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(54) Title: POCT DEVICE TO DETECT CERVICAL CANCER SPECIFIC BIOMARKER

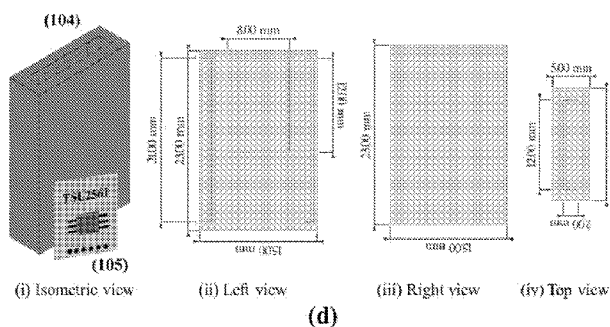


Figure 6

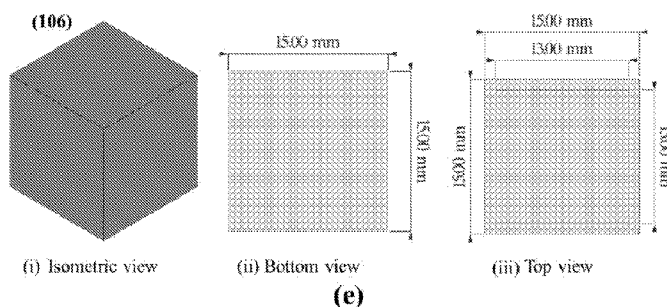


Figure 6

(57) Abstract: A biomarker sensor specific sensing of cervical cancer comprising gold nanoparticles with capture molecule immobilized thereon exhibiting LSPR for target, PP1Y2 biomarker and a non-invasive and point of care testing (POCT) device facilitating biomarker based cervical cancer detection system comprising an optical source; a sample stage holding said biomarker sensor comprising gold nanoparticles with capture molecule immobilized thereon adapted for exhibiting LSPR for target PP1Y2 biomarker in the analyte for LSPR based target biomarker detection and a signal processor means including an optical detector. The said POCT is based on the detection of the intensity change happens due to the variation in the refractive index at the surface of a Localized Surface Plasmon Resonance (LSPR) system owing to lock-and-key interaction of antibody and antigen.



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Title of Invention : POCT DEVICE TO DETECT CERVICAL CANCER SPECIFIC BIOMARKER.

FIELD OF INVENTION

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Present invention provides a biomarker sensor for sensing cervical cancer specific biomarker. More specifically, the said sensor comprises gold nanoparticles with capture molecule immobilized thereon exhibiting Localized Surface Plasmon Resonance (LSPR) for target protein, Protein
10 Phosphatase-1 gamma-2 (PP1Y2), a novel cancer biomarker (NCB). Present invention discloses a non-invasive point-of-care-testing (POCT) device facilitating biomarker based cervical cancer detection system comprising of a cervical cancer marker PP1Y2, present in the urine sample of the patients suffering from cervical cancer. The said device employs
15 colorimetric principles for the detection of the said marker in human urine, thus finds application in rapid and painless POCT diagnosis of cervical cancer.

BACKGROUND OF THE INVENTION

20

Worldwide, cervical cancer is the fourth most widely affected cancer type among women (Arbyn et al., "Estimates of incidence and mortality of cervical cancer in 2018: a worldwide analysis", The Lancet Global Health
8, e191; 2020) and is among the leading causes of malignancy of the
25 tumors present in the female reproductive system. Nearly 70% of all cervical cancer cases are caused due to sexually transmitted high-risk and oncogenic, Human Papillomavirus (HPV) infection, mostly by HPV 16 and 18 types (Clifford et al., "Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis", British journal of cancer 88,

63;2003). A few of the HPV infections are common and are not cancerous (Beutner et al., "Human papillomavirus and human disease", The American journal of medicine **102**, 9;1997). These types of infections are generally controlled by the inherent immune system and get cured within
5 1-2 years of occurrence (M. Schiffman, "Human papillomavirus and cervical cancer", The Lancet **370**, 890;2007). However, the transition of the infection may cause several changes in the cervical cells, if not get healed, and can eventually transform into precancerous lesions. The untreated precancerous lesions (Cervical Intraepithelial Neoplasia - CIN)
10 can progress to cervical cancer in 10-20 years. Thus, it is recommended to have routine cervical cancer screening every 3rd year for women younger than 21 years of age (D. M. Eddy, "Screening for cervical cancer", Annals of Internal Medicine **113**, 214;1990). The objective is to identify the disease in the precancerous stage or at the early stage of the
15 development of cervical cancer. It is found that the five-year survival rate for stage I cervical cancer patients is around 54%, which reduces to 40% for stage II cervical cancer patients and drops to around 18% for stage III and stage IV patients, respectively (N. A. Razak, "Estimating the five-year survival of cervical cancer patients treated in Hospital Universiti Sains
20 Malaysia", Asian Pacific Journal of Cancer Prevention **14**, 825;2013).

Hence, it is recommended for repeated Papanicolaou test (Pap test) to ensure successful identification of the fatal disease at early stages, leading to a reduced mortality rate (Schiffman et al., "Human
25 papillomavirus and cervical cancer", The Lancet **370**, 890;2007). Although these screening tests have the advantage of early diagnosis, the tests are expensive, extremely invasive and require highly skilled professionals and trained health care personnel for supervision.

There are several conventional procedures widely used for cervical cancer screening. HPV testing is one of the tests for the presence of high-risk infections in cervical cells (R. Sankaranarayanan, "HPV screening for cervical cancer in rural India," *New England Journal of Medicine* **360**, 1385;2009). Likewise, in Pap (Papanicolaou-stained) testing (Das et al., "Cytological pattern of papanicolaou smears and detection of cervical cancers: An experience from a tertiary care center of eastern zone of India", *Oncology Journal of India* **2**, 25;2018), Pap smear is used to screen for CIN, HPV/Pap co-testing (Hutchinson et al., "Utility of liquid-based cytology for cervical carcinoma screening: results of a population-based study conducted in a region of Costa Rica with a high incidence of cervical carcinoma", *Cancer Cytopathology: Interdisciplinary International Journal of the American Cancer Society* **87**, 48;1999) where the collected cell samples are monitored for high-risk HPV types and cervical cell changes, and visual inspection after application of acetic acid (VIA) or Lugol's iodine (VILI) in the cervical region which stains the cancer affected cells in the cervical region and helps in easy distinction of cancerous from noncancerous tissues (Duraismy et al., "Methods of detecting cervical cancer", *Advances in Biological Research* **5**, 226;2011).

In HPV inspection or Pap test methods, cervical cells are collected from the cervix using a swab such as wooden or plastic scraper and/or cervical brush and are stored in a liquid preservative vial for further examination for the presence of any HPV infection or cervical cell changes using Pap cytology test (Duraismy et al., "Methods of detecting cervical cancer", *Advances in Biological Research* **5**, 226;2011). An abnormal Pap smear result is trailed by colposcopic examination, biopsy and histological confirmation of the results (*The Lancet* **370**, 890;2007). The whole procedure is not only tiresome for the patient but also necessitates well-

trained health care professionals for monitoring the results accurately, which may vary even in experts' hands.

A lot of researches have already been performed directed towards both improved diagnosis and early detection of cervical cancer. A few recent works show enhanced, modern, and miniaturized diagnostic tools which can improve the colposcopic performance even in the less experienced hands (Das et al., "Elimination of specular reflection and identification of ROI: The first step in automated detection of Cervical Cancer using Digital Colposcopy", IEEE, 237;2011). A few other works report fluorescence (Tumer et al., "Ensembles of radial basis function networks for spectroscopic detection of cervical precancer", IEEE Transactions on Biomedical Engineering **45**, 953;1998), infrared (Wood et al., "Fourier transform infrared (FTIR) spectral mapping of the cervical transformation zone, and dysplastic squamous epithelium", Gynecologic oncology **93**, 59;2004) and Raman spectroscopic techniques (Kanter et al., "Application of Raman spectroscopy for cervical dysplasia diagnosis", Journal of biophotonics **2**, 81;2009) for the detection of precancerous lesions leading to early detection of positive cases of cervical cancer. Many groups have also reported auto-fluorescence technique (Benavides et al., "Multispectral digital colposcopy for in vivo detection of cervical cancer", Optics express **11**, 1223;2003), which can better differentiate the normal cells with cervical dysplasia as compared to the conventional technique of cervicography (Duraismy et al., "Methods of detecting cervical cancer", Advances in Biological Research **5**, 226;2011) where the cells are differentiated by the application of acetic acid. Apart from all these, a few developments have also done in the field of detection of HPV DNA as a tool for the early detection of cervical cancer (Benavides et al., "Multispectral digital colposcopy for in vivo detection of cervical cancer",

Optics express **11**, 1223;2003). Also, some groups have examined CA-125 antigen from blood serum for the early detection of cervical neoplasia (Porika et al., "Squamous cell carcinoma antigen and cancer antigen 125 in southern Indian cervical cancer patients", Asian Pac J Cancer Prev **11**, 1745;2010). However, many of the reported techniques either use bulky instruments or these are invasive in nature.

Since the primary causes of cervical cancer are governed by several complicated mechanisms, identification of a particular biomarker for this fatal disease has been elusive. There are number of reports available regarding different professed biomarkers of cervical cancer, such as, U.S. Pat. No. 7,157,233 has reported a number of cell cycle regulation genes, including p14^{ARF}, p21waf1, Topo2A and cyclin-E as the possible biomarkers for the identification of cervical dysplasia. Similarly, U.S. Patent No. 7,186,514 has discovered a new gene, MN gene, which is present in chromosomal DNA that signifies conditions of tumorigenicity in various tissues, including cervical tissues. In another work, U.S. Patent No. 20050186215A1 identified a human gene, CUDR, in cancer cells. However, these works represent a generalized route to the clinical detection of cervical cancer, as the genes mentioned above are not unique to cervical cancer owing to its expression in other tumor cells and minute levels in even normal cells too.

Furthermore, U.S. Patent No. 6,709,832 reported another cell cycle regulatory protein, p16^{INK4a}, as a potential biomarker for cervical cancer, which is overexpressed in biopsies of cervical dysplastic cells from CIN I, II, III and even more significant in invasive carcinomas. Later, in a U.S. Patent No. 7,306,926, authors formalized a procedure to measure p16^{INK4a} as a diagnosis tool for the detection of cervical cancer from the solubilized

cervical samples collected from the cervix of the affected patients. Thus far, p16^{INK4A} is the lone protein, which is the only commercially available biomarker for cervical cancer. In another patent, U.S. Patent No. 8,900,813, authors have claimed the discovery of CIP2A as a positive
5 biomarker for the detection of cervical cancer. It is reported that either CIP2A mRNA or CIP2A protein or both can be used with at least one additional biomarker such as MCM2, MCM5, p14^{ARF}, and p16^{INK4a} to serve better as a detection tool for cervical cancer.

10 In a similar line, the present invention claims for the advancement involving a potential and particular biomarker PP1Y2, secreted from the cervical dysplasia and neoplasia tissues as an NCB for the detection of cervical cancer (Chakrabarti et al., "Protein phosphatase PP1Y2 in sperm morphogenesis and epididymal initiation of sperm motility", Asian journal
15 of andrology **9**, 445;2007). The said biomarker also appears in the urine of the affected patients, and hence eliminates the need for the scrapping of cervical tissues for examination. It has been observed through examinations that the mentioned protein is detected in precancerous as well as all the cancerous stages of Cervical Cancer, irrespective of the
20 stage of cancerous lesions. However, the concentration varies with stages of cancer growth.

This invention also states the development of a point-of-care-testing (POCT) device, which will surely transform the screening process for
25 cervical cancer from tedious and highly invasive procedure to effortless and painless practice. In the present era, POCT devices have gained enormous popularity and have occupied a significant sector of the medical field, due to its advantages of in situ monitoring of health, reliability, real-time results, portability, and economic values (Luppa et al., "Point-of-care

testing (POCT): Current techniques and future perspectives", *TrAC Trends in Analytical Chemistry* **30**, 887;2011). There are a variety of commercially available biosensors categorized on the basis of the signal transduction technologies like optical (Damborský et al., "Optical biosensors", *Essays in biochemistry* **60**, 91 ;2016), electrochemical (U.S. Patent No. 5776324A), thermometric (Ramanathan et al., "Principles and applications of thermal biosensors", *Biosensors and Bioelectronics* **16**, 417;2001), piezoelectric (P. Skládal, "Piezoelectric biosensors", *TrAC Trends in Analytical Chemistry* **79**, 127 , 2016) or magnetic (J. B. Haun, "Magnetic nanoparticle biosensors", *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology* **2**, 291;2010). Among all these, exponential growth on research and development of the optical biosensors directed towards health care, environmental applications and biotechnology have been highly experienced over the last few decades.

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An optical biosensor system is a sensitive analytical device consisting of a bio recognition sensing element, such as enzyme, antibody, antigen, receptor, nucleic acids, whole cells or tissues, integrated with an optical transducer element aimed to produce signals proportionate to the concentration of the analyte. These systems are capable of diagnosing various biomarkers present in certain body fluids, for example serum, sputum, sebum, tear or urine, and among others, which has permitted the efficient detection of a number of fatal diseases. In lieu of its advantages in terms of direct, real-time, sensitive, selective, and label-free detection of miscellaneous chemical or biological substances over conventional analytical techniques, optical biosensors have empowered affordable diagnosis at patients' site. Further, among others, the immunosensors (La et al., "Immunosensing using nanoparticles", *Materials today* **13**, 24;2010) exploiting the colorimetric sensing

capacities are considered as the most consistent technique for the quantitative analysis of biomarkers present in various biological fluids owing to its nature of specific lock and key interaction between the sensor material and the analyte forming a visual color change on reaction.

5

Although the colorimetric sensing methods, which show a significant color change upon interaction with the required target, have been widely accepted and appreciated for its ease of examination with naked eye, they also possess quite a few drawbacks. At first, the development of considerable color upon interaction with the target present in the analyte necessitates a huge amount of analyte to be used to differentiate between the control and the affected cases. Also, if the concentration of the target in the analyte is very less, the result of the interaction of the analyte and the sensor may provide false results as there might not be significant color change. Thus, the dynamic range of the reaction limits to slightly over one order of magnitude. Also, a few of the enzyme based colorimetric sensing takes longer time to respond, hence difficult to incorporate for real time analysis.

Optical transduction of the colorimetric sensor responses, other than visual color change, can be analyzed with a number of spectroscopic techniques. The binding event between the capture molecule and the target can be optically observed by labeling the capture molecule with a tag for such as florescent tag as in Enzyme Linked Immunosorbent Assay (ELISA) used for fluorescent spectroscopy (Tian et al., "A copper based enzyme-free fluorescence ELISA for HER2 detection", Journal of immunological methods **451**, 78;2017) or Raman tag for Raman spectroscopy (Zong et al., "Surface-enhanced Raman spectroscopy for bioanalysis: reliability and challenges," Chemical reviews **118**,

4946;2018). However, these labeled tags can have adverse effects on assay results due to the steric hindrance to the attached biomolecules. Further, these methods are not compatible to real time testing as well as increase the device complexity and cost. Hence, label-free assays are more advantageous for real time analysis, providing improved performance and sensitivity, and can also be used for biomolecular interaction analysis. One such improved and label free transducer is metal nanoparticle. These are used as a tool to monitor binding events through a phenomenon known as Localized Surface Plasmon Resonance (LSPR) (Zhou et al., "The analysis of nobel metal nanoparticles LSPR phenomena", Chinese Journal of Sensors and Actuators **5**, 007;2010). Thus, this technique can be efficiently exploited for the early detection of innumerable fatal diseases, including various forms of cancer, even if the antigen is present in very minute amount. Such methods have certainly modernized effective patient management and, potentially, reduce mortality rate.

The development of biocompatible nanoparticles, such as gold, silver, copper, and among others have provided a proficient sensing probe for targeted therapy (Tiwari et al., "Functionalized gold nanoparticles and their biomedical applications", Nanomaterials **1**, 31;2011). The basic rationale is that the nanometer-sized-particles have larger surface to volume ratio which provides improved conjugation sites for multiple diagnostic and therapeutic agents over bulk materials. This improvement in the structural and functional properties has not only miniaturized the analyte volume required for the detection but also has provided scope for the economic portable devices. Also, these metal nanoparticles are capable of showing LSPR property, which shows specific sharp spectral absorbance peaks owing to the attachment of the biomolecules and

produces explicit electromagnetic near-field shifts with the change in the local refractive index in the surrounding of the nanoparticle (Zhou et al., "The analysis of nobel metal nanoparticles LSPR phenomena", Chinese Journal of Sensors and Actuators **5**, 007;2010). This property of the LSPR
5 phenomenon is exploited to determine the concentration of the particular target in the complex medium.

In the same direction, this work is also focused on the development of a clinical diagnostic tool for non-invasive and early identification of cervical
10 cancer, exploiting an improved spectroscopic metal nanoparticle-based sensing technique i.e. LSPR. This system will surely meet the demand of a reliable tool to ease the screening process of cervical cancer and will clear the deficiencies of all the prior arts.

15 **OBJECT OF INVENTION**

One of the basic objects of the present invention is to provide sensing of cervical cancer specific to bio-marker NCB (PP1Y2), wherein the over
expression of the said protein strongly indicates the occurrence of cervical
20 cancer in females.

Another object of the present invention is to measure the appearance of the said biomarker in urine of the subjects non-invasively.

25 A further object of the present invention is to provide antibodies against the NCB wherein the said antibody when used in Western Blot (WB) assay detects expression level of NCB.

Yet another object of the present invention provides a sensor comprising a colloidal solution of surface modified metal nanoparticles imparting LSPR property.

5 Still another object of the present invention provides surface modified metal nanoparticles by capping the surface of the nanoparticles with a chemical linker substance, which preferentially attract the specific capture molecules, antibodies, in subsequent addition.

10 Another object of the present invention is to provide a simple, non-invasive and user-friendly point of care testing (POCT) device for early and real time identification of the targeted protein biomarker for early detection of cervical cancers.

15 SUMMARY OF THE INVENTION

The basic aspect of the present invention is to provide a biomarker sensor for specific sensing of cervical cancer comprising gold nanoparticles with capture molecules immobilized thereon exhibiting LSPR for targeted PP1Y2
20 biomarker.

In another aspect, the present invention provides a biomarker sensor comprising said anti-PP1Y2 biomarker antibody, which is specific towards the target PP1Y2 biomarker, required to be detected in any biological
25 fluid-based analyte including urine samples, as the said capture molecule which is linked and immobilized on said gold nanoparticle.

A further aspect of the present invention is to provide a biomarker sensor wherein said PP1Y2 biomarker antibody as capture molecule is linked and

immobilized on said gold nanoparticle involving antibody to gold nanoparticle linkers preferably 3,3'-Dithiodipropionic acid di(N-hydroxysuccinimide ester) DTSP, 1-Ethyl-3-(3-dimethylamino propyl)carbodiimide (EDC)/N-hydroxysuccinimide EDC/NHS, cysteamine,
5 3,3-Dithio- bis-(sulfosuccinimidyl)propionate (DSP), 3,3'-Dithiobis (sulfosuccinimidyl propionate) (DTSSP) and the like.

In a still further aspect, the present invention provides the biomarker sensor comprising load of said PP1Y2 biomarker antibody as capture
10 molecule on said DTSP attached gold nanoparticles is in proportions of 60 μ L to 1.5 mL, respectively.

In a further aspect, the present invention provides the biomarker sensor wherein said gold nanoparticles comprises nanoparticles of ~ 5 nm to ~
15 10 nm in diameter having wine red color.

In another aspect, the present invention provides a non-invasive and point of care testing (POCT) facilitating biomarker based cervical cancer detection system comprising:

- 20 i) an optical source;
- ii) a sample stage including biomarker sensor comprising gold nanoparticles with capture molecules immobilized thereon adapted for exhibiting LSPR for targeting PP1Y2 biomarker in the analyte through LSPR based target biomarker detection;
- 25 iii) a signal processor means including an optical detector.

A further aspect of the present invention provides a non-invasive and point of care testing (POCT) facilitating biomarker based cervical cancer detection system comprising of said biomarker sensor specific towards the

target PP1Y2 biomarker required to be detected in any biological fluid based analyte preferably in urine samples including said gold nanoparticles of ~ 5 nm to ~ 10 nm in diameter having said capture molecule comprising anti-PP1Y2 biomarker antibody and wherein said
5 chemical linker to stably immobilize said capture molecule is selected from DTSP, EDC/NHS, cysteamine, DSP, DTSSP preferably DTSP.

Yet another aspect of the present invention provides a non-invasive and point of care testing (POCT) device facilitating biomarker based cervical
10 cancer detection system which is configured to allow said biomarker present in analyte/urine sample and the antibody immobilized on gold nanoparticles interaction and generation of output signals confirming LSPR intensity variation based thereon.

15 A still further aspect of the present invention provides a non-invasive and point of care testing (POCT) device facilitating biomarker based cervical cancer detection comprising said optical source such as a light emitting diode (LED) having narrow wavelength between ~ 515 nm and ~ 540 nm, comprising said optical detector, which is including a silicon photodiode
20 capable of sensing in the visible light wavelength range and capturing the variation in the transmitted light intensity compatible to the signal processing unit, with said sample stage including a cuvette, placed in between said the optical source and the said optical detector including photodetector, and wherein said signal processor means including
25 transmission intensity based generation of calibration plot and diagnosis of the unknown samples.

In another aspect, the present invention provides a non-invasive and point of care testing (POCT) device facilitating biomarker based cervical
30 cancer detection system comprising an optoelectronic device including:

an optical transduction set up comprising optical source housing for LSPR optical source preferably LED, an optical detector preferably a silicon photo detector with said sample stage including a glass cuvette placed in
5 between said optical source and the said photodetector, said signal processing means operatively connected to display, means for detection purposes.

A further aspect of the present invention provides a non-invasive and
10 point of care testing (POCT) device facilitating biomarker based cervical cancer detection system wherein the optical source illuminates the sensing material held by the glass cuvette, and the output transmitted signal is collected by the photodetector arranged in the apparatus set;
said signal processing means comprises of a microcontroller for
15 processing incoming transmitted analog signals from the photodetector to convert it into digital signals for subsequent display, wherein display means for easy end user detection, said microcontroller commanded with pre-loaded calibrated data of various concentration of the protein to be identified, to enable measuring of the correct level of the protein of
20 interest present in the analyte examined.

A still further aspect of the present invention provides a process for the manufacture of the biomarker sensor for specific sensing of cervical cancer comprising:
25 i) providing gold nanoparticles in the range of ~ 5 nm to ~ 10 nm;
ii) involving solution of the chemical linker to generate chemically linked gold nanoparticles;
iii) adding selectively 60 µL to 1.5 mL of anti-NCB antibody solution to the
DTSP linked gold nanoparticles to thereby provide the gold
30 nanoparticles with said anti-NCB antibody capture molecules

immobilized thereon capable of exhibiting LSPR for target PP1Y2 biomarker.

BRIEF DESCRIPTION OF THE ACCOMPANYING FIGURES

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Figure 1 Depicts gel showing the expression of NCB transcript in the cervico-vaginal fluid collected from clinically confirmed subjects of cervical cancer and precancerous stages. β -actin served as internal control for the reaction.

10

Figure 2 Depicts Immunofluorescence micrograph showing signal of NCB in the cells of the cervico-vaginal fluid collected from the clinically confirmed cervical cancer patients.

15

Figure 3 Depicts photo-micrograph showing co-expression of NCB and HPV in the cervico-vaginal fluid collected from early and advanced stages of cervical cancer patients. Red fluorescence depicts NCB while green shows HPV signal in the specimen. DAPI is showing blue staining for the nucleus of the cells.

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Figure 4 Depicts comparison of total secreted protein and detection of NCB in the urine samples of healthy, unhealthy females as well as cervical cancer patients. (a) Coomassie staining of gel shows separated urine proteins through SDS-PAGE. (b) Ponceau staining of blot shows blotted protein profile of various urine samples. (c) Western blot shows the shedding of NCB antigen in cervical cancer patients' urine. P1: recurrence of cervical cancer; P2: cervical cancer stage IIIB; UH: Unhealthy with abnormal pap but noncancerous cervix; F1 & F2: Healthy female urine sample.

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Figure 5 Depicts the detection of NCB in the urine samples collected from precancerous as well as various stages and groups of cervical cancer. (a) Ponceau staining of blots shows the protein profile of various urine samples. (b) Western blot analysis shows the shedding of NCB antigen in urine, where Lane 1 to 5 CaCx: Different stages of cervical cancer; Lane 5 & 6 Rec: Recurrence cases of cervical cancer; Lane 8 & 9 Tre: Follow-up under treatment patients; Lane 10 to 14 CIN: Different CIN stages; Lane 15 andf 16 UH: abnormal pap and unhealthy but noncancerous cervix.

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Figure 6 Shows the dimensional geometry of 3D printed device. (a) shows (i) Isometric (ii) Front (iii) Bottom (iv) Left (v) Right (vi) Top view of the 3D printed ndevice (100). (b) shows (i) Isometric (ii) Front (iii) Top (iv) Bottom (v) Left (vi) Right view of the cuvette housing (101) part of the 3D printed device. (c) shows (i) Isometric (ii) Left (iii) Right view of the LED housing (102) part of the 3D printed device and the schematic diagram of the LED (103). (d) shows (i) Isometric (ii) Left (iii) Right (iv) Top view of the photodetector casing (104) part of the 3D printed device and the schematic representation of the photodetector, TSL2561 (105). (e) shows (i) Isometric (ii) Bottom (iii) Top view of the cover of the cuvette (106).

Figure 7 Depicts the UV-Visible spectroscopy results for (a) pristine gold nanoparticles (AuNPs - brown dashed line), AuNPs after DTSP attachment (solid traffic green line), Antibody (Ab) attached DTSP linked AuNPs (blue square symbols), and after Antigen (Ag) attachment (protein separated from cervical cancer patients' urine) on Ab linked on the AuNPs (pink dash-dot line). (b) pristine AuNPs (blue dashed line), DTSP linked AuNPs (traffic green dash-dot line), Ab attached DTSP linked AuNPs (red

triangular symbols), and after Ag attachment (urine sample) on Ab-AuNPs composite (violet dash-dot-dot line). (c) shows change of UV-Vis absorbance intensity along with gradual blue shifts of spectra on successive additions of urine with respect to Ab stabilized on DTSP linked AuNPs absorption intensities. Legends 1 to 3 in the image (c) represent absorptions of AuNPs, AuNPs linked DTSP, and Ab stabilized on DTSP linked AuNPs, respectively.

Figure 8 Depicts the results of the interference study. Plot (a) shows UV-Vis spectra of AuNPs (black line with red box), DTSP immobilized AuNPs (solid light green line), Ab stabilized AuNPs (blue triangular symbols only), and on addition of increasing amounts of cervical cancer patient's urine sample (other lines after blue triangular symbols) to Ab stabilized AuNPs. Plot (b) shows UV-Vis spectra of DTSP immobilized AuNPs (solid light green line), Ab stabilized AuNPs (blue triangular symbols only), and on the addition of different amounts of healthy urine sample (other lines after blue triangular symbols) to Ab stabilized AuNPs. Plot (c) shows the UV-Vis response of Ab immobilized AuNPs (blue triangular symbols only) on addition of different amounts of healthy patient's urine sample (lines below the blue triangular symbols), initially, and on subsequent addition of increasing amounts of cervical cancer patient's urine sample (lines above the blue triangular symbols) in comparison to DTSP immobilized AuNPs (solid light green line), and Ab stabilized AuNPs (blue triangular symbols only).

25

Figure 9 Depicts the change in intensity and wavelength shift obtained from UV-Visible spectroscopic analysis of ten affected patients' urine samples. Plot (a) shows the hypsochromic shift of the UV-Vis spectra observed after the addition of ten different cervical cancer affected

patients' urine samples to the Ab stabilized AuNPs solution. Plot (b) shows the normalized intensity of absorbance of the UV-Vis spectra observed after the addition of ten different cervical cancer affected patients' urine samples to the Ab stabilized AuNPs solution.

5

Figure 10 Shows the circuit diagram of the electronic transducing set up. The image shows a microcontroller (Arduino Uno R3, ATmega328p) at the center, to the right of which, is a resistor (R) of 1 k and an LED (D). To the left of the microcontroller is the photodetector (TSL2561) and a display (OLED).

10

Figure 11 Depicts Mass spectrometry analysis showing the purity and integrity of NCB synthetic peptide of 23 amino acids (Mol wt ~ 2.5 kDa).

15 **Figure 12** Depicts the calibration curve obtained from the developed portable POCT device for cervical cancer detection.

DETAILED DESCRIPTION OF THE INVENTION

20 The present invention thus discloses a biomarker sensor for specific sensing of cervical cancer and a non-invasive, POCT device facilitating biomarker based cervical cancer detection system. The said system involves identification of cervical cancer biomarker, (PP1Y2), from the urine sample of a patient. The said system is based on the detection of
25 the intensity change due to the variation in the refractive index at the surface of a Localized Surface Plasmon Resonance (LSPR) system owing to lock-and-key interaction of antibody and antigen. The present invention also claims the development of a POCT system comprising one optical source, one photodetector, and metal nanoparticle-based sensor.

The said system comprises of an LSPR optical source, a dedicated photodetector and a geometrically confined cuvette placed in between the LSPR optical source and the photodetector. The cuvette is filled with the synthesized sensor material for the LSPR detection of the said target
5 protein. The sensor comprises the colloidal solution of modified metal nanoparticles, particularly for the detection of the targeted protein biomarker.

The mentioned terms shall have the meaning as described below,
10

As used herein, the term 'biological sample' is a body sample that can be used as an analyte to detect various biomarkers of certain diseases. A biological sample includes, but is not limited to, urine sample, cervical tissues, cervical sections, or biopsies of the cervix.

15 As used herein, the term 'cervical cancer' refers to the development of neoplasia or the abnormal growth of a mass of carcinogenic tissues in the cervix or cervical regions of a female.

20 As used herein, the term 'biomarker' refers to the naturally occurring molecule or gene or any characteristic by which a particular disease or pathological imbalance can be measured.

As used herein, the term 'cervix' refers to the lower region of the uterus
25 of a female reproductive system which encompasses uterine cervix, endocervix, and ectocervix.

As used herein, the term 'cervico-vaginal fluid' means the fluid collected from the excretion of the cervical tissues of a female.

As used herein, the term 'antibody' refers to the protective protein formed by the immune system of the body in response to any invasion from foreign body known as an antigen.

5

As used herein, the term 'protein' refers to a chain of amino acids.

As used herein, the term LED refers to light-emitting diode, which is an LSPR optical source used in the developed apparatus.

10

As used herein, the term photodiode refers to an optical detector, which changes its electrical properties when exposed to a light source.

As used herein, the term cuvette refers to a container made up of a transparent material such as glass, used in the developed apparatus to hold the sensing material.

As used herein, the term POCT refers to the reported point of care testing device, which is portable in nature and is aimed to provide easy and real time diagnosis of the disease.

The present invention as discussed herein before discloses PP1Y2 as a novel biomarker for the diagnosis of cervical cancer. A number of results generated in line with the clinically verified healthy and cervical cancer affected subjects have revealed that the expression level of PP1Y2 increases in all cases of cervical neoplasia. The increased expression level of PP1Y2 was also found throughout various stages of cancer of the cervix.

The expression of the reported NCB was verified in the cervico-vaginal fluid (CVF) samples collected from clinically confirmed cervical cancer patients affected with various stages of cervical cancer. The reverse transcriptase polymerase chain reaction (RT-PCR) results of the RNA,
5 extracted from the CVF samples collected from the cervical cancer affected patients, showed an elevated level of the reported NCB in all of the clinically confirmed cervical cancer cases.

Figure 1 shows the results of RT-PCR. The expression of NCB-PP1γ2 was
10 also validated in CVF samples of the patients having unhealthy but noncancerous cervix and also in the cases of patients showing recurrence of cervical cancer. The RT-PCR results of such cases show a diminished intensity of the bands as compared to the intensity of the bands obtained for various stages of cervical cancer cases. Apart from RT-PCR,
15 Immunofluorescence (IF) technique was also implemented to test the presence of the claimed NCB in the CVF samples collected from the clinically confirmed cervical cancer cases, as shown in **Figure 2**. The results show that the CVF samples containing NCB, confirmed a positive immune-reactivity when incubated with anti-NCB antibodies. However,
20 the CVF samples where NCB is absent, no immune-reactivity against anti-NCB antibodies was observed.

In another study, the presence of NCB was tested for the CVF samples, which were clinically confirmed for the presence of HPV infection. Co-
25 immuno localization of NCB and HPV for various stages of cervical cancer patients was done using dual immunostaining technique, as shown in **Figure 3**. The results show positive immuno-staining for both NCB and HPV in the collected CVF samples, indicated by red and green stain, respectively in the figure shown and blue is nucleus stained with DAPI.

Therefore, it can be concluded that the reported NCB is expressed in all the cases where HPV infection was clinically confirmed.

Present invention is related to the detection of the NCB, PP1 γ 2, in the urine samples of cervical cancer patients. For this work, urine samples were collected from different stages of clinically confirmed cervical cancer patients. Urine samples were also collected from patients having abnormal but noncancerous cervix and the patients showing recurrence of the disease. Two healthy individuals were also tested for the existence of NCB in the respective urine samples. The results of the testing are shown in **Figure 4**. Overall level of secretory proteins was negligible in healthy individuals as evident from coomassie and ponceau staining shown in **Figure 4(a)** and **Figure 4(b)**, respectively. No detectable signal of bands corresponding to NCB could have been observed in case of healthy volunteers whereas very intense bands were observed in cervical cancer patients when probed with anti-NCB specific antibodies. This data indicated that the anti-NCB antibody is specific to the claimed NCB and the Western Blot technique is efficient for the identification of the discovered protein.

20

The expression of the said NCB was examined in the urine samples of a number of subjects using the Western Blot technique, as shown in **Figure 5**. The results obtained from the Western Blot technique shows that the said NCB is found in all the clinically confirmed cervical cancer cases. It also shows that high-intensity bands were obtained for different stages of cervical cancer patients as compared to the intensity of the bands obtained for different CIN stages, patients showing recurrence of the disease, and also for the patients having unhealthy but noncancerous cervix.

25

The above results show that the NCB is a potential biomarker for the early identification of precancerous and cervical cancer cases from the urine samples of the subjects.

5

It is evident that the diagnosis of cervical cancer by painless non-invasive pathway is elusive for quite a long period. The said urinary protein, PP1Y2, which is specific to cervical cancer, is targeted to develop an LSPR based device exploiting antigen-antibody interactions for an easy
10 diagnosis of cervical cancer. Liquid phase LSPR technique further offers promising homogeneity of the solution and material light interactions in order to enhance the specificity and selectivity of sensing.

Figure 6(a) (i-vi) refers to the developed three-dimensional (3D)
15 apparatus for the detection/quantification system for cervical cancer diagnosis. The sensing system (**100**) is made up of a reusable 3D printed apparatus. **Figure 6(b) (i-vi)** shows a housing (**101**) placed at the middle of the 3D printed set up (**100**) to hold the cuvette. **Figure 6(c) (i-iii)** shows an optical source chamber (**102**) of the 3D printed set up
20 (**100**), to place the Light Emitting Diode (LED) (**103**). **Figure 6(d) (i-iii)** an optical detector chamber (**104**) at the other end of the 3D printed set up (**100**), to house the photodetector (**105**). **Figure 6(e) (i-iii)** shows the cuvette cover (**106**) used for capping the cuvette to minimize the interference of undesired external light. The transducing system
25 composes a central processing unit, which contains a microcontroller, accessory units like resistor, and a digital user interface. The optical source, photodetector unit, microcontroller, and the display can be electrically powered either by a portable rechargeable battery, low voltage power outlet system, or a combination of both. The optical source can be

a simple LED, or a controlled high-power laser, or any other suitable light source. The optical detector unit is any visible range photodetector module such as silicon photodiodes. The digital user interface unit can be any available display for example a Liquid Crystal Display, or an Organic
5 Light Emitting Diode (OLED) display.

Initially, the reagent, which is a suitable solution containing antibodies specific to NCB is taken into the cuvette. The chosen cuvette is of standard dimensions (1 cm × 1 cm). Subsequently, the test sample,
10 either a solution containing the required antigen or urine sample collected from the patients' having cervical cancer, is dropped to the cuvette externally by some suitable quantifiable transfer unit such as a micropipette, or a graduated dropper. The analyte solution or the urine sample should be mixed well before use to confirm the homogeneity of
15 the solution. After pouring the sample into the cuvette, it can directly be placed in the 3D printed housing. Once the cuvette is placed into the housing, the light source and the microprocessor board can be turned on using a suitable power delivery source, as mentioned previously and the cuvette is covered with the cuvette cover mentioned. The said device
20 works on the transmission mode. Hence, the light coming out of the light source can directly be passed through the cuvette containing the material to be sensed, and during the passage, a fraction of the incoming light is expected to be absorbed by the solution inside the cuvette.

25 The extent of absorption would proportionally vary with the concentration of the targeted NCB present in the analyte used. The light left out after the absorption is to fall on the photodetector, fixed at the other end of the developed 3D printed device. The amount of light falling on the detector can be digitally read with the help of a microcontroller-based central

processing unit. Further, programming can be set into the microcontroller to digitally process the incoming light intensity data into the corresponding concentration of the target protein present in the analyte used. A number of known concentrations or volume of test samples has
5 been tested with the fabricated device to generate a calibration curve for the quantitative estimation of the test sample.

The reagent that is used for the LSPR based sensing is made of metal nanoparticles such as gold with the subsequent attachment of specific
10 anti-NCB antibody by any chemical or physical attachment procedures. Further, the metal nanoparticles solution is prepared from a precursor salt. The precursor salt is advised to dissolve in Milli-Q water to prepare a relatively low and suitable concentration. Henceforth, a suitable reducing agent such as Sodium Borohydride or Trisodium citrate can be used to
15 synthesize metal nanoparticles from the precursor solution. The synthesis should be done under constant stirring with a suitable stirring rate to control the size of the synthesized metal nanoparticles. Optimization of synthesis parameters is required for synthesizing appropriate metal nanoparticles. Subsequently, anti-NCB antibodies, specific to the targeted
20 protein, can be attached to the surface of the metal nanoparticles. A chemical linker such as DTSP is used for the attachment of antibodies on the surface of metal nanoparticles. After the attachment, the solution should be kept in a suitable environment, under low specific temperature such as 4°C for further use.

25

The sensitivity of the developed sensor was examined with the help of UV-Visible spectrophotometer, and the results are shown in **Figure 7**. The metal nanoparticles solution was taken at first in a cuvette, and the UV-Vis absorbance response was examined. Afterward, UV-Vis absorbance

spectrum was examined for the anti-NCB antibody stabilized metal nanoparticles solution and on the addition of the analyte on the antibody stabilized metal nanoparticles solution. Two types of analytes were used for the study are: the total protein separated from the urine samples of the clinically confirmed cervical cancer affected patients and the crude urine samples collected from the clinically confirmed cervical cancer affected patients.

On addition of the total separated protein to the anti-NCB attached metal nanoparticle solution, an increase in UV-Vis absorbance intensity with a concurrent hypsochromic shift was observed, as shown in **Figure 7(a)**. The results in Figure 7(b) show the increase in UV-Vis absorbance intensity with a significant hypsochromic shift observed on the addition of 300 μ L of crude urine sample collected from the clinically confirmed cervical cancer patients. The results in **Figure 7(c)** show a monotonic increase in the intensity of the UV-Vis absorbance spectra with concurrent hypsochromic shift on increasing the volume of the crude urine samples added on the antibody stabilized metal nanoparticles solution.

The increase in the intensity of the absorbance spectra with concurrent hypsochromic shift is found to be a cause of the LSPR phenomenon. The increase in the intensity of the absorbance spectra is probably due to significant electronic changes occurred in the optical properties on the surface of metal nanoparticles owing to the interacting antibody and the reported NCB. The hypsochromic shift observed is hypothesized as a reason for charge accumulation on the surface of metal nanoparticles owing to antigen-antibody interaction.

The selectivity of the sensor was studied by examining the interaction of the anti-NCB antibody with proteins other than the claimed NCB. The results of the interference study are shown in **Figure 8**. For the interference study, healthy volunteers' urine samples, individuals not affected by cervical cancer, were collected and considered for the study. **Figure 8(a)** shows the increase in the intensity with subsequent hypsochromic shift (lines above blue triangular symbols and below red squared black line) on the addition of increasing volume of crude urine samples collected from the clinically confirmed cervical cancer patients to the anti-NCB attached gold nanoparticles solution (blue symbols). **Figure 8(b)** shows the UV-Vis absorbance results obtained after the addition of crude urine samples collected from healthy volunteers (lines above solid traffic green colored line and the blue symbols), to the anti-NCB attached gold nanoparticles solution. The results in **Figure 8(c)** the difference in the UV-Vis absorbance properties on the addition of healthy as well as affected patient's crude urine samples to the same sensor solution. It was observed that when a healthy patient's urine sample was dispensed to the sensor solution, there was neither any increase in intensity nor was any hypsochromic shift. However, on the addition of the cervical cancer affected patient's crude urine sample to the same sensor solution, both the increased intensity and hypsochromic shift properties of the LSPR phenomenon were observed. The results show that the LSPR property, as described above, of the metal nanoparticles is observed only for the cases containing NCB in the analyte whereas no such LSPR property, neither increase in the intensity of the absorbance spectra nor the hypsochromic shift, was observed in the healthy volunteers' urine sample where the claimed NCB is absent.

A total of ten (10) clinically confirmed cervical cancer patients' crude urine samples were tested with the developed sensing technique. Interestingly, all the affected cases showed LSPR phenomenon in UV-Vis spectrophotometer, as shown in **Figure 9**. **Figure 9(a)** shows the
5 hypsochromic wavelength shift observed for ten different cervical cancer affected patient's crude urine samples, when added to the sensor solution. **Figure 9(b)** shows the increased in intensity of the UV-Vis absorbance observed in the same experiment. The plot depicts that different cervical cancer patient's urine samples show different LSPR
10 effect. This is probably due to the presence of different concentrations of the NCB in various cervical cancer affected patients' urine samples owing to different stages of cervical cancer and/or probably due to different concentrations of urine at different times of a day based on the metabolic activity of the patient.

15

Therefore, the above studies demonstrate that the sensing technique proposed in the present work is competent enough in identifying the claimed NCB and thus can be recommended for the diagnosis of cervical cancer.

20

The illustration of various techniques and embodiments preferred for the present invention are mentioned as experimental studies in the below section. However, it should be noted here that the descriptions stated do not limit the scope of the present invention, as attributed in the claims.
25 Further modifications could be intended within the scope and spirit of the invention.

EXPERIMENTAL STUDIES

Example 1: Evaluation of NCB transcript in Cervico-vaginal fluid (CVF)

Cervico-vaginal fluid (CVF) was collected from the early as well as advanced stages of the clinically confirmed cervical cancer patients, and total RNA from the sample was extracted. Expression of NCB transcript was checked in these samples through conventional Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) using NCB specific primers. **Figure 1** shows the expression of NCB transcript in the cervico-vaginal fluid collected from clinically confirmed subjects of cervical cancer and various CIN stages. Here, β -actin is used as internal control for the reaction.

Detailed results for the expression of NCB in various clinically confirmed CVF samples tested with RT-PCR have been summarized in **Table 1**. A total of 115 patients were considered for the examination of the CVF through RT-PCR. It was found that out of fifty (50) clinically confirmed cervical cancer cases, forty-one (41) samples (82%) expressed NCB through RT-PCR of the CVF samples. Out of twenty-five (25) clinically verified CIN cases, NCB was detected in fifteen (15) cases (60%) through RT-PCR of the CVF samples. In a similar test, CVF samples of four (4) out of ten (10) (40%) treated patients and three (3) out of five (5) patients having recurrence of the disease, showed the existence of NCB when tested with RT-PCR of the CVF samples of the respective patients. In contrast, eleven (11) out of twenty-five (25) CVF samples of the patients having abnormal noncancerous pap was tested positive for the presence of NCB in the CVF sample.

Table 1: Detailed results along with clinico-pathological status of clinical CVF samples positive for the expression of NCB.

Clinico-Pathological Stage of Samples	Sample Size	Samples positive for NCB (RT-PCR)
Non-Cancerous Abnormal pap	25	11/25 (44%)
CIN	25	15/25 (60%)
Cervical cancer	50	41/50 (82%)
Treated	10	04/10 (40%)
Recurrence	05	03/05 (60%)
Total	115	74/115 (64%)

These results demonstrate that the transcript expression of NCB (~ 205 bp) was observed in all the clinically confirmed cervical cancer samples. High-intensity band signal was also observed in different CIN stages as well as faint bands were detected in fluid collected from unhealthy cervical conditions. The data indicate that the claimed NCB is frequently expressed in precancerous as well as different stages of cervical cancer.

Example 2: Assessment of NCB protein expression in Cervico-vaginal fluid by Immunofluorescence technique

The expression of NCB at the translational level in the CVF collected from the clinically confirmed cervical cancer patients was evaluated using the indirect immuno-fluorescence technique. Slides were prepared from the CVF, and after processing, it was incubated with anti-NCB specific antibody for immunostaining. The results in **Figure 2** demonstrate NCB expression at the translational level in the CVF samples of cervical cancer patients. The immunofluorescence photo-micrograph indicates that the control set did not show any immune-reactivity when incubated without anti-NCB primary antibody.

20

Example 3: Co-Immunostaining of NCB and HPV in the CVF of Pre-cancerous and various stages of cervical cancer subjects

Co-immuno-localization of NCB and HPV in the CVF collected from precancerous as well as various stages of cervical cancer patients was

done using dual immunostaining technique. CVF slides were probed with specific anti-NCB and anti-HPV primary antibodies. HPV antibody was against the consensus region of E6 oncoprotein of HPV 16 and 18 strains. Positive immuno-staining was observed for both NCB and HPV in the patient samples, which is indicated by red and green stain respectively in **Figure 3** and blue color indicates nucleus stained with 4',6-diamidino-2-phenylindole (DAPI). Photo-micrographs in the figure show co-expression of NCB and HPV in the cervico-vaginal fluid samples collected from early (CIN II), advanced stages of cervical cancer patients CaCx IB, IIA, IIIB, and negative sample. The data indicate that NCB is found in different stages of cervical cancers where the HPV infection is also present. Hence, it can be concluded here that the claimed protein is co-expressed with HPV infection even in precancerous and early stages of cervical cancer.

Example 4: Examination for the secretion of PP1Y2 protein in urine samples with Western Blot technique

Protein extracted from a biological fluid, urine sample, of the patient, were denatured with Laemmli buffer containing 10% Beta-mercaptoethanol (BME) by heating of samples at 95°C for 10 min on a hot plate. Now the denatured protein samples were loaded (30 g) in each well of 12.5% SDS-PAGE gel, and then the gel was electrophoresed in a Tris-Glycine and SDS buffer system at a constant voltage i.e. 50 V. The proteins separated through SDS-PAGE were transferred onto nitrocellulose membrane by electro-blotting. The blotted membrane was stained with ponceau stain to visualize the blotted proteins. Now the membrane was washed with 1X Phosphate buffer saline (PBS) buffer and kept for blocking in 2% Bovine serum albumin (BSA) in 0.1% Phosphate buffer saline with Tween-20 (PBST) buffer for 1 h over rocker shaker at room temperature (25°C). The blot was then incubated with NCB specific

primary antibody at certain standardized dilutions (1:3000) for overnight (12-14 hours) at 4°C. The membrane was then washed with 0.1% PBST for three times and with 1X PBS twice. Now secondary antibody tagged with horseradish peroxidase (HRP) was added, at a standardized dilution
5 (1:5000) for 4 h at room temperature (25°C). Thereafter, the membrane was washed with 0.1% PBST thrice followed by 1X PBS twice. Further, protein blots were developed through chemiluminescence reagent and the developed protein bands were detected through Chemidoc showing signals corresponding to the protein of interest.

10

Figure 4 shows the comparison of the total excreted proteins and the presence of the claimed NCB in the urine samples of the healthy and cervical cancer affected patients. Here, **P1** is represented for the urine sample of the patient undergoing recurrence of cervical cancer; **P2** is
15 represented for the urine sample of the patient affected by cervical cancer stage IIIB; **UH** is represented for the urine sample of an unhealthy patient with abnormal pap but noncancerous cervix; **F1 & F2** is represented for healthy female urine sample. **Figure 4(a)** shows the Coomassie Brilliant Blue staining to visualize the protein bands in the
20 12.5% acrylamide gel after the Electrophoresis. **Figure 4(b)** shows Ponceau (Pink) staining of protein bands on the nitrocellulose membrane after electro-blotting (transfer of protein from SDS-PAGE gel to nitrocellulose membrane) of various urine samples obtained in **Figure 4(a)**. **Figure 4(c)** depicts the specific bands on the membrane which was
25 probed with anti-NCB specific primary antibody followed by enzyme tagged secondary antibody and finally developed with substrate using chemiluminescence method to produce the specific signal showing the existence of NCB antigen in the urine sample of cervical cancer. In sum, these data indicate that PP1Y2 is not present in the urine sample of

healthy individuals but gets secreted in cervical cancer patients as evident from the western blot results. These results signify that the NCB is a definite protein found in the urine samples of the cervical cancer affected patients.

5

Similarly, western blot study was done with urine samples collected from different stages of cervical cancer patients, recurrence of cervical cancer (Rec), patients undergoing follow-up treatment (Tre), various stages of precancerous (CIN), and patients with unhealthy cervix but noncancerous conditions (UH) and examined for the existence of the NCB. **Figure 5** shows the results of the detection of NCB in the urine samples collected from various stages and groups of cervical cancer patients. **Figure 5(a)** shows the ponceau stained protein bands and **Figure 5(b)** shows the western blot results. The data shows that the intensity of protein band for NCB is not equal for all the cases examined. The variation in the intensity of the NCB bands observed in the WB results, allows distinction of different stages and groups of cervical cancer. It was observed that the intensity of the protein band in UH is less intense as compared to the advanced and other stages of cervical cancer.

20

The details of urine samples collected and tested for NCB through WB is summarized in **Table 2**. The samples were confirmed by the clinicians from King George Medical University, Lucknow for the cancer and their stage.

25

Table 2: Details of urine samples tested for NCB through WB.

Sample Type	No. of samples tested	No. of positive sample in WB
Healthy Volunteers	05	0/05 (0%)
Abnormal but noncancerous	30	22/30 (73%)

Pap		
CIN II/ III	50	43/50 (86%)
IA/ IB	05	04/05 (80%)
IIA/ IIB	42	39/42 (93%)
IIIA/ IIIB	33	31/33 (94%)
Follow-up treated cases	15	11/15 (73%)
Recurrence	05	05/05 (100%)
Total Urine Samples	185	155/185 (84%)

The table data show that none of the healthy urine samples was tested positive for the presence of NCB, which is in line with the clinical results for healthy samples. In contrast, for different stages of cervical cancer, the positivity of NCB presence was found to be much higher such as for 5 IA/IB stage of cervical cancer, four (4) out of the five (5) clinically confirmed cases were found to be positive with NCB in WB (80%), for IIA/IIB stage, thirty-nine (39) out of the forty-two (42) clinically confirmed cases were found to express NCB in WB (92%), and for 10 IIIA/IIIB stage, thirty-one (31) out of thirty-three (33) clinically verified cases were found to have high levels of NCB (93%) expressed in WB results of the urine samples.

Similarly, for CIN II/III cases, forty-three (43) out of fifty (50) clinically 15 positive cases showed detectable levels of NCB presence (86%) in WB study. Out of fifteen (15) clinically assured cases of patients undergoing treatment for cervical cancer, eleven (11) cases showed presence of NCB in WB. For recurrence cases, all the five (5) clinically verified cases were found to be positive with NCB in WB (100%). For patients with abnormal 20 cervix but noncancerous conditions, twenty-two (22) out of the thirty (30) clinically confirmed cases showed a light band in WB.

In summary, the results shown above suggest that PP1γ2 protein levels were elevated in urine samples of the cervical cancer patients. The

increased level of PP1Y2 protein could be detected in the patient's urine samples as early as in CIN I, thus providing an opportunity for early diagnosis of the disease. PP1Y2 protein expression was also examined in CVF samples through RT-PCR and urine sample through WB in patients inflicted with cervical intraepithelial neoplasia (CIN), various stages of cervical cancer (CaCx), patients undergoing treatment (Tre), patients showing recurrence of the disease and cases of abnormal noncancerous cervical pap.

Table 3 shows comparative results of the CVF samples tested through RT-PCR and urine samples of the same set of patients through WB for the assessment of NCB appearance profile, simultaneously. A total of fifty-two (52) patients were enrolled for this study, and both CVF and urine samples were collected from the cervical cancer sites of the respective subjects. Twelve (12) patients were clinically confirmed stage IIA/IIB cervical cancer, ten (10) patients from stage IIIA/IIIB cervical cancer, and thirteen (13) patients infected with CIN II/III stage. Seven (07) patients undergoing treatment for the disease were clinically found to be positive with cervical cancer. Two (2) patients were clinically diagnosed with a recurrence of cervical cancer, and eight (08) patients were found to have abnormal but noncancerous pap.

Table 3: Comparative results of the CVF samples.

Sample Type	Total no. of samples	Samples positive for NCB Expression	
		PAP (RT-PCR)	Urine (Western Blotting)
Non-Cancerous/ Abnormal	08	03/08 (37%)	04/08 (50%)
CIN II/ III	13	04/13 (31%)	10/13 (77%)

CaCx IIA/ IIB	12	06/12 (50%)	11/12 (92%)
CaCx IIIA/ IIIB	10	06/10 (60%)	09/10 (90%)
Treated	07	02/07 (29%)	06/07 (86%)
Recurrence	02	01/02 (50%)	02/02 (100%)
Total	52	22/52 (42%)	42/52 (81%)

Out of twelve (12) stage IIA/IIB cervical cancer patients, only six (06) (50%) patients showed the presence of NCB in respective CVF, examined through RT-PCR. In contrast, the presence of NCB was confirmed in urine samples of eleven (11) (92%) patients when examined through WB technique. Similarly, out of ten (10) number of stage IIIA/IIIB patients, NCB presence in CVF was found only six (06) (60%) patients, when examined through RT-PCR whereas the number increased to nine (09) (90%) when the NCB presence was examined in the urine samples of the patients using WB technique. Of all the thirteen (13) CIN II/III cases, four (04) (31%) patients' CVF samples confirmed the presence of NCB in RT-PCR, whereas NCB was validated in urine samples of ten (10) (77%) patients through WB technique. Among the seven (07) patients undergoing treatment, two (02) (29%) showed the presence of the NCB in CVF in RT-PCR wherein the number was found to be six (06) (86%) when NCB examination was done with the urine samples of the respective patients through WB technique. For the two (02) recurrence cases, one (01) (50%) showed the presence of NCB in the CVF collected through RT-PCR, however both the two (02) (100%) urine samples expressed the presence of NCB in WB. Amid all the CVF and urine samples collected from eight (08) patients having abnormal non-cancerous pap, three (03) (31%) CVF samples confirmed the NCB presence in RT-PCR. In contrast, four (04) (50%) urine samples confirmed the presence of NCB in WB.

The clinical studies designed in the mentioned examination suggest PP1Y2 as a reliable and sensitive biomarker dedicated for the early identification

of the cervical cancer cases regardless of the stage of the development of the cancer. The above-mentioned data illustrate that PP1Y2 is expressed in high percentages in the urine sample of the patients suffered from cervical cancer diseases, any stage or group, whereas the healthy
5 volunteers' urine samples do not express any PP1Y2 protein. In abstract, these data suggest that PP1Y2 is a potential and reliable biomarker for cervical cancer.

**Example 5: UV-Visible Spectrophotometer results of antibody-
10 antigen interaction**

UV-Vis spectrum of the gold nanoparticles was obtained by mixing 1.5 mL of synthesized gold nanoparticles with 1 mL of Mili-Q water as shown in **Figure 7(a)**. Afterwards, UV-Vis spectrum of 1.5 mL solution of chemical linker, DTSP, linked gold nanoparticles diluted with 1 mL of Mili-Q water
15 were observed. Furthermore, 60 μ l of anti-NCB antibody solution, prepared in PBST buffer was added to the DTSP linked gold nanoparticles. In one study, 300 μ l of total protein solution containing the protein of interest, which was separated from urine samples collected from clinically confirmed cervical cancer patients, was added, and the UV-Vis spectra
20 were recorded, as shown in **Figure 7(a)**. In another study, 300 μ l of the crude urine samples collected from the clinically confirmed cervical cancer patient was added, and UV-Vis spectra were recorded, as shown in **Figure 7(b)**.

25 **Figure 7(c)** shows the variation in the absorbance intensity and the subsequent hypsochromic shifts with progressive addition of different volumes of the crude urine samples of the cervical cancer patient ranging from 20 μ L to 300 μ L. The experiment suggests as long as the unbound paratope sites are available on the anti-NCB antibodies present on the

surface of the gold nanoparticles, the epitope site on the reported NCB, present in the affected urine sample, selectively binds to the antibodies on the gold nanoparticles via lock and key mechanism.

5 **Example 6: Interference study with healthy analyte using UV-Visible Spectrophotometer**

An interference study was performed comparing the binding affinity of the anti-NCB with the reported NCB present in medically verified cervical cancer affected urine samples and healthy volunteer's urine samples. UV-
10 Vis spectroscopic response of the gold nanoparticles solution was taken by adding 1.5 mL of the synthesized gold nanoparticles solution with 1 mL of Milli-Q water. Afterward, UV-Vis spectrum was recorded for 1.5 mL of DTSP linked gold nanoparticles solution diluted with 1 mL of Milli-Q water. To this solution, 60 μ L of anti-NCB antibody solution, prepared in PBST
15 buffer, was added, and the UV-Vis spectrum was recorded. Furthermore, different volumes of the required analyte were added to the anti-NCB antibody stabilized gold nanoparticles solution, and the UV-Vis spectra were recorded.

20 **Figure 8(a)** shows the UV-Vis spectrophotometric response for the interaction of anti-NCB antibodies with cervical cancer affected patients' urine samples (where the complimentary antigen, NCB, is present). The results show the LSPR effect on the addition of different volumes of cervical cancer affected patient's urine sample to the solution containing
25 anti-NCB antibodies stabilized gold nanoparticles solution. On the contrary, after the addition of the healthy volunteer's urine sample (collected from a healthy woman, where the complementary antigen is absent) to the anti-NCB antibodies stabilized gold nanoparticles solution, no such LSPR effect was observed as it can be depicted in **Figure 8(b)**.

The results in the plot show either a decrease or no significant change in the intensity of the absorbance spectra on the addition of the healthy urine samples. This is probably due to the absence of any interaction between the reported NCB, which is absent in a healthy urine sample, with the corresponding anti-NCB antibodies stabilized on the gold nanoparticles.

Figure 8(c) shows the UV-Vis spectra obtained after the addition of cervical cancer affected patient's urine samples and healthy volunteer's urine samples alternatively. The results show that the developed sensor responds only if the analyte contains the reported NCB, as the intensity of absorbance increases, with respect to the UV-Vis spectrum for antibody stabilized gold nanoparticles solution, in that case only, whereas it is not sensitive towards healthy volunteer's urine sample, as the intensity is not changing with respect to the UV-Vis spectrum for antibody stabilized gold nanoparticles solution.

Here, it can be depicted from the spectra that on the addition of cervical cancer positive patients' urine sample to anti-NCB antibodies stabilized gold nanoparticles solution, the antibodies bind to the particular NCB of interest present in the urine sample. This lock-and-key binding shows an LSPR shift with a parallel intensity increment. However, no such response of LSPR or intensity increment was observed on addition of healthy patient's urine sample due to absence of the claimed NCB to which the anti-NCB antibodies are supposed to bind. Thus, from the above results, it can be concluded that the proposed sensing technique is potentially capable of identifying cervical cancer patients, non-invasively from the urine sample of the patients.

Example 7: Case study with cervical cancer positive urine samples

A case study with ten (10) different clinically confirmed cervical cancer affected patient's urine samples (U1-U10) was done with the developed sensing technique, as shown in **Figure 9**. It was observed that all the cases show the LSPR effect on the addition of the affected patient's urine sample on the anti-NCB stabilized gold nanoparticles solution.

Figure 9(a) shows the hypsochromic change ($\Delta\lambda$) observed due to LSPR effect on the addition of the affected patient's urine sample on the anti-NCB stabilized gold nanoparticles solution for ten (10) different urine samples (U1-U10). The $\Delta\lambda$ was calculated by measuring the difference in the wavelength between the peak of UV-Vis spectra of the anti-NCB antibodies stabilized gold nanoparticles and the peak of the UV-Vis spectra after the addition of the cervical cancer affected patient's urine sample.

Figure 9(b) shows the change in the intensity of the absorbance spectra (A_N) observed due to LSPR effect on the addition of the affected patient's urine sample on the anti-NCB stabilized gold nanoparticles solution for ten (10) different urine samples (U1-U10). The A_N was calculated by measuring the difference in the intensity of absorbance observed for the UV-Vis spectra of the anti-NCB antibodies stabilized gold nanoparticles solution and the intensity of the absorbance observed for the UV-Vis spectra after the addition of the clinically confirmed cervical cancer patients' urine sample.

Example 8: Transmittance based POCT System for quantification of NCB

The detailed description of the transmittance-based system for quantification of the biomarker of interest is as follows,

- (a) The detection unit comprises of a glass cuvette filled with the transformed sensing solution wherein the mentioned anti-NCB antibodies
5 are expected to interact with the protein of interest present in the analyte to generate quantifiable optical signal corresponding to the concentration of the protein present in the analyte.
- (b) The three-dimensional (3D) design of the developed optoelectronic device, as shown in **Figure 6**, shows the components of the developed
10 apparatus. It consists of a sample stage (**101**) an optical transduction set up (**102**), (**103**), (**104**), (**105**), and a signal processing unit programmed to convert the incoming signals for display,
- (i) wherein **Figures 6(a) (i-vi)** represents the isometric, front, bottom, left, right, top view of the aforementioned developed apparatus;
- 15 (ii) wherein the sample stage (**101**) (**Figures 6(b) (i-vi)**) is arranged to place the glass cuvette holding the sensor solution;
- (iii) wherein the optical transduction set up comprises of an optical source housing (**102**) (**Figures 6(c) (i-iii)**) to hold the LSPR optical source such as a LED (**103**), an optical detector chamber (**104**) (**Figures 6(d)**
20 (**i-iii**)) to hold the optical detector such as photodetector (**105**) along with a glass cuvette placed in between the optical source and the photodetector;
- (iv) wherein the optical source illuminates the sensing material held by the glass cuvette, and the output transmitted signal is collected by the
25 photodetector arranged in the apparatus set up;
- (v) wherein the signal processing unit comprises of a microcontroller which is programmed to process the incoming transmitted analog signals from the photodetector to convert it into digital signals which is then displayed for easy legibility of the end user. The microcontroller is also

commanded with calibrated data of various concentration of the protein to be identified, obtained experimentally, to measure the correct level of the protein of interest exists in the analyte examined.

- 5 The detailed component arrangement of the optical transduction set up is shown in **Figure 6**. Here, black polymer is used to design the entire casing of the device to create dark environment in order to minimize the interference of external diffused light on the sensing set up. Two hollow spaces are arranged to mount the optical source, such as LED and the
10 photodetector. The holes to house the LED and the photodetector are done in accordance with the dimensions of the component used. Along with these, a frame for positioning the glass cuvette is also arranged in the apparatus set up. The optical source is connected to a power supply such as battery through a resistor. And the photodetector is connected
15 externally to the signal processing unit.

- The signal processing unit is developed employing the open source Arduino Uno R3 development board. It consists of Arduino Integrated Development Environment (IDE) software platform used for programming
20 and interfacing Arduino board with the external circuitry through microcontroller fitted with the Arduino Uno R3 board. The microcontroller embedded is ATmega328p and is fitted with command to convert the analog input signals from the photodetector to digital output readings.

- 25 The ATmega328p microcontroller of the Arduino Uno R3 board consists of 6 analog pins and 14 digital pins used as input or output pins. It is regulated at 16 MHz clock pulse using ceramic resonator. Also, it has a USB jack which can be used both as a power supply unit and for uploading the program. It has 10-bit ADC (Analog to Digital Converter)

for digitizing the analog input from the photodetector. The input from the photodetector is set to any analog pins on the development board.

The circuit diagram of the developed device is illustrated in **Figure 10**. The figure shows the internal circuitry of the open source Arduino Uno R3 development board to which the other components are interfaced. The positive end of the LED used is connected to the 5V pin of the Arduino Uno via a 1 k Ω resistor and the negative pin of the LED is connected to GND pin of the Arduino Uno. For the connection of the photodetector included, two analog pins are required for inter IC (integrated circuit) connection. The serial clock (SCL) and serial data (SDA), pins of the photodetector are connected to A4 and A5 pins of the Arduino Uno development board. The V_{cc} and GND pins of the photodetector are connected to the 3.3 V and GND pins of the Arduino Uno board. For the display board connection, the serial clock (SCL) and serial data (SDA) pins are connected to the A4 and A5 analog pins of the Arduino Uno board. And the V_{cc} and GND pins of the display are connected to the 3.3 V and GND pins of the Arduino Uno board.

20 **Example 9: Experimental Methods and Protocols**

I. Preparation of reagents

(a) Pre-treatment of crude urine samples

The mid-stream 10-45 mL crude urine samples at any random time of the day were collected from the patients in 50 mL sterile conical centrifuge tubes and placed immediately in the cool box. The samples were immediately transported to the lab. Further, the urine sample was filtered through the Whatmann paper (8 μ m) to remove the mucus and contaminant followed by the centrifugation at 1000*g for 5 min at 4°C to

remove any other contaminant left, which might not remove through filtration.

(b) Separation of NCB from urine samples

5 After centrifugation, supernatant was collected in another fresh tube, and an equal volume of pre-chilled acetone was added and incubated at -20°C for 40- 45 min to mediate acetone precipitation followed by centrifugation at 12000* g for 5 min at 4°C. The supernatant was discarded, and the pellet was re-suspended with 100 L to 200 L of protein extraction RIPA
10 buffer containing 1X Protease and Phosphatase inhibitors. After protein quantification through Bradford assay, samples were aliquoted and stored at -80°C for further analysis.

(c) Synthesis of synthetic peptide specific of PP1Y2

A peptide corresponding to 23 amino acids specific to the C-terminal of
15 NCB was synthesized commercially from **LifeTein, LLC, Hillsborough, New Jersey, USA**, for the antibody generation. Mass Spectrometry analysis was done to assess the integrity of the synthetic peptide as given in **Figure 11**.

(d) Generation of Antibody in Rabbit

20 Synthetic peptide specific to the NCB protein i.e. PP1Y2 was dosed to the female rabbit at optimized time point to raise the polyclonal antibodies in two female rabbits. Pre-immune sera were collected from these animals; afterward these animals were immunized with the first dose of 200 g of purified peptide with complete Freund's adjuvant and boosted five times
25 with 100 g of the same peptide at intervals of 7 days in incomplete Freund's adjuvant. 500 L of serum was collected after every two dosages of peptide injection and the complete serum was collected from the final bleed, which was affinity-purified through the same synthetic peptide. The

specificity of the affinity-purified antibody was tested against the 200 ng of this peptide and 50 g of mouse testis extract.

(e) Preparation of PBS Buffer

For 1 L 10X PBS, 25.6 g of Di-Sodium Hydrogen Phosphate (Na₂HPO₄·7H₂O), 80 g of Sodium Chloride (NaCl), 2 g of Potassium Chloride (KCl), and 2 g of Potassium Di-Hydrogen Phosphate (KH₂PO₄) reagents were dissolved properly in Milli-Q water and the final volume was made up-to 1 litre. This 10X PBS buffer was stored at 4°C for further use and diluted to 1X at the time of using this buffer.

10

(f) Preparation of PBST Buffer

PBST buffer was prepared by dissolving 0.1% (v/v) of Tween-20 surfactant to the required amount of prepared 1X PBS buffer, and 4°C storage condition was maintained.

(g) Preparation of Antibody Solution

1% (w/v) Bovine Serum Albumin (BSA) solution was prepared with a previously prepared PBST solution. 2.44 mg/mL stock solution of antibody was diluted, maintaining a ratio of 1:3000 in freshly prepared 1% BSA solution. The stock solution of the antibody (**Reagent I**) was stored initially at -20°C. For dilution purposes, the antibody stock solution was thawed at 4°C. The diluted antibody solution was then kept again at -20°C until further use.

(h) Preparation of Laemmli buffer

Laemmli sample buffer (2X) was prepared by using 100 mM of Tris HCL (pH 6.8), 0.02% Bromophenol Blue, 20% β-mercaptoethanol, 2% Sodium Dodecyl Sulphate (SDS) and 20% Glycerol in double distilled/RO water.

(i) Preparation of Tris-Glycine and SDS buffer system

Tris-Glycine running buffer (1X) was prepared by dissolving 25 mM Tris, 192 mM Glycine and 0.1% SDS in double distilled/RO water.

Transfer Buffer (2X) was prepared by dissolving 50 mM Tris Base, 384 mM Glycine, and 10% Methanol in double-distilled/RO water.

5 **(j) Preparation of RIPA buffer**

Radio-immunoprecipitation assay buffer (RIPA buffer) was commercially purchased from Sigma Aldrich (Cat. No. R0278).

(k) Preparation of gold nanoparticles solution

0.25 mM aqueous Tetrahydrochloroaurate (HAuCl_4) solution (**Reagent II**) was prepared by dissolving 10 mg of HAuCl_4 in 100 mL of Milli-Q water with stirring to mix the solution well. 0.1 M of aqueous reducing agent solution (**Reagent III**) was prepared by dissolving 0.38 g of Sodium Borohydride (NaBH_4) in 100 mL of Milli-Q water. Afterward, 20 mL of **Reagent II** was taken in a beaker and it was stirred at 1030 rpm in a hotplate. Then, 100 μL of **Reagent III** was added, drop-wise, to the stirring **Reagent II**. The change in the color of the stirring solution from yellow to orange at first and subsequently to wine red in nearly 10 min indicates the formation of gold nanoparticles. The gold nanoparticles solution (**Reagent IV**) thus synthesized were stored at 4°C in an airtight container for further use.

(l) Preparation of DTSP (3,3'-Dithiodipropionic acid di(N-hydroxysuccinimide ester) solution

DTSP solution of 5 mM strength was prepared by dissolving 2 mg of solid DTSP powder in 1 mL of Milli-Q-water. For further usage, 1:10 dilution of the prepared DTSP solution in Milli-Q water was done. This diluted solution (**Reagent V**) was kept at 4°C in an airtight container for subsequent use.

II. Preparation of sensor

The preparation of the sensor goes through two processes, as mentioned below,

(a) Attachment of DTSP with gold nanoparticles

DTSP attached gold nanoparticles solution was obtained by adding 1300
5 μL of diluted DTSP solution (**Reagent V**) was added to 7 mL of gold nanoparticles solution (**Reagent IV**). This solution was kept in the dark for an hour and was then filtered using a syringe filter of 0.45 μm in diameter. The prepared solution of DTSP attached gold nanoparticles solution thus obtained (**Reagent VI**) was stored at 4°C for further use.

10 **(b) Attachment of Antibody on DTSP attached gold nanoparticles solution**

To obtain anti-NCB antibody attached DTSP capped gold nanoparticles (**Reagent VII**), 60 μL of the prepared antibody solution (**Reagent I**) was added to 1.5 mL of **Reagent VI**. The immobilization of antibody on
15 DTSP linked gold nanoparticles solution was confirmed with the help of UV-Vis spectroscopy. This solution was then stored at 4°C.

The **Reagent VI** is the required sensor used for further analysis of the analyte.

20 **III. Procedure of testing an analyte**

For testing the analyte for the presence of the protein of interest (NCB), following steps were followed,

(a) 1.5 mL of the sensor solution, **Reagent VI**, was taken into a glass cuvette.

25 (b) The sensor solution was illuminated with the optical source and the transmitted signal reading was observed from the device. This reading is counted as the baseline value for the examination of the NCB in the provided analyte.

(c) After recording the baseline reading, 300 μ L of the analyte (urine sample of the patient) is added to the cuvette containing the sensor solution and mixed well.

(d) Again, the transmitted signal reading of the analyte added sensor solution was recorded and examined for the positivity or the negativity of the test.

IV. Preparation of Calibration curve

In order to map the variation in transmitted signal intensity with the concentration of NCB in the analyte, obtained from the developed device, a calibration curve was prepared, as shown in **Figure 12**, by plotting the normalized transmitted intensity value to the amount of urine solution added to the sensor.

The calibration curve, mentioned in **Figure 12**, shows the variation of the normalized intensity of the transmitted signal collected by the photodetector with the change in the amount of NCB present in the urine sample of the subject. The normalized transmitted intensity, as collected in the photodetector, is calculated by, $I_N = (I_t - I_i) / I_i$ where I_N represents the normalized transmitted signal intensity, I_i represents the baseline transmitted signal intensity obtained by illuminating the sensor solution before addition of urine sample. I_t represents the transmitted signal intensity obtained by illuminating the sensor solution after the addition of urine sample.

It can be inferred from the calibration curve that the normalized transmittance intensity decreases with increase in the volume of the urine sample added to the sensor solution. The reason for such observations is as follows, initially when only the sensor solution was analyzed, where

there is absence of NCB and thus there is no interaction of antibody and antigen takes place, the absorbance of incident light is low and hence the transmittance intensity is high. Similarly, with the addition of NCB from the analyte to the sensor, the interaction between the antibodies
5 stabilized on the sensor and the NCB from the urine sample of the patient which increases the absorbance of the incident light and thus the transmittance signal intensity decreases. The transmittance signal intensity decreases gradually with increase in the addition of NCB to the sensor solution until the phenomenon reaches a saturation point. The
10 transmittance signal intensity obtained in the photodetector is then converted into legible signal is monitored by the signal processing unit as depicted in **Figure 12**.

As explained in Example 8, a calibration curve is fitted into the
15 microcontroller system to map the increase in the volume of the urine sample collected from the cervical cancer affected subject, which is equivalent to the increase in NCB concentration, to the change in the normalized transmittance signal intensity as observed in the photodetector arranged in the device. Thus, when the device reports any
20 positive increase in the transmitted signal intensity on the addition of the analyte to the sensor solution, as compared to the transmittance signal intensity of the pristine sensor solution without addition of analyte, the microcontroller is capable to process the incoming signal, map it to the calibration curve and inform the severity of the disease.

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Thus, the LED/LDR arrangement translates the variation in the LSPR signal of the liquid sample in the cuvette during the specific epitope-paratope antigen-antibody reaction into the variation in the intensity of the transmitted light. Subsequently, the presence of the marker and the

amount of the marker can be quantified with the help of the variation in the intensity of the transmitted light, as discussed with the figures 9 and 12. Importantly the low-cost POCT system/method was found to be at par with high end and costly UV-Vis spectra.

5

Thus, the present invention provides for a simple and cost-effective biomarker sensor specific sensing of cervical cancer through targeting PP1Y2 biomarker and a non-invasive point of care testing (POCT) facilitating biomarker based cervical cancer detection system, which finds application for screening of cervical cancer non-invasively from the urine sample of the subject. Developed device will not only ease the tiresome process of conventional screening methods, but also simplify the need of the high skilled health care professionals and eradicate the boundaries of socio-economic limitations.

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WE CLAIM

1. A biomarker sensor for specific sensing of cervical cancer comprising gold nanoparticles with capture molecules immobilized thereon exhibiting
5 LSPR for target PP1Y2 biomarker.
2. A biomarker sensor as claimed in claim 1 comprising anti-said PP1Y2 biomarker antibody, which is specific towards the target PP1Y2 biomarker, required to be detected in any biological fluid-based analyte including
10 urine samples, as the said capture molecule, which is linked and immobilized on said gold nanoparticles.
3. A biomarker sensor as claimed in any one of claims 1 or 2 wherein said PP1Y2 biomarker antibody as capture molecule is linked and immobilized
15 on said gold nanoparticle involving antibody to gold nanoparticles linkers preferably selected from 3,3'-Dithiodipropionic acid di(N-hydroxysuccinimide ester) DTSP, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/N-hydroxysuccinimide (NHS), cysteamine, 3,3-Dithio- bis-(sulfosuccinimidyl) propionate (DSP), 3,3'-
20 Dithiobis (sulfosuccinimidyl propionate) (DTSSP) and the like.
4. A biomarker sensor as claimed in any one of claims 1 to 3 comprising load of said PP1Y2 biomarker antibody as capture molecule on said DTSP attached gold nanoparticles is in proportions of 60 μ L to 1.5 mL,
25 respectively.
5. The biomarker sensor as claimed in anyone of claims 1 to 4 wherein said gold nanoparticles comprises nanoparticles of $\sim$ 5 nm to $\sim$ 10 nm in diameter having wine red color.

6. A non-invasive and point of care testing (POCT) device facilitating biomarker based cervical cancer detection system comprising:

i) an optical source;

5 ii) a sample stage including biomarker sensor comprising gold nanoparticles with capture molecule immobilized thereon adapted for exhibiting LSPR for target PP1Y2 biomarker in the analyte for LSPR based target biomarker detection;

iii) a signal processor means including an optical detector.

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7. A non-invasive and point of care testing (POCT) device facilitating biomarker based cervical cancer detection system as claimed in claim 6 comprising of said biomarker sensor specific towards the target PP1Y2 biomarker required to be detected in any biological fluid based analyte
15 preferably in urine samples including said gold nanoparticles of ~ 5 nm to ~ 10 nm in diameter having said capture molecule comprising anti-said PP1Y2 biomarker antibody and wherein said chemical linker to stably immobilize said capture molecule is selected from DTSP, EDC/NHS, cysteamine, DSP, DTSSP preferably DTSP .

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8. A non-invasive and point of care testing (POCT) device facilitating biomarker based cervical cancer detection system as claimed in any one of claims 6 or 7, which is configured to allow said biomarker present in analyte/urine sample and the antibody immobilized on gold nanoparticles
25 interaction and generation of output signals confirming LSPR intensity variation based thereon.

9. A non-invasive and point of care testing (POCT) device facilitating biomarker based cervical cancer detection system as claimed in any one

of claims 6 to 8 comprising said optical source comprising a light emitting diode (LED) having narrow wavelength between ~ 515 nm and ~ 540 nm, said optical detector including a silicon photodiode capable of sensing in the visible light wavelength range and capturing the variation in the transmitted light intensity compatible to the signal processing unit, with
5 said sample stage including a cuvette, placed in between said optical source and the said optical detector including photodetector, and wherein said signal processor means including transmission intensity based generation of calibration plot and diagnosis of the unknown samples.

10

10. A non-invasive and point of care testing (POCT) device facilitating biomarker based cervical cancer detection system as claimed in any one of claims 6 to 9 comprising an optoelectronic device including:

an optical transduction set up comprising optical source housing for LSPR
15 optical source preferably LED, an optical detector preferably a photodetector with said sample stage including a glass cuvette placed in between said optical source and said photodetector, said signal processing means operatively connected to display means for detection purposes.

20 11. A non-invasive and point of care testing (POCT) device facilitating biomarker based cervical cancer detection system as claimed in any one of claims 6 to 10 wherein the optical source illuminates the sensing material held by the glass cuvette, and the output transmitted signal is collected by the photodetector arranged in the apparatus set up;

25 said signal processing means comprises of a microcontroller for processing incoming transmitted analog signals from the photodetector to convert it into digital signals for subsequent display, in display means for easy end user detection,

30 said microcontroller recommended with preloaded calibrated data of various concentration of the protein to be identified, to enable measure of

the correct level of the protein of interest present in the analyte examined.

12. A process for the manufacture of the biomarker sensor for specific
5 sensing of cervical cancer as claimed in any one of claims 1 to 5 comprising:

- i) providing gold nanoparticles in the range of ~ 5 nm to ~ 10 nm ;
- ii) involving solution of the chemical linker to generate chemical linked gold nanoparticles;
- 10 iii) adding selectively 60 μ L to 1.5 mL of anti-NCB antibody solution to the chemical linked gold nanoparticles to thereby provide the gold nanoparticles with said anti-NCB antibody capture molecules immobilized thereon capable of exhibiting LSPR for target PP1 γ 2 biomarker.

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AMENDED CLAIMS

received by the International Bureau on 09 December 2021 (09.12.2021)

WE CLAIM:

1. A biomarker sensor for specific sensing of cervical cancer comprising gold nanoparticles having linked and immobilized thereon capture molecules comprising anti PP1Y2 biomarker antibody immobilized thereon with load of said anti PP1Y2 biomarker antibody as capture molecule attached to said gold nanoparticles in proportions of 60 μ L to 1.5 mL, said anti PP1Y2 biomarker antibody being specific towards the target PP1Y2 biomarker, required to be detected in any biological fluid-based analyte including urine samples by exhibiting LSPR for target PP1Y2 biomarker.
2. A biomarker sensor as claimed in claim 1 wherein said anti -PP1Y2 biomarker antibody as capture molecule is linked and immobilized on said gold nanoparticle involving antibody to gold nanoparticles linkers preferably selected from 3,3'-Dithiodipropionic acid di(N-hydroxysuccinimide ester) DTSP, 1-Ethyl-3-(3- dimethylaminopropyl) carbodiimide (EDC)/N-hydroxysuccinimide (NHS), cystemine, 3,3-Dithio-bis-(sulfosuccinimidyl) propionate (DSP), 3,3'-Dithiobis (sulfosuccinimidyl propionate) (DTSSP) and the like.
3. The biomarker sensor as claimed in anyone of claims 1 or 2 wherein said gold nanoparticles comprises nanoparticles of $\sim$ 5 nm to $\sim$ 10 nm in diameter having wine red color.
4. A non-invasive and point of care testing (POCT) device facilitating biomarker based cervical cancer detection system comprising:
 - i) an optical source;
 - ii) a sample stage including a cuvette containing a biomarker sensor comprising gold nanoparticles having linked and immobilized thereon capture molecules comprising anti PP1Y2 biomarker antibody immobilized thereon with load of said anti PP1Y2 biomarker antibody as

capture molecule attached to said gold nanoparticles in proportions of 60µL to 1.5 mL, said anti PP1γ biomarker antibody being specific towards the target PP1γ2 biomarker, required to be detected in any biological fluid-based analyte including urine samples by exhibiting LSPR for target PP1γ2 biomarker

iii) a microcontroller based signal processor means including optical detector;

said sample stage including said cuvette, placed in between said optical source and the said optical detector including photodetector, and wherein said signal processor means include transmission intensity based generation of calibration plot for diagnosis of the unknown samples.

5. A non-invasive and point of care testing (POCT) device facilitating biomarker based cervical cancer detection system as claimed in claim 4 comprising of said biomarker sensor specific towards the target PP1γ2 biomarker including said gold nanoparticles of ~ 5 nm to ~ 10 nm in diameter having said capture molecule comprising anti-said PP1γ2 biomarker antibody and including said chemical linker to stably immobilize said capture molecule selected from DTSP, EDC/NHS, cysteamine, DSP, DTSSP preferably DTSP .

6. A non-invasive and point of care testing (POCT) device facilitating biomarker based cervical cancer detection system as claimed in any one of claims 4 or 5 which is configured to allow said biomarker present in analyte/urine sample and the antibody immobilized on gold nanoparticles interaction and generation of output signals confirming LSPR intensity variation based thereon.

7. A non-invasive and point of care testing (POCT) device facilitating biomarker based cervical cancer detection system as claimed in any one of claims 4 to 6 comprising said optical source comprising a light emitting

diode (LED) having narrow wavelength between ~ 515 nm and ~ 540 nm, said optical detector including a silicon photodiode capable of sensing in the visible light wavelength range and capturing the variation in the transmitted light intensity compatible to the signal processing unit.

8. A non-invasive and point of care testing (POCT) device facilitating biomarker based cervical cancer detection system as claimed in any one of claims 4 to 7 comprising an optoelectronic device including:

an optical transduction set up comprising optical source housing for LSPR optical source preferably LED, an optical detector preferably a photodetector with said sample stage including a glass cuvette placed in between said optical source and said photodetector, said signal processing means operatively connected to display means for detection purposes.

9. A non-invasive and point of care testing (POCT) device facilitating biomarker based cervical cancer detection system as claimed in any one of claims 4 to 8 wherein the optical source illuminates the sensing material held by the glass cuvette, and the output transmitted signal is collected by the photodetector arranged in the apparatus set up;

said signal processing means comprises of said microcontroller for processing incoming transmitted analog signals from the photodetector to convert it into digital signals for subsequent display, in display means for easy end user detection, said microcontroller including preloaded calibrated data of various concentration of the protein to be identified, to enable measure of the correct level of the protein of interest present in the analyte examined.

10. A process for the manufacture of the biomarker sensor for specific sensing of cervical cancer as claimed in any one of claims 1 to 3 comprising:

i) providing gold nanoparticles in the range of ~ 5 nm to ~ 10 nm;
ii) involving solution of the chemical linker selected from DTSP,
5 EDC/NHS, cysteamine, DSP, DTSSP preferably DTSP to generate
chemical linked gold nanoparticles;
iii) adding selectively 60 μ L to 1.5 mL of anti-NCB antibody solution to the
chemical linked gold nanoparticles to thereby provide the gold
nanoparticles with said anti-NCB antibody capture molecules immobilized
10 thereon capable of exhibiting LSPR for target PP1Y2 biomarker.

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STATEMENT UNDER ARTICLE 19 (1)

Amended claims are directed to clarify and qualify inventive aspects of the present invention which provides a biomarker sensor for specific sensing of cervical cancer comprising gold nanoparticles having linked and immobilized thereon capture molecules comprising anti PP1Y2 biomarker antibody immobilized thereon with load of said anti PP1Y2 biomarker antibody as capture molecule attached to said gold nanoparticles in proportions of 60 μ L to 1.5mL, said anti PP1Y2 biomarker antibody being specific towards the target PP1Y2 biomarker, required to be detected in any biological fluid-based analyte including urine samples by exhibiting LSPR for target PP1Y2 biomarker.

The present invention also provides for the first time a non-invasive and point of care testing (POCT) device facilitating biomarker based cervical cancer detection system comprising:

an optical source; a sample stage including a cuvette containing the said biomarker sensor; and a microcontroller-based signal processor means including an optical detector; said sample stage including said cuvette, is placed in between said optical source and the said optical detector including photodetector, and wherein said signal processor means include transmission intensity based generation of calibration plot for diagnosis of the unknown samples.

Importantly, the present invention specifically provides the point of care testing device for the detection of cervical cancer non-invasively from the urine sample of the patient utilizing the LSPR property of gold nanoparticles as a probe, where anti- PP1Y2 antibodies are attached to the gold nanoparticles and are allowed to interact with PP1Y2 antigen present in the urine sample of the patient has been explored. The change in the refractive index of the surrounding medium of the gold nanoparticles occurred due to the antigen-antibody reaction (between anti- PP1Y2 antibodies and PP1Y2 antigen on top of gold nanoparticles) is then detected using the non-invasive and point of care testing (POCT) device of the present invention.

The above distinctive aspects of the present advancement have been clearly and sufficiently brought out in the amended claims.

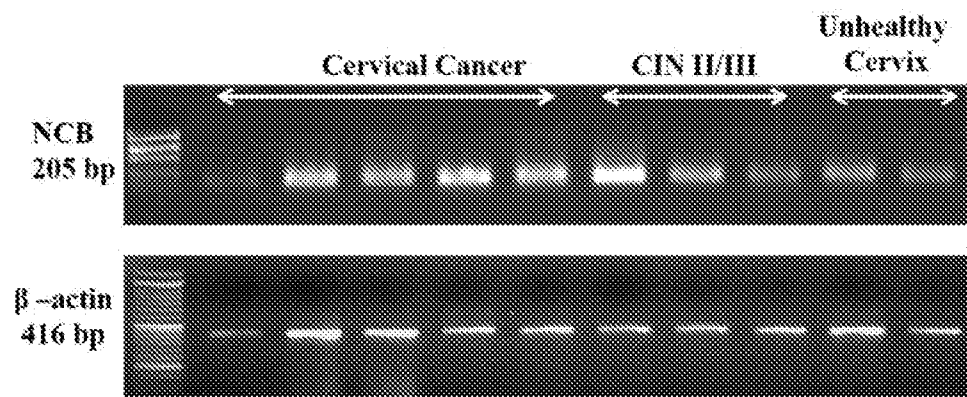


Figure 1

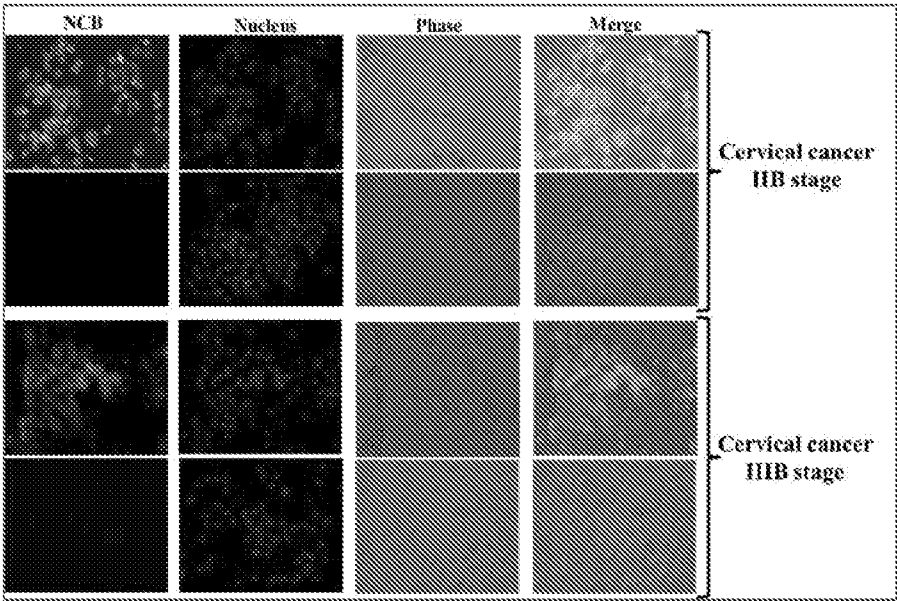


Figure 2

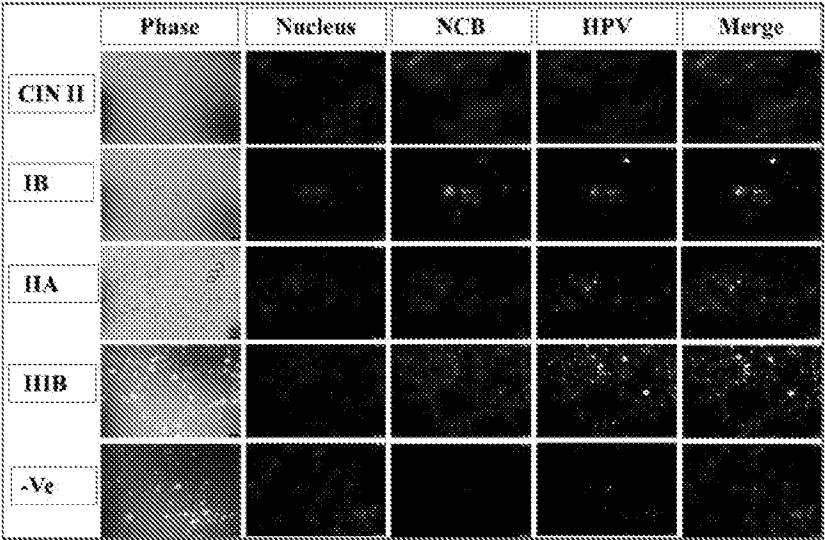


Figure 3

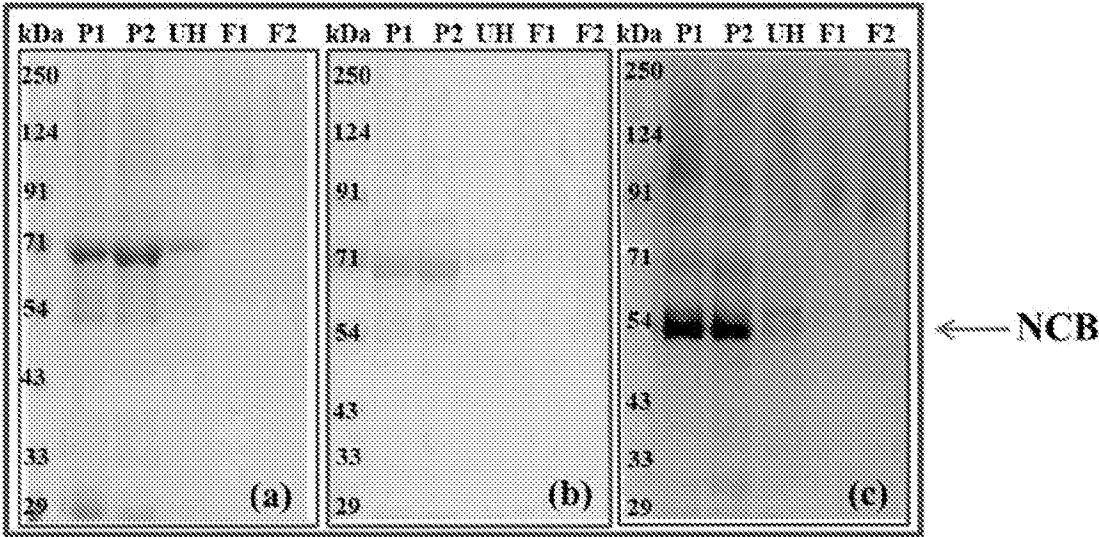


Figure 4

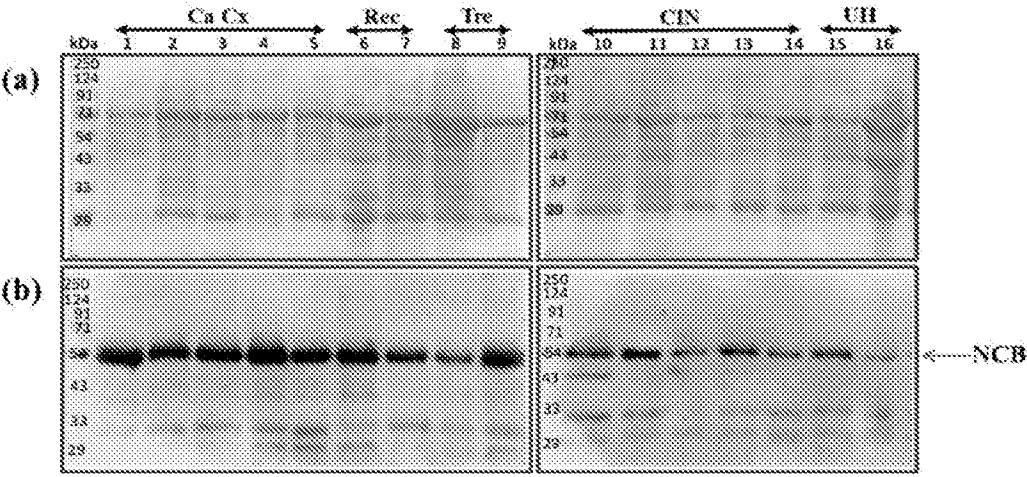


Figure 5

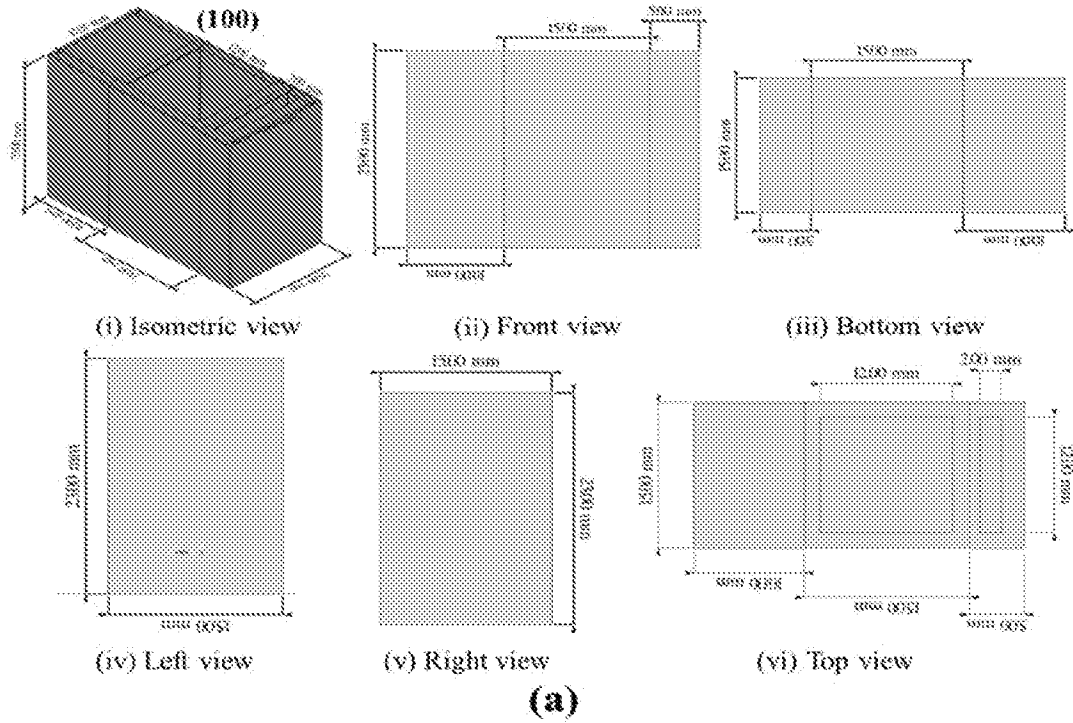


Figure 6

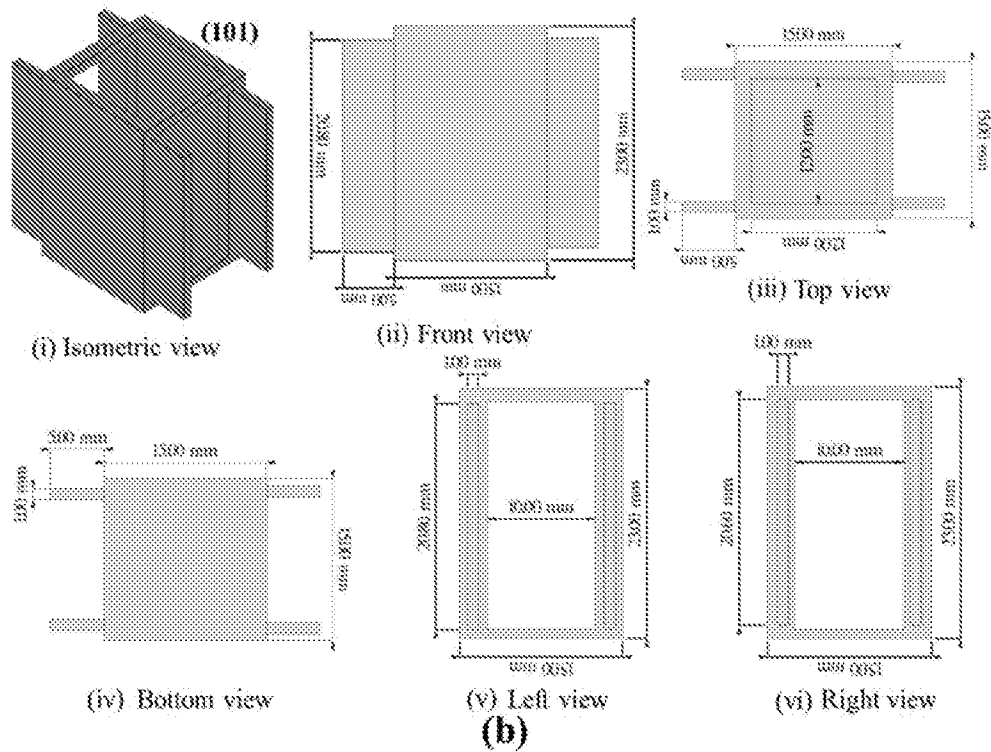


Figure 6

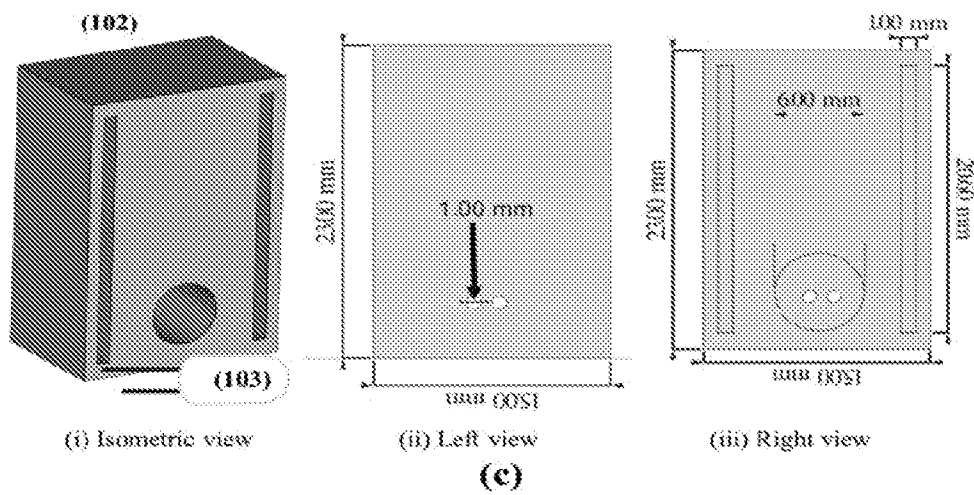


Figure 6

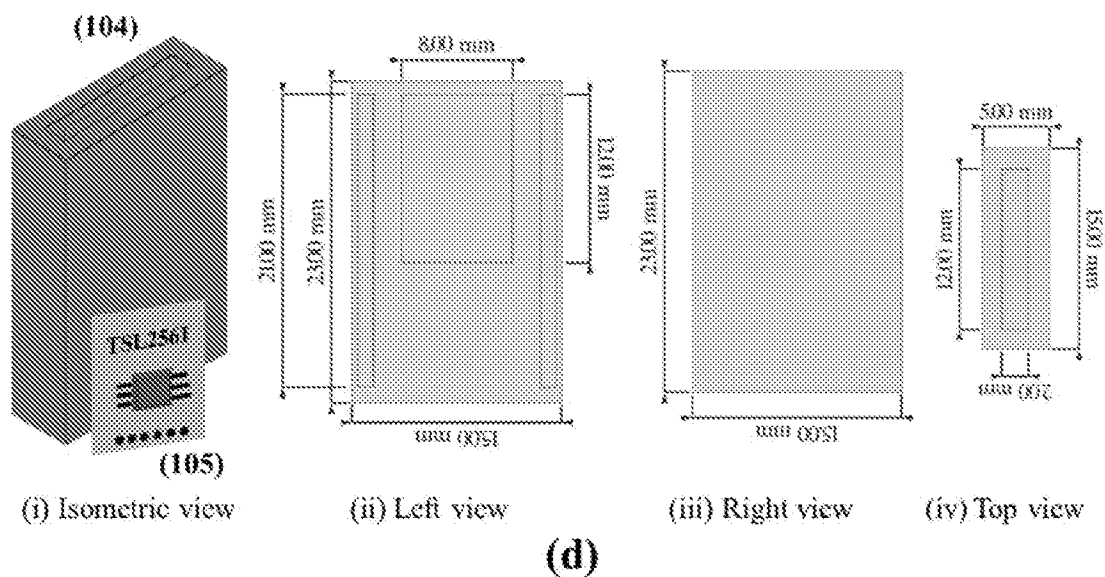


Figure 6

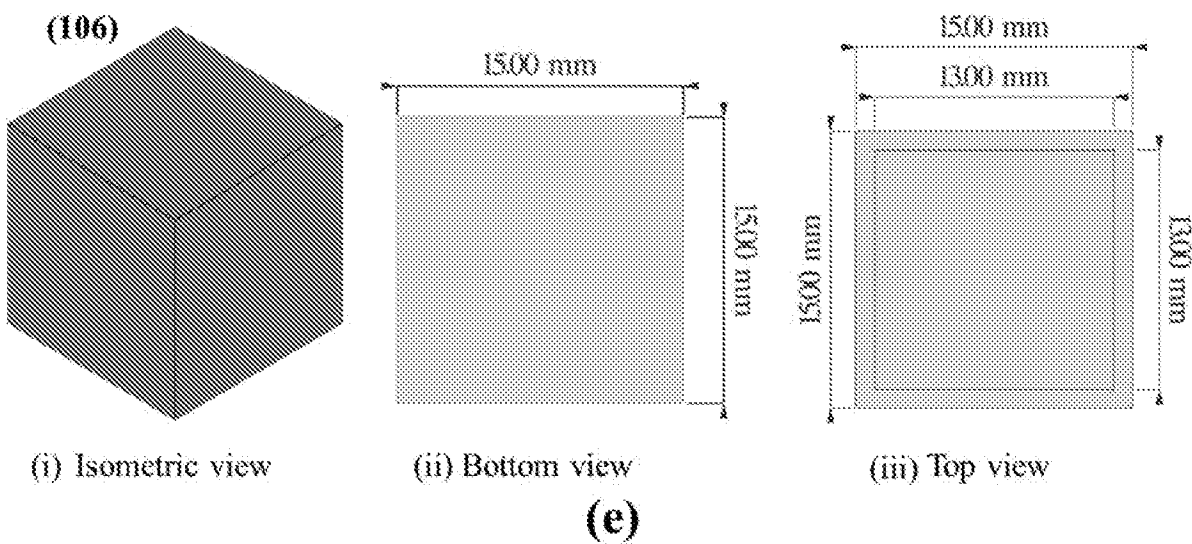
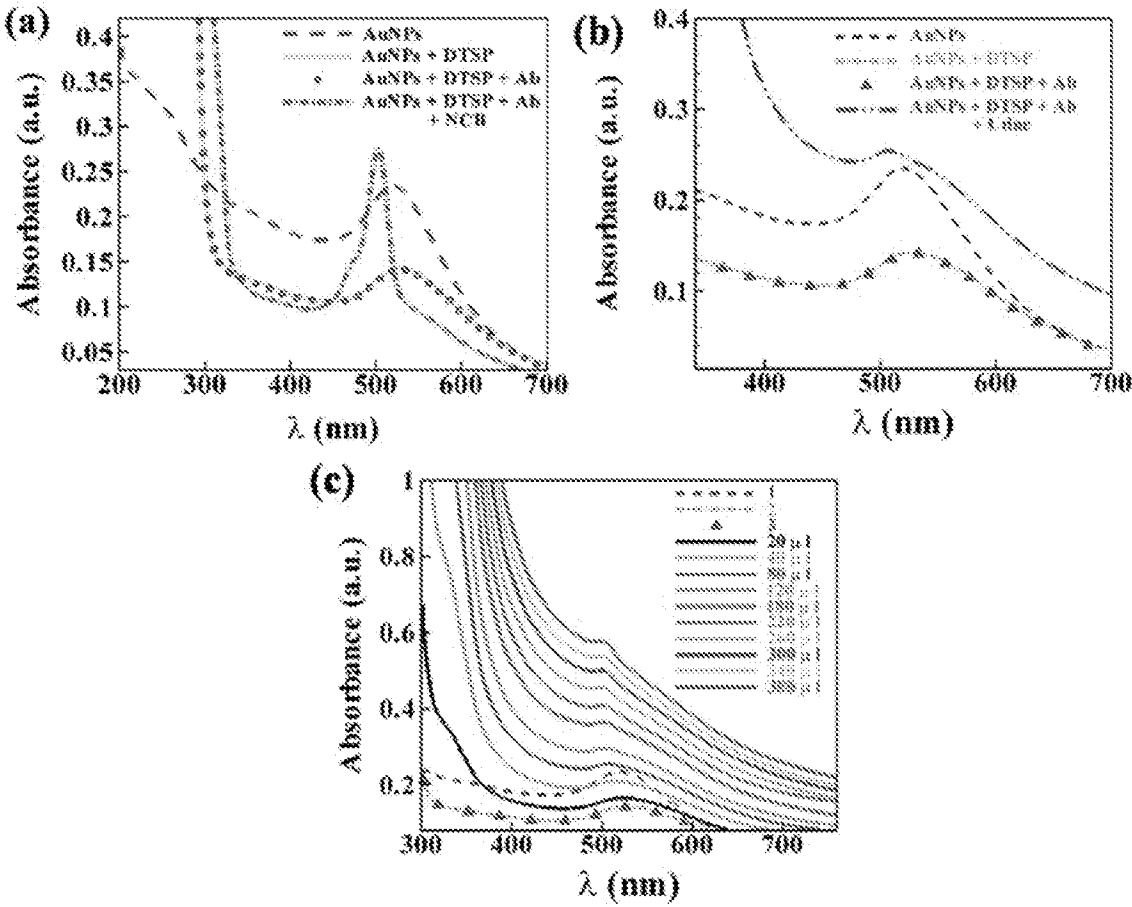


Figure 6



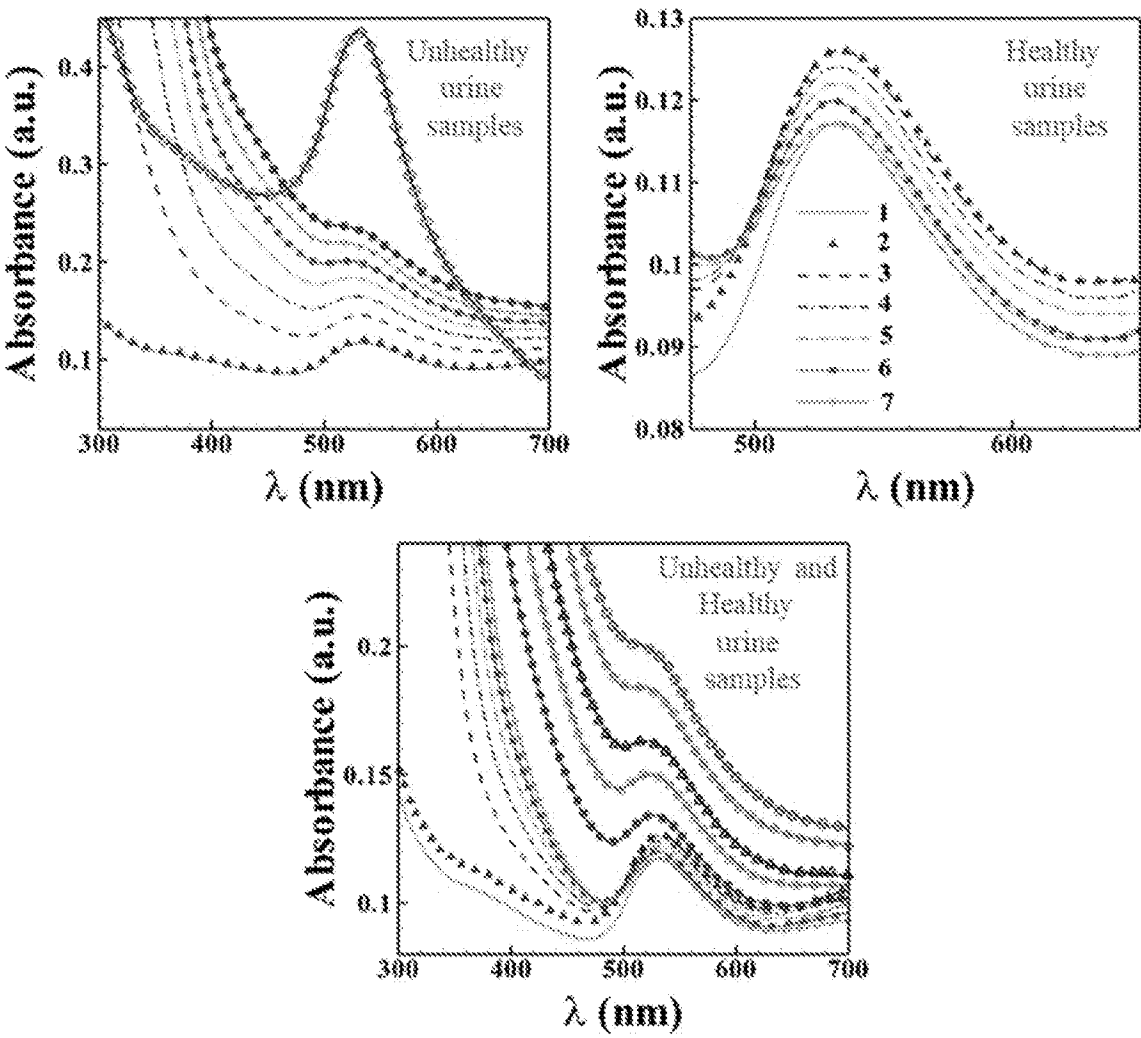


Figure 8

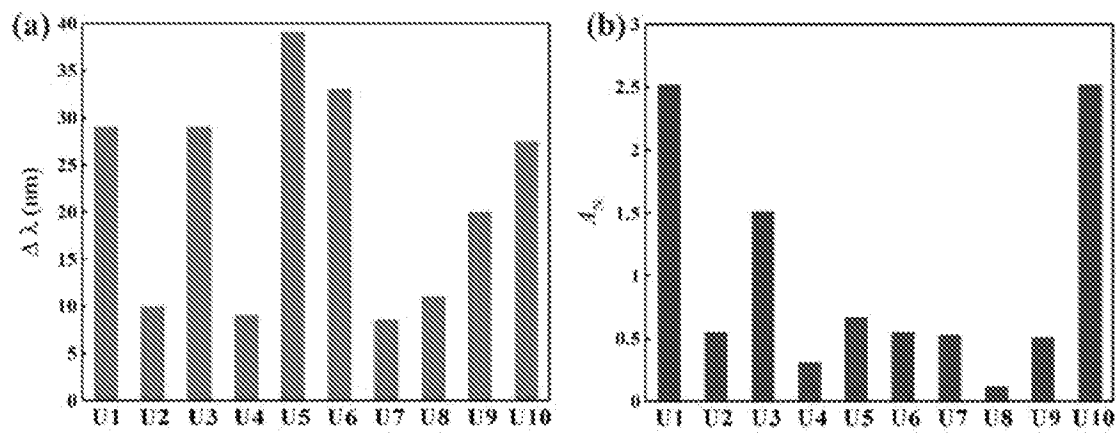


Figure 9

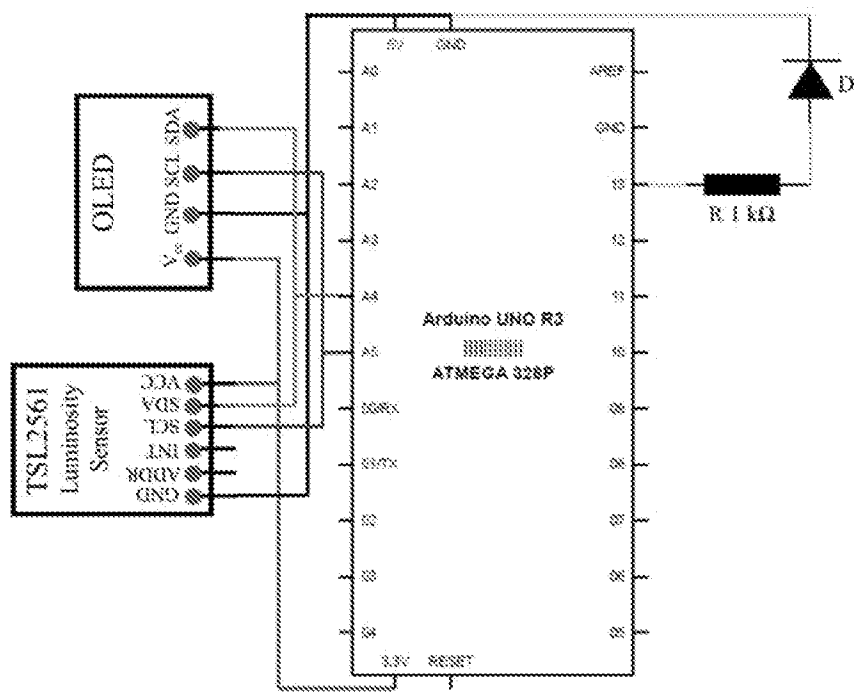


Figure 10

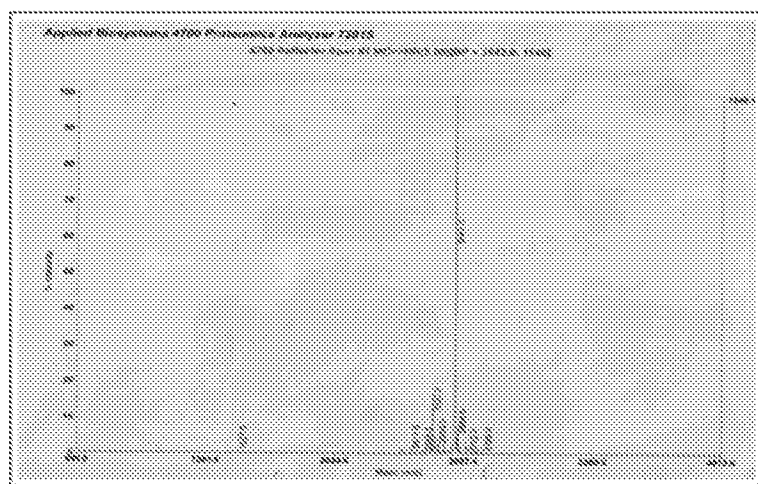


Figure 11

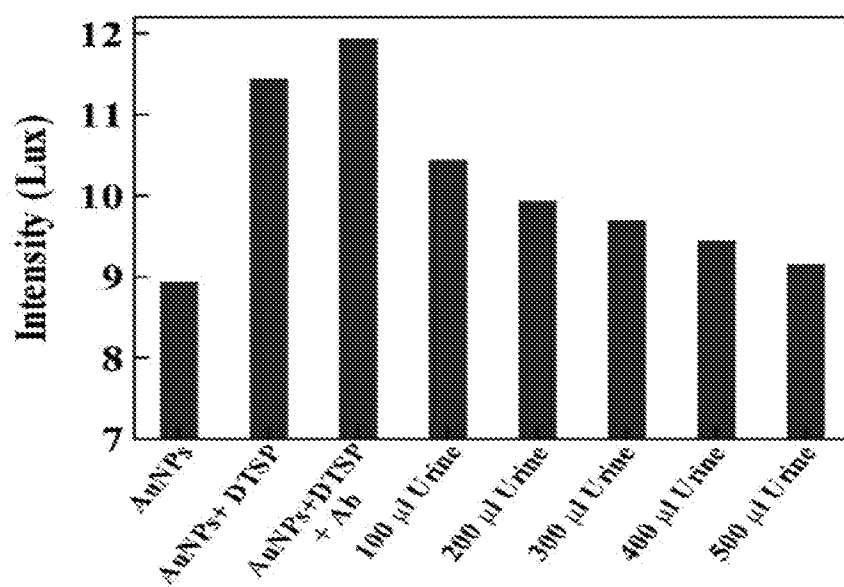


Figure 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2020/051023

A. CLASSIFICATION OF SUBJECT MATTER
G01N33/53 Version=2021.01

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	CDRI Compendium of products & services - CSIR-CDRI, 2019. & Annual report 2016-2017-CSIR-CDRI, 2019. page 28 & page 58 respectively	1-12
Y	Parmita Kar et al: "A protocol for stripping and reprobing of Western blots originally developed with colorimetric substrate TMB", Electrophoresis, 2012 Oct, vol.33, no.19-20; pp.3062-3065. page 3063, column 1; fig.1	1-12
Y	Hong Y et al: "Localized surface plasmon resonance based nanobiosensor for biomarker detection of invasive cancer cells", J. of Biomedical Optics, 2013, vol.19, no.5, pp.1-6. whole document	1-12
Y	Onur Tokel et al: "Advances in Plasmonic Technologies for Point of Care Applications", Chemical Reviews, 2014, vol. 114, PP.5728-5752. page 5741-5743; fig.10	1-12



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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

08-04-2021

Date of mailing of the international search report

08-04-2021

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2020/051023

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.