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सं. \ No. 1427/KOL/2010

दिनांक \ Dated the 17/01/2018

सेवा मे, \ To :

Address of Service:- K. JAYASREE C/O A-180-181, PHASE-1, OKHLA INDUSTRIAL AREA, NEW DELHI-20, INDIA
Email Id:-

विषय :- पेटेंट आवेदन संख्या 1427/KOL/2010 के संबंध मे अधिनियम की धारा 43 के तहत पेटेंट अनुदान तथा पेटेंट रजिस्टर मे प्रविष्टि की सूचना
Sub :- Intimation of the grant and recordal of patent under section 43 of the Act in respect of patent application no. 1427/KOL/2010

महोदय/महोदया,
Sir/Madam,

आपको सूचित किया जाता है कि पेटेंट अधिनिय, 1970 की धारा 12 व 13 तथा उस आधार पर बने नियम के तहत उपर्युक्त पेटेंट आवेदन के परीक्षण [व 24/10/2017 को हुई सुनवाई] के उपरांत एतद्द्वारा पेटेंट अनुदान किया जाता है। तथा पेटेंट अनुदान की प्रविष्टि 17/01/2018 को पेटेंट रजिस्टर मे कर दी गयी है।

This is to Inform you that following the examination of above mentioned patent application under section 12 and 13 of The Patents Act, 1970 and Rules made thereunder [and hearing held on 24/10/2017] a patent is hereby granted and recorded in the Register of Patents on the 17/01/2018. The Patent Certificate is enclosed herewith.

पेटेंट संख्या \ Patent No	: 291804
आवेदक का नाम \ Name Of Applicant	: MAHAJAN, LALIT
पेटेंट दिनांक \ Date of Patent	: 22/12/2010
पूर्विक्ता तिथि \ Priority Date	: 22/12/2010
परीक्षण हेतु अनुरोध दाखिल करने की तिथि \ Filling date of Request for examination	: 18/01/2012
शीर्षक \ Title	: A CHEMILUMINESCENCE IMMUNOASSAY KIT FOR EARLY DETECTION OF DENGUE IgM ANTIBODIES IN HUMAN SERUM OR PLASMA
दावों की संख्या \ Number of claims	: 1-7

उपर्युक्त पेटेंट के अनुदान का प्रकाशन अधिनियम की धारा 43 के तहत पेटेंट कार्यालय के आधिकारिक जर्नल मे किया जाएगा।
The grant of above mentioned patent will be published in the Official Journal of the patent Office under section 43 of the Act.

पेटेंट अधिनियम 1970 यथा संशोधित पेटेंट (संशोधन) नियम, 2005/ पेटेंट नियम, 2003 यथा संशोधित पेटेंट (संशोधन) नियम, 2016 की धारा 142 की उप-धारा (4) के प्रावधानों के तहत उपरोक्त प्रविष्टि की तिथि से 3 माह के भीतर इस कार्यालय मे नवीकरण शुल्क जमा किया जाना चाहिए।
The payment of renewal fee is required to be made at this office within three(3) months from the aforesaid date of recording according to the proviso in sub-section(4) of Section 142 of The Patents Act,1970, as amended by The Patents (Amendment) Act, 2005 / The Patents Rules, 2003 as amended by The Patents (Amendment) Rules, 2016.

Rajendra Lohiya
(नियंत्रक पेटेंट)
Controller of Patents

टिप्पणी / Note :

1. संशोधित नवीकरण शुल्क हेतु कृपया महानियंत्रक पेटेंट, अभिकल्प एवं व्यापार चिह्न की आधिकारिक वैबसाइट www.ipindia.gov.in पर उपलब्ध पेटेंट (संशोधन) नियम 2016 की प्रथम अनुसूची (शुल्क) देखें।
For revised renewal fees kindly refer to the First Schedule (fees) of The Patents (Amendment) Rules 2016 available on the official website of Controller General of Patents, Designs and Trade Marks www.ipindia.gov.in
2. कार्यालय द्वारा पेटेंट प्रमाणपत्र की कोई भी कागजी प्रति अलग से जारी नहीं की जाएगी।
No hard copy of Patent Certificate shall be issued separately by the office.

I Claim

1. A chemiluminescence immunoassay ***(CLIA)*** kit for analyzing the presence of dengue IgM antibody in human serum or plasma comprising enzyme conjugate, Enzyme conjugate diluent, wash buffer, negative control , positive control, calibrator, luminol substrate, substrate diluent, sample diluent and micro titer wells immobilized with anti-human IgM antibody.

2. A chemiluminescence immunoassay kit as claimed in claim 1 wherein the microtiter wells are prepared by :
   add 1µg/ml to 5 µg/ml anti-human IgM antibodies with 0.01M to 0.2M Phosphate buffer of pH 5 to 7 containing 0.05M to 0.2M Sodium Chloride;
   vortex the mixture to get a homogeneous solution;
   dispense 100µl to 200 µl of the above solution in to each well of the microtiter plate;
   incubate the wells for overnight at 2°C to 8°C;
   wash the wells with wash buffer;
   block the wells with blocking buffer;
   incubate the wells for 30 minutes to 2 hrs;
   dry the wells at 37°C.

3. A ***chemiluminescence immunoassay*** kit as claimed in claims 1 and 2 wherein the conjugate used is dengue antigen conjugated with HRPO.

4. A ***chemiluminescence immunoassay*** kit as claimed in claim 1 and 3 wherein the dengue antigens used herein refers to dengue native viral antigen or recombinant dengue envelop antigen or synthetic peptide of dengue envelop antigen or a combination there of.

5. A ***chemiluminescence immunoassay*** kit as claimed in claims 1 and 3 wherein the 10X enzyme conjugate is such that the concentration of dengue viral antigen in enzyme conjugate is 1.0µg/ml to 20µg/ml, concentration of recombinant dengue envelop antigen in enzyme conjugate is 1.0µg/ml to 20µg /ml, and concentration of synthetic peptide of dengue envelop antigen in enzyme conjugate is 1.0µg/ml to 20µg /ml or a combination there of.

6. A ***chemiluminescence immunoassay*** kit as claimed in claims 1, 3 and 4 wherein the conjugate diluent comprises of 50mM to 500mM TRIS Buffer of pH 7 to 8, 0.5% to 2% BSA and 0.05% to 0.2% Proclin.

7. A ***chemiluminescence immunoassay*** kit as claimed in claim 1 wherein the sample diluent comprises of 10mM to

100mM TRIS buffer of pH 7.0 to 8.0, 0.1M to 1M Sodium Chloride, 0.5% to 1% Casein, 0.05% to 0.1% Sodium Azide and 0.001% to 0.005% Methylene Blue Dye.

8. ***A chemiluminescence Immunoassay kit as claimed in claim 1 wherein the substrate used is luminal substrate.***

Dated this 20th day of December 2010.

K. Jayasree
Agent for the applicant

I Claim

1. A chemiluminescence immunoassay ***(CLIA)*** kit for analyzing the presence of dengue IgM antibody in human serum or plasma comprising enzyme conjugate, Enzyme conjugate diluent, wash buffer, negative control , positive control, calibrator, luminol substrate, substrate diluent, sample diluent and micro titer wells immobilized with anti-human IgM antibody.

~~2. A chemiluminescence immunoassay kit as claimed in claim 1 where in the microtiter wells are immobilized with anti-human IgM antibody.~~

3. A chemiluminescence immunoassay kit as claimed in claim 1 wherein the microtiter wells are prepared by :
   add 1µg/ml to 5 µg/ml anti-human IgM antibodies with 0.01M to 0.2M Phosphate buffer of pH 5 to 7 containing 0.05M to 0.2M Sodium Chloride;
   vortex the mixture to get a homogeneous solution;
   dispense 100µl to 200 µl of the above solution in to each well of the microtiter plate;
   incubate the wells for overnight at 2°C to 8°C;
   wash the wells with wash buffer;
   block the wells with blocking buffer;

incubate the wells for 30 minutes to 2 hrs;
dry the wells at 37°C.

4. A ***chemiluminescence immunoassay*** ~~diagnostic~~ kit as claimed in claims 1 and 2 wherein the conjugate used is dengue antigen conjugated with HRPO.

5. A ***chemiluminescence immunoassay*** ~~diagnostic~~ kit as claimed in claim 1 and 3 wherein the dengue antigens used herein refers to dengue native viral antigen or recombinant dengue envelop antigen or synthetic peptide of dengue envelop antigen or a combination there of.

6. A ***chemiluminescence immunoassay*** ~~diagnostic~~ kit as claimed in claims 1 and 3 wherein the 10X enzyme conjugate is such that the concentration of dengue viral antigen in enzyme conjugate is 1.0µg/ml to 20µg/ml, concentration of recombinant dengue envelop antigen in enzyme conjugate is 1.0µg/ml to 20µg /ml, and concentration of synthetic peptide of dengue envelop antigen in enzyme conjugate is 1.0µg/ml to 20µg /ml or a combination there of.

7. A ***chemiluminescence immunoassay*** ~~diagnostic~~ kit as claimed in claims 1, 3 and 4 wherein the conjugate diluent comprises of 50mM to 500mM TRIS Buffer of pH 7 to 8, 0.5% to 2% BSA and 0.05% to 0.2% Proclin.

8. A ***chemiluminescence immunoassay*** ~~diagnostic~~ kit as claimed in claim 1 wherein the sample diluent comprises of 10mM to 100mM TRIS buffer of pH 7.0 to 8.0, 0.1M to 1M Sodium Chloride, 0.5% to 1% Casein, 0.05% to 0.1% Sodium Azide and 0.001% to 0.005% Methylene Blue Dye.

9. ***A chemiluminescence Immunoassay kit as claimed in claim 1 wherein the substrate used is luminal substrate.***

Dated this 20th day of December 2010.

K. Jayasree
Agent for the applicant

FORM- 2

THE PATENT'S ACT, 1970

(39 OF 1970)

&

The Patents Rules, 2006

COMPLETE SPECIFICATION

(See Section 10 and rule 13)

A chemiluminescence Immunoassay kit for the detection of dengue IgM antibodies in human serum or plasma

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The following specification particularly describes the invention and the manner in which it is to be performed:

Field of Invention

The present invention relates to Dengue IgM CLIA kit for analyzing the presence of dengue IgM antibody present in human serum or plasma. More specifically, the present invention is an in vitro qualitative chemiluminescent immunoassay for the detection of dengue IgM antibody present in human serum or plasma. Further more the present invention is used for the early diagnosis of dengue by detecting dengue IgM antibodies present in human serum or plasma.

More specifically it is a Microwell chemiluminescence immunoassay for the detection of Dengue IgM antibody present in human serum or plasma.

The present kit is intended for clinical diagnostic testing.

It is a highly sensitive kit which is used for the early detection of dengue IgM antibodies present in human serum or plasma.

Background of invention

Dengue fever is the most common viral disease transmitted by mosquitoes which has become a major international public health concern. It is caused by four serotypes of dengue virus (DEN): DEN-1, DEN-2, DEN-3 and DEN-4. Dengue fever is the most important arthropod-borne viral disease affecting humans.

Dengue is transmitted by the bite of an Aedes mosquito infected with any one of the four dengue viruses. It occurs in

tropical and sub-tropical areas of the world. Symptoms appear 3—14 days after the infective bite.

Dengue viruses are transmitted to humans by Stegomyia aegypti (formerly Aedes Aegypti) mosquitoes and cause a wide range of symptoms from unapparent or mild disease to severe hemorrhagic form. Dengue virus belongs to the Flavivirus genus of the flaviviridae family. Flaviviruses are enveloped, single stranded RNA viruses. The genomic RNA is approximately 11kb in size and encodes three structural proteins: C (Core protein), M (membrane protein) and E (envelope protein). The non structural proteins are (NS): NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5.

The mosquito borne dengue viruses cause dengue fever, a severe flu like illness. The disease is prevalent in third world topical regions and spreading to subtropical developed countries including US. WHO estimates that 50 – 80 million cases of dengue fever occur worldwide each year including a potentially deadly form of disease called dengue hemorrhagic fever (DHF) and dengue shock syndrome(DSS). Primary infection with dengue virus results in a self limiting disease characterized by mild fever lasting 3 to 7 days, severe headaches with pain behind the eyes, muscles and joint pain , rash and vomiting. Secondary infection is the more common form of disease in many parts of the southeast Asia and South America. Early diagnosis of DSS is particularly important , as the patients may die within 12 to 24 hrs if appropriate treatment is not administered.

Dengue virus infections is currently detected by means of several biological tests: virus isolation on mosquito cells , viral RNA detection by RT- PCR, or serological tests such as IgM capture ELISA. RT-PCR is very expensive and its use in clinical labs are very difficult. MAC- ELISA cannot be used for early diagnosis because the IgM does not become detectable until 5 to 10 days after the onset of illness in case of primary dengue infection and until 4 to 5 days after the onset of illness in secondary infection. So we have invented a new diagnostic tool for the detection of dengue IgM antibody detection in human serum using chemiluminescence Immunoassay.

In primary infections, circulating IgM antibody to the viral coat proteins is detected 5-6 days after the onset of illness, and gradually decreases within 1-2 months of onset (6, 7). IgG antibody to dengue virus is detected approximately 14 days after onset in primary infections, and by day 2 in secondary infections. In secondary infections, IgM antibody may reappear but gradually diminishes, while IgG antibody persists, often at high titer. These patterns of dengue antibody development permit serological differentiation of primary and secondary infections. Characteristically, acute patients with primary infections have a higher IgM: IgG ratio than are found in secondary infections. Patients with secondary infections generally have higher IgG levels. Acute or recent infections are identified by a rise in antibody titer as well as high IgM levels (7).

Chemiluminescence immunoassay is now established as one of the best alternatives to conventional radioimmunoassay for the quantitation of low concentrations of analytes in complex samples. CLIA has been shown to be more sensitive than the conventional colorimetric methods and does not require long incubation or the addition of stopping reagents. Among various enzyme assays that employ light emitting reactions, one of the most successful assays is enhanced chemilumnescent immunoassay involving HRPO labeled antibody or antigen and a mixture of chemi- substrate , H_2O_2 and enhancers.

The CLIA kits are designed to detect glow based chemiluminescent reactions. The kits provide a broader dynamic assay range, superior low- end sensitivity and a faster protocol than the conventional methods.

Earlier diagnosis of dengue reduces the risk of complication such as DHF or DSS especially in countries where dengue is endemic.

Prior Arts

CN 101308138

The invention relates to a dengue virus antibody enzyme-linked immunity diagnostic kit. The kit includes an enzyme labeling board, a negative control serum, a positive control serum, an enzyme marker, a sample diluent, a concentrated washing liquid, a substrate chromogenic liquid, a stop solution and a sealing plate glue, wherein the enzyme marker contains a goat anti-human IgM-HRP; an envelope antigen

on the enzyme labeling board includes: a recombinant dengue virus type 1 envelope protein specific antigen with the amino acid sequence of SEQ ID No.1; a recombinant dengue virus type 2 envelope protein specific antigen with the amino acid sequence of SEQ ID No.3; a recombinant dengue virus type 3 envelope protein specific antigen with the amino acid sequence of SEQ ID No.5; and a recombinant dengue virus type 4 envelope protein specific antigen with the amino acid sequences of SEQ ID No .7. An early diagnosis can be made to patients infected with dengue fever for the first time through detecting dengue virus IgM antibodies in serum.

The cited prior art is entirely different from the present invention because, the cited prior art is an indirect ELISA method for the detection of dengue virus IgM antibodies in serum. Where as the present invention is a capture chemiluminescence immunoassay kit for the early diagnosis of dengue IgM antibodies present in human serum or plasma. The principle itself is different. In the cited prior art, dengue antigen is immobilized on the wells and then the anti human IgM antibody – HRPO conjugate is added. Where as in the present invention, anti human IgM antibody is immobilized on the wells and then the dengue antigen HRPO conjugate is added. The substrate used is also entirely different. The present invention is more sensitive than the cited prior art.

CN 101281197

HIV-1 p24 antigen acridine ester chemiluminescence immunity analyzing testing method pertains to the clinical blood testing analysis method technique field. The prior HIV-1

p24 antigen testing method has problems of low sensitivity, rigmarole operations and the like. The invention adopts a double antibody/antigen sandwich method, uses hydrogen peroxide and horse radish peroxidase marked on the HIV-1 p24 antigen to catalyze luminol series compounds to irradiate, and executes a qualitative and/or quantitative analysis to the HIV-1 p24 antigen. The invention has a good correlativity with the general ELISA testing method, which has a correlation coefficient of 0.996 at the instance of P no more than 0.01; the sensitivity for testing the HIV-1 p24 antigen achieves 0.5pg/ml, which reaches the testing level of the overseas advanced kit; has wide detection range in 5-6 magnitude order; conforms to the requirement of the national reference article standard of the HIV-1 p24 antigen; and the invention also has advantages of short reaction time, easier operation and the like.

The cited prior art is entirely different from the present invention because, the cited prior art is an acridine ester chemiluminescence immunoassay for the detection of HIV-1 p24 antigen. Where as the present invention is a luminol based chemiluminescnce immunoassay for the detection of dengue IgM antibodies present in human serum or plasma.

CN 101377507

The invention discloses a chemiluminescent kit for detecting a herpes simplex virus I-type IgM antibody, which comprises a negative control, a positive control, a solid-phase vector which is coated by an anti-human Mu-chain monoclonal antibody, a neutralizing antigen, a herpes simplex virus I-type

IgM antibody marked by enzyme, a chemiluminescent substrate solution and a concentrated washing solution; wherein, the enzyme is alkaline phosphatase, the chemiluminescent substrate solution contains 1, 2-diarylethane derivatives. With the kit of the invention to detect the herpes simplex virus I-type IgM antibody, the operation is simple and convenient, the sensitivity is high, the specificity is good, and the detection result coincides with those of other detection methods. The invention also discloses a preparation method of the chemiluminescent kit for detecting a herpes simplex virus I-type IgM antibody.

The cited prior art is entirely different from the present invention because, in the cited prior art, anti human enzyme conjugate is prepared and then it is added to the antigen and then the complex formed is added to the well immobilized with anti human antibody. Further, the enzyme used is Alkaline Phophatase. Where as in the present invention, anti human IgM antibodies are immobilized on the wells and then dengue antigen conjugated with HRPO are added . Further in the present invention, the luminol is used where as in the cited prior art, 1,2-diarylethane derivatives are used instead of luminol. Hence the present invention is entirely different from the cited prior art.

CN 101221170

The invention relates to an enzymatic chemiluminescence substrate system which is highly efficient and stable. The enzymatic chemiluminescence substrate system is composed of two components of A solution and B solution, the A

solution contains a chemiluminescence substrate, the B solution contains a compound enhancer, the A solution and the B solution are mixed by the equal volumes before using; wherein, the chemiluminescence substrate in the A solution is Luminol sodium salt or the derivative thereof, and the compound enhancer in the B solution is 3-chloro-4-hydroxy acetanilide or sodium perborate tetrahydrate. The substrate system has various advantages of strong luminous signal, rapid onset of action, ability to achieve a luminous platform within 2 minutes, long luminous duration, and so on. In addition, the invention also provides a chemiluminescence immunoassay kit which contains the substrate system.

The cited prior art is entirely different from the present invention because in the cited prior art, the chemiluminescence substrate in the A solution is Luminol sodium salt or the derivative thereof, and the compound enhancer in the B solution is 3-chloro-4-hydroxy acetanilide or sodium perborate tetrahydrate. The enhancer solution is entirely different from the present invention.

JP 2008013777

To provide a new composition for chemiluminescence, facilitating the utilization as a display, and having excellent long-term storage stability and large chemiluminescent effects, and to provide a method of chemiluminescence by using the composition.; The composition for chemiluminescence contains an oxalic acid ester, a fluorescent material, hydrogen peroxide and an organic strong acid, and has pH 3.6-4.2, wherein the reaction of the

oxalic acid with the hydrogen peroxide is inhibited. The composition for chemiluminescence contains the oxalic acid ester, the hydrogen peroxide and the organic strong acid, and has pH 3.6-4.2, wherein the reaction of the oxalic acid with the hydrogen peroxide is inhibited.

The cited prior art is entirely different from the present invention because, in the cited prior art, oxalic acid ester, a fluorescent material and Hydrogen peroxide and an organic strong acid are used as luminol which is having pH 3.6 to 4.2. Where as the present invention of the applicant is using a luminol which is Phenol based and is having a pH of 7.0 to 7.6.

CN200986546

The utility model belongs to the field of medical diagnosis and detection technologies, and discloses a novel enzyme-aided chemiluminescence immune detection system. A frame is orderly provided with a series of in-vitro diagnosis kits, a vibrator, an isothermal incubation device, a plate washing device, a chemiluminescence detection device and a fitted control and analysis managing device. No hazardous reagent is used in the test with this utility model, which prevents pollution and injury to operators of radionuclide, and greatly shortens detection period. The utility model is characterized in high sensitivity, easy operation and convenient standardized operation, and can be used for biology and medicine research and clinical test diagnosis.

The cited prior art is entirely different from the present invention because the cited prior art comprises of diagnostic kits, vibrator, an isothermal incubation device, a plate washing device , a chemilminescence detection device and a fitted control and analysis managing device. It does not disclose the disease for which it is used or the antigen or antibodies used for the preparation of the test device. It does not disclose the luminol and the enhancers used in the present invention.

CN 1880957

The invention discloses a synthetic method and system of enzymatic chemical luminous substrate in the immune diagnosis technological domain, which consists of A and B two groups of component, wherein group A is composed of enzymatic chemical luminous substrate, reinforcer and amino acid; group B is composed of amino acid oxidase and stabilizer; the general formula of enzymatic chemical luminous substrate is as follows, wherein R1 is halogen; R2 is alkyl carboxylase, alkyl mahogany sulfonate or alkyl ammonium salt with 3-8 C atoms. The invention possesses strong luminous signal, long lasting time, which lengthens the reserving period at normal temperature.

In the cited prior art, the group A solution comprises of luminous substrate, re- inforcer and amino acid and the group B composed of amino acid oxidase and stabilizer. Where as in the present invention of the applicant, Group A comprises of luminol and enhancer and group B comprises

of Perborate or Hydrogen peroxide. Hence the present invention is entirely different from the cited prior art.

CN 1880958

The invention discloses an enzymatic chemical luminous immune detecting system in the medical diagnosis technological domain, which comprises the following parts: computer, printer, washing machine, 37 deg.c incubator, mini-sized turbonator, serial external detecting diagnosis agent, high-effective stable enzymatic chemical luminous immune detecting system and chemical luminous device based on standard 96 holes transparent microporous board, wherein the serial external diagnosis detecting agent contains first-function series, tumour series, gonadal hormone series, contagion series, myocardial symbolic material series, diabetic series, aristogenic fertilitatic series and other forty or more kinds. The invention can finish qualitative quantitative detection, which can print the detected result of different items printed on the same assay list.

The cited prior art is entirely different from the present invention because in the cited prior art, the detecting system comprises of computer, printer, washing machine , incubator, mini sized turbonator, serial external detecting diagnosis agent, high effective stable enzymatic chemical luminous immune detecting system and chemical luminous device based on standard 96 holes transparent microporous board. It does not disclose the antigen or antibody immobilized on the device or the disease for which the kit is used.

Where as the present invention is a chemiluminescence immunoassay kit for the detection of the dengue IgM antibodies present in the human serum or plasma. In the present invention, the antihuman IgM antibodies are immobilized on the wells. The conjugate used is dengue antigen conjugated with HRPO. It uses a luminol and the phenol based enhancers and the Hydrogen peroxide.

JP 11253197

To provide a method for immunologically assaying an antigen or antibody by a chemiluminescence method using a peroxidase as marker. This method, an enzyme immunoassay using a chemiluminescence method with a peroxidase as marker, comprises immunologically assaying an antigen or antibody in high sensitivity using a chemiluminescence method which uses a chemiluminescence reagent prepared by contacting an N,N'-disubstituted-9,9'-bisacridinium salt of the general formula (R<1> and R<2> are each an alkyl, aryl or halcaryl; R<3> , R<4> , R<5> and R<6> are each H, an alkyl, aryl, alkoxy, aryloxy or halogen; X is an n-valent anion; (n) is 1 or 2) with a reducing agent in the presence of an N,N-disubstituted carboxylic acid amide compound and formic acid.

The cited prior art is entirely different from the present invention because, in the cited prior art, the luminol used is N,N' – disubstituted – 9,9'- bisacridinium salt with a reducing agent in the presence of N,N- disubstituted carboxylic acid amide compound and formic acid.

Where as the present invention of the applicant is a chemiluminescence immunoassay kit for the detection of dengue IgM antibodies present in human serum or plasma. In the present invention, the antihuman IgM antibodies are immobilized on the wells. The conjugate used is dengue antigen conjugated with HRPO. The kit contains luminol, enhancers and Hydrogen peroxide. Hence the present invention is entirely different from the cited prior art.

JP 10090186

To measure a quantity of emitted light with reference to the amount of an enzyme with good reproducibility in the measurement of the quantity of emitted light of chemiluminescence generated by the reaction of a dioxetane compound with the enzyme which is obtained by using the compound as a substrate by a method wherein the surface of a reaction liquid is covered with another liquid whose specific gravity is lower than that of the liquid and the reaction is performed. A reaction which generates chemiluminescence may be performed in such a way that, e.g. 1,2-dioxetane and, e.g. the substrate of an alkali phosphate are mixed with an aqueous solution and that the surface of a reaction liquid is covered with a liquid whose specific gravity is lower than that of the reaction liquid.; The liquid which covers the surface may be a liquid which is transparent to an extent that the measurement of chemiluminescence is not disturbed, its more preferable boiling point is 120 deg.C or higher, the liquid is desirably nonvolatile, and a mineral oil, silicone oil or the like is enumerated as a specific example. The measuring condition of a temperature or the like may be suitably

according to a substance to be measured, etc., and 30 to 40 deg.C is especially preferable. The quantity of emitted light of chemiluminescence may be measured by a measuring apparatus, a measuring implement or the like which is used normally to measure the quantity of emitted light generally.

The cited prior art is entirely different from the present invention because, in the cited prior art, the reaction which generates the chemiluminescence may be performed in such a way that 1, 2- dioxetae and the substrate of an alkali phosphate are mixed with an aqueous solution and that the surface of the reaction liquid is covered with a liquid whose specific gravity is lower than that of the reaction liquid. The liquid which covers the surface may be a liquid which is transparent to an extent that the measurement of chemiluminescnece is not disturbed. The quantity of the emitted light of chemiluminescence may be measured by a measuring apparatus. Where as the present invention is a luminol based chemiluminescence method which uses enhancer solution and Hydrogen peroxide. In the present invention, the antihuman IgM antibodies are immobilized on the wells. The conjugate used is dengue antigen conjugated with HRPO. It is user friendly and is more sensitive than the conventional methods.

EP 0516948

A chemiluminescent method using a protected enhancer compound which is triggered by a hydrolytic enzyme and then enhances a chemiluminescent reaction is described. The reaction involves an amino substituted acylhydrazide which is

activated to produce light by a peroxide compound and a peroxidase in the presence of the activated enhancer compound. The result is enhanced generation of light from the reaction. The hydrolytic enzyme is present alone or as a label in an immunoassay, DNA probe assay or other assay wherein the hydrolytic enzyme is bound to a reporter molecule.

The cited prior art is entirely different from the present invention because in the cited prior art, the buffer used is Phosphate buffer which is entirely different from the present invention. Further to that in the cited prior art, Mg(OAc)2 and p- phenylphenol acetate are used which are not at all used in the present invention. The kit comprises of an amino substituted acylhydrazide which is activated to produce light by a peroxide compound and a peroxidase in the presence of the activated enhancer compound. The enhancer solution used in the cited prior art is also entirely different.

JP 5018971

To obtain an antigen/antibody measuring method which can largely change the light emitting amount in corresponding to the concentration variation of antigens or antibodies to be measured and is improved in measurement accuracy. This antigen/antibody measuring method performs antigen-antibody reaction by dipping an antibody electrode 1 with antibodies 2 being fixed to its surface in a solution to be inspected and again performs the antigen-antibody reaction by adding marked antibodies 5 marked with a chemiluminescent acridinium derivative 4 to the solution to be

inspected, and then, fixes the marked antibodies 5 to the antigens 3 fixed to the surface of the electrode 1. Then, after washing the surface of the electrode 1 and separating unfixed antibodies 5, the concentration of the antigens 3 in the solution to be inspected is measured by causing chemiluminescence by electrochemically reducing the oxygen dissolved in an electrolyte by using the electrode 1 as a working electrode and measuring the emitted quantity of light. This method is effective for a solution containing antigens at a concentration of about 10<-11>-10<-5>g/ml and the emitted quantity of light can be changed largely against the variation of the antigen concentration and the measurement accuracy is remarkably improved.

The cited prior art is entirely different from the present invention because in the cited prior art, the antigen / antibody measuring method performs antigen – antibody reaction by dipping an antibody electrode with antibodies being fixed to its surface in a solution to be inspected and again performs the antigen – antibody reaction by adding marked antibodies marked with a chemiluminescent acridinum derivative to the solution to be inspected and then fixes the marked antibodies to the antigens fixed to the surface of the electrode. Then after washing the surface of the electrode 1 and separating unfixed antibodies, the concentration of the antigen in the solution to be inspected is measured by causing chemiluminescence by electrochemically reducing the oxygen dissolved in an electrolyte using the electrode 1 as a working electrode and measuring the emitted quantity of light. Where as the present invention is a chemiluminescence

immunoassay using luminol, enhancer solution and Hydrogen per oxide. In the present invention, the antihuman IgM antibodies are immobilized on the wells. The conjugate used is dengue antigen conjugated with HRPO It is a more sensitive test than the conventional methods.

Dengue virus infection is currently detected by means of several biological tests: virus isolation on mosquito cells, viral RNA detection by RT- PCR, or serological tests such as IgM capture ELISA. RT-PCR is very expensive and its use in clinical labs is very difficult.

In early acute phase of infection, the specific IgM class antibodies are the first to appear in serum or plasma 1 to 4 week after the onset of the symptoms and lasts for up to 3 months. IgG class antibodies appear later and remain elevated throughout the patient's life. Detection of IgM is indicative of the recent current infection and IgG is a marker for past exposure to the causative agent. Specific IgM & IgG responses to a particular infectious agent can be measured by antibody based diagnostic test such as CLIA.

So we have invented a new diagnostic tool for the early detection of dengue IgM antibody in human serum or plasma using Chemiluminescence Immuno Assay. It is having high sensitivity and specificity for detecting the presence of dengue IgM antibodies. Development of sensitive, type specific dengue diagnostic system is crucial because of the explosive growth in dengue transmission.

Objectives

It is an object of the present invention to develop a chemiluminescence immuno assay diagnostic kit for the diagnosis of dengue IgM antibodies present in human serum or plasma.

The other object of the present invention is to provide a new diagnostic and qualitative immunoassay kit for the detection of dengue IgM antibodies present in human serum or plasma.

It is another object of the present invention to develop a kit for the diagnosis of dengue IgM antibodies with high sensitivity and specificity.

Yet another object of the present invention is to develop an inexpensive and simple diagnostic kit for the detection of dengue IgM antibodies without compromising the sensitivity and specificity.

Yet another object of the present invention is to provide a simple kit with long shelf life.

Summary of the invention

Dengue IgM CLIA is a sensitive and accurate chemiluminescence immunoassay for the qualitative detection of dengue IgM antibodies present in human serum or plasma. It is designed for the in vitro- qualitative detection of Dengue IgM antibodies in human serum or plasma and is used as a screening test for testing of collected blood

samples suspected of dengue. The kit detects all four subtypes: DEN1, DEN2, DEN3 and DEN 4 of dengue virus.

According to the present invention, the kit for analyzing the presence of dengue IgM antibodies present in human serum or plasma comprises of 8 X 12 matrix of 96 wells , each of which are about 1cm high and 0.7cm in diameter made of polystyrene. The micro titer wells are prepared by immobilizing anti human IgM antibodies on the microtiter wells. The coating buffer consists of Phosphate buffer of pH 5 – 7 containing Sodium Chloride.

In an embodiment of the present invention, the dengue IgM CLIA is prepared by adding antihuman IgM antibody in Phosphate buffer solution of pH 5.0 to 7.0 followed by mixing it thoroughly to obtain a homogeneous solution and there after dispensing the antibody solution in the well. Incubate the wells overnight at 2 to 8°C.

Wash the wells with PBST (Phosphate buffer and NaCl and T-20). Then block the wells with blocking solution consisting of blockers like BSA or Casein or detergent or sera or a combination there of. Then incubate for 3 hrs at room temperature (20°C to 30°C). Discard the solution and wash with PBS and then dry the plates at 2°C to 8°C.

Then enzyme conjugate is prepared by conjugating dengue antigen with enzyme such as HRPO. The antigens used for the conjugation are dengue native viral antigen or recombinant dengue envelop antigen or synthetic peptide of

dengue envelop antigen or a combination there of. The conjugate of each antigen is separately prepared by conjugating the antigen with HRPO.

Sample and controls are added to the wells. Dengue IgM antibodies if present in the sample binds to the immobilized antihuman IgM antibodies on the microwells. The plates are then washed to remove unbound material. Horse radish peroxidase. (HRPO) conjugated dengue antigen is added to each well. Then cover the plates and incubate at 37°C . The excess of the enzyme conjugate is again removed with the diluted wash buffer. This dengue antigen conjugate will bind to dengue specific IgM antibodies which will complex with anti human IgM antibodies.

In the next step, the freshly prepared substrate solution containing luminol and Hydrogen peroxide are added to the wells and incubated for 5 minutes. Light intensity will develop in proportion to the amount of dengue IgM antibodies present in the specimen. The chemiluminescent substrate reaction is read by the microplate luminometer for RLU (Relative Light Unit)

The CLIA kit for the detection of the dengue IgM antibody is having a sensitivity of 97.15% and specificity of 99.84%.

Brief description with drawing

Figure 1 shows the principle of the test.

Figure 2 shows the microtiter wells.

Detailed description

Dengue IgM CLIA is a chemiluminescent Immuno assay kit for the early detection of dengue IgM antibodies present in human serum or plasma. Dengue IgM CLIA kit for the early detection of dengue IgM antibodies present in human serum or plasma comprises of 8X12 matrix of 96 wells (Figure 2) about 1 centimeter high and 0.7 centimeter in diameter made of white polystyrene. The kit for the detection of dengue IgM antibodies comprises of a microtiter well immobilized with anti human IgM antibodies, enzyme conjugate, conjugate diluent, substrate solution, substrate diluent, calibrator, negative control, positive control , diluent buffer and sample diluent. The microtiter wells are prepared by immobilizing anti human IgM antibodies. Specimens and controls are added to the well. Dengue IgM antibodies if present in the sample will bind to the anti-human IgM antibody on the surface of the well. Then enzyme conjugate is added to each well. The conjugate will bind to the anti human IgM antibody – dengue IgM antibody complex. Finally the substrate solution containing luminol and hydrogen peroxide is added to the wells and incubated. The light intensity will develop in proportion to the amount of dengue IgM antibodies present in the specimen. The chemiluminescent substrate reaction is read by the luminometer for RLU(relative light unit).

Preparation and chemical composition of the microwells

In an embodiment of the present invention, the microtiter wells are prepared by adding 1µg/ml to 5 µg/ml of anti human IgM antibody with 0.01M to 0.2M Phosphate buffer of pH 5 to pH 7 which contains 0.05M to 0.2M Sodium Chloride. Vortex

the mixture to get a homogeneous solution and then filter the solution. Then dispense 100 µl to 200 µl of the solution into each well of the microtiter plate. Incubate the wells for overnight at 2 to 8°C for 16 to 18 hrs. Then wash the wells with the wash solution: PBST (Phosphate buffer, NaCl and T-20) of pH 7.0 to 8.0 and then block the wells with blocking buffer consisting of BSA, Casein etc. Then incubate the wells for 30 minutes for 2 hrs and then dry at 37°C.

Preparation and chemical composition of enzyme conjugate

The enzyme conjugate is prepared by conjugating dengue antigen with HRPO. The dengue antigens used are dengue native viral antigen or recombinant dengue envelop antigen or synthetic peptide of dengue envelop antigen or a combination there of. These antigens are conjugated separately with HRPO.

For conjugation of dengue native viral antigen, take 10mg to 30mg of enzyme such as HRPO and dissolve in 1.0ml of distilled water. Add 0.005M to 0.05M Sodium per iodide and mix with HRPO. Shake the solution well and incubate the mixture for 10 to 200minutes. Mix the activated HRPO solution with 1mg/ml to 10mg/ml of dengue Native viral antigen. Allow the solution to maturize for 2hrs at ambient temperature. Add 20mM to 500mM Borohydride solution with the above solution and leaving it to maturize for 30 minutes to 300 minutes at ambient temperature. Then add 50mM to 500mM Ethanol amine solution. Allow the solution to maturize for 60 minutes followed by dialyzing. Then dilute the solution

obtained in Enzyme conjugate Diluent buffer in a ratio of 1:2 to 1:200;

For conjugation of recombinant dengue envelop antigen, take 10mg to 30mg of enzyme such as HRPO and dissolve in 1.0ml of distilled water. Add 0.005M to 0.05M Sodium per iodide and mix with HRPO. Shake the solution well and incubate the mixture for 10 to 200minutes. Mix the activated HRPO solution with 1mg/ml to 10mg/ml of recombinant dengue envelop antigen. Allow the solution to maturize for 2hrs at ambient temperature. Add 20mM to 500mM Borohydride solution with the above solution and leaving it to maturize for 30 minutes to 300 minutes at ambient temperature. Then add 50mM to 500mM Ethanol amine solution. Allow the solution to maturize for 60 minutes followed by dialyzing. Then dilute the solution obtained in Enzyme conjugate Diluent buffer in a ratio of 1:2 to 1:200;

For conjugation of synthetic peptide of dengue envelop antigen, take 10mg to 30mg of enzyme such as HRPO and dissolve in 1.0ml of distilled water. Add 0.005M to 0.05M Sodium per iodide and mix with HRPO. Shake the solution well and incubate the mixture for 10 to 200minutes. Mix the activated HRPO solution with 1mg/ml to 10mg/ml of synthetic peptide of dengue envelop antigen. Allow the solution to maturize for 2hrs at ambient temperature. Add 20mM to 500mM Borohydride solution with the above solution and leaving it to maturize for 30 minutes to 300 minutes at ambient temperature. Then add 50mM to 500mM Ethanol amine solution. Allow the solution to maturize for 60 minutes

followed by dialyzing. Then dilute the solution obtained in Enzyme conjugate Diluent buffer in a ratio of 1:2 to 1:200.

Preparation of 10X Enzyme conjugate

In an embodiment of the present invention, 10 X enzymes conjugate is prepared by mixing a unique concentration of the above mentioned conjugates with enzyme conjugate diluent buffer. The 10X enzyme conjugate is prepared such that the concentration of dengue native viral antigen in enzyme conjugate is 1.0µg/ml to 20 µg/ml; the concentration of recombinant dengue envelop antigen in enzyme conjugate is 1.0µg/ml to 20 µg/ml and the concentration of synthetic peptide of dengue envelop antigen in enzyme conjugate is 1.0µg/ml to 20 µg/ml.

Preparation and chemical composition of Enzyme conjugate diluent buffer.

The enzyme conjugate diluent buffer is prepared by mixing 10mM to 100mM PBS, 0.01% to 1% Phenol, 0.1% to 5% BSA , 1% to 10% FCS(Fetal Calf Serum) ; the final pH of the solution is 7.0 to 7.5.

Preparation and chemical composition of Conjugate diluent

The conjugate diluent buffer comprises of 50mM to 500mM TRIS Buffer of pH 7 to 8, 0.5% to 2% BSA, 0.05% to 0.2% Proclin.

Preparation and chemical composition of Luminol substrate

Further embodiment of the present invention is chemiluminescent substrate solution which is prepared as follows.

Take 100mM to 500mM Tris buffer solution of pH 7.0 to 7.6. To this add, 0.5mM to 100mM Luminol, 1% to 5% DMSO (Dimethyl Sulphoxide), 0.1mM to 10mM 4-Methoxy Phenol or 0.1mM to 10mM 4 – Hydroxy bi phenyl or 0.1mM to 10mM 4 – (1 Hydroxy Pyrrol-1-yl) Phenol or 0.1mM to 10mM 2-Hydroxy Cinnamic acid or 0.1 mM to 10mM 4-Bromophenyl Boronic Acid or a mixture there of. Mix well to get a homogeneous solution. Then add 0.1mM to 10mM EDTA and 0.01% to 0.1% 2-chloro acetamide.

Preparation and chemical composition of Luminol diluent

In further embodiment of the present invention, the luminol substrate diluent is prepared as follows.

To the 0.01M to 0.2M Citrate Phosphate Buffer solution of pH 5.0 to 6.0, add 0.006% to 0.06% H_2O_2. To this solution, add preservatives like Gentamycin Sulphate (1µg to 100µg/ml) and 2- Chloro acetamide(0.01% to 0.1%) .

Preparation and chemical composition of Sample diluent

In an embodiment of the present invention, the sample diluent comprises of 10mM to 100mM TRIS buffer, 0.1M to 1M Sodium Chloride, 0.5% to 1% Casein, 0.05% to 0.1% Sodium Azide and 0.001% to 0.005% Methylene blue dye.

Wash buffer (25X)

In further embodiment of the present invention, wash buffer is prepared by mixing 100mM to 500mM Phosphate Buffer Saline with 0.25% - 1.25% Tween -20 and pH of 1X is between 7.0 – 7.8.

Preparation and chemical composition of diluent for negative control/ positive control / Calibrator

Prepare the diluent solution by mixing 10mM to 100mM TRIS buffer of pH 7.0 to 8.5, 0.05% to 0.5% Sodium Chloride, 1% to 5% Ethylene Glycol, 0.1% to 0.5% BSA and 0.05% to 0.2 % Sodium Azide.

Preparation of negative control

To the above diluent solution, add normal human serum in the ratio 1: 10. i.e. 1 ml human serum with 10 ml diluent. Mix well to get a homogeneous solution.

Preparation of Calibrator

Calibrator is prepared by mixing the positive sera with above diluent in the ratio 1:100 to 1: 500.

Preparation of Positive control

Positive control is prepared by mixing the positive sera with the above diluent in the ratio 1:10 to 1:50.

Principle

Dengue IgM CLIA is a chemiluminescence Immuno Assay based on Capture Immunoassay principle (Figure 1). The microwells are immobilized with anti human IgM antibodies.

The sample and controls are added to the microtiter wells and incubated. Dengue IgM antibodies if present in the sample will bind with the antihuman IgM antibodies adsorbed on the surface of the wells. Then the plates are washed to remove unbounded material. Horse Radish Peroxidase (HRPO) conjugated with dengue antigen (sero type 1-4) is added to each well. This dengue antigen conjugate will bind to the dengue specific IgM antibodies which will complex with antihuman IgM antibodies. Finally substrate solution containing Luminol and Hydrogen peroxide is added to the wells and incubated. Light intensity will develop in proportion to the amount of Dengue IgM antibodies present in the specimen. The chemiluminescence substrate reaction is read by Luminometer for RLU.

Test procedure:

To the microwell, add negative control. To the other two wells, add positive control. Add calibrator in another two wells, Add sample diluent in each well followed by the addition of sample. Cover the plate and then incubate in an incubator at 37°C. After the incubation, take out the plate and then wash the wells with working wash buffer. Then enzyme conjugate is added to the wells. Then cover the wells and incubate at 37°C. Aspirate the wells and then add substrate solution in each well. Incubate at room temperature in dark for 5 minutes. Read the RLU value within 3 to 10 minutes after the addition of the substrate.

Test Validity

1) RLU of NCx/ cut off value must be < 0.3. If it is not so, then run is valid and must be repeated.

2) RLU of PCx/ cut off must be > 10.0. If it is nt so, the run is invalid and must be repeated.

a) Cut off value = mean RLU of calibrator x factor (f).

Eg. If the factor is 0.5 , then,

Cut off value = 6530X0.5 = 3265

b. Calculation of Sample index Ratio: Calculate the sample Index ratio as follows.

$$\text{Sample Index Ratio} = \frac{\text{RLU of sample}}{\text{Cut off value}}$$

e.g. RLU of sample = 155655

Cut off value = 3265

Sample Index Ratio = 155655/ 3265 = 47.67

Interpretation of results.

Test specimens with RLU value less than the cut off value are non- reactive and may be considered as negative for Dengue IgM antibodies .

Test specimens with RLU value greater than or equal to the cut off value are reactive for Dengue IgM antibodies.

Test specimens with RLU value within 10% below the cut off should be considered suspect for the presence of dengue IgM antibodies and should be retested in duplicate.

Specimens with RLU Value equal to or greater than the cut off value are considered initially reactive by the kit criteria. Original specimen should be retested in duplicate.

If both duplicate retest sample RLU value is less than the cut off value, the specimen is considered non reactive.

If any one of the duplicates retest sample RLU value is equal to or greater than the cut off or both duplicate retest value are equal to or greater than the cut off, the specimen is considered reactive by the Kit criteria.

The invention can be described more clearly with reference to the following examples but these are for illustrative purpose only and same should not be construed to restrict the scope of the invention.

Working examples

Preparation of microtiter wells

The microtiter wells are prepared by adding 2.0µg/ml of anti human IgM antibody with 0.1M Phosphate buffer of pH 6.0 containing 0.15M Sodium Chloride. Vortex the mixture to get a homogeneous solution, filter the solution and dispense 100µl of the above solution in the microtiter plate wells. Incubate the wells for overnight at 2°C to 8°C. Then wash the wells three times with PBST(Phosphate buffer , NaCl and T-20) of pH 7.0 and then block the wells with PBS, BSA. Then incubate the wells for 30 minutes for 2 hrs and then dry at 37°C.

Preparation and chemical composition of enzyme conjugate

The enzyme conjugate is prepared by mixing the conjugates obtained by conjugating 5mg/ml of dengue native viral

antigen with HRPO and 5mg/ml of recombinant dengue envelop antigen with HRPO.

Take 10mg of enzyme such as HRPO and dissolve in 1.0ml of distilled water. Add 0.01M Sodium per iodide and mix with HRPO. Shake the solution well and incubate the mixture for 60 minutes. Mix the activated HRPO solution with 5mg/ml dengue Native viral antigen. Allow the solution to maturize for 2hrs. at ambient temperature. Add 20mM to 500mM Borohydride solution with the above solution and leaving it to maturize for 30 minutes at ambient temperature. Then add 50mM to 500mM Ethanol amine solution. Allow the solution to maturize for 60 minutes followed by dialyzing. Then dilute the solution obtained in Enzyme conjugate Diluent buffer in a ratio of 1:2 to 1:200;
And

Take 10mg of enzyme such as HRPO and dissolve in 1.0ml of distilled water. Add 0.01M Sodium per iodide and mix with HRPO. Shake the solution well and incubate the mixture for 60 minutes. Mix the activated HRPO solution with 5mg/ml of recombinant dengue envelop antigen. Allow the solution to maturize for 2hrs. at ambient temperature . Add 20mM to 500mM Borohydride solution with the above solution and leaving it to maturize for 30 minutes at ambient temperature. Then add 50mM to 500mM Ethanol amine solution. Allow the solution to maturize for 60 minutes followed by dialyzing. Then dilute the solution obtained in Enzyme conjugate Diluent buffer in a ratio of 1:2 to 1:200.

Preparation of 10X Enzyme conjugate

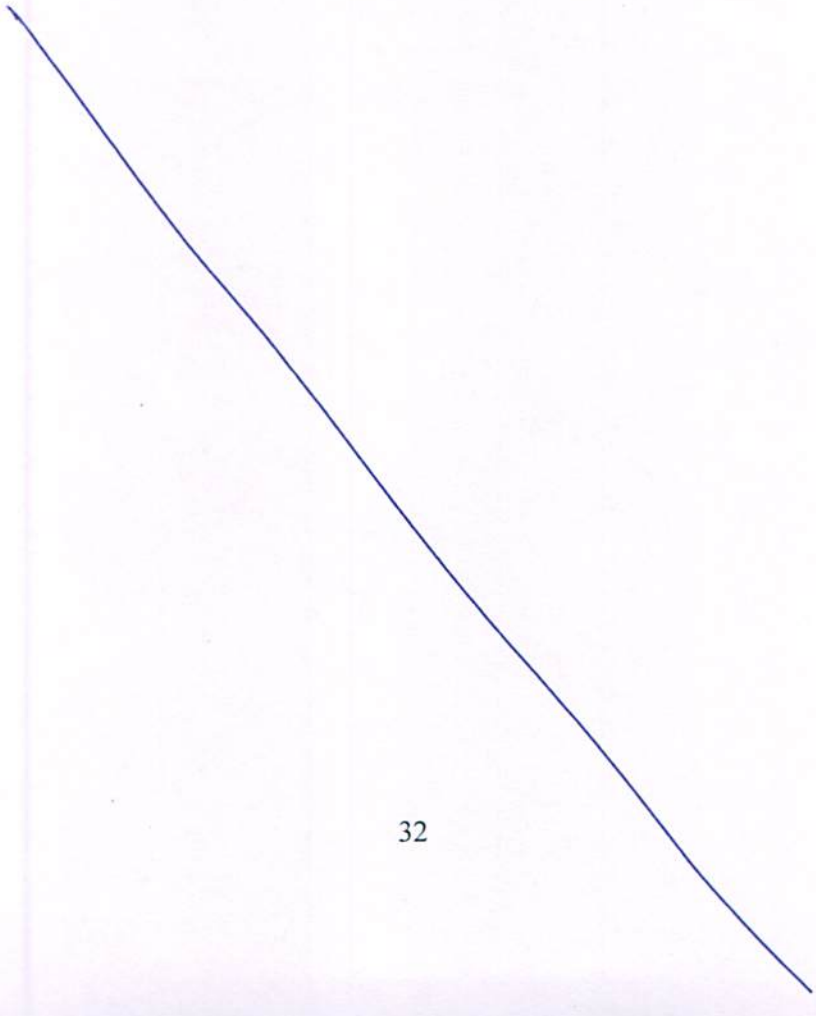
For 10X enzyme conjugate, the concentration of the basic components of conjugate such as dengue native viral antigen in HRPO conjugate is 15µg/ml, and concentration of recombinant dengue envelop antigen in HRPO conjugate is 15µg/ml.

Preparation and chemical composition of conjugate diluent

Conjugate diluent comprises of 100mM TRIS buffer of pH 8.0, 1% BSA and 0.1% Proclin.

Preparation and chemical composition of Sample diluent

The sample diluent of the present invention comprises of 10mM TRIS buffer, 0.1M Sodium Chloride, 0.5% Casein, 0.05% Sodium Azide and 0.002% Methylene blue dye.



Abstract

A chemiluminescence Immunoassay kit for the detection of dengue IgM antibodies in human serum or plasma

The present invention, dengue IgM CLIA kit is designed for the invitro qualitative detection of dengue IgM antibodies present in human serum or plasma. It is an in vitro qualitative chemiluminescence Immunoassay for the detection of dengue IgM antibodies present in human serum or plasma. It is a highly sensitive technique which detects the dengue IgM antibodies present in human serum or plasma. The kit comprises of a microwell which is immobilized with anti human IgM antibodies. The kit further comprises of enzyme conjugate, sample diluent, conjugate diluent, Enzyme conjugate diluent buffer, luminol substrate, substrate diluent , positive control, negative control , calibrator and wash buffer.

Lalit Mahajan

Applicant

LALIT MAHAJAN

IP/1427/KOL/2010

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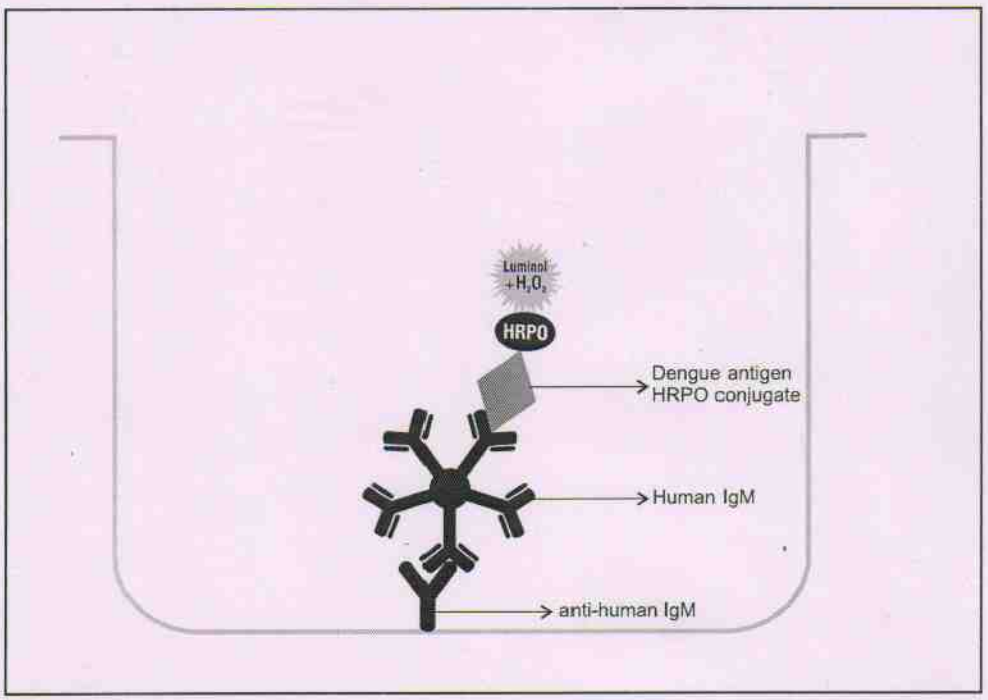

(Fig. 1)

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K. Jayasree
Registered Patent Agent

LALIT MAHAJAN

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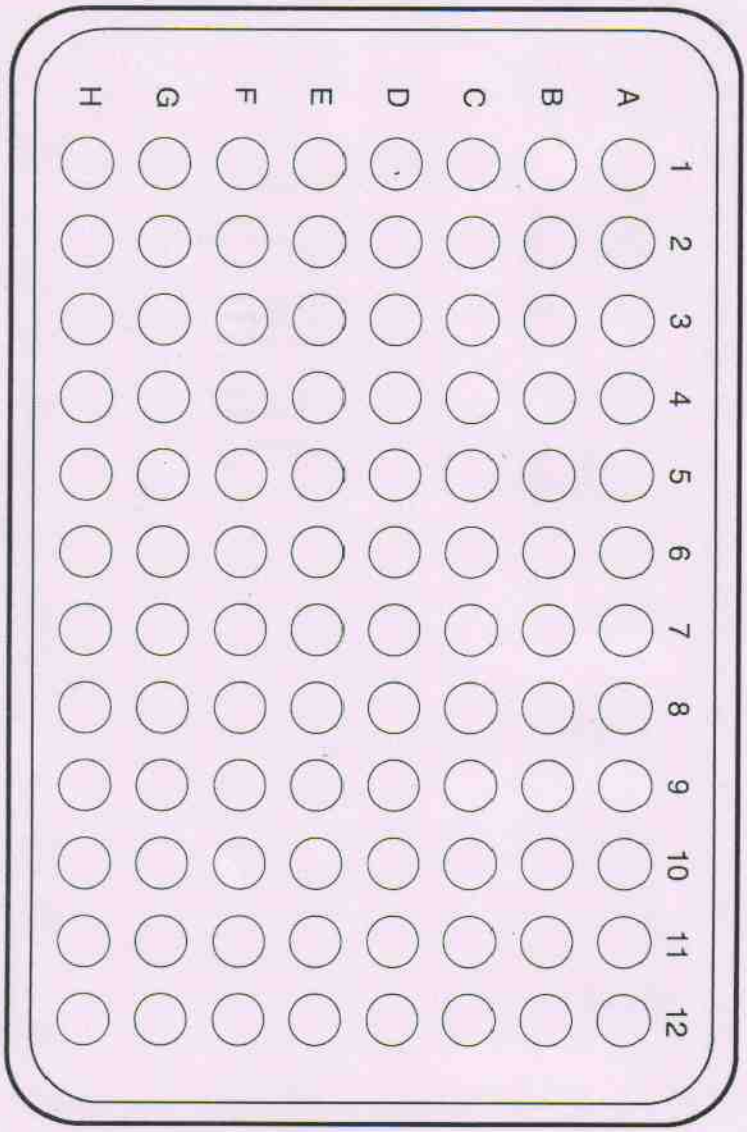

Figure-2

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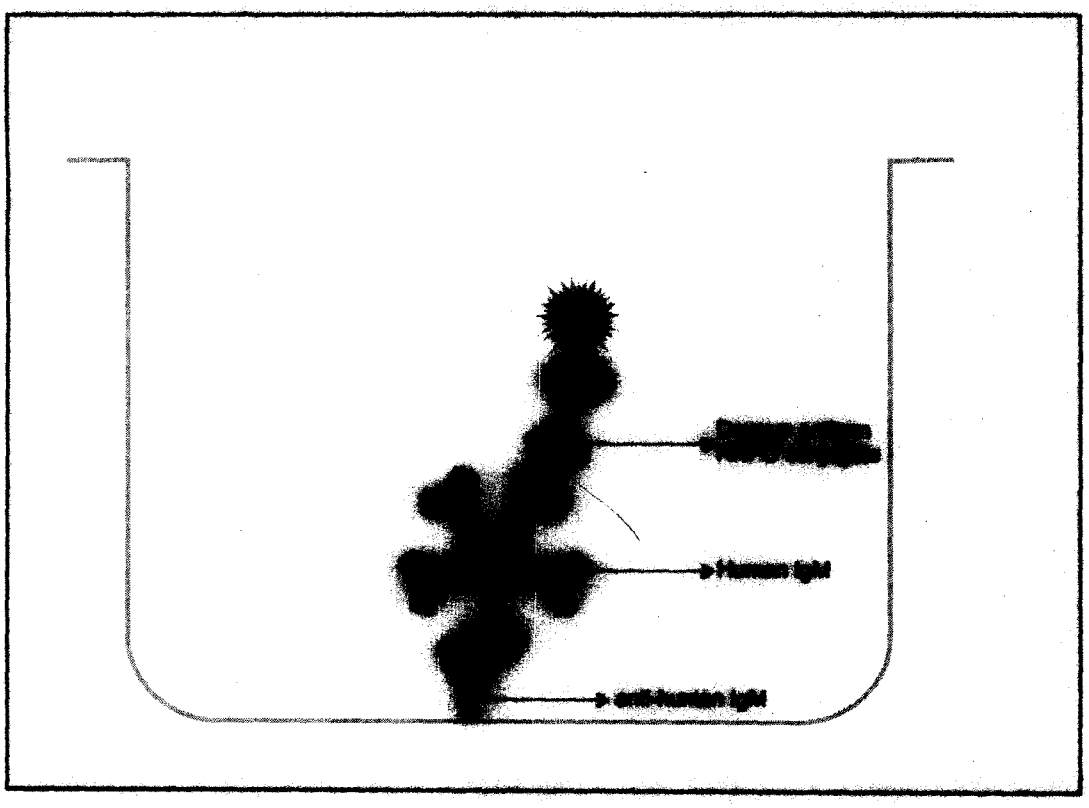
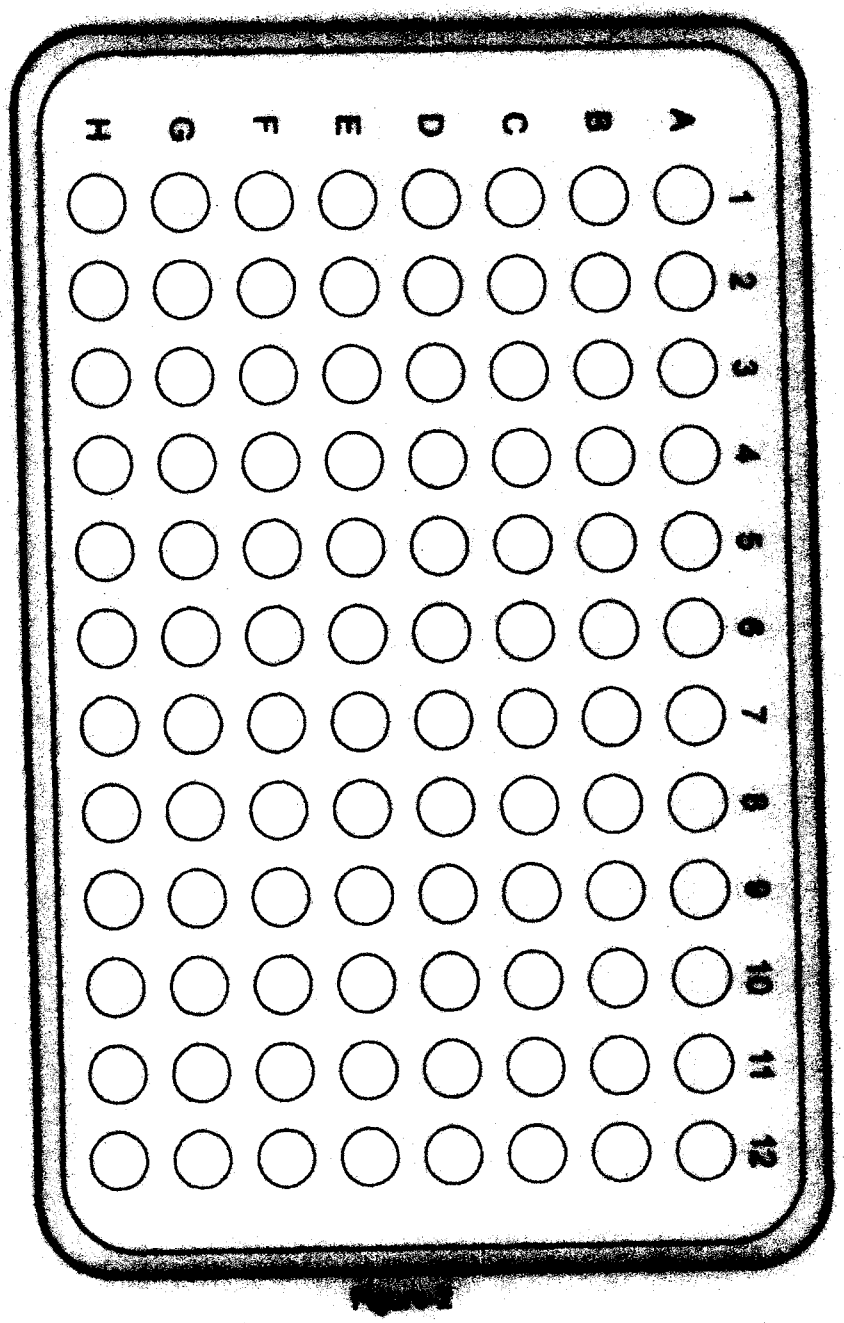
Registered Patent Agent



(Fig. 1)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