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पेटेंटी / Patentee	:	MGM Institute Of Health Sciences (MGMIHS), Deemed University u/s 3 of UGC Act, 1956

प्रमाणित किया जाता है कि पेटेंटी को उपरोक्त आवेदन में यथाप्रकटित QUANTUM DOT POWERED IP-10 ANTIBODY BASED KIT FOR LATENT TB AND TB ANTIGEN DETECTION नामक आविष्कार के लिए, पेटेंट अधिनियम, १९७० के उपबंधों के अनुसार आज तारीख 15th day of November 2017 से बीस वर्ष की अवधि के लिए पेटेंट अनुदत्त किया गया है।

It is hereby certified that a patent has been granted to the patentee for an invention entitled QUANTUM DOT POWERED IP-10 ANTIBODY BASED KIT FOR LATENT TB AND TB ANTIGEN DETECTION as disclosed in the above mentioned application for the term of 20 years from the 15th day of November 2017 in accordance with the provisions of the Patents Act,1970.

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अनुदान की तारीख : 22/11/2019
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पेटेंट नियंत्रक
Controller of Patent

टिप्पणी - इस पेटेंट के नवीकरण के लिए फीस, यदि इसे बनाए रखा जाना है, **15th day of November 2019**को और उसके पश्चात प्रत्येक वर्ष मे उसी दिन देय होगी।

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6th year	--	--	--	--	--	--	--	--	--
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(54) Title: QUANTUM DOT POWERED IP-10 ANTIBODY BASED KIT FOR LATENT TB AND TB ANTIGEN DETECTION

(57) Abstract: The present invention provides an antibody quantum dot complex comprising *Mycobacterium tuberculosis* anti-IPlO antibody and biogenic quantum dots for detection of Latent tuberculosis (LTB) antigens and TB antigens and a process for the synthesis thereof. Further, the present invention provides a diagnostic kit comprising the anti-IPIO antibody for detection of LTBI antigens and active tuberculosis antigens, and a method of assaying the concentration of the *M. tuberculosis* IP-10 antigen in an individual recovering from tuberculosis or exhibiting symptoms thereof.

"QUANTUM DOT POWERED IP-10 ANTIBODY BASED KIT FOR LATENT TB AND TB ANTIGEN DETECTION"

TECHNICAL FIELD OF THE INVENTION:

The present invention relates to an antibody quantum dot complex comprising *Mycobacterium tuberculosis* anti-IP 10 antibody and biogenic quantum dots for detection of Latent tuberculosis (LTB) antigens and active TB antigens and a process for synthesis thereof.

Further, the present invention relates to a diagnostic kit to differentiate the latent TB and active *Mycobacterium tuberculosis* (MTB) antigen on the basis of IP-10 antigen concentration *in vitro* and a method of assay thereof.

BACKGROUND AND PRIOR ART OF THE INVENTION:

For the diagnosis of tuberculosis, discovery of *Mycobacterium tuberculosis* (MTB) specific immuno-dominant antigens have led to a significant new avenue in diagnostics. Several antigens have been identified as biomarkers for detection of active *Mycobacterium tuberculosis.* Numerous kits have also been developed in this direction and have been commercialized.

Advances in genomics, molecular biology and immunology have led to T cell based, *in vitro*, interferon-γ (IFN-γ) assays. Early work had shown that a test that assayed the *in vitro* production of interferon gamma (IFN-γ) by T cells in response to defined MTB antigens had potential to replace the conventionally used Tuberculin Skin Test (TST). The IFN-γ assay is an *in vitro* diagnostic aid working on the principle that T cells of individuals sensitized with *M. tuberculosis* release IFN-γ on countering mycobacterial antigens. A high level of IFN-γ response is likely to indicate previous sensitization with *M. tuberculosis,* but does not necessarily imply active disease. In this respect, the IFN-γ assay is similar to the TST. It cannot easily distinguish between latent infection and active disease *(M. Pai, Indian Journal of Medical Microbiology, (2005) 23 (3):151-158).* In 2001, the

QuantiFERON-TB test (QFT) based on this concept was approved by the USFDA as an aid for detecting latent *Mycobacterium tuberculosis* infection. However, since this version of IFN-γ assay employed Purified Protein Derivative (PPD) as the stimulating antigen, it became clear that such assays will be encumbered with specificity issues observed with the PPD based TST.

Around the same time, comprehensive research lead to the discovery of highly immunogenic antigens such as TB7.7, early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) that improved specificity of the IFN-γ assay significantly. These antigens are encoded within the "region of difference 1 (RD1)" of a pathogen and are consequently absent in most non-tuberculous mycobacterial (exceptions include *Mycobacterium kansasii*, *Mycobacterium marinum*, *Mycobacterium szulgai*) and all Bacille Calmette Guerin (BCG) vaccine strains. IFN-γ responses to overlapping peptides of the RD1 encoded antigens ESAT-6, CFP-10, TB7.7 forms the basis for the detection of MTB infection in three licensed and commercially available tests. Tests based on these TB specific antigens are called RD1 based IFN-γ assays. With the introduction of RD1 based assays, PPD based IFN-γ assays are already being phased out.

QuantiFERON-TB Gold (Cellestis Limited, Carnegie, Victoria, Australia), a whole blood enzyme-linked immunoassay (ELISA) has European CE mark approval and received ETSFDA approval in 2005 for the detection of both latent TB infection (LTBI) and Tuberculosis disease. In October 2007, the ETS FDA gave its approval for the Quantiferon TB Gold In Tube to be marketed in the ETS. Other assays including T-SPOT. TB assay (only uses ESAT-6 and CFP10) (Oxford Immunotec, Oxford, ETζ) is the second assay to reach commercial development. It is an enzyme-linked immunospot assay (ELISPOT) that uses peripheral blood mononuclear cells and has European CE mark approval and was approved for use in Canada in 2005.

In India, both QuantiFERON-TB Gold and T SPOT-TB assays have been evaluated in research settings, in rural and urban populations. However, these assays are currently not used in clinical practice in India.

However, the limitations of the currently available tests are as follows:

- The sensitivity may be impaired in immunosuppressed individuals (such as HIV positive or patients receiving immunosupressing medication),
- In some situations a relatively large volume of blood is necessary (3ml per QuantiFERON test and 8ml for T-SPOT-TB), which may limit its use in infants, severely ill and anaemic children,
- The tests do not discriminate between active, latent and recent infection,
- The tests have not been demonstrated to be able to predict the probability of the patient progressing from recent or latent TB to active TB (*M. Pai, 2005).*

Most of the test limitations are due to measurement of the effect parameter IFN-γ at very low levels, close to the limit of even the most sensitive method (QuantiFERON test measures IFN-γ at 0.35 IU/ml (l7.5pg/ml) and the T-SPOT.TB assay: 5 spots/field). Decreasing cut-off to enhance sensitivity will eventually result in impaired specificity of the tests. Pai M. *et al*, 2004 stated that the repeated testing of people with test results in the lower range of IFN-γ varies around the cut-off level which underlines the potential risk of false positive and false negative results of the QuantiFERON(QFT) test. To increase the sensitivity of the test, additional *M.tuberculosis* specific antigens have been introduced in the third generation QuantiFERON assay(QFT), the antigen TB7.7 (p4) has been added in QuantiFERON In tube test (QFT-IT) and the potentially sensitivity has been improved, but it still depends on measurements at very low IFN-γ levels.

Morten Ruhwald *et al.*, 2008 found out Interferon-γ inducible protein-lO (IP-10) to be expressed in high levels compared to the QFT-IT. For diagnosis of *M.tuberculosis*, IP-10 and possibly IL-2 were found to be alternative markers to

IFN- γ. Yoshihiro Kobashi *et al.,* 2015 have found high levels of (IP-10) and Monocyte induced interferon-γ (MIG) in the supernatant of active TB patients than patients with non-TB disease. IP-10 and MIG using a supernatant stimulated with MTB-specific antigens showed similar results to Interferon-Gamma Release Assays (IGRAs). IP-10 showed a stronger association with risk factors for LTBI [Wang S, 2012]. Plasma IP-10 appeared to be the most consistent of the biomarkers studied, as it was the only marker that significantly differentiated active TB from both the LTBI and QFT negative control groups and significantly declined during anti-TB chemotherapy (I. Wergeland, 2015, *J Infect. 2015 Apr;70(4):381-91).Yet* no sensitive and specific markers to replace IFN-γ for diagnosis of TB infection have been identified in the presently published literature. Various studies have disclosed IP-10 in connection to infections, but not as a marker in the diagnosis of infection with prior antigen stimulation.

In the last decade, fluorescent colloidal nanoparticles or quantum dots (QDs) have been intensively studied for both basic and applied research due to their unique size-dependent properties (Kovalenko MV et al., 2015) QDs are nanometer scale semiconductor crystals composed of groups II to VI or III to V elements and are defined as particles with physical dimensions smaller than the exciton Bohr radius (Chan WC et al., 2002) Quantum dots have increasingly attracted the attention due to their unique optical properties as compared to conventional organic fluorescent dyes. Their much greater brightness, rock-solid photo stability and unique capabilities for multiplexing, combined with their intrinsic symmetry and narrow emission bands, have made them far better substitute for organic dyes used in existing diagnostic assays. Quantum dot a type of fluorescence nanoparticles has been considered highly sensitive substitutes for organic dyes in existing diagnostic assays. They exhibit an extraordinary capability to detect molecule at ultra-low concentration which can be used for very early detection of disease progression. These QDs are mainly produced by chemical routes. Although some biosynthesis protocol of quantum dots have been reported, the synthesis of *M. tuberculosis* IP-10 antibody conjugated to quantum dots is still a relatively new

and less explored area both in the terms of purely green synthesis, scalability, ease of purification and toxicity.

Therefore, the present inventors have aimed to develop an efficient antibody i.e. *Mycobacterium tuberculosis* anti-IP 10 antibody based kit for the identification of *Mycobacterium tuberculosis* at ultra-low concentration, wherein the said antibody is complexed with highly efficient biocompatible QDs as a detection ligand which can be used for differentiation of LTB and TB infection states as well as the progression of the disease.

OBJECT OF THE INVENTION:

It is an object of the present invention to provide an assay to differentiate Latent TB infection (LTBI) from the active diseased state of tuberculosis.

It is another object of the present invention to provide a *Mycobacterium tuberculosis* anti-IP 10 antibody and biogenic quantum dot conjugate for detection of LTB and TB antigens and a process for preparation thereof.

It is yet another object of the present invention to provide a diagnostic kit comprising the antibody-quantum dot conjugate for differentiation of LTB and TB infection states as well as the progression of the disease.

SUMMARY OF THE INVENTION:

In keeping with the object of the present invention, the present invention provides a *Mycobacterium tuberculosis* anti-IP 10 antibody and biogenic quantum dot (QDs) complex for detection of LTB and TB antigens, wherein the quantum dots are selected from Cerium oxide (Ce02).

In an aspect, the present invention provides an antibody quantum dot complex selected from anti-IP 10 antibody-CeCbQDs, anti-IP 10 antibody-Si02 QDs, anti-IP 10 antibody-ZnSQDs and anti-IP 10 antibody-Zn02 QDs.

In another aspect, the present invention provides a process for the preparation of anti-IP 10 antibody quantum dot complex, the said process comprising;

(a) biotinylating anti-IP 10 antibody;
(b) conjugating streptavidin to the quantum dots and purifying by column chromatography;
(c) reacting biotinylated IP-10 antibody of step (a) and streptavidin conjugated quantum dots of step (b) in a ratio ranging from about 0.5:2.5 to about 1:2 at a temperature ranging from about 20°C to about 40°C to obtain anti-IP 10 antibody quantum dot complex in the reaction mixture;
(d) centrifuging the reaction mixture of step (c) at 8000 to 20000 rpm for 15 minutes and collecting the pellet followed by suspending the pellet in PBS buffer; and
(e) purifying the resultant anti-IP 10 antibody quantum dot complex by repeated centrifugation in reaction mixture (2 times).

In yet another aspect the present invention provides a diagnostic kit for the detection of LTB and TB antigens in biological fluids of an individual recovering from tuberculosis or exhibiting symptoms of tuberculosis, the said kit comprising;

(a) anti-IP-10 antibody-quantum dot complex in an amount ranging from about 50µ1to about 200 µï;
(b) IP-10 antigen in concentrations ranging from about lOng, 50pg, 25pg and 500 fg and in an amount ranging from about 10 µï to about 100 µï;
(c) Microtiter plate/eppendorf tubes for incubation of the said complex with biological fluid and a Quartz cuvette/ cuvette for the quantitative determination of the IP-10 antigen; and
(d) a chart depicting the intensity of the photoluminescence bands formed at excitation wavelength of 365 nm of anti-IP-10 antibody -quantum dot complexes formed with IP-10 antigen at concentrations ranging from about 0.5pg/ml to about l0,000pg/ml.

In one aspect, the present invention provides an assay for detecting the Latent *Mycobacterium tuberculosis* TB and active TB antigens in biological fluids using the diagnostic kit, the said method comprising;

(a) incubating about 100 µï to about 200µ1 of anti-IP-10 antibody-CeCbQDs complex for 15 minutes at temperature ranging from about 20°C to about 40°C with 10 to IOOµI of the biological fluid;

(b) simultaneously incubating about IOOµI to about 200µ1 anti-IP-10 antibody-CeCkQDs complex with the IP-10 antigen in concentrations ranging from about 0.5pg/ml to about l0,000pg/ml; and

(c) measuring the emission band and photoluminescence (PL) intensity of the reaction complex formed in steps (a) and (b) at an excitation wavelength of 365 nm to quantify the presence of the IP-10 antigen and determine the progression of tuberculosis, or

(d) visually detecting the IP-10 antigen conjugated to anti-IP 10 antibody-CeCkQDs complex using UV light, wherein a positive result is represented by a greenish color indicating the presence of the IP-antigen.

In a further aspect, the present invention provides the present quantum dot-antibody complex for detection of latent *Mycobacterium tuberculosis* antigens in an individual recovering from tuberculosis or in individuals exhibiting symptoms of tuberculosis. More specifically, the anti-IP-lO antibody-CeCbQDs complex is used for detection of IP-10 antigen, at ultralow concentrations of 0.5pg/ml. The detection of the complex is based on fluorescence characteristics of quantum dots and was performed by spectrofluorophotometer and UV light.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS:

Figure 1 depicts a flow diagram for the preparation of anti-IPlO antibody-CeOiQDs complex;

Figure 2 depicts a diagrammatic representation for the preparation of anti-IP 10 antibody- CeCbQDs complex and fluorescence changes occur when anti-IP 10 antibody- CeOiQDs complex binds to IP-10 antigen;

Figure 3 shows the confirmation of binding on 10% Native PAGE stained by silver staining in which Lane 1: Molecular weight Marker (10-245 kD), Lane 2: Biotinylated Anti-IP 10 Antibody, Lane 3: Biotinylated Anti-IP 10 Antibody, Lane 4: Cerium oxide quantum dot (CeCkQDs), Lane 5: Streptavidin, Lane 6: Streptavidin attached CeCbQDs, Lane 7: Anti-IP 10 Antibody- CeCbQDs complex, Lane 8: Anti-IPlO Antibody- CeCkQDs complex + IP-10 Antigen (50pg/ml), Lane 9: Anti-IPlO Antibody- CeCbQDs complex + IP-10 Antigen(500fg/ml), Lane 10: IP-10 Antigen;

Figure 4 represents the photoluminescence intensity of various preparation observed under black UV-light excited at 365 nm in UV-transilluminator, wherein, 1: Anti-IPlO Antibody- CeCbQDs complex, 2: Anti-IPlO Antibody-CeChQDs complex + IP-10 Antigen (50pg/ml), 3: Anti-IPlO Antibody- CeCbQDs complex + IP-10 Antigen (500fg/ml), 4: IP-10 Antigen, 5: Bovine Serum Albumin (BSA);

Figure 5 represents the Emission band and photoluminescence intensity various preparation at 365nm excitation wavelength Anti-IPlO Antibody- CeCbQDs complex with different concentration of IP-10 Antigen with respective controls;

Figure 6 depicts TEM micrograph of cerium dioxide quantum dots at 20nm scale;

Figure 7 depicts 3D spectra of cerium dioxide quantum dots.

DETAILED DESCRIPTION OF THE INVENTION:

The invention will now be described in detail in connection with certain preferred and optional embodiments, so that various aspects thereof may be more fully understood and appreciated.

The monoclonal antibody anti-Human IP-10 was purchased from Peprotech, USA.

In a preferred embodiment, the present invention provides an antibody - quantum dot complex for detection of Latent Tuberculosis (LTB) and TB antigens, the said complex comprising *Mycobacterium tuberculosis* anti-IP 10 antibody and quantum dot (QDs).

The quantum dots are selected from the group consisting of Cerium oxide (CeO_2), Silicon dioxide (S1O2), Zinc Sulfide (ZnS), and other suitable QDs such as Zinc oxide (ZnCh), Nickel Oxide (NiO) and Graphene oxide.

The particle size of the quantum dots is ranging from about lnm to about 10 nm (Figure 6).

The antibody - quantum dot complex is selected from the group consisting of anti-IP 10 antibody- Ce02 QD complex, anti-IP 10 antibody- S1O2 QD complex, anti-IP 10 antibody- ZnS QD complex, anti-IP 10 antibody- Zn02 QD complex, anti-IP 10 antibody- NiO QD complex and anti-IP 10 antibody- Graphene Oxide QD. More preferably, the antibody - quantum dot complex is selected from the group consisting of anti-IPlO antibody- Ce02 QD complex, anti-IPlO antibody- S1O2 QD complex, anti-IP 10 antibody- ZnS QD complex and anti-IP 10 antibody - Zn02 QD complex. Most preferably, the antibody - quantum dot complex is anti-IP 10 antibody- Ce02 QD complex.

Each of the components of the antibody - quantum dot complex is modified to enhance its binding affinity to each other, such that the reactivity of the antibody and the quantum dots is not modified. Accordingly, the *Mycobacterium tuberculosis* anti-IP 10 antibody is biotinylated to enhance its binding with quantum dot moiety. About 5pg/ml to 20pg/ml of the anti-IP 10 antibody in buffer was biotinylated. 10µ1 of the antibody was used for biotinylation. The present invention employs an optimized protocol for biotinylation of the anti-IPlO antibody. Further, the quantum dots are conjugated to streptavidin to facilitate a non-covalent reaction between the biotin conjugated anti-IP 10 antibody and the quantum dots. The process for preparation of the present anti-IP 10 antibody-Ce02 complex is detailed in Figure 1. The detail procedure explained in example section.

In an embodiment, the present invention provides an antibody - quantum dot complex for detection of Latent Tuberculosis (LTB) and TB antigens, wherein the particle size of the quantum dot is ranging from about lnm to about 5nm (Figure 6)·

The binding of the biotinylated anti-IP 10 antibody and the streptavidin-quantum dot complex was confirmed on 10% Native PAGE stained by silver staining (Figure 3). Accordingly, the anti-IPlO Antibody- CeCbQDs complex and anti-IPlO Antibody- CeCbQDs complex + IP-10 Antigen were confirmed on Native PAGE stained by silver staining compared with their respective controls and molecular weight marker. The complex formed possessed an increased molecular weight and therefore the band corresponding to the complex appeared to be above the control.

In figure 3, the run of the protein bands in Lanes 7, 8 and 9 corresponding to the anti-IP 10 Antibody- CeCbQDs complex, antilPlO Antibody- CeCbQDs complex + IP-10 Antigen (50pg/ml)and anti-IP 10 antibodyCeChQDs complex + IP-10 Antigen(500fg/ml), indicate the binding of the complex of the present invention as well as the complex to the IP antigen to be detected.

In another preferred embodiment, the present invention provides a process for the preparation of anti-IP 10 antibody quantum dot complex, the said process comprising;

(a) biotinylating anti-IP 10 antibody;
(b) conjugating streptavidin to the quantum dots and purifying by by column chromatography;
(c) reacting biotinylated IP-10 antibody of step (a) and streptavidin conjugated quantum dots of step (b) in a ratio ranging from about 0.5:2.5 to about 1:2 at a temperature ranging from about 20°C to about 40°C to obtain anti-IP 10 antibody quantum dot complex in the reaction mixture;
(d) centrifuging the reaction mixture of step (c) at 8000 to 20000 rpm for 15 minutes and collecting the pellet followed by suspending the pellet in PBS buffer; and

(e) purifying the resultant anti-IP 10 antibody quantum dot complex by repeated centrifugation in reaction mixture (2 times).

In accordance with the foresaid embodiment, the quantum dots, preferably CeCbQDs were synthesized by *Cajanus cajan* protein beads and characterized by UV-Vis spectroscopy. The present invention employs a process of green synthesis for production of the quantum dots. The biologically synthesized CeCbQDs were characterized by UV-Vis spectroscopy, Transmission Electron Microscope (TEM), Fourier transform infrared spectroscopy (FTIR), and its fluorescence property was analysed by Spectrofluorophotometer.

The process of conjugating streptavidin to the quantum dots produced by green synthesis comprises the following steps:

(a) adding 150 µï to 300 µï of quantum dots to 300 µï to 600 µï of reaction buffer comprising O.lmM Sodium borate buffer, pH 7.4 at room temperature;

(b) 10 to 30µ1 of streptavidin from a lmg/ml stock solution was mixed well with preparation of step (a);

(c) 50µ1to IOOµI of 2mg/ml 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) solution was added to step (b) reaction mixture and mixed thoroughly at room temperature for 2hrs with gentle shaking;

(d) adding 5µ1to 20µï of quenching buffer comprising 1M Glycine, pH 6.5to step(c) and incubating the reaction solution for 15 minutes at room temperature to obtain Streptavidin-quantum dot complex; and

(e) purifying the Streptavidin-quantum dot complex obtained in step(d) by gel filtration chromatography.

More specifically, the process of conjugating streptavidin to the CeCbQD comprises:

(a) adding 250µ1 of characterized CeCbQDs to 500µ1 of the reaction buffer comprising O.lmM Sodium borate buffer, pH 7.4 at room temperature;

(b) 25µï of streptavidin was taken from a 1mg/ml stock solution and mixed with step(a) preparation;

(c) 75µï of 2mg/ml 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) solution was prepared using reaction buffer (O.lmM Sodium borate buffer, pH 7.4) was added into the step (b) reaction mixture and mixed thoroughly at room temperature for 2hrs with gentle shaking.

(d) adding 10µ1 of quenching buffer comprising 1M Glycine, pH 6.5 to step(c) and incubating the reaction solution for 15 minutes at room temperature to obtain Streptavidin- CeCbQDs complex;

(e) purifying the streptavidin- CeChQD complex obtained in step(d) by gel filtration chromatography.

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) solution was prepared using reaction buffer comprising O.lmM Sodium borate buffer, pH7.4. Further, the process of purification using gel filtration chromatography was performed using the Sephadex gel G-25 column.

In one preferred embodiment, the biotinylated anti- IP-10 antibody and purified streptavidin-CeChQDs complex in a 1:2 ratio was allowed to react for 2hrs. The ambient temperature used at the time reaction is in the range from about 20°C to 40°C. After incubation, the reaction mixture was centrifuged at 10000 rpm for 15 minutes and the pellet was collected. This pellet was suspended in PBS buffer comprising 200 mM NaCl, 2.7 mM KC1, 8.2 mM Na2HP0 $_4$, 4.2 mM NaH $_2$P04, and 1.15 mM K2HPO4, pH 7.4. To further eliminate free antibodies and small molecules from the antibody IP-10 and CeCbQDs complex formed, the suspended pellet is once again centrifuged as described previously and the pellet of the IP-lO-CeCbQDs complex is collected and dispersed in PBS buffer (pH 7.4) which was stored at 4°C.The purified anti-IP-1O antibody -CeCbQDs complex was used for IP-10 antigen detection in the diagnostic kit.

The procedure of preparing the antibody-quantum dot complex and its use in detection of the IP-antigen is provided in Figure 2.

In yet another preferred embodiment, the present invention provides a diagnostic kit for the detection of LTB and TB antigens in biological fluids of an individual recovering from tuberculosis or exhibiting symptoms of tuberculosis, the said kit comprising;

(a) anti-IP-10 antibody-quantum dot complex in an amount ranging from about 50µ1to about 200 µï;
(b) IP-10 antigen in concentrations ranging from about lOng, 50pg, 25pg and 500 fg in an amount ranging from about 10 µï to about 100 µï;
(c) a microtiter plate/eppendorf tube for incubation of the said complex with biological fluid and a Quartz cuvette/ cuvette for the quantitative determination of the IP-10 antigen; and
(d) a chart depicting the intensity of the photoluminescence bands formed at excitation wavelength of 365 nm of anti-IP-10 antibody -quantum dot complexes formed with IP-10 antigen at concentration ranging from about 0.5pg/ml to about l0,000pg/ml.

The antibody - quantum dot complex used in the diagnostic kit of the present invention is selected from the group consisting of anti-IPlO antibody- CeCk QD complex, anti-IP 10 antibody- SiCk QD complex, anti-IP 10 antibody- ZnS QD complex, anti-IP 10 antibody- ZnCk QD complex, anti-IP 10 antibody- NiO QD complex and anti-IP 10 antibody- Graphene OxideQD

The anti-IP-10 antibody-quantum dot complex used in the diagnostic kit is preferably the anti-IP-10 antibody-CeCkquantum dot complex. Further, the biological fluids used for detection of the tuberculosis antigen in the present invention include sputum samples/saliva samples/ urine samples/ sweat samples/ tear samples or blood samples.

In one preferred embodiment, the present invention provides an assay for detecting the Latent *Mycobacterium tuberculosis* TB and active TB antigens in biological fluids using the diagnostic kit provided herein, the said method comprising;

(a) incubating about 100 µï to about 200µ1 of anti-IP-10 antibody-CeCbQDs complex for 15 minutes at room temperature with 10 to IOOµI of the biological fluid;

(b) simultaneously incubating about 100 µï to about 200µ1 anti-IP-10 antibody-CeCbQDs complex with the IP 10 antigen in concentrations ranging from about 0.5pg/ml to about l0,000pg/ml;

(c) measuring the emission band and photoluminescence (PL) intensity of the reaction complex formed in steps (a) and (b) at an excitation wavelength of 365 nm to quantitatively determine the presence of the IP antigen; and/or

(d) visually detecting the IP-10 antigen conjugated to anti-IP 10 antibody-CeCbQDs complex using UV light, wherein a positive result is represented by a greenish color indicating the presence of the IP-antigen.

The emission band shifted towards the shorter wavelength and photoluminescence intensity increased with the increasing concentration of IP-10 antigen (Figure 5). The IP-10 antigen conjugated to Anti-IPlO Antibody-CeCbQDs complex can be visually detected using UV light. A distinguished greenish color was observed when IP-10 Antigen binds to Anti-IPlO Antibody-CeCbQDs complex (Figure 4).

In one embodiment, the present invention provides the antibody - quantum dot complex comprising the *Mycobacterium tuberculosis* anti-IP 10 antibody and quantum dot (QDs) for use in the detection of Latent Tuberculosis (LTB) and TB antigens.

In one more embodiment, the present invention provides compositions comprising *Mycobacterium tuberculosis* anti-IP 10 antibody bound to quantum dots for use in biological applications including imaging, labeling and sensing.

The following examples, which include preferred embodiments, will serve to illustrate the practice of this invention, it being understood that the particulars shown are by way of example and for purpose of illustrative discussion of preferred embodiments of the invention.

EXAMPLES

Example 1: Anti-Human IP-10

Monoclonal antibody anti-Human IP-10 was purchased from Peprotech, USA (Catalog Number: 500-M60, Source: *Monoclonal Mouse,* Produced in BALB/c mice) using highly pure recombinant Human IP-10 as the immunizing antigen. This antibody was purified from cell culture by protein affinity chromatography. Immunogen: *E.coli* derived Recombinant Human IP-10 (CXCL10, PeproTech catalog #300-12)

Example 2: Biotinylation of Anti-Human IP-10

Biotinylation of Anti-IP 10 antibody were carried out by using one step Antibody Biotinylation kit (MACS Miltenyi Biotec Order no. 130-093-385) according to the procedure described by the supplier. Resuspend the lyophilized powder fully by mixing thoroughly. Incubate the mixture at a controlled room temperature for 24 hour. After incubation, the antibody is ready for direct use. Accordingly the required volume of antibody was biotinylated. The biotinylation process was done with 10pg/ml Anti-IPlO antibody in PBS in well optimized well. 10µï of the antibody was added in well for biotinylation. Figure 2 provides a scheme showing the process for preparation of the anti-IP 10 Antibody-CeOiQDs complex.

Example 3: Ce0 $_2$ quantum dot (Ce0 $_2$QDs) used for complex

The green synthesized pure Ce0 $_2$QDs were synthesized by *Cajanus cajan* protein beads and characterized by UV-Vis spectroscopy, Transmission Electron Microscope (TEM), Fourier transform infrared spectroscopy (FTIR). The Fluorescence property was analysed by Shimadzu Spectrofluorophotometer RF-

6000 (Indian Patent Application ηo.:201721018037) were used in this protocol for synthesis of Streptavidin-CeCbQDs.

Example 4: Streptavidin-Ce0 $_2$QDs binding

250µ1 of characterized CeCbQDs was added to 500µ1 of reaction buffer (O.lmM Sodium borate buffer, Ph 7.4) at room temperature in step 1. 25µ̈ of streptavidin was taken from a 1mg/ml stock solution and mixed well with step-l preparation designated as step 2. 2mg/ml 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) solution was prepared using reaction buffer (O.lmM Sodium borate buffer, pH 7.4). For the binding assay, 75µ1 of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) solution was added into the step 2 reaction mixture and mixed thoroughly step 3. This reaction was performed at room temperature for 2hrs with gentle shaking. 10µ1 of quenching buffer (1M Glycine, pH 6.5) was added in step3 and incubated for 15 minutes at room temperature. After incubation, Streptavidin-CeCbQDs complex was purified by gel filtration chromatography (Sephadex gel G-25, 2ml column)

Example 5: Antibody IP-10 and CeCbQDs complex formation

Antibody IP-10 and CeCbQDs complex was made by incubating biotinylated IP-10 antibody and streptavidin attached CeCbQDs for 2h at room temperature in the ratio of 1:2. After incubation, reaction mixture was centrifuged at 10000 rpm for 15 minutes and pellet was collected. This pellet was suspended in PBS buffer (200 mM NaCl, 2.7 mM KCl, 8.2 mM NaiHPCb, 4.2 mM NaH_2P0_4, and 1.15 mM K2HPO4, pH 7.4). In view to remove free antibodies and small molecules from Antibody IP-10 and CeCbQDs complex formed, suspended pellet was once again centrifuged as described previously and pellet of the IP-lO-CeCbQDs complex was finally collected and dispersed in PBS buffer (pH 7.4) which was stored at 4°C.

Example 6: Recombinant Human IP-10 (CXCL10)

Recombinant Human IP-10 (CXCL10) purchased from Peprotech, USA (Catalog Number: 300-12) Synonyms: γ-Interferon Inducible Protein 10, CXCL10, crg-2, Source: *E.coli* AA Sequence: **VPLSRTVRCT CISISNQPVN PRSLEKLEII PASQFCPRVE IIATMKKKGE KRCLNPESKA IKNLLKAVSK ERSKRSP,** Purity: Greater than 98% by SDS-PAGE gel and HPLC analyses.) The different concentration of antigens i.e. lOng/ml, 50pg/ml, 25pg/ml and 5pg/ml were prepared.

Example 7: Confirmation of formation of biotinylated IP-10 and CeChQDs-streptavidin binding complex on 10%Native PAGE

The difference between IP-10 monoclonal antibody and biotinylated antibody were confirmed on 10% Native PAGE under silver staining as the biotinylated antibody band appeared above the monoclonal antibody. Similarly, CeCbQDs-streptavidin binding were confirmed on 10% Native PAGE under silver staining as the quantum dots under silver staining not observed any band on PAGE. The streptavidin band appeared at approximately 63kD (52.8kD) and CeCbQDs-streptavidin band did not appear on PAGE. Similarly, biotinylated IP-10 and CeChQDs-streptavidin binding complex showed only one band of antibody on 10% Native PAGE. Similarly the antigen (IP-10) and complex bound to antigen were analyzed on 10% Native PAGE (Figure 3).

Example 8: IP-10 antigen detection under UV light using anti-IP-10-CeO_2QDs complex on glass slide

One drop (~l μϋ) of each of preparation was taken on a glass slide in following order: anti- IP-l0-CeO2QDs complex (samplel), reaction of lOng of IP-10 antigen and anti-IP-l0-CeO2QDs complex (sample-2), reaction of 0.5 pg of IP-10 antigen anti-IP-l0-CeO2QDs complex (sample-3), IP-10 antigen (Sample-4) and Bovine Serum Albumin (Sample-5). Kept for 20 minutes at room temperature for evaporation and dried sample was observed visually under day light and UV light (365nm). Under UV light the fluorescence were observed in sample- 2 and

sample -3 which represents reaction of lOng of IP-10 antigen and anti-IP-10-CeChQDs complex and reaction of 500 fg of IP-10 antigen anti-IP-lO-CeCLQDs complex (Figure 5).

Example 9: Spectrofluorophotometric antigen IP-10 detection using anti-IP-lO-CeChQDs complex

50µl of different concentration (i.e. lOng, 50pg, 25pg and 500 fg) of IP-10 antigen was incubated with the 150µl of anti-IP-lO-CeCLQDs complex for 15 minutes at room temperature and the emission band and photoluminescence (PL) intensity was observed at 365nm excitation wavelength. It was observed that the band shifted towards the short wavelength and the intensity of the shifted emission peak increased with the increasing concentration of antigen. The emission was consistently shifted towards left (shorter wavelength) which is distinctly different from the major component of the natural fluorescence spectrum of these QDs. This emission peak can grow upon conjugation to antibodies and subsequent binding to antigens. The present inventors have concluded that the wavelength shift is due to changes in the chemical environment of QD complexes when they encounter the antigen and may be due to physical deformation of the QD that changes the quantum confinement state.

Sr. No.	**Sample**	**λ_{em} (nm)=365 at λ_{ex}=365 nm**	**Intensity at λ_{ex}=365 nm**
1.	Anti-IP-10 antibody	415.0	515.0
2.	Biotinylated anti-IP-10 Ab	416.0	245.1
3.	IP-10 antigen	416.0	340.4
4.	Streptavidin	415.0	208.3
5.	CeO_2QDs	450.0	31677.12
6.	Streptavidin-CeO_2QDs	435.0	63154.32
7.	Anti-IP-10Ab-CeO_2QDs	434.0	73329.84
8.	10ngIP-10Ag + anti-IP-	429.0	168654.96

9.	50pgIP-10Ag + anti-IP-	427.0	114970.32
10	25pgIP-10Ag + anti-IP-	430.0	105121.24
11.	500fgIP-10Ag+ Anti-IP-	422.0	58966.2

Example 10: Diagnostic kit for detection of latent and active *Mycobacterium tuberculosis* antigens

The diagnostic kit for the detection of LTB and TB antigens in biological fluids (sputum samples/saliva samples/ urine samples/ sweat samples/ tear samples or blood samples) of an individual recovering from tuberculosis or exhibiting symptoms of tuberculosis was prepared comprising ;

(a) anti-IP-lO antibody-quantum dot complex in an amount of 100 to 200 µï;

(b) IP-10 antigen in concentrations ranging from about lOng, 50pg, 25pg and 500 fg in an amount of 50 to 100 µï;

(c) microtiter plate/ eppendorf tube for incubation of the said complex with biological fluid and a Quartz cuvette/ cuvette for the quantitative determination of the IP-10 antigen; and

(d) a chart depicting the intensity of the photoluminescence bands formed at excitation wavelength of 365 nm of anti-IP-10 antibody -quantum dot complexes formed with IP-10 antigen at concentrations ranging from about 0.5pg/ml to about l0,000pg/ml.

Advantages of the present invention:

- The present complex was able to detect pure IP-10 antigen at ultralow concentration i.e. 0.5pg/ml and specific to *Mycobacterium tuberculosis* antigen within 20 minutes using spectrofluorophotometer.
- This highly sensitive detection/diagnostic kit of the present invention is able differentiate the latent TB and active *Mycobacterium tuberculosis* (MTB) on the basis of the IP-10 antigen concentration *in vitro.*
- The conjugate of *Mycobacterium tuberculosis* anti-IP 10 antibody and biogenic cerium oxide quantum dot (CeCbQDs) as kit can be used a point of care screening of TB on the basis of Yes or No fluorescence simply by mixing serum and antibody-quantum dot conjugate at room temperature.

We claim,

1. An antibody - quantum dot complex for detection of Latent Tuberculosis (LTB) and TB antigens comprising *Mycobacterium tuberculosis* anti-IPlO antibody and quantum dot (QDs).

2. The antibody - quantum dot complex according to claim 1, wherein the quantum dot is selected from the group comprising Cerium oxide (CeCh), Silicon oxide (S1O2), Zinc sulfide (ZnS), Zinc oxide (ZnCL), Nickel Oxide (NiO) and Graphene oxide.

3. The antibody - quantum dot complex according to claim 2, wherein the antibody - quantum dot complex is selected from the group consisting of anti-IP 10 antibody- Ce02 QD complex, anti-IP 10 antibody- S1O2 QD complex, anti-IP 10 antibody- ZnS QD anti-IP 10 antibody- Zn02 QD complex, anti-IP 10 antibody -NiO QD complex and anti-IP 10 antibody - Graphene oxide QD complex.

4. The antibody - quantum dot complex according to claim 3, wherein the antibody - quantum dot complex is preferably anti-IP 10 antibody- Ce02 QD complex.

5. The antibody - quantum dot complex according to claim 1, wherein the latent TB antigen is IP- 10 antigen.

6. The antibody - quantum dot complex according to claim 1, wherein the *Mycobacterium tuberculosis* anti-IP 10 antibody is biotinylated.

7. The antibody - quantum dot complex according to claim 1, wherein the quantum dot is conjugated to streptavidin or avidin.

8. The antibody - quantum dot complex according to claim 2, wherein the quantum dots particle size is ranging from about lnm to about 10 nm.

9. The antibody - quantum dot complex according to claim 1, wherein the ratio of the biotinylated IP-10 antibody of step (a) and streptavidin conjugated quantum dots of step (b) is ranging from about 0.5:2. 5 to about 1:2

10. A process for the preparation of an anti-IP 10 antibody quantum dot complex, the said process comprising;
    (a) biotinylating anti-IP 10 antibody;
    (b) conjugating streptavidin to the quantum dots;
    (c) reacting biotinylated IP-10 antibody of step (a) and streptavidin conjugated quantum dots of step (b) in a ratio ranging from about 0.5:2. 5 to about 1:2 at a temperature ranging from about 20°C to about 40°C to obtain anti-IP 10 antibody quantum dot complex in the reaction mixture;
    (d) centrifuging the reaction mixture of step (c) at 8000 to 20000 rpm for 15 minutes and collecting the pellet followed by suspending the pellet in PBS buffer; and
    (e) purifying the resultant anti-IP 10 antibody quantum dot complex present in the pellet by column chromatography.

11. The process for the preparation of anti-IP 10 antibody quantum dot complex according to claim 10, wherein the anti-IP 10 antibody quantum dot complex selected from the group consisting of anti-IP 10 antibody- CeO_2 QD complex, anti-IP 10 antibody- $S1O2$ QD complex, anti-IP 10 antibody- ZnS QD anti-IP 10 antibody- ZnO_2 QD complex, anti-IP 10 antibody -NiO QD complex and anti-IP 10 antibody-Graphene oxide QD complex.

12. The process for the preparation of anti-IPlO antibody quantum dot complex according to claim 10, wherein biotinylated IP-10 antibody of step (a) and

streptavidin attached quantum dots of step (b) is allowed to react in a ratio of 1:2, respectively.

13. A diagnostic kit for detection of Latent Tuberculosis (LTB) and diseased TB antigens in biological fluid of an individual recovering from tuberculosis or exhibiting symptoms of tuberculosis, the said kit comprising;
    (a) anti-IP-10 antibody-quantum dot complex according to claim 1, in an amount ranging from about 50µ1to about 200 µï;
    (b) IP-10 antigen in concentrations ranging from about lOng, 50pg, 25pg and 500 fg in an amount ranging from about 10 µï to about 100 µï;
    (c) microtiter plate/ eppendorf tubes for incubation of the said complex with biological fluid (sputum samples/saliva samples/ urine samples/ sweat samples/ tear samples or blood samples) and a Quartz cuvette/ cuvette for the quantitative determination of the IP-10 antigen; and
    (d) a chart depicting the intensity of the photoluminescence bands formed at excitation wavelength of 365 nm of anti-IP-10 antibody -quantum dot complexes formed with IP-10 antigen at concentrations ranging from about 0.5pg/ml to about l0,000pg/ml.

14. A diagnostic kit for detection of Latent Tuberculosis (LTB) and diseased TB antigens according to claim 13, wherein the said anti-IP-10 antibody-quantum dot complex is selected from the group consisting of anti-IPlO antibody- CeCkQD complex, anti-IP 10 antibody- $S1O_2$ QD complex, anti-IP10 antibody- ZnSQD complex, anti-IPlO antibody- ZnO_2 QD complex, anti-IP 10 antibody -NiO QD complex and anti-IP 10 antibody-Graphene oxide QD complex.

15. A method for detecting of Latent *Mycobacterium tuberculosis* TB and active TB antigens in pure form and biological fluids (sputum samples/saliva samples/ urine samples/ sweat samples/ tear samples or blood samples) using the diagnostic kit of claim 14, the said method comprising;

(a) incubating about 100 µï to about 200µ1of anti-IP-lO antibody-CeCbQDs complex for 15 minutes at a temperature ranging from about 20°C to about 40°C with 10 to 100 µï of the biological fluid;

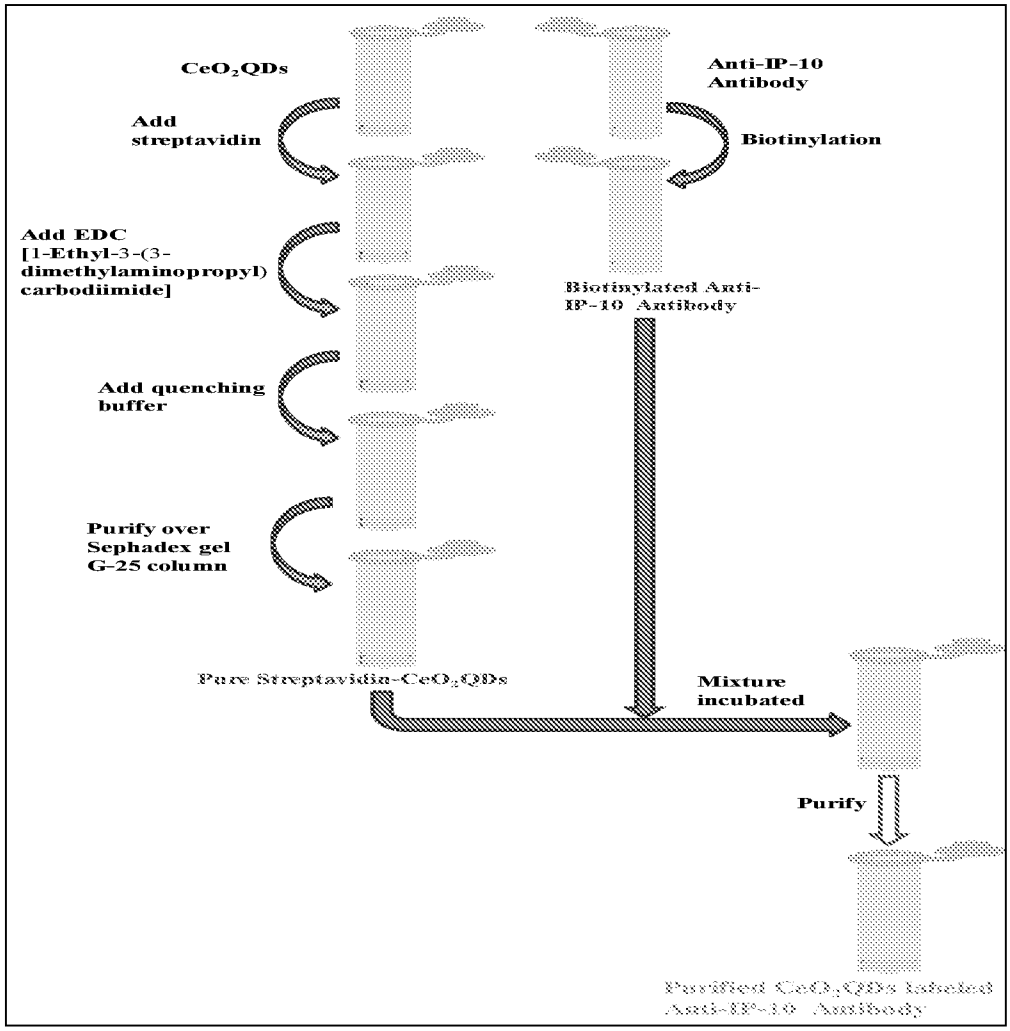
(b) simultaneously incubating about 100 µï to about 150µ1 of anti-IP-lO antibody-CeChQDs complex with the IP 10 antigen in concentrations ranging from about 0.5pg/ml to about l0,000pg/ml;

(c) measuring the emission band and photoluminescence (PL) intensity of the reaction complex formed in steps (a) and (b)at an excitation wavelength of 365nm to quantitatively determine the presence of the IP antigen; and

(d) visually detecting the IP-10 antigen conjugated to anti-IPlO antibody-CeChQDs complex using UV light, wherein a positive result is represented by a greenish color indicating the presence of the IP-antigen.

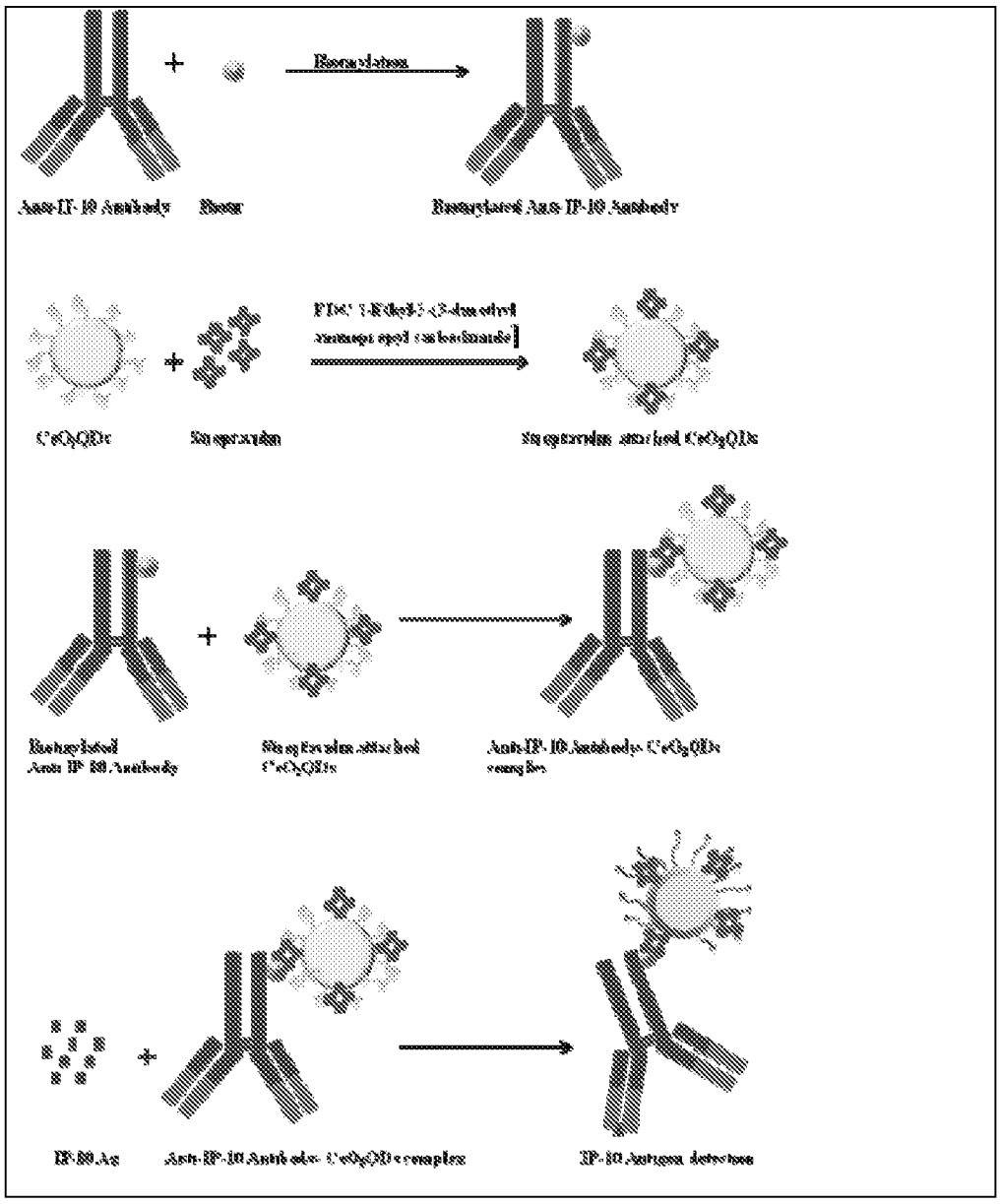
I/VI

Figure 1


CeO_2QDs
Add streptavidin
Add EDC [1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide]
Add quenching buffer
Purify over Sephadex gel G-25 column
Pure Streptavidin-CeO_2QDs
Anti-IP-10 Antibody
Biotinylation
Biotinylated Anti-IP-10 Antibody
Mixture incubated
Purify
Purified CeO_2QDs labeled Anti-IP-10 Antibody

II/VI

Figure 2



III/VI

Figure 3

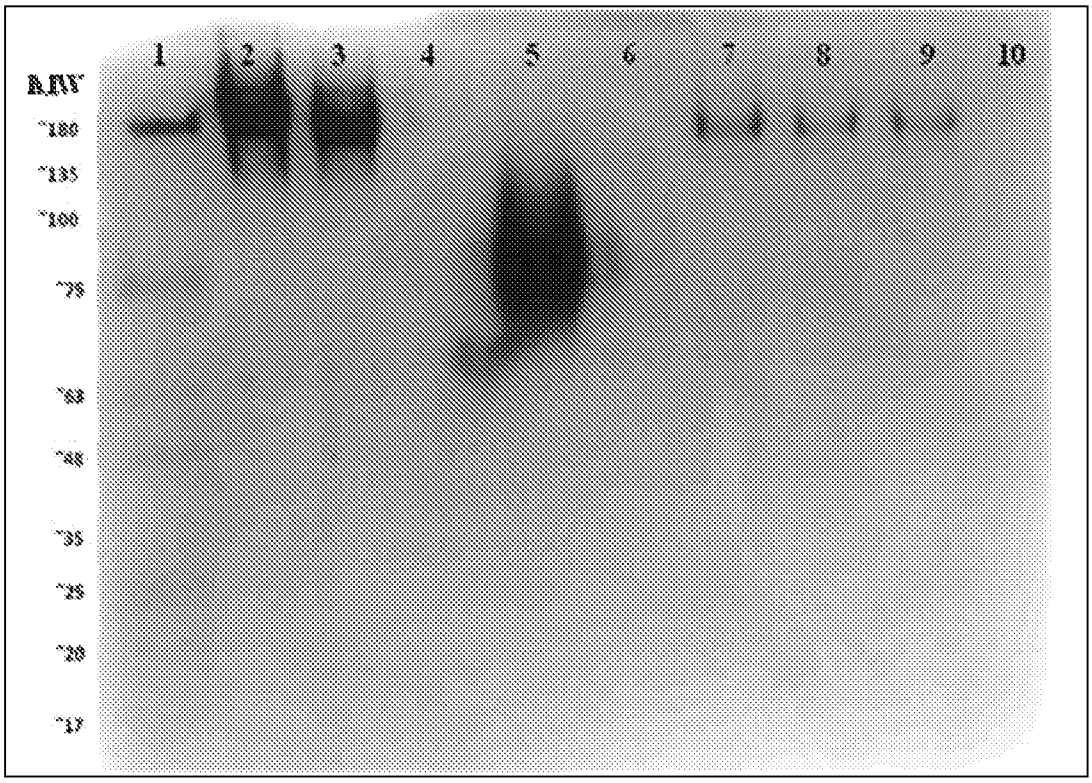


Figure 4

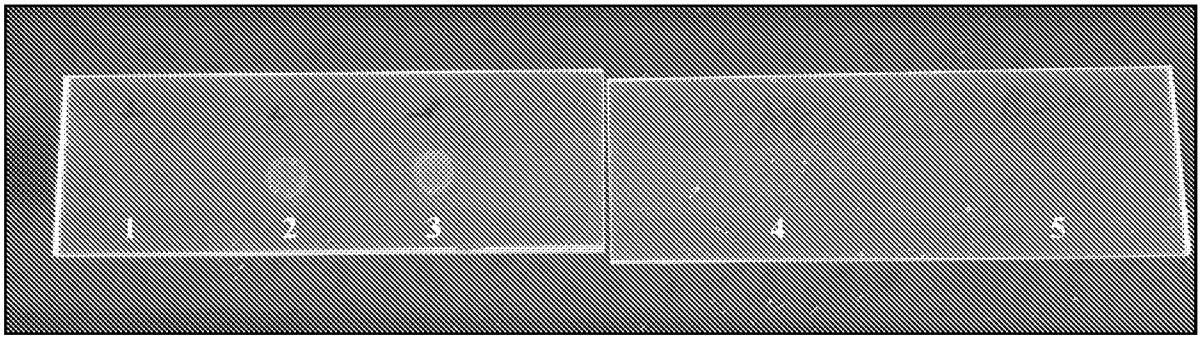
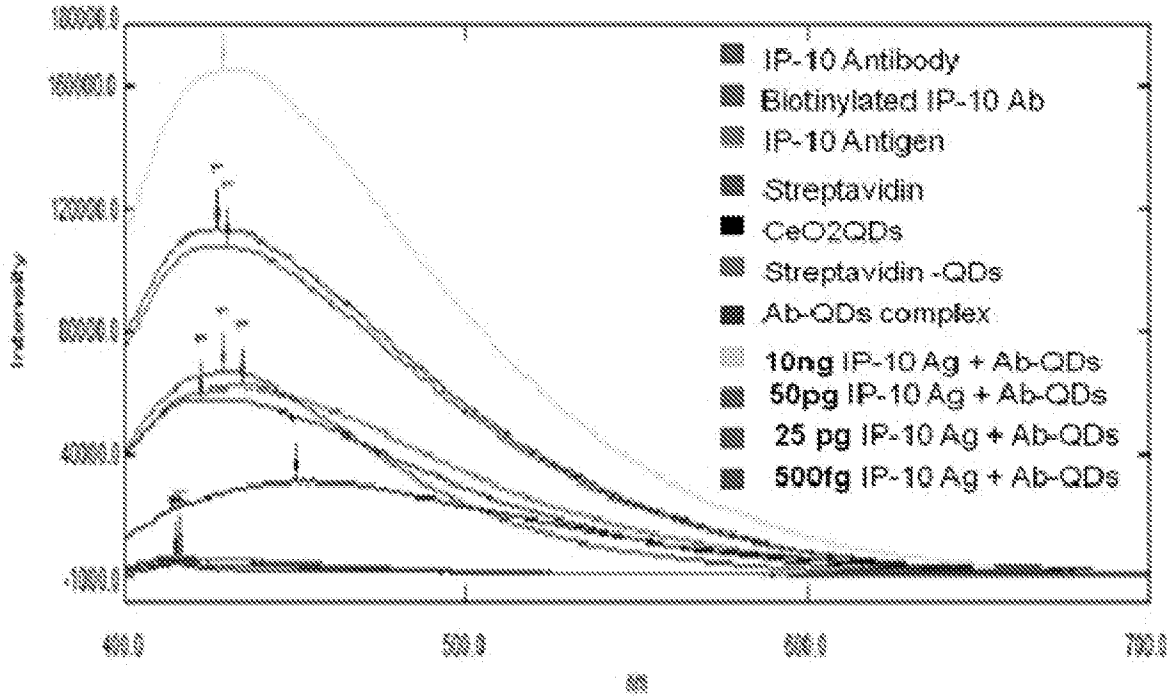
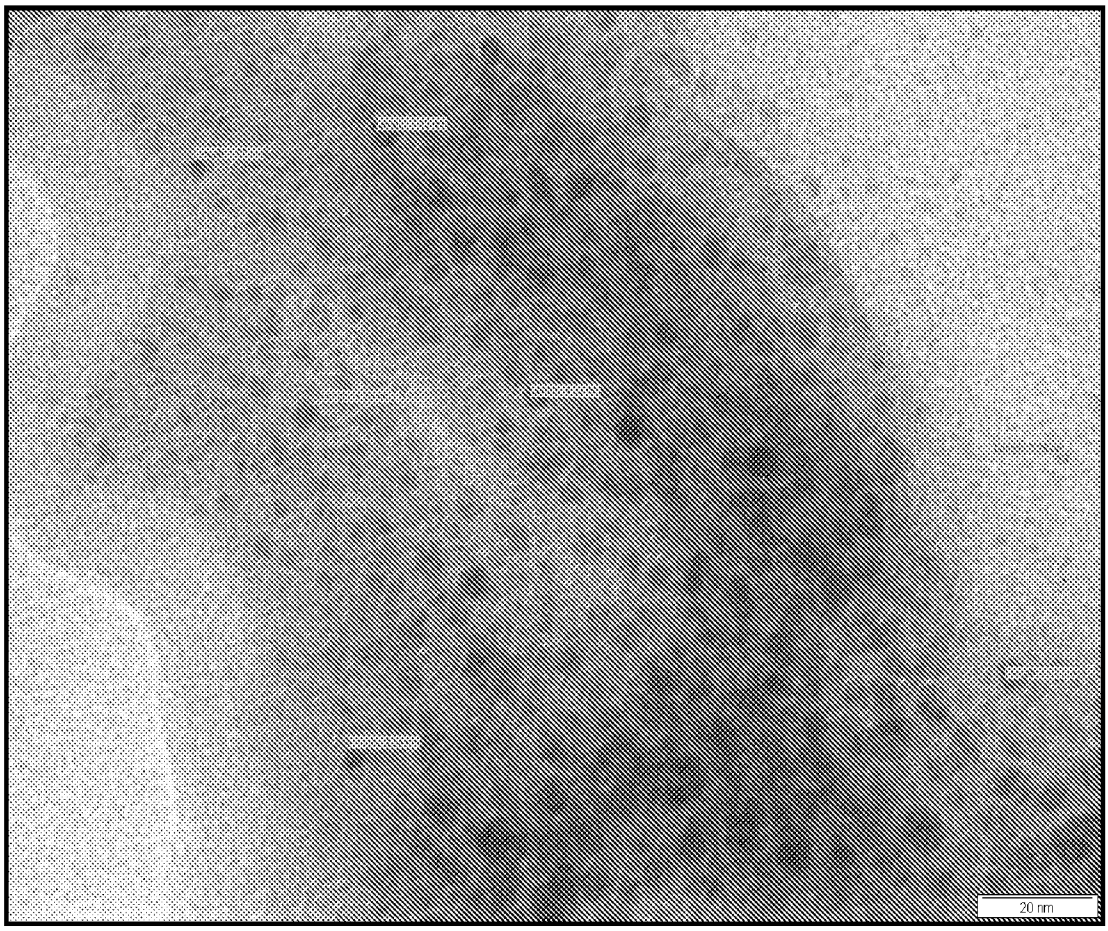


Figure 5


IP-10 Antibody
Biotinylated IP-10 Ab
IP-10 Antigen
Streptavidin
CeO2QDs
Streptavidin -QDs
Ab-QDs complex
10ng IP-10 Ag + Ab-QDs
50pg IP-10 Ag + Ab-QDs
25 pg IP-10 Ag + Ab-QDs
500fg IP-10 Ag + Ab-QDs

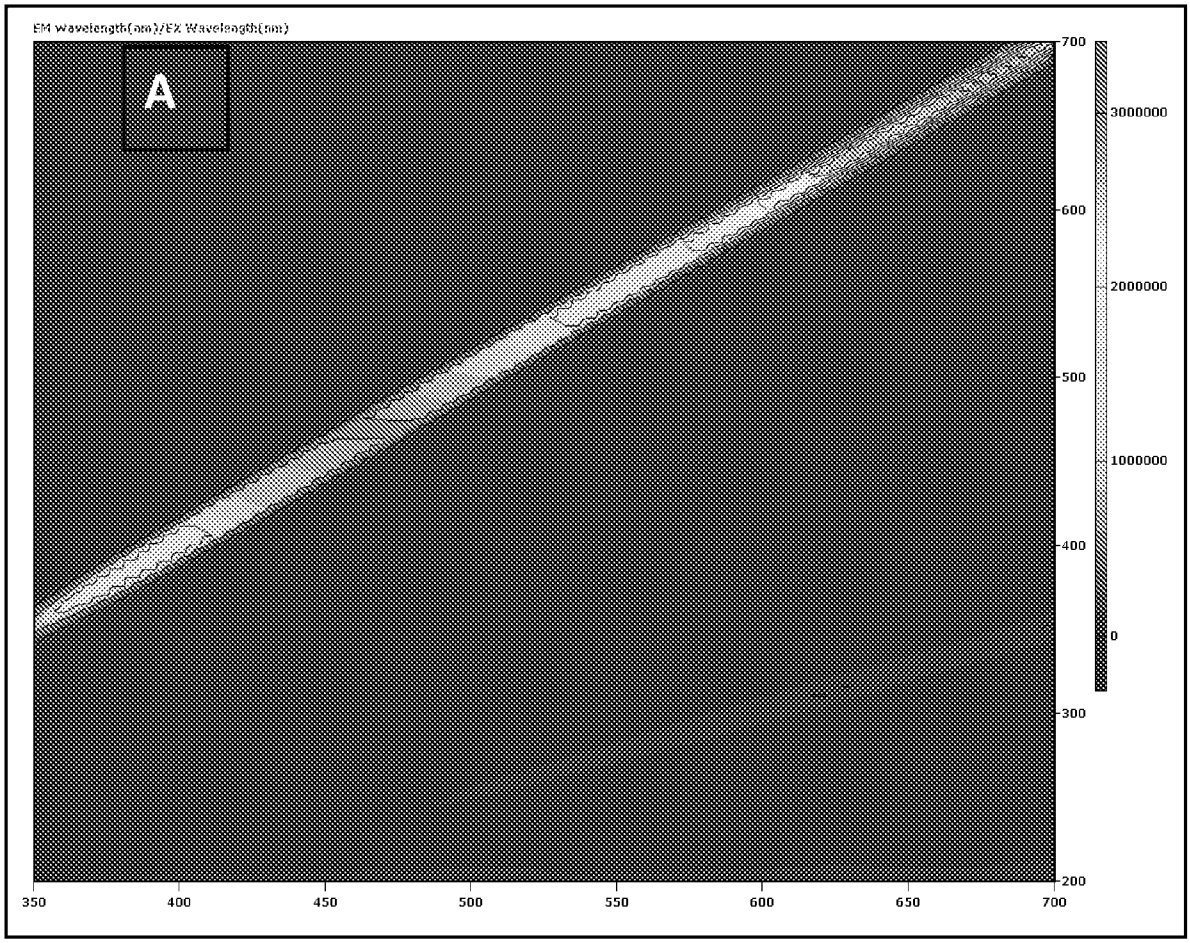
V/VI

Figure 6



VI/VI

Figure 7



INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2018/050688

A. CLASSIFICATION OF SUBJECT MATTER
G01N33/569 Version=2019.01

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

TotalPatent One, IPO Internal Database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KR20130121601A , (A&R THERAPEUTICS CO., LTD.), 6 November 2013 (06-11-2013), paragraphs [0117]-[0120].	1-15
Y	WO2010070581A1, (UNIVERSITY OF CAPE TOWN), 24 June 2010 (24-06-2010), paragraphs [0022], [0038]; claims 1, 2, 9, 10, 15, 17, 19, 20, 21, 40, 45.	1-15
Y	US20080317768A1 (BOEING COMPANY), 25 December 2008 (25-12-2008), paragraphs [0051], [0080], [0096], [0125].	1-3, 5-14
Y	IN201721018037A (MGM INSTITUTE OF HEALTH SCIENCES), 16 June 2017 (16-06-2017), abstract; page 17; claims 12, 15, 16.	1-15
Y	CN101519696A1 (THE FIRST AFFILIATED HOSPITAL OF THIRD MILITARY MEDICAL UNIVERSITY OF PLA), 2 September 2009 (02-09-2009), example 1 (2) and example 2 (3); paragraph [0068].	6, 7, 9, 10, 12

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11-01-2019	11-01-2019

Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT
Information on patent faintly members

International application No.
PCT/IN2018/050688

Citation	Pub. Date	Family	Pub. Date
WO 2010070581 A1	24-06-2010	ZA 201104416 A	28-08-2013
		AP 201105772 A	30-06-2011