
7
6
5
4
3
2
log10pfu/ml
24
48
72
96
120
144
168
Horas post infección
17D/14
17D/E200
17D/8
17D/1
17D/13

Figura 15



Virus Fiebre Amarilla

Figura 16

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Art 8 and Rules 3 and 44)

applican or agen reference	FOR FURTI ER see Not cation ransmittal of ternational Search Report ACTION (Form PCT/ISA/220) as wel as, where app rcable, tem below	
P1255		
nternational application No.	International ng date (day/month/year)	(Earlie) Priority Date (day/month/year)
PCT/BR 02/00036	8 March 2002 (08 03 2002)	9 M rch 2001 (09 03 2001)
Applicant		
F ndaca Oswald Cruz F ocruz		

Th ternational search repo has been prepared by this International Searching Authority and transmitted to the applican accord ng to A le 8 A copy being ransm ted to he International Bureau

Th terna on l search repo consist f l f 4 sheets.

☐ l also accompanied by copv f each prio art documen ted in th report.

Bas f report

W th regard to the la g ag the ternationa search was carried ou on the basis f the ternational application the language h ch i as f led less otherwise ndicated under h tem.

☐ he ternat onal search as carried out on the basis of translation f the international application furnished to th ho ty (Rule 23 (b))

b W h regard to any leotid nd/o ino acid seq ence d sclosed the ternational appl catio the internat nal search was carried on he basis f he sequence l ng

☒ contained he tern nal appl cation tten form.

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☐ furn shed subsequentl to th Authority computer readable form

☐ the statement that he subsequentlv furn shed tten sequence listing does not go beyond he disclosure the nternat on l applicat on as f led has been f rn shed.

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2 ☐ Certai laims were eare bl (See Box I).

3 ☐ U ty f ven i lacking (See Box II)

With regard to he tit

☒ the tex approved as subm ted by he applicant.

☐ the text has been estab shed b\ h ion to read as f llows

5 W th regard he betra

☒ he ex approved as subm ted by th ppl can

☐ the text has been established, accord ng to Rul 38.2(b), by th A hority as appears Box III The applican may th one mon h from he date f ma l ng of th nternational search report, subm commen to th Authority

6. The f gure of the drawi gs be published wi h he abstract Figure N 1

☒ as suggested by he applican ☐ None f the figures.

☐ because he applican fai ed to suggest figure.

☐ because th figure better characterizes invention.

Form PCT SA/2 0 (f rst sheet) (July 998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/BR 02/00036

CLASSIFICATION OF SUBJECT MATTER

IPC C12N 15/40 15/86 A61K 39/00 39/015

Accord ng to I tern ional Paten Classif catio (IPC) to both nat onal lass f cation and PC

B FIELDS SEARCHED

M mum documentation searched (classification system followed by lassification symbols)

IPC C12N A61K

Documentation searched other than m mum documentation to the extent that such documents are included the f lds searched

Electro data base consulted d ring the international search (name of data base and, where practicabl search terms used)

WPI CAS STN-registry BLAST (NCBI)

C DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation f document, with ndication, where appropriate, of the relevant passages	Relevant to l m N
Y	US 6171854 B1 (GALLER et al) 9 January 2001 (09 01 01) *column 6 lines 40-64*	1-3 6 7 10 15 19 22 24 25 27 28 37 39 42 43 46 53 57 58
Y	WO 00/15823 A1 (GENVEC INC) 23 March 2000 (23 03 00) *cla ms*	1 3 6 7 10 15 19 22 24 25 27 28 37 39 42 43 46-53 57 58

☒ Further documen are listed the continuation f Box C ☒ See patent family annex

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- A document def ng the general state of the art which not considered be of particular relevance
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- & document member f the same patent family

Date f the actual completion f the nternational search	Date f mail ng of the nternational search report
7 June 2002 (07 06 2002)	20 June 2002 (20 06 2002)
Name and ma l ng adress of the ISA/ T Austrian P tent Office Kohlmarkt 8 10 A 1014 V enna Facs m le No 1/53424/535	Autho zed fficer MOSSER T lephone N 1/53424/437

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INTERNATIONAL SEARCH REPORT

ternation applica N
PCT/BR 02/00036

C (Con uation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation f documen wi h ndica ion. where appropriate. of the relevan passages	Relevan to laim
Y	EP 0872553 B1 (THE UNITED STATES OF AMERICA AS REPRESENTED BY THE SECRETARY DEPARTMENT OF HEALTH AND HUMAN SERVICES) 15 November 2000 (15 11 00) *claims*	1-3 6 7 10 15 19 22 24 25 27 28 37 39 42 43 46 53 57 58
Y	BONALDO M C et al The yellow fever 17D vaccine virus as a vector for the expression of foreign proteins development of new live flavivirus vaccines Mem Inst Oswaldo Cruz 2000 95 Suppl 1 pages 215-223 Medline-abstract [online] [retrieved on 7 June 2002 (07 06 02)] Retrieved from the Internet URL http //www ncbi nlm nih gov/entrez/query fcgi?cmd=Retrieve&db =PubMed&list_uids=11142718&dopt=Abstract *abstract*	1 3 6 7 10 15 19- 22 24 25 27 28 37 39 42 43 46 53 57 58
A	US 5736148 A (SUMIYOSHO et al) 7 April 1998 (07 04 98) *claims*	1 58
A	RODRIGUES M et al Influenza and vaccinia viruses expressing malaria CD8 T and B cell epitopes Comparison of their immunogenicity and capacity to induce protective immunity' J Immunol (1994) 153(10) pages 4636-4648 (abstract) CA [online] Chemical Abstracts online [retrieved on 7 June 2002 (07 06 02)] Retrieved from CA Database STN International Karlsruhe (DE) CA Accession no 122 7436 *abstract*	1 15 20 2 28 37 52 53
A	MIYAHIRA Y et al Induction of CD8+ T cell-mediated protective immunity against Trypanosoma cruzi" International Immunology (1999) 11(2) pages 133 141 (abstract) CA [online] Chemical Abstracts online [retrieved on 7 June 2002 (07 06 02)] Retrieved from CA Database STN International Karlsruhe (DE) CA Accession no 130 324090 *abstract*	1 15 18 20 27 28 31 37 52 53 56

INTERNATIONAL SEARCH REPORT

Information on paten family members

ICT/BR 0 00036 0

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Patent document cited search report	Publication date	ten fam member( )		Pu cation da
DK				
A1				
		AU	/	
DK		AU		
			T2	
			A1	
			A4	
			A1	
		ES		
		ES		
		GR		
		JP	T2	
		WO		
		US	BA	
US			A2	
US BA		BR		
		CA	AA	
			A2	
			A3	
		ZA		
WO			none	

(2) NTERN TIONAL PPLIC O UB SHED UN T A NT COO RA O TREATY (PCT)

(19) World I tellect I Property Orga ization
Intern onal B rea

(43) I tern tional Publ catio Date
19 Septembe 2002 (19 09.2002)

PCT

(10) I t rn t I Publ catio N mbe
WO 02/072835 A1

(51) I tern tion l Pate Classifica ion C12N 15/40
5/86 A6 K 39/00, 39/015

(2) I tern tion pplication N mber PCT/BR02/00036

(22) tern tion Fi g Date March 2002 (08.03 .002)

(25) Filing La g age Eng

(26) P blicatio La guage En sh

(30) Priority Data
0 05877.5 9 March 200 (09.03 2001) GB

(71) Applica (for all designated States except US)
FIOCRUZ, Fundação, Oswaldo, Cruz [BR/BR]
venida Brasil 4365 Manguinhos, 045-900, Rio de aneiro (BR)

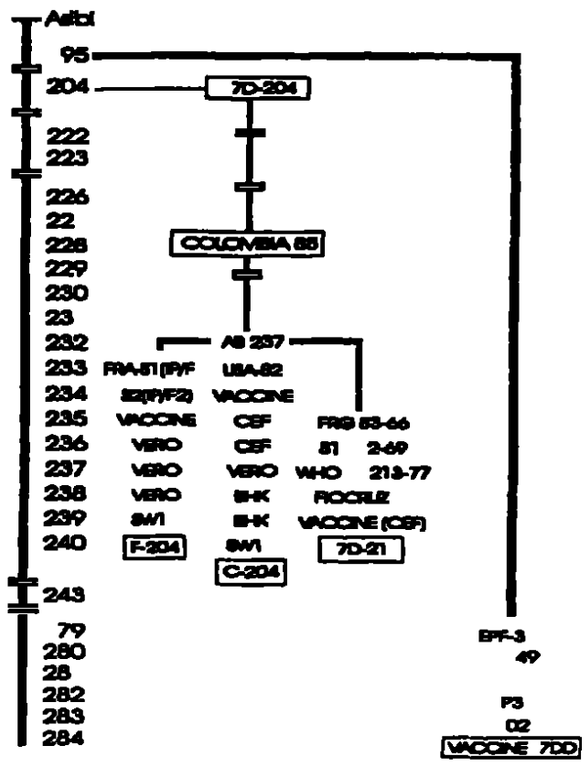
(72) Inventors d
(75) Inventors/Applica (for US only) BONALDO, M rna, C [BR/BR] vi da ra 4365 1.05 045-900, Ma g hos R de Jan ro (BR G LLER, Ricard [R/BR] ve da rasi 4365 1 05 045-900, Man g hos, Rio de Janeiro (BR) FREIRE, Marcos d Silva [R/BR] venida Bras 4365 05, 045-900 Mang nhos R de J neiro (BR) GARRAT Rich rd, C. [GB/BR] Sebas ão de Morae 60 São Paul (B

(4) gen LM DA, Kát Feru des he ng lmeida Associados Rua Bened tinos 6, 1 ndar CEP 2008 -050 Rio de Janeiro (BR)

(8) Designated St tes (national) E, AG I AM, AT U AZ, BA, BB BG BR, BY BZ, CA CH CN, CO, CR, CU CZ, DE, DK DM DZ, EC, EE, ES FI GB GD GE, GH GM, R, HU ID. IL, IN IS, JP KE, KG KR, KZ, LC, LK, LR, LS LT LU L M MD MG MK, MN MW MX MZ, NO, NZ, OM PH PL, PT RO RU SD SE, SG SI SK SL, TJ TM TN TR, TT TZ, U UG US UZ, VN YU ZA ZM ZW

[Cont ued on next page]

(54) Title USE OF FLAVIVIRUS FOR THE EXPRESSION OF PROTEIN EPITOPES AND DEVELOPMENT OF NEW LIVE TTENU TED VACCINE VIRUS TO IMMUNE AG INST FLAVIV US ND OTHER INFECTIOUS AGENTS



(57) betrac The presc nven on relates to vaccine agamst nfec ns caused by flavivirus More particularly to the se f the YF vaccine virus (7D) to express the level of envelope, protei ptopes from the pathogens hich wi l l specif mmune response to the paren pathogen.

WO 02/072835 A1

(84) Desig ted States *(regional)* PO paten (G GM KE, LS MW MZ, SD S SZ, U /M /W), Euras paten (M A/ BY C KZ, MD. TM), European patent (T BE, CH CY DE, DK ES FI FR. GB GR, IE, IT LU MC NL, PT SE, TR) OAPI paten (BF BJ CF CG CI CM G GN GQ GW ML, MR NE, SN TD TC

Publis ed
h nternat onal search por

For two-le er codes and othe abbrevi ons refe the "G id- nce Notes on Code and bbrevat ons appearing at he beg n- ng of each regular ue of he PCT Gaze

SUPERINDUSTRIA Y COMERCIO
Rad acion 03000274 00000000 Folios 286
Fecha (AMD) 2003-01-03 17 09 55
Tramite 011 PCT-PATENT 379 FASE NACI 411 PRESENTAC
Remitente: PAPO TUTISTAN DE MARIANA CORPORATIONS

286

REQUISITOS NECESARIOS PARA PRESENTACION Y AGILIZACION DEL TRAMITE

	USUARIO	RADICADOR
PATENTE DE INVENCIÓN **MODELO DE UTILIDAD**	( )	( )
I dicación d que se solicita la concesión de una patente.		( )
Datos de identificación del solicitante de la persona que se presenta la solicitud		( )
Descripción de la invención.		( )
Dibujos de ser estos pertinentes.		( )
Comprobante de Pago de las tasas establecidas.		
COMPLETA ( ) **INCOMPLETA**	( )	( )
DISEÑO INDUSTRIAL ( )		
Indicación de que se solicita el registro de Diseño Industrial	( )	( )
Datos de identificación del solicitante o de la persona que presenta la solicitud	( )	( )
Representación gráfica y fotográfica del Diseño Industrial o muestra del Material que incorpora el diseño	( )	( )
Comprobante de pago de las tasas establecidas	( )	( )
COMPLETA ( ) **INCOMPLETA**	( )	( )
ESQUEMA DE TRAZADO ( )		
Indicación de que solicita el registro de un Esquema de trazado	( )	( )
Datos de identificación del solicitante o de la persona que presenta La solicitud	( )	( )
Representación gráfica del esquema de trazado	( )	( )
Comprobante de pago de las tasas establecidas	( )	( )
COMPLETA ( ) **INCOMPLETA**	( )	( )

Firma Responsable. ANDREA POSSE

Fecha

287

Industria y Comercio
N C A

2020
Bogotá D C

Doctor
LAURA MICHELSEN NIÑO
Apoderado
FUNDACAO OSWALDO CRUZ FIOCRUZ

Oficio N° 05556

ASUNTO	Radicación N	03-00274
	Solicitud Internacional	PCT/BR2002/00036
	Fecha Inicio fase nacional	
	Trámite	011
	Evento	379
	Actuación	330
	Folios	001

Como resultado del examen de forma, realizado con fundamento el el artículo 38 de la Decisión 486 de la Comisión de la Comunidad Andina, artículo 27 del Tratado de Cooperación en Materia de Patentes (PCT) regla 51 bis del Reglamento y Circular Unica de la Superintendencia de Industria y Comercio se le comunica que:

- La solicitud no contiene la traducción de los anexos del reporte de examen preliminar internacional (Art. 36.2.b) del tratado PCT)
- La solicitud no contiene la copia del documento en que consta la cesión del derecho a la patente del inventor al solicitante o a su causante (Art. 26-k) Decisión 486)
- El solicitante debe aclarar si la solicitud corresponde al capítulo I o II del PCT por que la indicación en el petitorio no es concordante con los documentos del expediente en caso necesario adjuntar los documentos respectivos.

En cumplimiento de lo dispuesto por el artículo 39 de la Decisión 486 se le requiere para que dentro del término de dos meses siguientes a la fecha de notificación del presente oficio, complete los requisitos anteriormente enunciados. En caso de no dar cumplimiento al presente requerimiento, la solicitud se considerará abandonada y perderá su prelación

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2 literal e) del capítulo quinto del título primero de la Circular Única N° 10 de 2001

ALIX CARMENZA ESPEDES DE VERGEL
Jefe de la División de Nuevas Creaciones.

El anterior requerimiento fue notificado por fijación en lista de fecha 18 JUL 2003

202051