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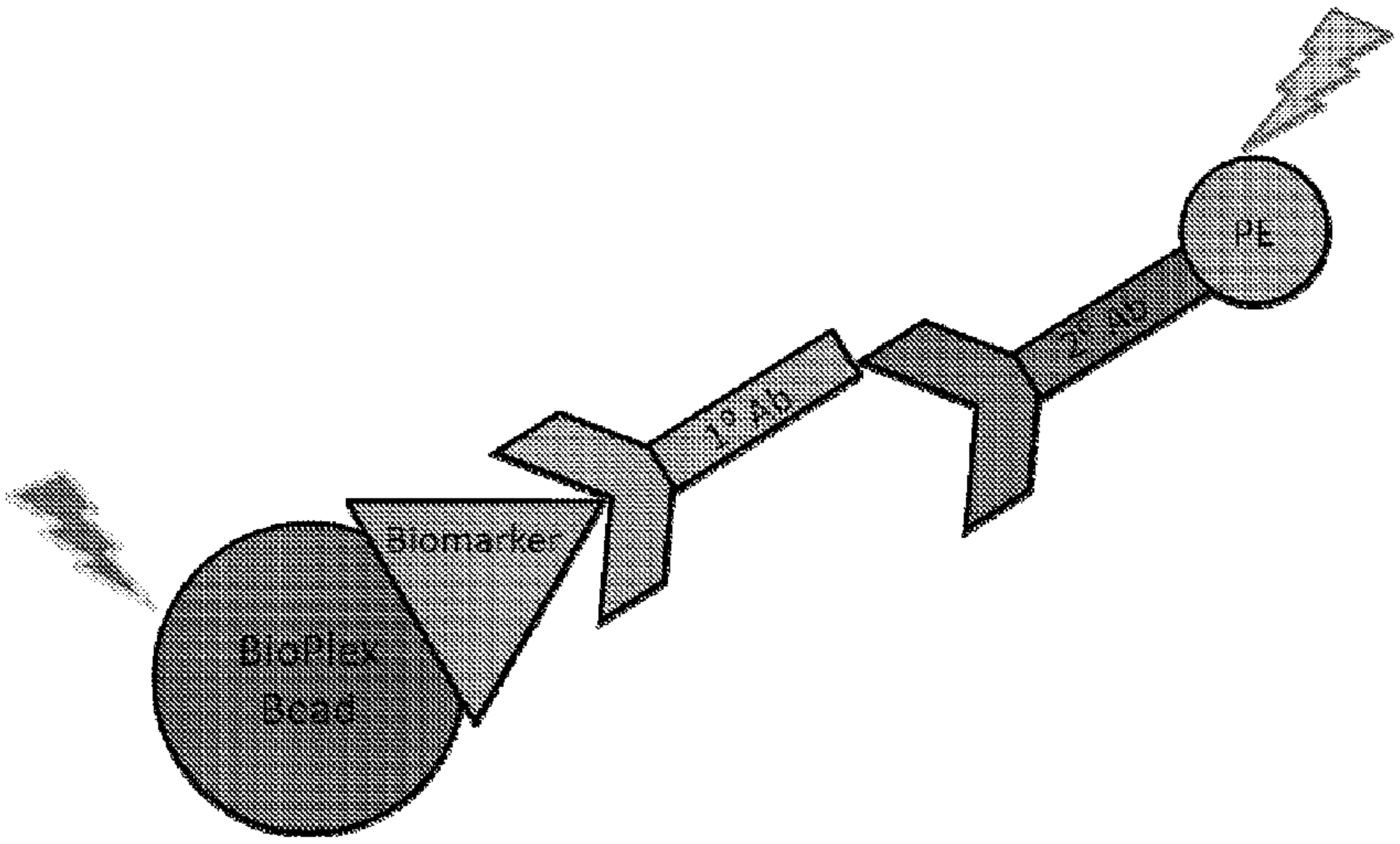
(54) **Titre :** ENSEMBLE DE BIOMARQUEURS SPECIFIQUES POUR LE DIAGNOSTIC NON INVASIF D'UN CANCER DU FOIE
(54) **Title:** SPECIFIC BIOMARKER SET FOR NON-INVASIVE DIAGNOSIS OF LIVER CANCER



(57) **Abrégé/Abstract:**

Cells within liver tumour mass comprise a unique set of proteins/tumour antigens when compared to the normal liver tissues epithelial cells juxtaposed to the tumour. The presence of tumour antigens couples the production of auto-antibodies against these tumour antigens. The present invention relates to the identification and elucidation of a protein set that can act as a novel marker set for liver cancer diagnosis and prognosis. Specifically, it relates to a kit that enables diagnostic and prognostic measurement of auto-antibodies in serum of liver cancer patients. The present invention provides a non-invasive, specific, sensitive, and cost effective detection and quantification method by evaluating a set of validated liver cancer proteins/tumour antigens, which includes Bmi-1, VCC1, SUMO-4, RhoA, TXN, ET-1, UBE2C, HDGF2, FGF21, LECT2, SOD1, STMN4, Midkine, IL-17A or IL26, to complement the conventional diagnostic methods.

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(54) Title: SPECIFIC BIOMARKER SET FOR NON-INVASIVE DIAGNOSIS OF LIVER CANCER

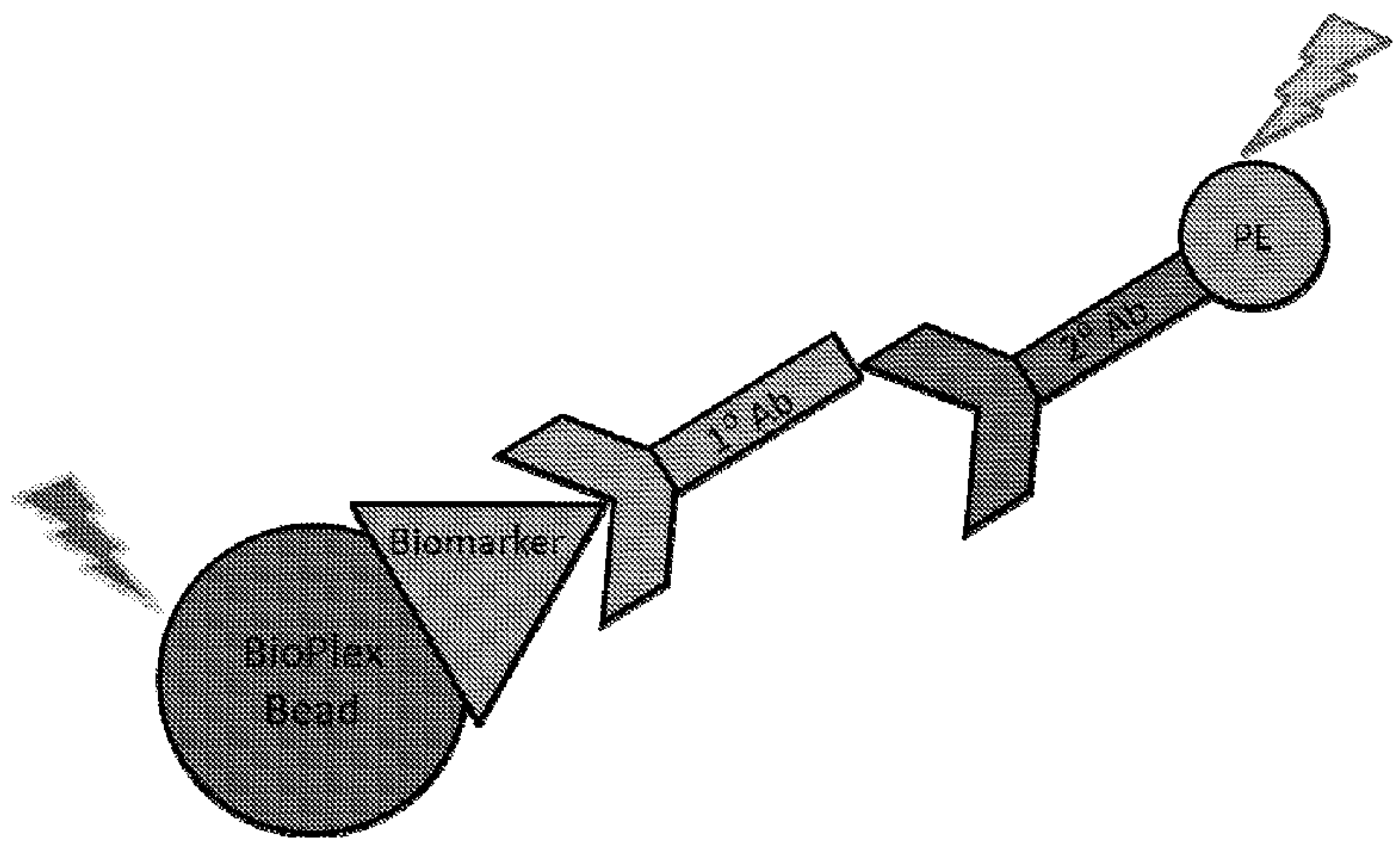

FIG. 7

(57) Abstract: Cells within liver tumour mass comprise a unique set of proteins/tumour antigens when compared to the normal liver tissues epithelial cells juxtaposed to the tumour. The presence of tumour antigens couples the production of auto-antibodies against these tumour antigens. The present invention relates to the identification and elucidation of a protein set that can act as a novel marker set for liver cancer diagnosis and prognosis. Specifically, it relates to a kit that enables diagnostic and prognostic measurement of auto-antibodies in serum of liver cancer patients. The present invention provides a non-invasive, specific, sensitive, and cost effective detection and quantification method by evaluating a set of validated liver cancer proteins/tumour antigens, which includes Bmi-1, VCC1, SUMO-4, RhoA, TXN, ET-1, UBE2C, HDGF2, FGF21, LECT2, SOD1, STMN4, Midkine, IL-17A or IL26, to complement the conventional diagnostic methods.

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SPECIFIC BIOMARKER SET FOR NON-INVASIVE DIAGNOSIS OF LIVER CANCER

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Technical Field

[0002] The present disclosure describes a detection and quantification method for a list of specific and novel Hepatocellular Carcinoma (HCC) tumor biomarkers, by measuring the corresponding auto-antibodies in liver cancer patients' sera. The set of biomarkers comprises Bmi1, VCC1, SUMO-4, RhoA, TXN, ET-1, UBE2C, HDGF2, FGF21, LECT2, SOD1, STMN4, Midkine, IL-17A and IL26. More specifically, this disclosure further describes a design of a high throughput and sensitive test kit readily available to take patients' peripheral serum samples for detecting liver cancers early and in a non-invasive manner by measuring the auto-antibodies against at least one of the biomarkers selected from the biomarker set. The present disclosure further allows identification of signature biomarker patterns for staging, as well as the detection of recurrences during a monitoring period of post-chemotherapeutic treatment. The present disclosure would support automatic data analysis.

Background of Invention

[0003] Hepatocellular carcinoma (HCC) is the second most prevalent cancer in China, which covers 5.7 % of the total population [1]. Most HCC patients have rapid tumor progressing resulting in high mortality rate. In order to improve the overall survival, early diagnosis of the disease becomes essential. Currently, the most common way of detecting HCCs are blood tests that measure level of HCC tumor markers such as alpha fetoprotein (AFP). AFP is a plasma protein produced by yolk sac and liver during the development of fetus serving as a form of serum albumin. In normal condition, AFP level gradually decreases after birth and remain in low level in adults. Increased level of tumor markers indicates probability of liver cancers. However, the major problem of the AFP test is excessive false positive. It is because HCC is not the only cause for the AFP level elevation, but alcoholic hepatitis, chronic hepatitis or cirrhosis also associates with increase of AFP.

[0004] Despite AFP test is commonly suggested for diagnosis of liver cancers, its result is not conclusive. Suspected patients will need to go through ultrasound imaging, CT scans or contrast MRI scans for further confirmation. Liver biopsy will be taken to distinguish whether the tumor is benign or malignant. However, conventional detection of HCCs comes with several limitations: (a) About 20% of liver cancers does not produce elevated level of the commonly used HCC tumor markers [2]. (b) Viral cirrhosis produces false positive results on the blood tests [3]. (c)

Ultrasound is not able to detect small tumors [4]. (d) CT scans require high radiation dose and are insensitive to tumors less than 1 cm [5]. (e) MRI scans are expensive and the procedure is time consuming. Due to these limitations, there are needs to develop novel biomarkers screen with higher sensitivity and specificity for the purpose of early diagnosis of HCC and/or determining a prognosis of HCC to complement the conventional methods.

[0005] HCC tumor cells tend to produce a unique set of proteins when compared to the normal liver epithelial cells juxtaposed to the tumor. Evaluation of validated HCC tumor biomarkers has great potential to facilitate the diagnosis of HCC. However, not all biomarkers themselves can be found in serum or urine for convenient diagnosis. Alternatively, the auto-antibodies which are specifically against the biomarkers provide an opportunity to evaluate the expression of the biomarkers. It has been demonstrated in many cancers that the presence of tumor biomarkers couples the production of auto-antibodies against these tumor antigens [6-8]. Detection on auto-antibodies in patients' sera would allow us to examine the presence of biomarkers more efficiently. Ideally, examination of auto-antibodies from peripheral blood would be a testament for detecting liver cancers early, and in a non-invasive manner. One common hurdle hindering clinical use of biomarkers is that they have not been validated after discovery. But once validated, such test would be cost effective and accurate. The design of the prototype also supports high-throughput screening. This may alleviate the cost required for conventional liver cancer diagnosis.

[0006] There follows a list of references that are occasionally cited in the specification.

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[8] Wang YQ, Zhang HH, Liu CL, Xia Q, Wu H, Yu XH, Kong W. Correlation between auto-antibodies to survivin and MUC1 variable number tandem repeats in colorectal cancer. Asian Pac J Cancer Prev. 2012;13(11):5557-62.

Summary of Invention

[0007] In the present invention, a detection and quantification method measuring the auto-antibodies against a list of specific tumor biomarker aiming for diagnosing and staging cancers is provided. Comparing to the normal liver epithelial cells, HCC tumor cells tend to produce a unique set of proteins. The evaluation of the unique protein set, biomarkers, will complement the conventional diagnostic methods and facilitate early detection of cancers.

[0008] By using a Two-Dimensional/Mass Spectrometry based method, a set of liver cancer biomarkers from paired patients' biopsies (tumor biopsy versus juxtaposed normal tissue) is identified in the present invention comprising Bmi1, VCC1, SUMO-4, RhoA, TXN, ET-1, UBE2C, HDGF2, FGF21, LECT2, SOD1, STMN4, Midkine, IL-17A and IL26.

[0009] Specificity and accuracy of this set of liver caner biomarkers are then validated and taken together for diagnosis of liver cancers. In the present invention, proteins of the listed biomarkers are expressed from cDNA clones, purified and coupled to fluorescent microsphere beads with different emission wavelengths. Auto-antibodies present in patients' sera against the proteins immunologically bind to the protein-bead conjugate. The auto-antibodies subsequently interact with PE-conjugated secondary antibodies. The specific fluorescence signal of the microsphere beads serves as an identifier for the conjugated biomarkers. By measuring the fluorescent intensity given by the PE-conjugated secondary antibodies at the complex, it allows the detection and quantification of the auto-antibodies. Since the auto-antibodies are produced in the patients' sera in proportion to the abundance of the biomarkers at HCC tumor cells, the higher fluorescent intensity resulted from higher concentration of auto-antibodies indicates the higher expression of the corresponding biomarkers. The lowest detection limit of each biomarker to the total serum auto-antibodies is about 0.15 ng/mL.

[0010] Comparing to sera from healthy subjects, the level of auto-antibodies against the target biomarkers is at a higher concentration in cancer patient. Moreover, comparing different sera from liver cancer patients at different stages, signature patterns for staging may be generated. Thus, the present invention allows the non-invasive evaluation of the targeted liver cancer biomarker. This enables the detection of HCC at early stages and the identification of signature biomarker patterns for staging, as well as the detection of recurrences during a monitoring period of post-chemotherapeutic treatment.

Brief Description of the Drawings

[0011] Embodiments of the present invention are described in more detail hereinafter with reference to the drawings, in which:

[0012] **FIG. 1** shows the difference in protein expression pattern between tumor biopsy and juxtaposed normal tissue by two-dimensional/mass spectrometry leading to the identification of 15 specific biomarkers up-regulated in liver cancer; arrows indicate location of spots identified on a 2-D gel of the mass spectrometry.

[0013] **FIG. 2** shows the set of 15 validated liver cancer biomarkers and their corresponding molecular weight targeted and measured in the present invention.

[0014] **FIG. 3** shows the workflow of expressing the biomarkers from cDNA clones.

[0015] **FIG. 4** shows the workflow of purification of the biomarkers expressed from *E. coli.*

[0016] **FIG. 5** shows the workflow of measuring the auto-antibodies by BioPlex™ system.

[0017] **FIG. 6** shows the conjugation of biomarker protein to BioPlex™ bead.

[0018] **FIG. 7** shows illustration of the complex of biomarker-]BioPlex™ bead conjugate immunoreacting with primary antibody and PE-conjugated secondary antibody.

[0019] **FIG. 8** shows the gel electrophoresis of the DNA insert released from plasmid cut by restriction enzymes HindIII and BamH1.

[0020] **FIG. 9** shows the Coomassie Blue stained SDS-PAGE verifying the IPTG induction of (a) Bmi1, (b) SOD1, (c) IL-17A, (d) TXN and (e) Midkine biomarkers.

[0021] **FIG. 10** shows the elution profile of (a) Bmi1, (b) SOD-1 and (e) IL-17A in AKTA.

[0022] **FIG. 11** shows the Coomassie Blue stained SDS-PAGE verifying the purification of His-tagged (a) Bmi1, (b) SOD-1 and (d) IL-17A biomarkers; Fraction A is bacteria without IPTG induction; Fraction B is bacteria with IPTG induction; Fraction C is bacterial lysate.

[0023] **FIG. 12** shows the standard curve showing the fluorescence intensity against the concentration of anti-Bmi1 antibody.

[0024] **FIG. 13** is a schematic diagram showing the design of the test: Patient serum containing auto-antibodies are mixed to a well containing 15 types of beads corresponding to the 15 biomarkers of the biomarker set, followed by the addition of PE-conjugated secondary antibody.

Definitions

[0025] The term "biomarker" refers to the protein uniquely expressed or up-regulated in the tumor comparing to the normal epithelial cells.

[0026] The term "biomarker set" refers to the specific combination of the biomarkers identified from paired patients' biopsies (tumor biopsy versus juxtaposed normal tissue) and is the target of the measurement in the present invention.

[0027] The term "auto-antibodies" refers to the anti-bodies produced by the patient body coupling to the expression of the tumor biomarker and it is present in the circulation and can be collected in the peripheral serum.

[0028] Bmi1 (Polycomb Ring Finger) is a protein component of a Polycomb Group (PcG) multiprotein PRC1-like complex. It is responsible for maintaining the transcriptionally repressive state of many genes, including Hox genes, throughout development. The regulation is via monoubiquitination of histone H2A 'Lys-119', which modifies histone and remodels chromatin, rendering the expression.

[0029] VCC1 or CXCL17 (Chemokine (C-X-C Motif) Ligand 17) has an essential role in angiogenesis and possibly in the development of tumors. It is also suggested that it is a housekeeping chemokine regulating the recruitment of non-activated blood monocytes and immature dendritic cells into tissues. It may also play a role in the innate defense against infections. Malfunction of VCC1 is associated with duodenitis and cholera.

[0030] SUMO-4 (Small Ubiquitin-Like Modifier 4) belongs to the family of small ubiquitin-related modifiers and located in the cytoplasm. It covalently attaches to the target protein, IKBA, in order to control its subcellular localization, stability, or activity. This eventually leads to a negative regulation of NF-kappa-B-dependent transcription of the IL12B gene.

[0031] RhoA (Ras Homolog Family Member A) regulates the signaling pathway linking plasma membrane receptors to the assembly of focal adhesions and actin stress fibers. It also involves in microtubule-dependent signaling essential during cell cycle cytokinesis, and other signaling pathways involved in stabilization of microtubules and cell migrations and adhesion.

[0032] TXN (Thioredoxin) forms homodimer and is involved in redox reactions through the reversible oxidation of its active center dithiol to a disulfide and catalyzes dithiol-disulfide exchange reactions. It has been reported to be associated with breast mucinous carcinoma.

[0033] ET-1 (Endothelin 1) is a potent vasoconstrictor produced by vascular endothelial cells. It binds to endothelin receptors widely expressed in all tissues, including non-vascular structure like epithelial cells, glia, and neurons. Apart from the main role in maintenance of vascular tone, it is also suggested to have co-mitogenic activity and potentiate the effects of other growth factors.

[0034] UBE2C (Ubiquitin-Conjugating Enzyme E2C) belongs to the family of E2 ubiquitin-conjugating enzyme. This is one of the three enzymes involved in ubiquitination, which is an important cellular mechanism for targeting abnormal proteins for degradation. More specifically, UBE2C is required for the targeted degradation of mitotic cyclins and for cell cycle progression. Thus, it is believed that this protein may be also involved in cancer progression.

[0035] HDGF2 is called hepatoma-derived growth factor 2. This protein which is highly expressed in a variety of tumors has been reported to play a pivotal role in the development and progression of several tumors. Although the mechanism is yet to be identified, it is suggested that HDGF2 has mitogenic, angiogenic, neurotrophic and antiapoptotic activity.

[0036] FGF21 (Fibroblast Growth Factor 21) is a family member of the FGF family which is involved in vary biological processes including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion. More specifically, FGF21 stimulates glucose update in differentiated adipocytes via the induction of glucose transporter SLC2A1/GLUT1 expression. It has been found that FGF21 is associated with fatty liver disease.

[0037] LECT2 (Leukocyte Cell Derived Chemotaxin 1) is a secretory protein acts as a chemotactic factor to neutrophils and stimulates the growth of chondrocytes and osteoblasts. This protein is associated with acute liver failure.

[0038] SOD1 (Superoxide Dismutase 1) is a Cu/Zn-containing antioxidant enzyme responsible for destroying free superoxide radicals into molecular oxygen and hydrogen peroxide in the cytosol, the nucleus, and the intermembrane space of the mitochondria. It is important for maintaining low levels of superoxide in the cytosol, thus protecting the cell from oxidative stress and subsequent cell death.

[0039] STMN4 (Stathmin-Like 4) is a small regulatory protein which is believed to have a role in relaying integrating diverse intracellular signaling pathways, which in turn, controls cell proliferation, differentiation and functions. It is also shown that this protein contributes to the control of microtubule dynamics by inhibiting the polymerization of microtubules and/or favoring their depolymerization.

[0040] Midkine or NEGF2 (Neurite Growth-Promoting Factor 2) is a secretory growth factor that binds heparin and responsive to retinoic acid. Midkine promotes cell growth, migration and angiogenesis, in particular during tumorigenesis. It has already been demonstrated to be associated with breast adenocarcinoma and soft tissue sarcoma.

[0041] IL-17A (Interleukin 17A) is a proinflammatory cytokine produced by the activated T cells. It regulates the activity of NF-kappaB and mitogen-activated protein kinases, stimulates the expression of IL6 and cyclooxygenase-2, and enhances the production of nitric oxide. Several chronic inflammation and sclerosis are usually associated with IL-17A elevation.

[0042] IL-26 (Interleukin 26) belongs to the IL-10 cytokine family and is produced by the activated T cells and targets epithelial cells for signal transduction. It binds strongly to glycosaminoglycans such as heparin, heparan sulphate, and dermatan sulfate on cellular surfaces which act similarly to coreceptors in order to enrich IL-26 on the surface of producer and target cells.

Detailed Description of Invention

[0043] In the following description, the biomarker/biomarkers, the corresponding embodiments of the detection/validation/identification/quantification methods are set forth as preferred examples. It will be apparent to those skilled in the art that modifications, including additions and/or substitutions, may be made without departing from the scope and spirit of the invention. Specific details may be omitted so as not to obscure the invention; however, the disclosure is written to enable one skilled in the art to practice the teachings herein without undue experimentation.

[0044] In the present invention, the set of liver tumor biomarkers for detection and quantification of liver cancer is first identified by two-dimensional/mass spectrometry resolving the difference in the pattern of proteins expression between the paired patients' biopsies (tumor biopsy versus juxtaposed normal tissue) (FIG. 1). The biomarkers are validated by immunohistochemical staining on paraffin-sectioned HCC blocks, and Western Blotting in HCC patients' sera. This results in a finalized list of 15 biomarkers to be evaluated in the present invention for the liver cancer diagnosis purpose (FIG. 2).

[0045] Based on the amino acid sequences of the targeted biomarkers, commercially synthesized cDNA clones are employed for the expression of the biomarker set (FIG. 3). Proteins expressed from the cDNA clones are then subjected to a series of steps of purifications (FIG. 4). The purified biomarkers are subsequently conjugated via stable amide bonds with BioPlex™ beads (FIGS. 5, 6), a type of fluorescent microsphere beads and available in a panel which give unique fluorescent signals individually for identification at a multiplex set up. The biomarkers on the beads are recognized by the specific primary antibodies, which are subsequently bound by an anti-human secondary antibody conjugated with PE (FIG. 7). Thus the BioPlex™ machine simultaneously measures two signals from the complex. The fluorescence given by the BioPlex™ beads serves as an identifier, while the signal from the PE indicates the presence of the biomarker in the complex. This also helps differentiating the biomarker-bead conjugates bound by the anti-body cascade from those with no immuno-reactivity with antibodies.

[0046] To prove the significance of the biomarkers in the present invention, the cDNA clones are confirmed by restriction enzyme cut (FIG. 8). The transformed bacteria is induced by IPTG to express the biomarker proteins. The protein expression verified by SDS-PAGE and Coomassie Blue staining reveals the protein bands (FIG. 9 a-e). The His-tagged Bmi1, SOD1 and IL-17A proteins are purified by AKTA (FIG. 10 a-c) and then verified by SDS-PAGE and Coomassie Blue staining (FIG. 11 a-c).

[0047] Sensitivity of the test is measured by spiking in a serial dilution of the antibodies. The lowest concentration of the antibody added that can give signal suggests the sensitivity of that particular biomarker. Meanwhile a standard curve is constructed showing the fluorescence intensity of the PE against the serial dilutions of the antibodies (FIG. 12). The standard curve will be used for estimating the concentration of the biomarker specific auto-antibodies in the patient sera by comparing the PE intensity.

[0048] In the present invention, a multiplex of 15 different BioPlex™ beads individually giving unique fluorescence are conjugated with the biomarker set and preloaded in the wells of a plate (FIG. 13). To a well, patient serum containing auto-antibodies is loaded and allowed to interact with the biomarker conjugates. The PE-conjugated secondary antibodies are then added and bind to the auto-antibodies. In the machine, the excess secondary antibodies are washed away, the complex comprising the biomarker-bead conjugate and cascade of antibodies are measured individually. The unique fluorescence signal of the BioPlex™ bead identifies the biomarkers, while the PE signal from the same complex indicates the presence of the auto-antibodies as the primary antibody (FIG. 7). Taken together, the measurement will suggest the presence of auto-antibodies and the relative concentration in the presents' sera.

[0049] In a standard randomized trial design, the mean of the relative level of auto-antibodies between the healthy group and patients diagnosed with liver cancer is compared. Student T test is used to analyze the variation significance. The significant difference indicates that the biomarker is specific for liver cancer. After the verification trials, ranges of the concentration of biomarker specific auto-antibodies will be obtained for the liver cancer positive and negative patients and serve as reference point for the future diagnosis. Meanwhile, expression pattern of the auto-antibodies is also compared between liver cancer patients of different stages. The signature patterns of the biomarker expressions will indicate the HCC staging.

[0050] Taken together, the measurement of the relative auto-antibodies level and the expression pattern of the biomarkers, the present invention represents a different avenue to complement conventional liver cancer diagnosis. The present invention further enables non-invasive detection of auto-antibodies against the validated targets in patients' sera of the present invention, identifying the extent and the characteristics of the disease. Apart from early detection for stage I liver cancers, the present invention also enables the generation of signature patterns for staging, and the detection of recurrences during a monitoring period of post-mastectomy or post-chemotherapeutic treatment.

Examples

[0051] The following examples are provided by way of describing specific embodiments of this invention without intending to limit the scope of this invention in any way.

[0052] **Example 1a**

[0053] **Protein extraction from patients' biopsies**

[0054] 500 mg of the paired patients' biopsies (tumor biopsy versus juxtaposed normal tissue) are collected and washed with PBS. The tissues are frozen by submerging into liquid nitrogen and immediately homogenized with pestle and mortar. To the homogenized samples, lysis solution (8M Urea, 4% CHAPS, 2% IPG Buffer, 0.2mg/ml PMSF) is added, then vortex for at least 5 min until the tissues are completely dispersed. The lysates are then clarified by centrifugation at 14,000 rpm for 10 minutes at 4 °C. The supernatants are further cleaned up by 2D Clean Up kit (Amersham) to remove the salt and impurities. The pellets are resuspended with minimum volume of Rehydration Solution (No DTT & IPG Buffer added). The protein concentrations are then measured by Bio-Rad protein assay and aliquots of 200 g/per tube are stored at -70 °C.

[0055] **Example 1b**

[0056] **Resolving proteins by two-dimensional electrophoresis**

[0057] To 1 ml rehydration stock solution, 2.8 mg DTT, 5 µl pharmalyte or IPG Buffer, and 2 µl bromophenol blue are added. 50 – 100 µg of protein sample is added to the 13 cm Immobiline DryStrip (IPG strip) containing 250 µl of rehydration solution. After removing the protective cover, the IPG strip is positioned in the strip holder with the gel side facing down, and overlaid with Cover Fluid to prevent dehydration during electrophoresis. The strip is then placed on to Ettan IPGphor (Amersham) for isoelectric focusing (first dimensional electrophoresis).

[0058] After the first-dimensional electrophoresis, the IPG strip is equilibrated with equilibrate solution (6 M Urea 2 % SDS, 50 mM Tris HCl pH 6.8, 30 % Glycerol, 0.002 % Bromophenol blue, 100 mg DTT per 10 ml buffer and 250 mg IAA per 10 ml buffer), and then washed with 1x SDS running Buffer for 4 - 5 times. The IPG strip is placed on top of the second-dimension gel and overlaid with sealing solution (0.5 % Low Melting agarose, 0.002 % Bromophenol Blue in 1 × SDS running Buffer). The second-dimensional electrophoresis is then carried out at 30 mA for first 15 min followed by 60 mA for 3 - 4 h.

[0059] Upon the completion of the second dimensional electrophoresis, the gel is removed from the cassette, fixed and stained with silver nitrate. 15 spots representing 15 up-regulated proteins are identified (FIG. 1). To identify the proteins (FIG. 2), the silver stained gel slices are destained and trypsinized to release the protein from the gel for MALDI-TOF analysis.

[0060] **Example 2a (SEQ ID NO.1)**

[0061] **Amino acid sequence of Bmi1**

[0062] MHRTTRIKITELNPHLMCVLCGGYFIDATTIIECLHSFCKTCIVRYLETSKYCPICD VQVHKTRPLLNIRSDKTLQDIVYKLVPGLFKNEMKRRRDFYAAHPSADAANGSNEDRG EVADEDKRIITDDEIISLSIEFFDQNRLDRKVNKDKEKSKEEVNDKRYLRCPAAMTVMHL RKFLRSKMDIPNTFQIDVMYEEEPLKDYYTLMDIAYIYTWRRNGPLPLKYRVRPTCKRM

KISHQRDGLTNAGELESDSGSDKANSPAGGIPSTSSCLPSPSTPVQSPHPQFPHISSTMNGT SNSPSGNHQSSFANRPRKSSVNGSSATSSG

[0063] **Example 2b (SEQ ID NO.2)**

[0064] **Amino acid sequence of VCC1**

[0065] MKVLISSLLLLLPLMLMSMVSSSLNPGVARGHRDRGQASRRWLQEGGQECECK DWFLRAPRRKFMTVSGLPKKQCPCDHFKGNVKKTRHQRHHRKPNKHSRACQQFLKQC QLRSFALPL

[0066] **Example 2c (SEQ ID NO.3)**

[0067] **Amino acid sequence of SUMO-4**

[0068] MANEKPTEEVKTENNNHINLKVAGQDGSVVQFKIKRQTPLSKLMKAYCEPRGLS VKQIRFRFGGQPISGTDKPAQLEMEDEDTIDVFQQPTGGVY

[0069] **Example 2d (SEQ ID NO.4)**

[0070] **Amino acid sequence of RhoA**

[0071] MAAIRKKLVIVGDGACGKTCLLIVFSKDQFPEVYVPTVFENYVADIEVDGKQVE LALWDTAGQEDYDRLRPLSYPDTDVILMCFSIDSPDSLENIPEKWTPEVKHFCPNVPIILV GNKKDLRNDEHTRRELAKMKQEPVKPEEGRDMANRIGAFGYMECSAKTKDGVREVFE MATRAALQARRGKKKSGCLVL

[0072] **Example 2e (SEQ ID NO.5)**

[0073] **Amino acid sequence of TXN**

[0074] MVKQIESKTAFQEALDAAGDKLVVVDFSATWCGPCKMIKPFFHSLSEKYSNVIF LEVDVDDCQDVASECEVKCMPTFQFFKKGQKVGEFSGANKEKLEATINELV

[0075] **Example 2f (SEQ ID NO.6)**

[0076] **Amino acid sequence of ET-1**

[0077] MDYLLMIFSLLFVACQGAPETAVLGAELSAVGENGGEKPTPSPPWRLRRSKRCS CSSLMDKECVYFCHLDIIWVNTPEHVVPYGLGSPRSKRALENLLPTKATDRENRCQCAS QKDKKCWNFCQAGKELRAEDIMEKDWNNHKKGKDCSKLGKKCIYQQLVRGRKIRRSS EEHLRQTRSETMRNSVKSSFHDPKLKGNPSRERYVTHNRAHW

[0078] **Example 2g (SEQ ID NO.7)**

[0079] **Amino acid sequence of UBE2C**

[0080] MASQNRDPAATSVAAARKGAEPSGGAARGPVGKRLQQELMTLMMSGDKGISA FPESDNLFKWVGTIHGAAGTVYEDLRYKLSLEFPSGYPYNAPTVKFLTPCYHPNVDTQG NICLDILKEKWSALYDVRTILLSIQSLLGEPNIDSPLNTHAAELWKNPTAFKKYLQETYSK QVTSQEP

[0081] **Example 2h (SEQ ID NO.8)**

[0082] **Amino acid sequence of HDGF2**

[0083] MARPRPREYKAGDLVFAKMKGYPHWPARIDELPEGAVKPPANKYPIFFFGTHET AFLGPKDLFPYKEYKDKFGKSNKRKGFNEGLWEIENNPGVKFTGYQAIQQQSSSETEGE GGNTADASSEEEGDRVEEDGKGKRKNEKAGSKRKKSYTSKKSSKQSRKSPGDEDDKDC KEEENKSSSEGGDAGNDTRNTTSDLQKTSEGT

[0084] **Example 2i (SEQ ID NO.9)**

[0085] **Amino acid sequence of FGF21**

[0086] MDSDETGFEHSGLWVSVLAGLLLGACQAHPIPDSSPLLQFGGQVRQRYLYTDDAQQTEAHLEIREDGTVGGAADQSPESLLQLKALKPGVIQILGVKTSRFLCQRPDGALYGSLHFDPEACSFRELLLEDGYNVYQSEAHGLPLHLPGNKSPHRDPAPRGPARFLPLPGLPPALPEPPGILAPQPPDVGSSDPLSMVGPSQGRSPSYAS

[0087] **Example 2j (SEQ ID NO.10)**

[0088] **Amino acid sequence of LECT2**

[0089] MFSTKALLLAGLISTALAGPWANICAGKSSNEIRTCDRHGCGQYSAQRSQRPHQGVDVLCSAGSTVYAPFTGMIVGQEKPYQNKNAINNGVRISGRGFCVKMFYIKPIKYKGPIKKGEKLGTLLPLQKVYPGIQSHVHIENCDSSDPTAYL

[0090] **Example 2k (SEQ ID NO.11)**

[0091] **Amino acid sequence of SOD1**

[0092] MATKAVCVLKGDGPVQGIINFEQKESNGPVKVWGSIKGLTEGLHGFHVHEFGDNTAGCTSAGPHFNPLSRKHGGPKDEERHVGDLGNVTADKDGVADVSIEDVISLSGDHCIIGRTLVVHEKADDLGKGGNEESTKTGNAGSRLACGVIGIAQ

[0093] **Example 2l (SEQ ID NO.12)**

[0094] **Amino acid sequence of STMN4**

[0095] MTLAAYKEKMKELPLVSLFCSCFLADPLNKSSYKYEADTVDLNWCVISDMEVIELNKCTSGQSFEVILKPPSFDGVPEFNASLPRRRDPSLEEIQKKLEAAEERRKYQEAELLKH

LAEKREHEREVIQKAIEENNNFIKMAKEKLAQKMESNKENREAHLAAMLERLQEKDKH AEEVRKNKELKEEASR

[0096] **Example 2m (SEQ ID NO.13)**

[0097] **Amino acid sequence of Midkine**

[0098] MQHRGFLLLTLLALLALTSAVAKKKDKVKKGGPGSECAEWAWGPCTPSSKDCG VGFREGTCGAQTQRIRCRVPCNWKKEFGADCKYKFENWGACDGGTGTKVRQGTLKKA RYNAQCQETIRVTKPCTPKTKAKAKAKKGKGKD

[0099] **Example 2n (SEQ ID NO.14)**

[00100] **Amino acid sequence of IL-17A**

[00101] MTPGKTSLVSLLLLLSLEAIVKAGITIPRNPGCPNSEDKNFPRTVMVNLNIH NRNTNTNPKRSSDYYNRSTSPWNLHRNEDPERYPSVIWEAKCRHLGCINADGNVDYHM NSVPIQQEILVLRREPPHCPNSFRLEKILVSVGCTCVTPIVHHVA

[00102] **Example 2o (SEQ ID NO.15)**

[00103] **Amino acid sequence of IL-26**

[00104] MLVNFILRCGLLLVTLSLAIAKHKQSSFTKSCYPRGTLSQAVDALYIKAA WLKATIPEDRIKNIRLLKKKTKKQFMKNCQFQEQLLSFFMEDVFGQLQLQGCKKIRFVE DFHSLRQKLSHCISCASSAREMKSITRMKRIFYRIGNKGIYKAISELDILLSWIKKLLESSQ

[00105] **Example 3a**

[00106] **Expression of biomarker set**

[00107] His tagged plasmids containing cDNA inserts encoding the biomarker set is transformed into DH5 competent cells (**301**, FIG. 3). Single colony is picked and allowed to grow in bacterial culture (**302**). The number of plasmid is expanded and extracted from the bacteria by miniprep. The plasmid is further transformed into BL21DE3 or BL21DE3pLysS competent cells. Transformed bacteria are selected and grew in 2 X 100 ml LB medium. When the bacterial culture reaches the optical density of 0.06, 200 μM of IPTG is added to 100 ml bacterial culture (**303**). Another 100 ml of bacterial culture without IPTG is used as negative control. The bacterial cultures are incubated at 30 °C with shaking. 500 μl of the bacterial cultures are saved and stored at -20 °C 3 h after the incubation and in the next morning after incubating overnight.

[00108] Bacterial cultures with and without IPTG induction are mixed together in a 500 ml centrifuge bottle. Bacterial cells are collected by centrifugation at 9000 rpm for 20 min at 4 °C (**304**). 500 μl of supernatant is saved as another negative control and the remaining supernatant is discarded. The bacterial cultures and negative controls collected in different points are run on a SDS-PAGE to resolve the protein (**305**). The gel is then stained with Coomassie Blue overnight. After destaining the gel, the protein induction can be confirmed by checking the size and comparing with the negative controls.

[00109] Example 3b

[00110] Protein purification for biomarker set

[00111] The bacterial cell pellets are resuspended in 10 ml solubilization buffer by vortex at room temperature. Keeping the resuspended cells in 50 ml centrifuge tube on ice, the cells are completely lysed by sonication at amplitude 70 % 10 rounds of 30 s with interval of 30 s (**401**, FIG. 4). The lysed cells are centrifuged at 10,000 rpm for 1 h at 4 °C (**402**). Supernatants are

transferred into dialysis tubing and submerged in 1 L unfiltered starting buffer for 4 - 6 h at 4 °C with constant stirring **(403)**. Dialysis is continued with another 1 L starting buffer overnight. The supernatant is further filtered with 0.22 μm filter disc and syringe. To the AKTA machine equipped with 0.1M Nickel sulfate charged HiTrap chelating column **(404)**, filtered samples are loaded **(405)**. A program is set at the AKTA machine that the eluent is collected in fractions automatically **(406)**. Proteins purified from different fractions are checked by SDS-PAGE analysis **(407)**.

[00112] **Example 4a**

[00113] **Protein coupling with BioPlex™ beads**

[00114] The purified proteins of the biomarker set are coupled with BioPlex™ beads (Bio-Rad) **(501)** according to the manufacturer's manual. In brief, uncoupled bead is vortexed for 30 s and then sonicated for 15 s. 1,250,000 beads are collected in a reaction tube by centrifugation of 100 μl bead at maximum speed for 4 min. After washing with 100 μl bead wash buffer by centrifugation, the beads are resuspended in 80 μl bead activation buffer. To the beads 10 μl 50 mg/ml freshly prepared S-NHS and 10 μl 50 mg/ml freshly prepared EDAC are added, followed by 20 min incubation in dark at room temperature (FIG. 6). The beads are then washed with 150 μl PBS twice.

[00115] To the washed beads, 10 μg proteins are added and the total volume is topped up with PBS to 500 μl, and allowed to incubate for 2 h with shaking in dark. Supernatant is removed after centrifugation at maximum speed for 4 min. 250 μl blocking buffer is added to the beads and shook in dark for 30 min, followed by centrifugation at maximum speed for 4 min and removal of supernatant. The beads are briefly washed and then resuspended in the storage buffer for storage at 4 °C. The numbers of the beads are counted with a hemocytometer.

[00116] **Example 4b**

[00117] **Validation of protein-bead coupling**

[00118] To a HTS 96 well plate, 50 µl of conjugated BioPlex™ beads (100 beads / µl) is added to react with primary followed by secondary antibodies (**502**). A serial dilution of the commercially available primary anti-bodies against the biomarker set is prepared as 8,000, 4,000, 1,000, 250, 62.5, 15.625, 3.906, 0.977, 0.244 and 0.061 ng/ml. 50 µl of each dilution is added to each well. Two negative controls are performed by excluding the primary antibodies, and both primary and secondary antibodies in the wells. The plate is then sealed with a foil and kept on a shaker for 30 min at 350 rpm, avoiding exposure to light.

[00119] After incubation, the beads are washed three times with 150 µl PBS. 50 µl of PE-conjugated secondary antibody (8,000 ng/ml) is added into each well except negative controls. The plate is sealed again and incubated in dark for 30 min with shaking. Excess antibodies are then washed away by PBS. The BioPlex™ machine is calibrated with the calibration kit and validation kit. After the HTS plate is loaded to the machine, signals from both the BioPlex™ beads and the PE conjugated at the secondary antibodies (**503**) are measured (schematic diagram is shown in FIG. 7). A calibration curve is generated by Logistic-5PL.

[00120] **Example 4c:**

[00121] **Collection of serum samples and measurement of auto-antibodies by BioPlex™ system**

[00122] Whole-blood samples are clotted by standing at 37 °C for 1 h. Sera containing the auto-antibodies is collected at the supernatant after centrifugation at 1000g room temperature for 10 min. The serum samples are diluted with PBS when necessary. To a HTS plate preloaded with

BioPlex™ beads conjugated with biomarker set, the serum samples are loaded and incubated for 30 min with shaking (FIG. 13). Similar to the steps described in Example 4b, to the PBS washed beads, 50 μl of PE-conjugated secondary antibody (8000 ng/ml) is added, followed by shaking for another 30 min. After three rounds of washing, the plate is loaded to the BioPlex™ machine and the fluorescence signal is measured (**504**). The concentration of the auto-antibodies can then be calculated from the standard curves.

[00123] The foregoing description of the present invention has been provided for the purposes of illustration and description. It is not intended to be exhaustive or to limit the invention to the precise forms disclosed. Many modifications and variations will be apparent to the practitioner skilled in the art.

[00124] The embodiments are chosen and described in order to best explain the principles of the invention and its practical application, thereby enabling others skilled in the art to understand the invention for various embodiments and with various modifications that are suited to the particular use contemplated.

What is claimed is:

1. A method for detecting the presence of hepatocellular carcinoma (HCC) biomarkers in a subject suspected of having HCC, the method comprising:

(a) providing a serum sample from the subject suspected of having HCC and measuring the serum for the presence of primary biomarker auto-antibodies against a set of biomarkers, wherein the set of biomarkers comprises RhoA, TXN, HDGF2, SOD1, IL-17A and IL26; and

(b) detecting the presence of the HCC biomarkers in the subject suspected of having HCC, the method comprising the steps of:

(i) mixing the serum sample with the set of biomarker conjugates to allow the primary biomarker auto antibodies, if present in the serum sample, to bind to the set of biomarker conjugates and washing away any unbound antibodies;

wherein the set of biomarker conjugates comprises each of the biomarkers in the set of biomarkers conjugated via an amide bond to a unique fluorescent microsphere bead, wherein each unique fluorescent microsphere bead associated with a specific particular biomarker in the set of biomarkers has a different emission wavelength for each biomarker,

wherein the biomarker conjugates are capable of being bound by a specific primary biomarker auto antibody present in the subject's serum sample,

(ii) adding to the mixture formed in step (i) anti-human secondary antibodies conjugated with phycoerythrin (PE), which are capable of binding primary biomarker auto antibodies; and allowing the anti-human secondary antibodies conjugated with PE to bind to specific primary antibodies bound to biomarker conjugates to form a fluorescent bead-biomarker-auto antibody-PE conjugated antibody cascade, and washing away any unbound antibodies; and

(iii) measuring the mixture formed in step (ii) for the presence fluorescent bead-biomarker-auto antibody-PE conjugated antibody cascade to determine whether the subject's serum contained primary biomarker auto antibodies.

2. The method of claim 1, wherein the set of biomarkers comprises Bmi-1, VCC1, SUMO-4, RhoA, TXN, ET-1, UBE2C, HDGF2, FGF21, LECT2, SOD1, STMN4, Midkine, IL-17A and IL26.

3. The method of claim 1 or 2, wherein the unique fluorescent signal from the microsphere beads serves to identify which biomarker in the set of biomarkers is present and wherein the signal from the PE indicates the presence of the biomarker conjugate.

4. The method of claim 3, wherein fluorescent intensity given by the PE-conjugated secondary antibodies in the fluorescent bead-biomarker-auto antibody-PE conjugated antibody cascade is measured to allow the detection and quantification of the primary biomarker auto antibodies.

5. The method of claim 1, wherein the set of biomarkers consists of RhoA, TXN, HDGF2, SOD1, IL-17A and IL26.

6. The method of claim 1, wherein the set of biomarkers consists of Bmi-1, VCC1, SUMO-4, RhoA, TXN, ET-1, UBE2C, HDGF2, FGF21, LECT2, SOD1, STMN4, Midkine, IL-17A and IL26.

7. The method of claim 1, wherein the set of biomarkers comprises HDGF2.

8. A method for detecting the presence of hepatocellular carcinoma (HCC) biomarkers in a plurality of subjects having HCC at different stages, the method comprising:

(a) providing a serum sample from a plurality of subjects having HCC at different stages and measuring the serum for the presence of primary biomarker auto-antibodies against a set of biomarkers, wherein the set of biomarkers comprises RhoA, TXN, HDGF2, SOD1, IL-17A and IL26;

(b) detecting the presence of the HCC biomarkers in the plurality of subjects having HCC at different stages, the method comprising the steps of:

(i) mixing the serum sample with the set of biomarker conjugates to allow the primary biomarker auto antibodies, if present in the serum sample, to bind to the set of biomarker conjugates and washing away any unbound antibodies;

wherein the set of biomarker conjugates comprises each of the biomarkers in the set of biomarkers conjugated via an amide bond to a unique fluorescent microsphere bead, wherein each unique fluorescent microsphere bead associated with a specific particular biomarker in the set of biomarkers has a different emission wavelength for each biomarker,

wherein the biomarker conjugates are capable of being bound by a specific primary biomarker auto antibody present in the subject's serum sample,

(ii) adding to the mixture formed in step (i) anti-human secondary antibodies conjugated with phycoerythrin (PE), which are capable of binding primary biomarker auto antibodies; and allowing the anti-human secondary antibodies conjugated with PE to bind to specific primary antibodies bound to biomarker conjugates to form fluorescent bead-biomarker-auto antibody-PE conjugated antibody cascade, and washing away any unbound antibodies; and

(iii) measuring the mixture formed in step (ii) for the presence fluorescent bead-biomarker-auto antibody-PE conjugated antibody cascade to determine whether the plurality of subjects' serum contained primary biomarker auto antibodies.

9. The method of claim 8, wherein the set of biomarkers comprises Bmi-1, VCC1, SUMO-4, RhoA, TXN, ET-1, UBE2C, HDGF2, FGF21, LECT2, SOD1, STMN4, Midkine, IL-17A and IL26.

10. The method of claim 9, wherein the set of biomarkers comprises HDGF2.

11. A method for detecting the presence of hepatocellular carcinoma (HCC) biomarkers in a subject suspected of having HCC, the method comprising:

(a) providing a serum sample from the subject suspected of having HCC and measuring the serum for the presence of at least one primary biomarker auto-antibodies against a set of biomarkers, wherein the set of HCC biomarkers comprises RhoA, TXN, HDGF2, SOD1, IL-17A and IL26; and

(b) detecting the presence of at least one of the HCC biomarkers in the subject suspected of having HCC, the method comprising the steps of:

(i) mixing the serum sample with the set of biomarker conjugates to allow the primary biomarker auto antibodies, if present in the serum sample, to bind to the set of biomarker conjugates and washing away any unbound antibodies;

wherein the set of biomarker conjugates comprises each of the biomarkers in the set of biomarkers conjugated via an amide bond to a unique fluorescent microsphere bead, wherein each unique fluorescent microsphere bead associated with a specific particular biomarker in the set of biomarkers has a different emission wavelength for each biomarker,

wherein the biomarker conjugates are capable of being bound by a specific primary biomarker auto antibody present in the subject's serum sample,

(ii) adding to the mixture formed in step (i) anti-human secondary antibodies conjugated with phycoerythrin (PE), which are capable of binding primary biomarker auto antibodies; and allowing the anti-human secondary antibodies conjugated with PE to bind to specific primary antibodies bound to biomarker conjugates to form a fluorescent bead-biomarker-auto antibody-PE conjugated antibody cascade, and washing away any unbound antibodies; and

(iii) measuring the mixture formed in step (ii), for the presence fluorescent bead-biomarker-auto antibody-PE conjugated antibody cascade to determine whether the subject's serum contained at least one primary biomarker auto antibodies.

12. The method of claim 11, wherein the set of biomarkers consists of RhoA, TXN, HDGF2, SOD1, IL-17A and IL26.

13. The method of claim 11, wherein the set of biomarkers comprises Bmi-1, VCC1, SUMO-4, RhoA, TXN, ET-1, UBE2C, HDGF2, FGF21, LECT2, SOD1, STMN4, Midkine, IL-17A and IL26.

14. The method of claim 13, wherein the set of biomarkers comprises HDGF2.

15. A method for detecting the presence of hepatocellular carcinoma (HCC) biomarkers in a plurality of subjects having HCC at different stages, the method comprising:

(a) providing a serum sample from a plurality of subjects having HCC at different stages and measuring the serum for the presence of at least one primary biomarker auto-antibodies against a set of biomarkers, wherein the set of biomarkers comprises RhoA, TXN, HDGF2, SOD1, IL-17A and IL26; and

(b) detecting the presence of the at least one of the HCC biomarkers in the plurality of subjects having HCC at different stages, the method comprising the steps of:

(i) mixing the serum sample with the set of biomarker conjugates to allow the primary biomarker auto antibodies, if present in the serum sample, to bind to the set of biomarker conjugates and washing away any unbound antibodies;

wherein the set of biomarker conjugates comprises each of the biomarkers in the set of biomarkers conjugated via an amide bond to a unique fluorescent microsphere bead, wherein each unique fluorescent microsphere bead associated with a specific particular biomarker in the set of biomarkers has a different emission wavelength for each biomarker,

wherein the biomarker conjugates are capable of being bound by a specific primary biomarker auto antibody present in the subject's serum sample,

(ii) adding to the mixture formed in step (i), anti-human secondary antibodies conjugated with phycoerythrin (PE), which are capable of binding primary biomarker auto antibodies; and allowing the anti-human secondary antibodies conjugated with PE to bind to specific primary antibodies bound to biomarker conjugates to form a fluorescent bead-biomarker-auto antibody-PE conjugated antibody cascade, and washing away any unbound antibodies; and

(iii) measuring the mixture formed in step (ii) for the presence of the fluorescent bead-biomarker-auto antibody-PE conjugated antibody cascade to determine whether the plurality of subjects' serum contained primary biomarker auto antibodies.

16. The method of claim 15, wherein the set of biomarkers comprises Bmi-1, VCC1, SUMO-4, RhoA, TXN, ET-1, UBE2C, HDGF2, FGF21, LECT2, SOD1, STMN4, Midkine, IL-17A and IL26.

17. The method of claim 15, wherein the unique fluorescent signal from the microsphere beads serves to identify which biomarker in the set of biomarkers is present and wherein the signal from the PE indicates the presence of the biomarker conjugate.

18. The method of claim 17, wherein fluorescent intensity given by the PE-conjugated secondary antibodies in the fluorescent bead-biomarker-auto antibody-PE conjugates antibody cascade is measured to allow the detection and quantification of the primary biomarker auto antibodies.

19. The method of claim 15, wherein the set of biomarkers consists of RhoA, TXN, HDGF2, SOD1, IL-17A and IL26.

20. The method of claim 15, wherein the set of biomarkers consists of Bmi-1, VCC1, SUMO-4, RhoA, TXN, ET-1, UBE2C, HDGF2, FGF21, LECT2, SOD1, STMN4, Midkine, IL-17A and IL26.

21. A kit for detecting HCC biomarker auto-antibodies to a plurality of hepatocellular carcinoma (HCC) biomarkers in a patient's serum, the kit comprising:

(a) a set of 15 hepatocellular carcinoma (HCC) biomarker conjugates comprising at least one HCC biomarker protein set forth in SEQ ID NOs: 1-15, wherein each protein set forth in SEQ ID Nos: 1-15 is coupled to a different fluorescent microsphere bead having a different emission wavelength; and

(b) a PE-conjugated secondary antibody capable of binding to all of the HCC biomarker auto-antibodies to the plurality of HCC biomarkers wherein the HCC biomarkers are at least one of Bmi-1, VCC1, SUMO-4, RhoA, TXN, ET-1, UBE2C, HDGF2, FGF21, LECT2, SOD1, STMN4, Midkine, IL-17A and IL26 as set forth in SEQ ID NOs: 1-15.

22. The kit of claim 21, wherein the plurality of HCC biomarkers comprises HDGF2.

23. The kit of claim 22, wherein the plurality of HCC biomarkers comprises Bmi-1, VCC1, SUMO-4, RhoA, TXN, ET-1, UBE2C, HDGF2, FGF21, LECT2, SOD1, STMN4, Midkine, IL-17A and IL26.

24. The kit of claim 23, wherein the plurality of HCC biomarkers comprises at least one of RhoA, TXN, HDGF2, SOD1, IL-17A and IL26.

25. The kit of claim 24, wherein the plurality of HCC biomarkers comprises RhoA, TXN, HDGF2, SOD1, IL-17A and IL26.

26. The kit of claim 21, wherein the kit is capable of detecting an auto-antibody to any one of the hepatocellular carcinoma (HCC) biomarker present in the patient's serum when the auto-antibody is present at an amount as low as about 0.15 ng/mL.

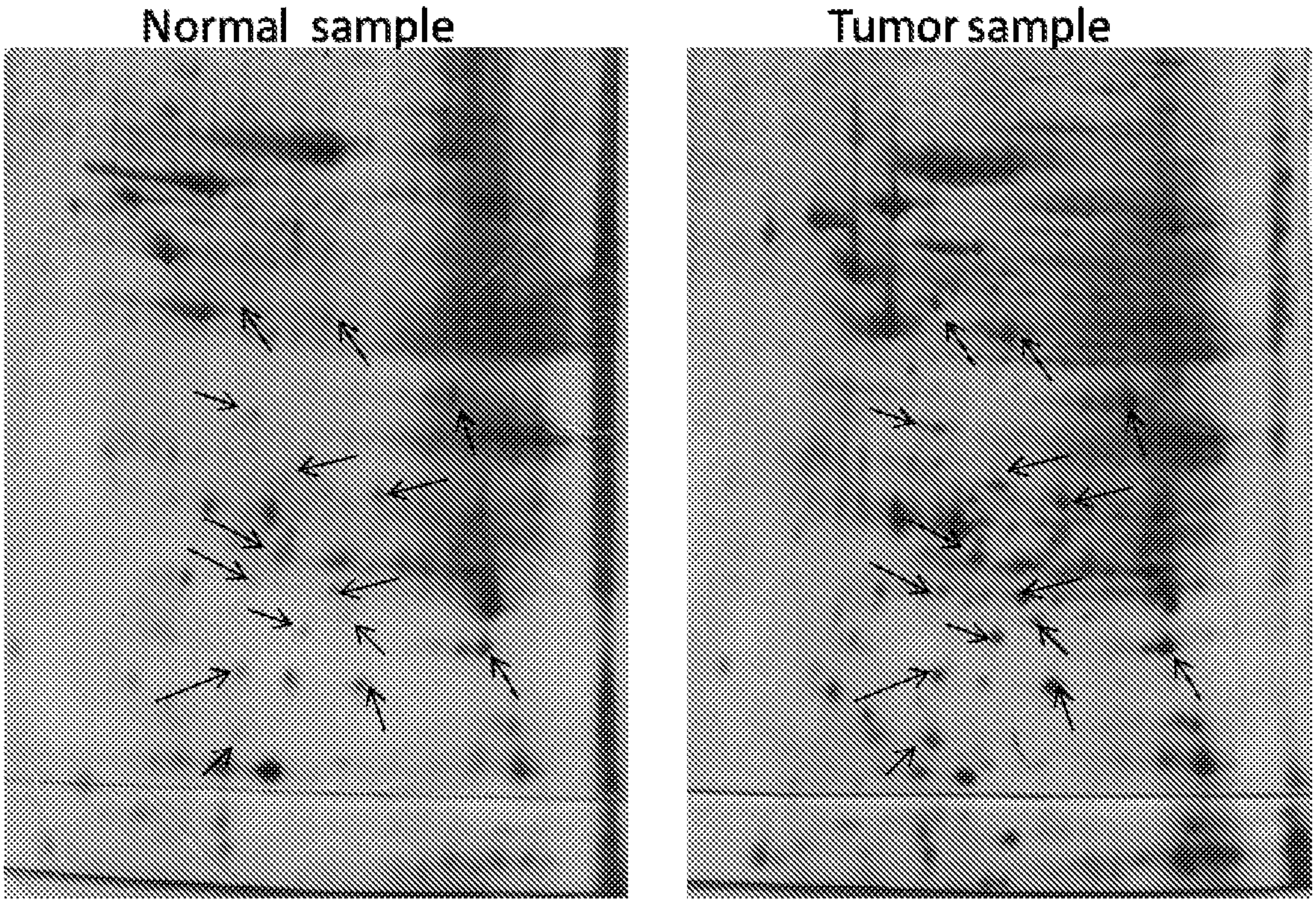


FIG. 1

Spot	Protein	MW (kD)
1	Bmi1	37
2	VCC1	13.6
3	SUMO-4	11
4	RhoA	22
5	TXN	12
6	ET-1	24
7	UBE2C	20
8	HDGF2	74
9	FGF21	20
10	LECT2	16
11	SOD1	16
12	STMN4	22
13	Midkine	19
14	IL-17A	17.5
15	IL-26	20

FIG. 2

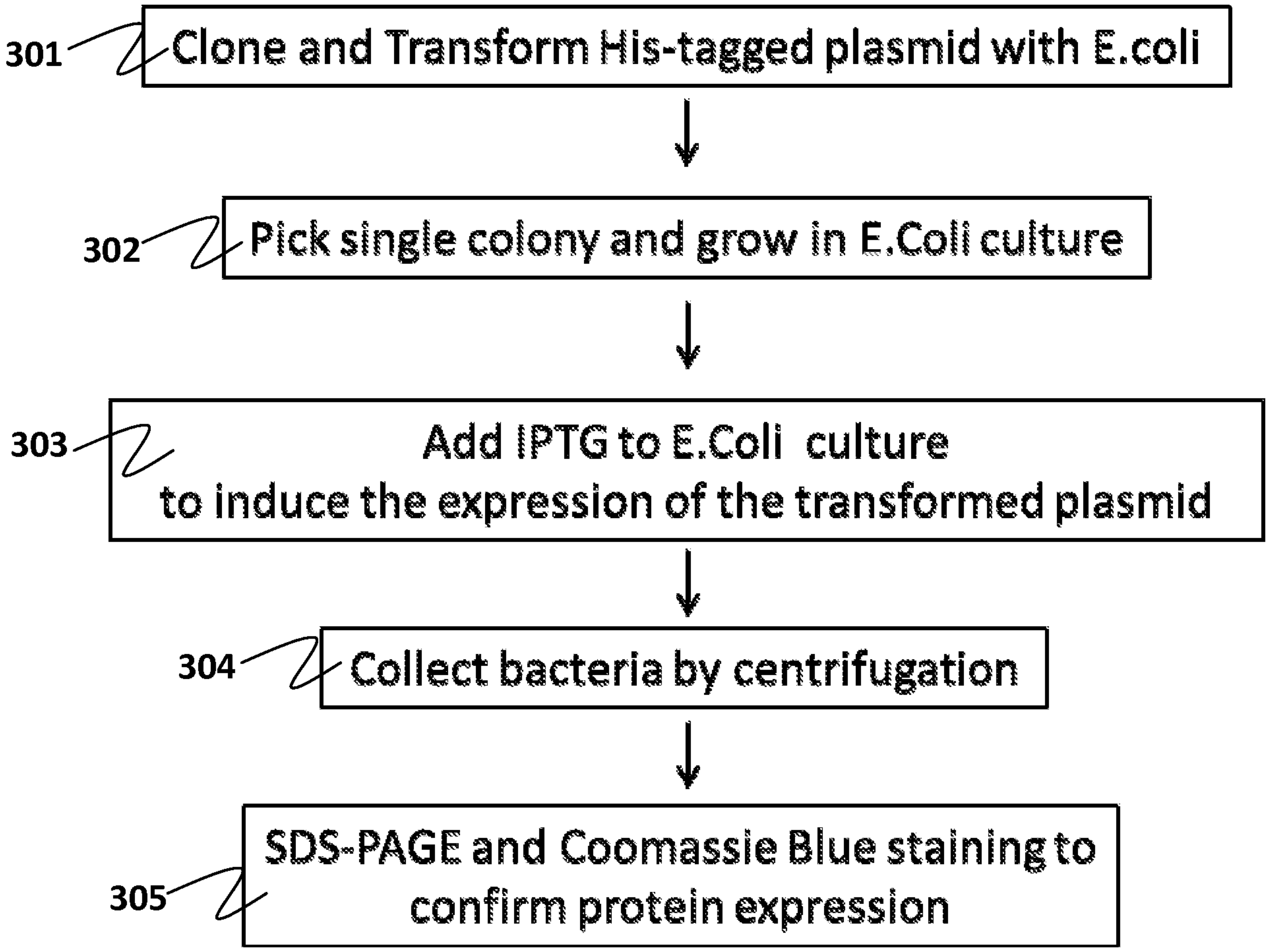

301
Clone and Transform His-tagged plasmid with E.coli
302
Pick single colony and grow in E.Coli culture
303
Add IPTG to E.Coli culture
to induce the expression of the transformed plasmid
304
Collect bacteria by centrifugation
305
SDS-PAGE and Coomassie Blue staining to
confirm protein expression

FIG. 3

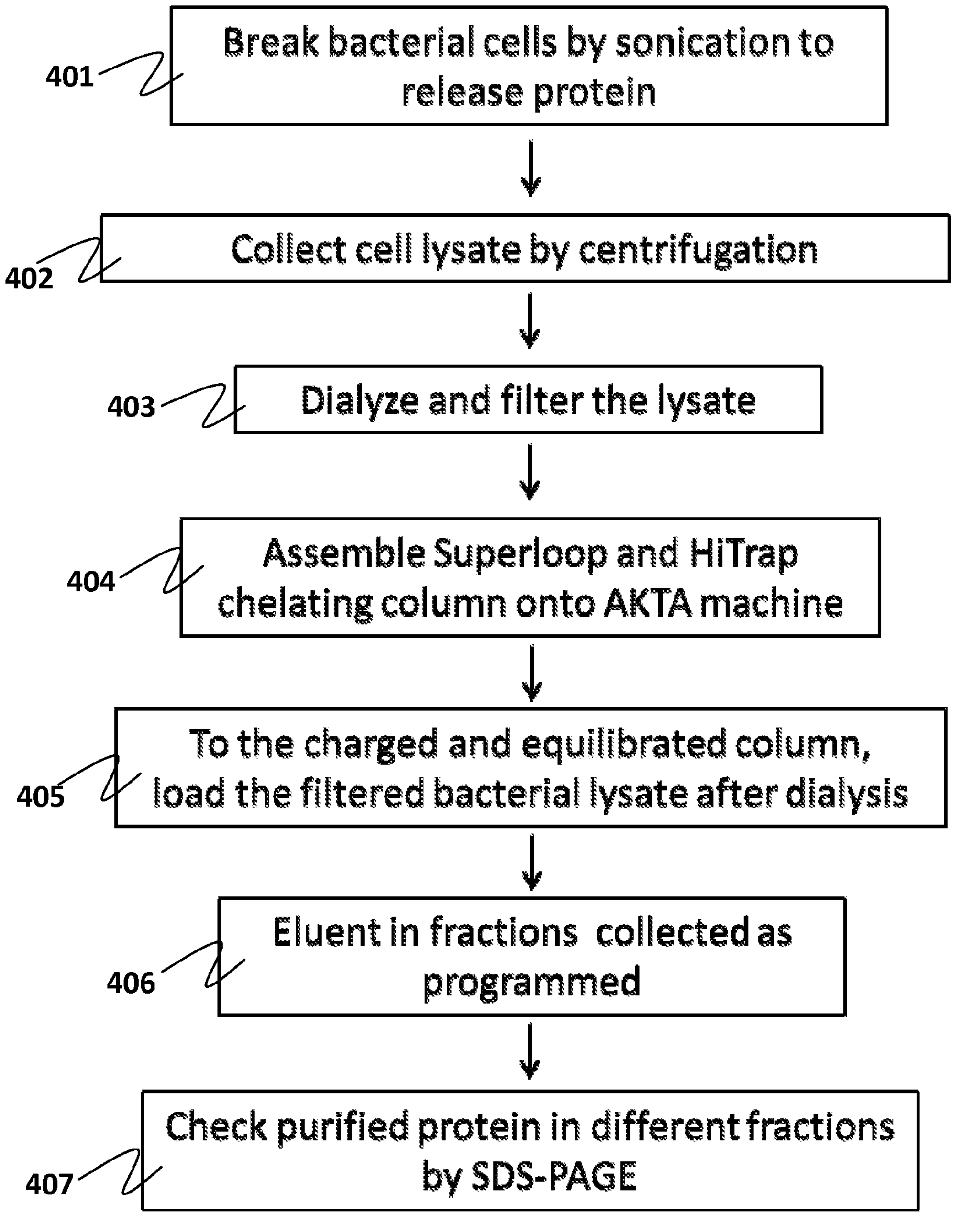

401
Break bacterial cells by sonication to release protein
402
Collect cell lysate by centrifugation
403
Dialyze and filter the lysate
404
Assemble Superloop and HiTrap chelating column onto AKTA machine
405
To the charged and equilibrated column, load the filtered bacterial lysate after dialysis
406
Eluent in fractions collected as programmed
407
Check purified protein in different fractions by SDS-PAGE

FIG. 4

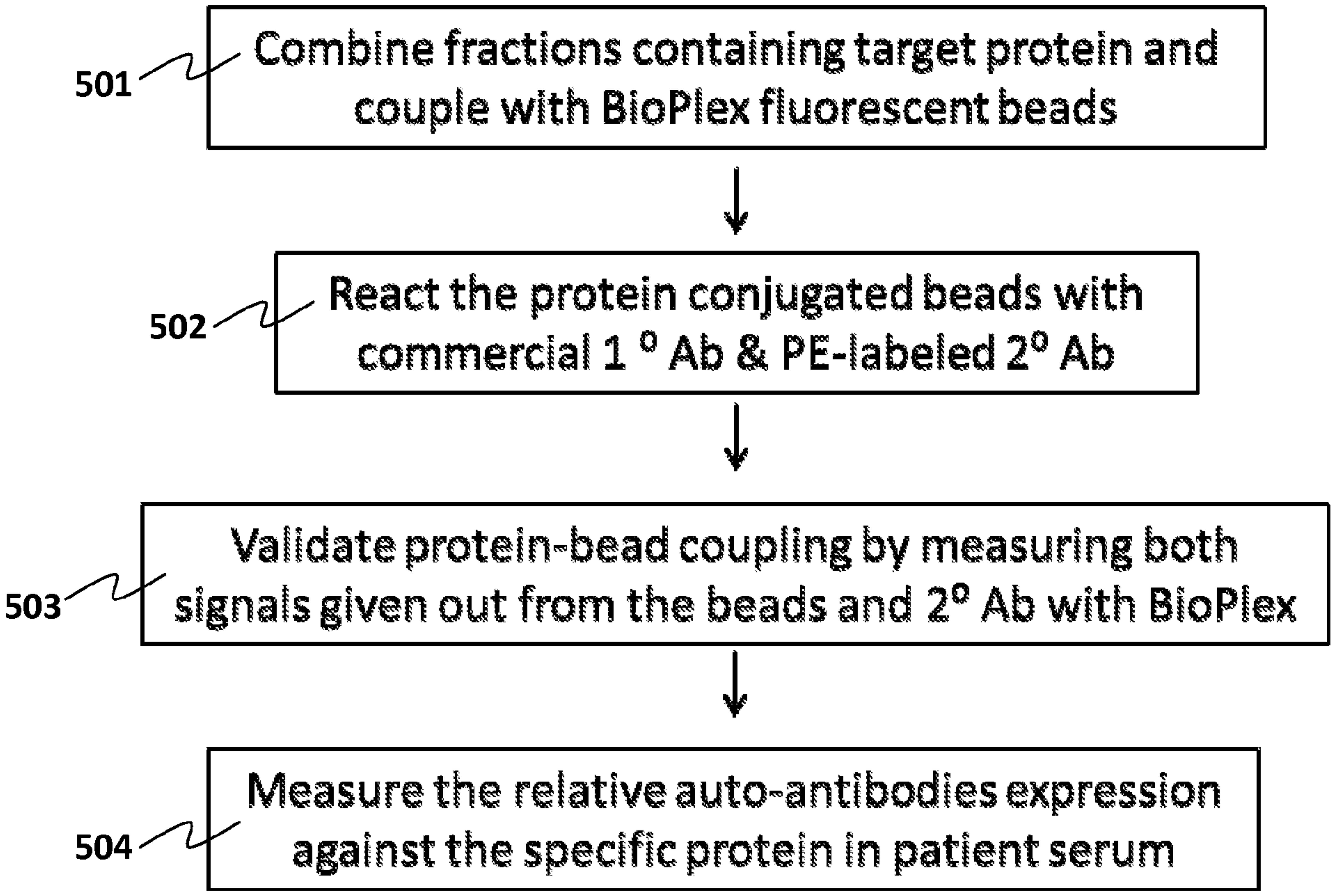

501
Combine fractions containing target protein and couple with BioPlex fluorescent beads
502
React the protein conjugated beads with commercial 1° Ab & PE-labeled 2° Ab
503
Validate protein-bead coupling by measuring both signals given out from the beads and 2° Ab with BioPlex
504
Measure the relative auto-antibodies expression against the specific protein in patient serum

FIG. 5

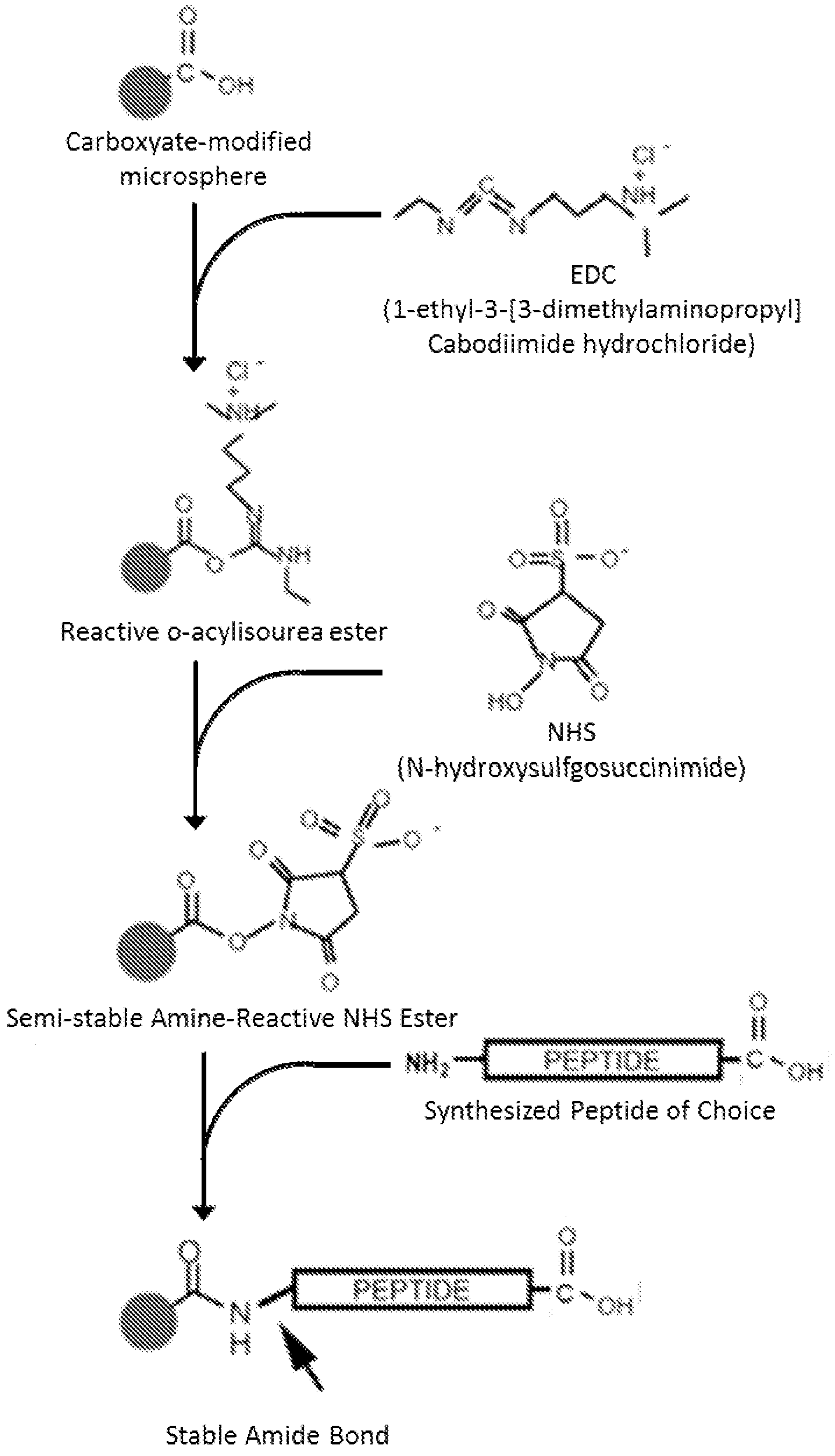

O
C
OH
Carboxyate-modified microsphere
Cl⁻
NH⁺
N
C
N
EDC
(1-ethyl-3-[3-dimethylaminopropyl]
Cabodiimide hydrochloride)
Cl⁻
NH⁺
O
O
NH
Reactive o-acylisourea ester
O
S
O⁻
O
N
HO
O
NHS
(N-hydroxysulfgosuccinimide)
O
S
O⁻
O
O
N
O
O
Semi-stable Amine-Reactive NHS Ester
NH₂
PEPTIDE
O
C
OH
Synthesized Peptide of Choice
O
N
H
PEPTIDE
O
C
OH
Stable Amide Bond

FIG. 6

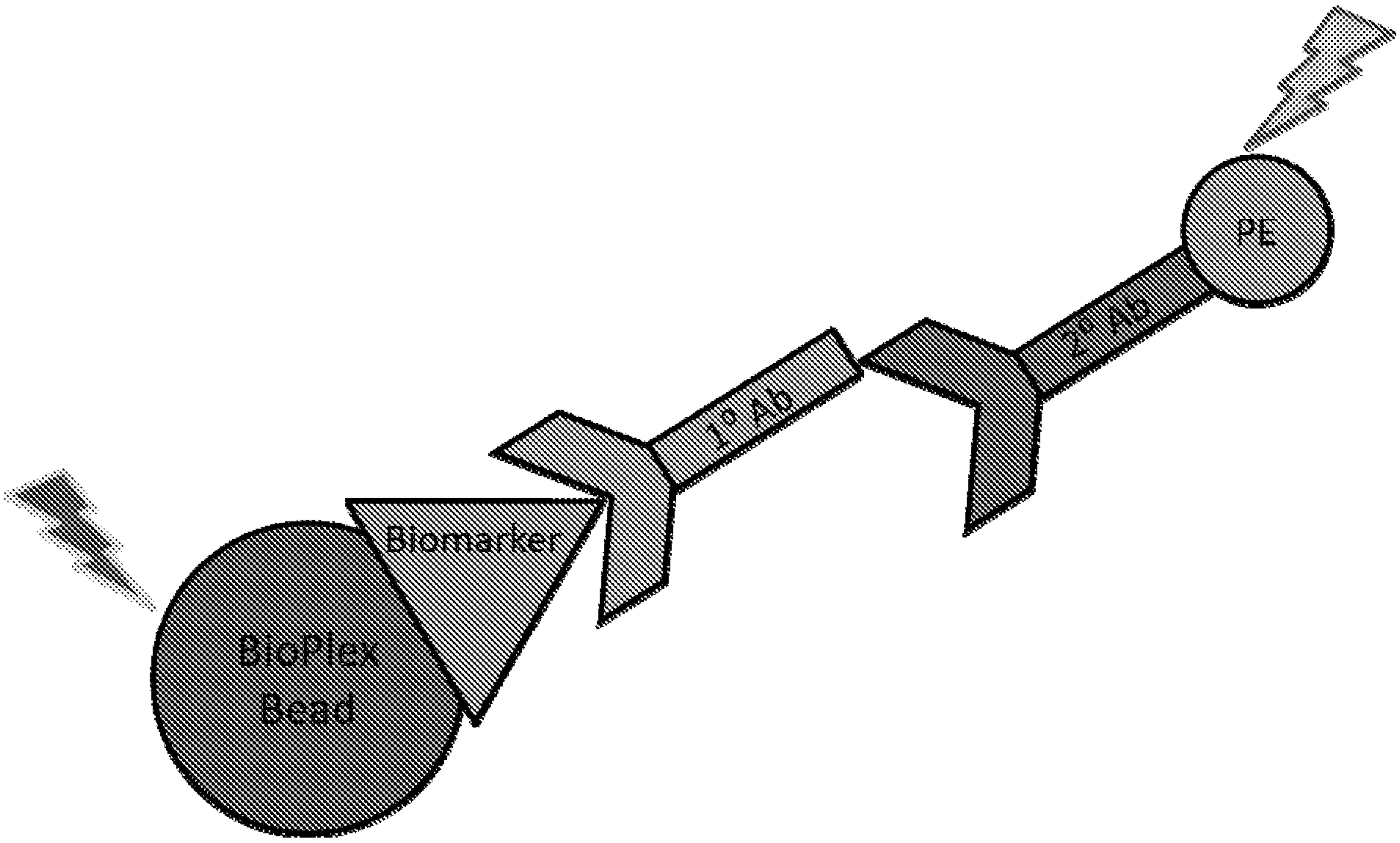


FIG. 7

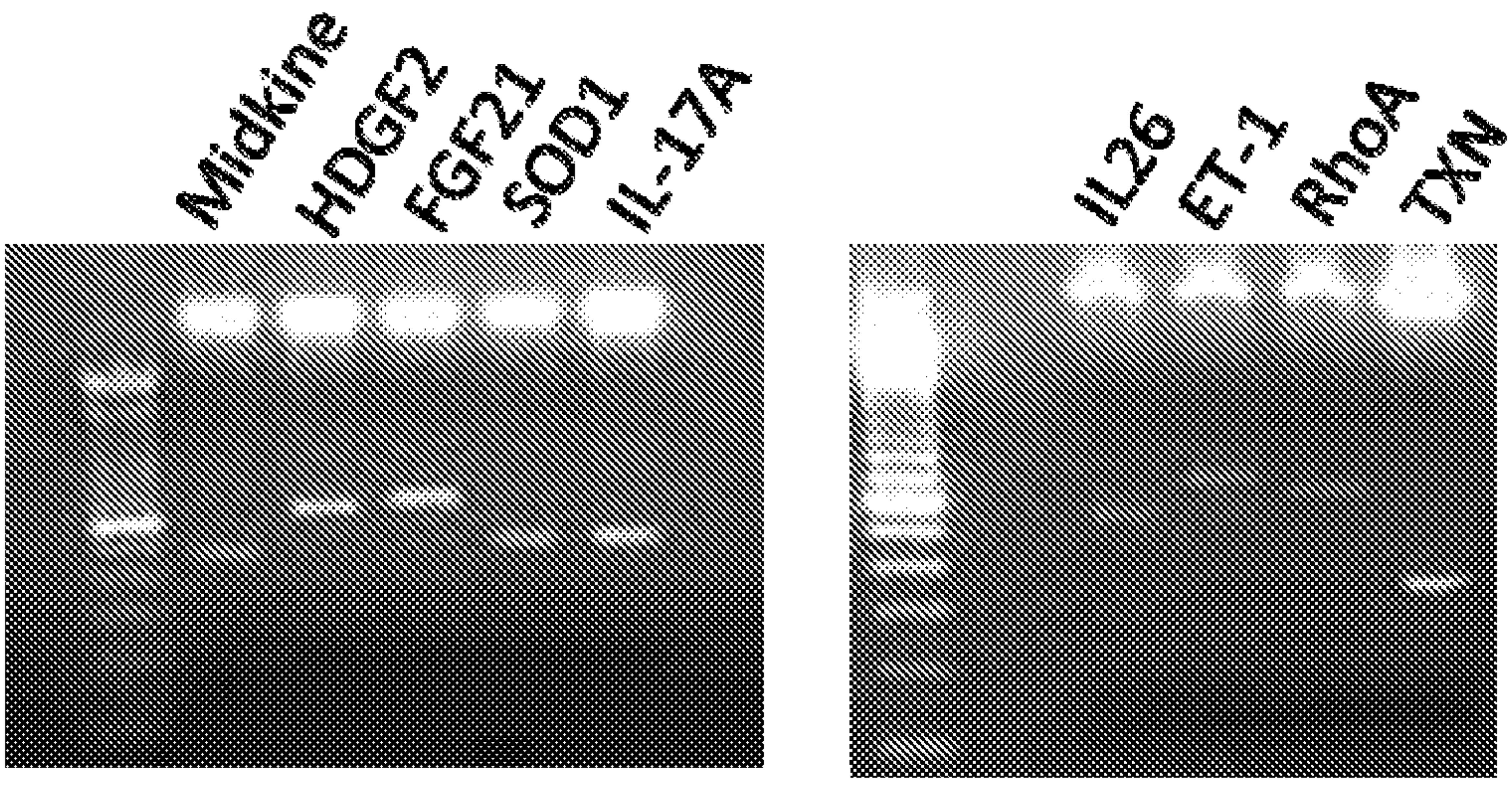


FIG. 8

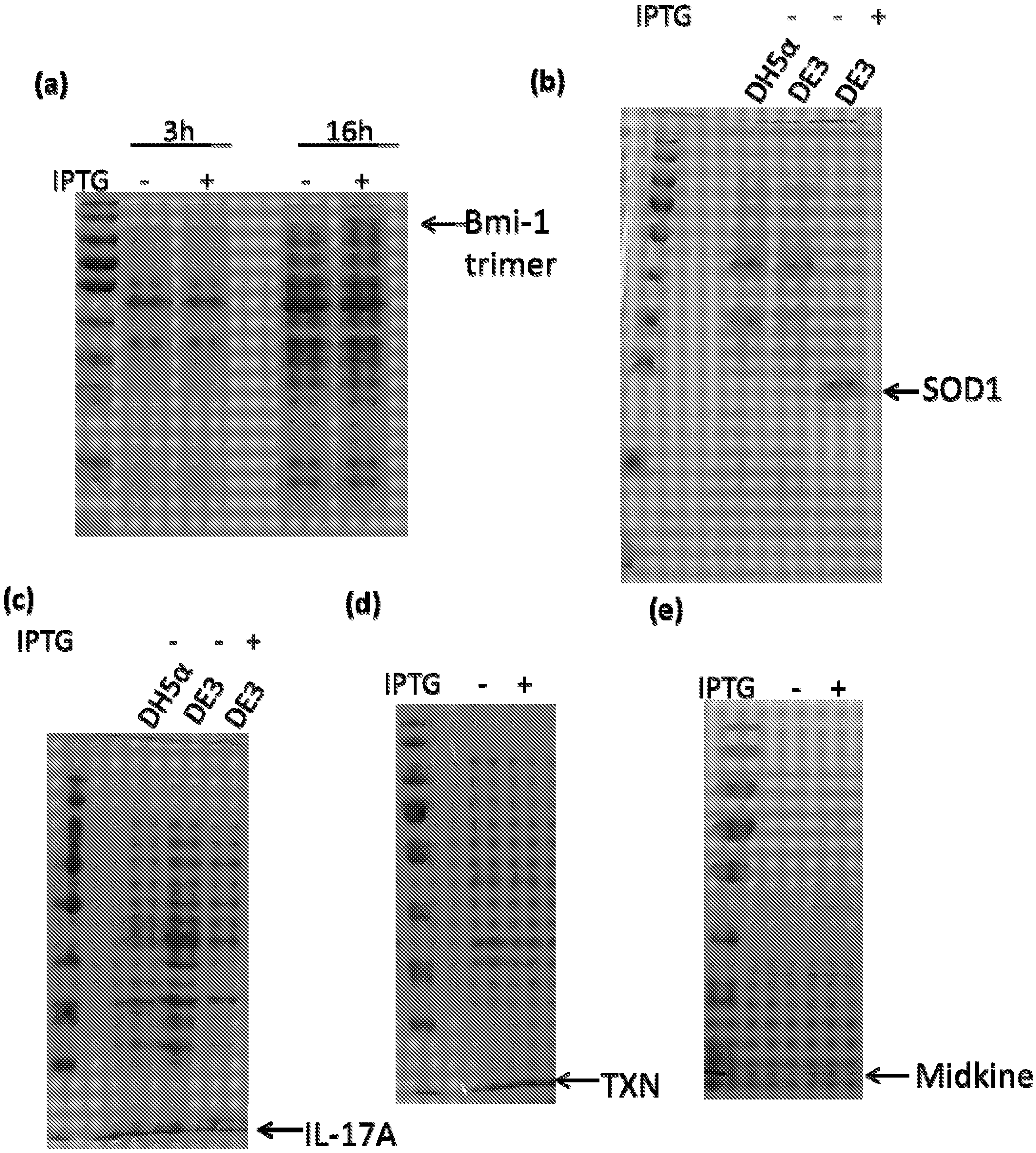

(a)
3h
16h
IPTG - + - +
Bmi-1 trimer
(b)
IPTG - - +
DH5α
DE3
DE3
SOD1
(c)
IPTG - - +
DH5α
DE3
DE3
IL-17A
(d)
IPTG - +
TXN
(e)
IPTG - +
Midkine

FIG. 9

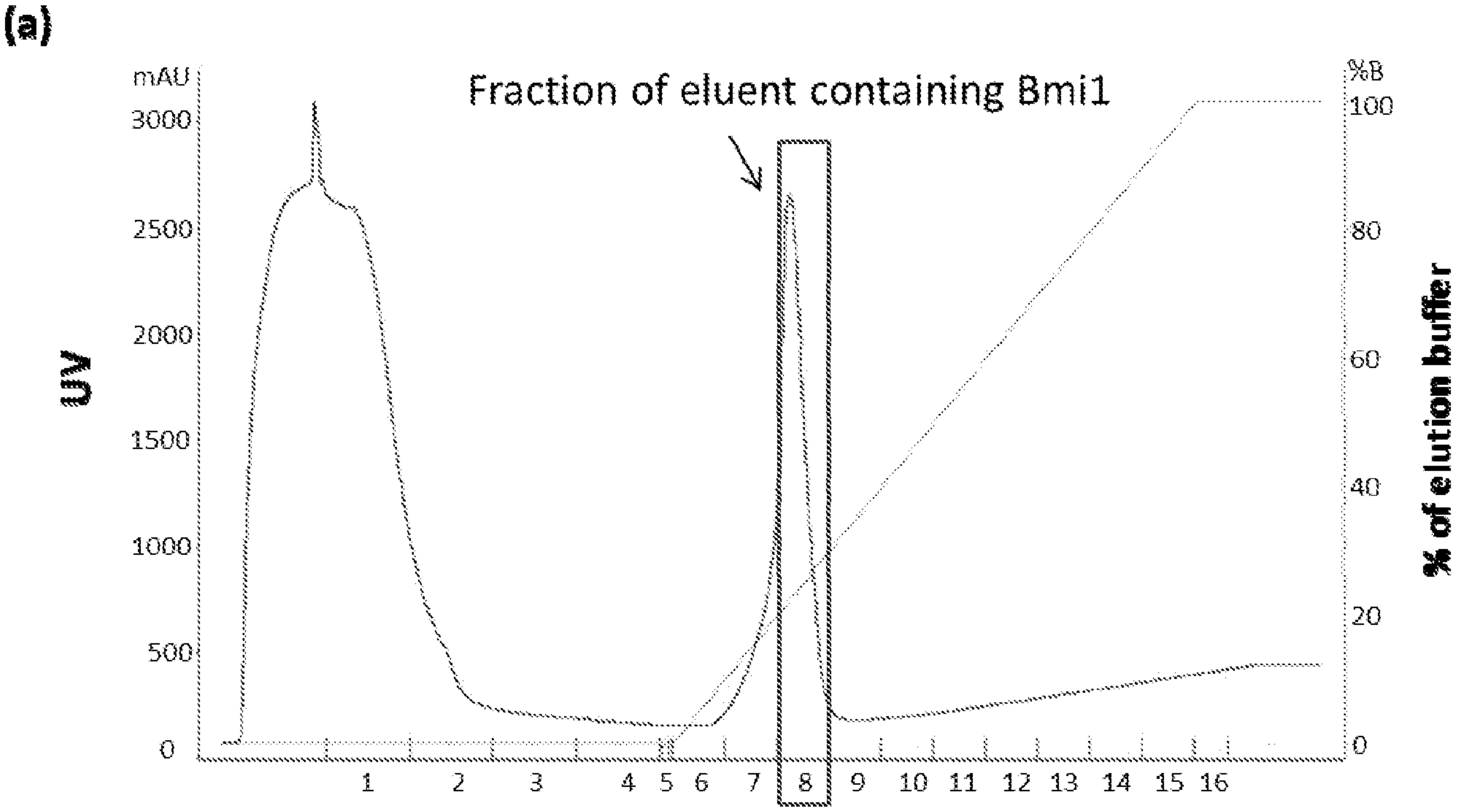

(a)
mAU
3000
2500
2000
1500
1000
500
0
UV
Fraction of eluent containing Bmi1
%B
100
80
60
40
20
0
% of elution buffer
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16

FIG. 10

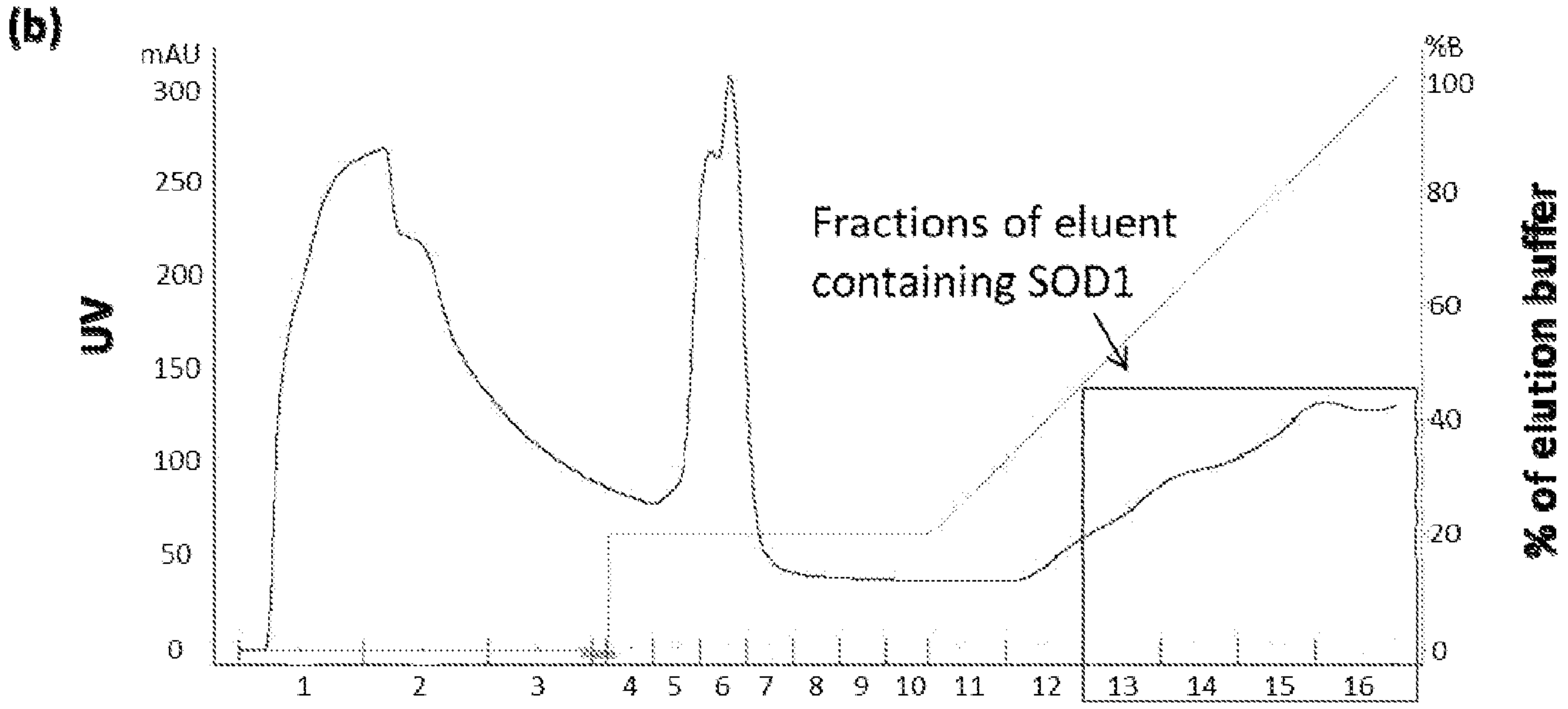

(b)
mAU
300
250
200
150
100
50
0
UV
%B
100
80
60
40
20
0
% of elution buffer
Fractions of eluent containing SOD1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16

FIG. 10 (cont'd)

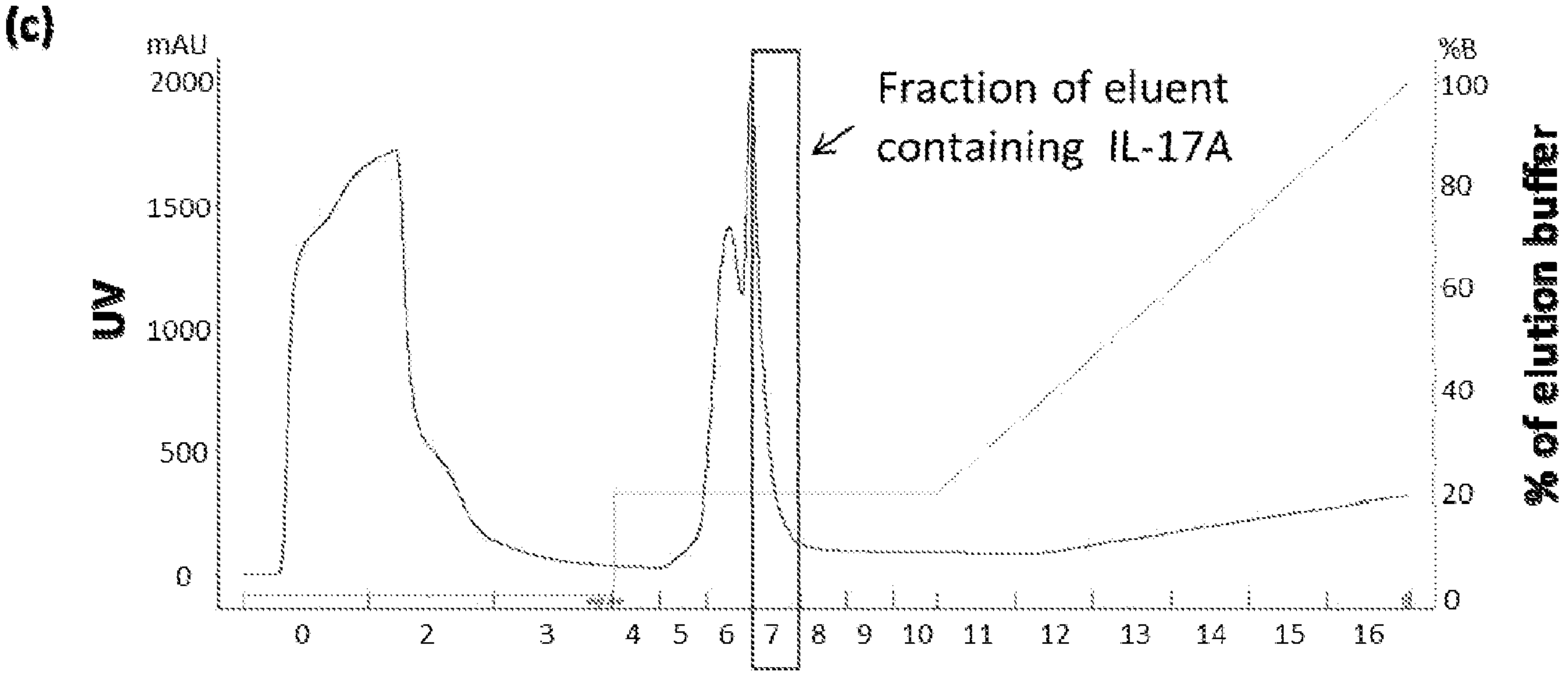

(c)
mAU
2000
1500
1000
500
0
UV
%B
100
80
60
40
20
0
% of elution buffer
Fraction of eluent containing IL-17A
0
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16

FIG. 10 (cont'd)

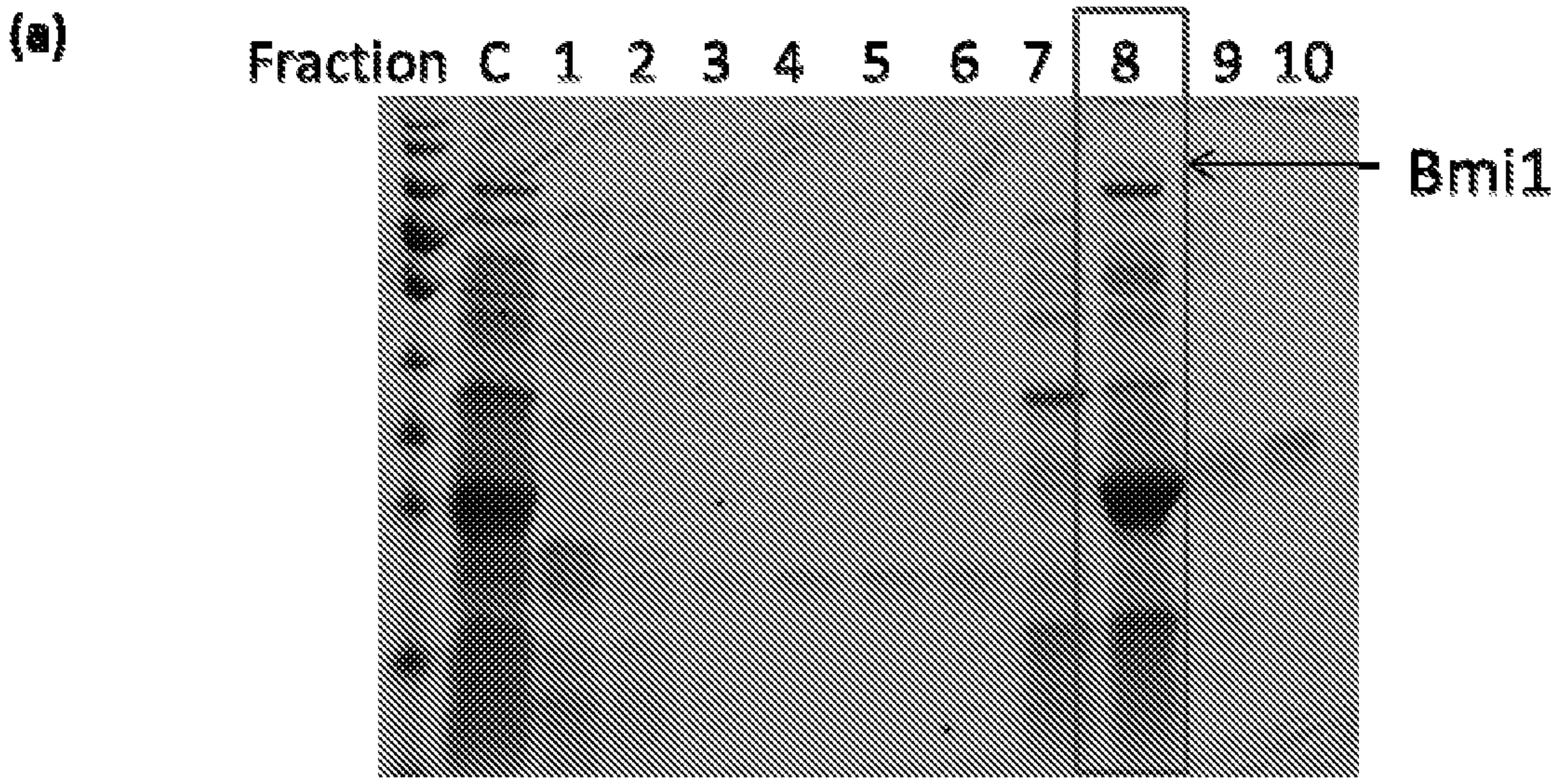


FIG. 11

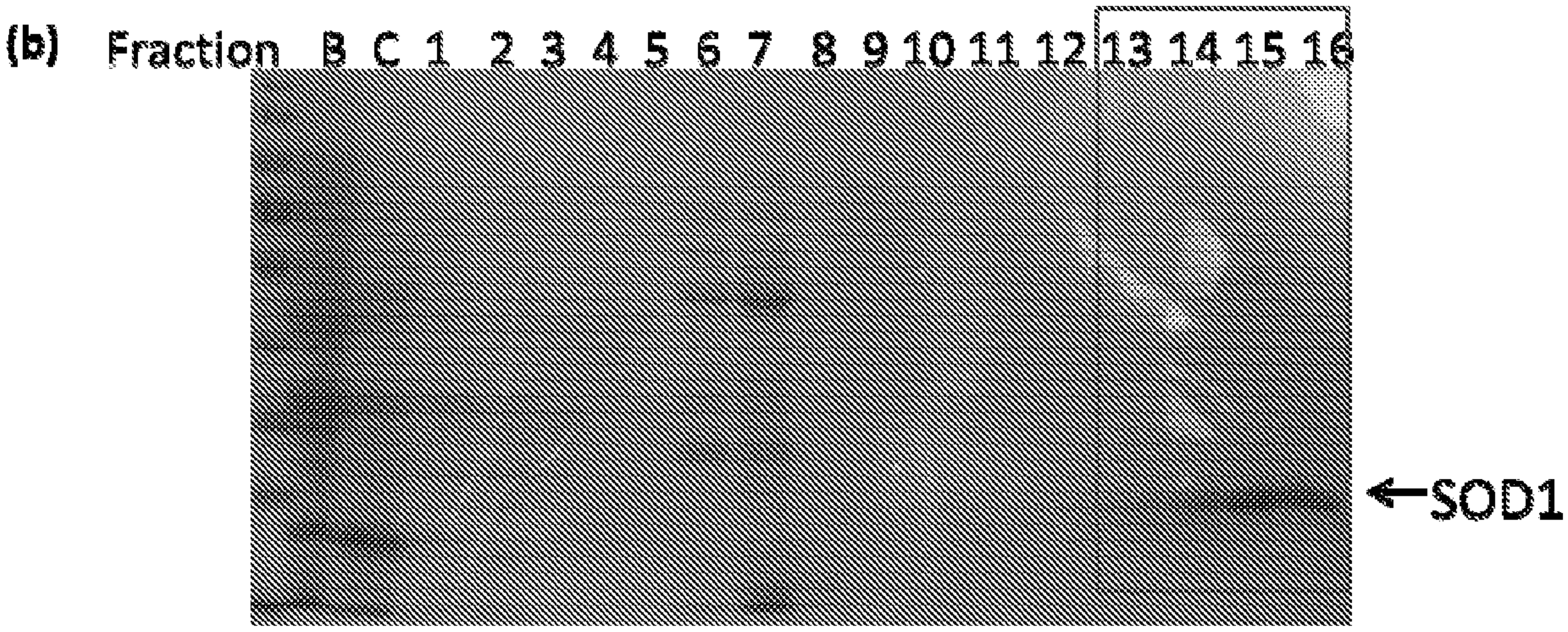


FIG. 11 (cont'd)

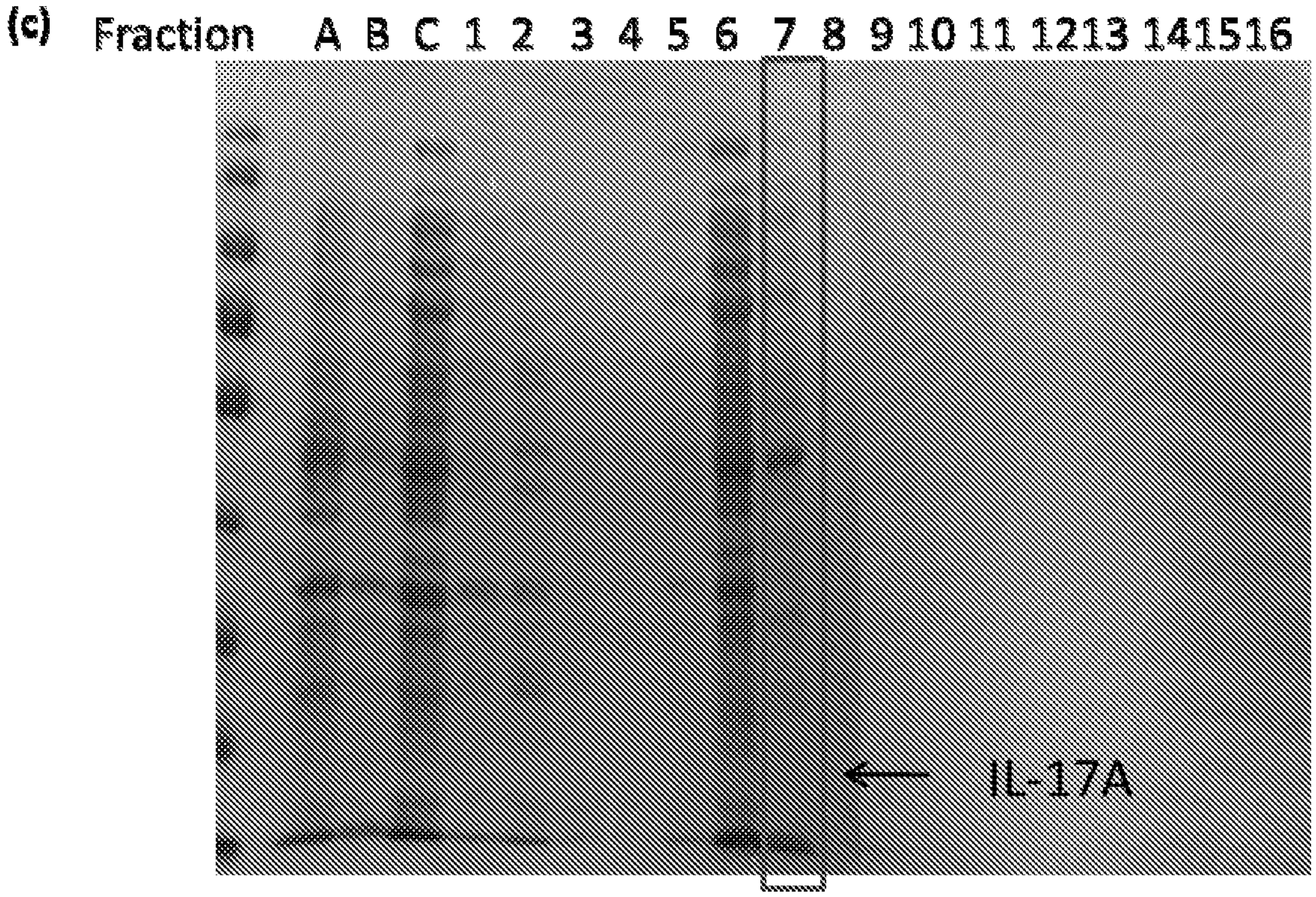


FIG. 11 (cont'd)

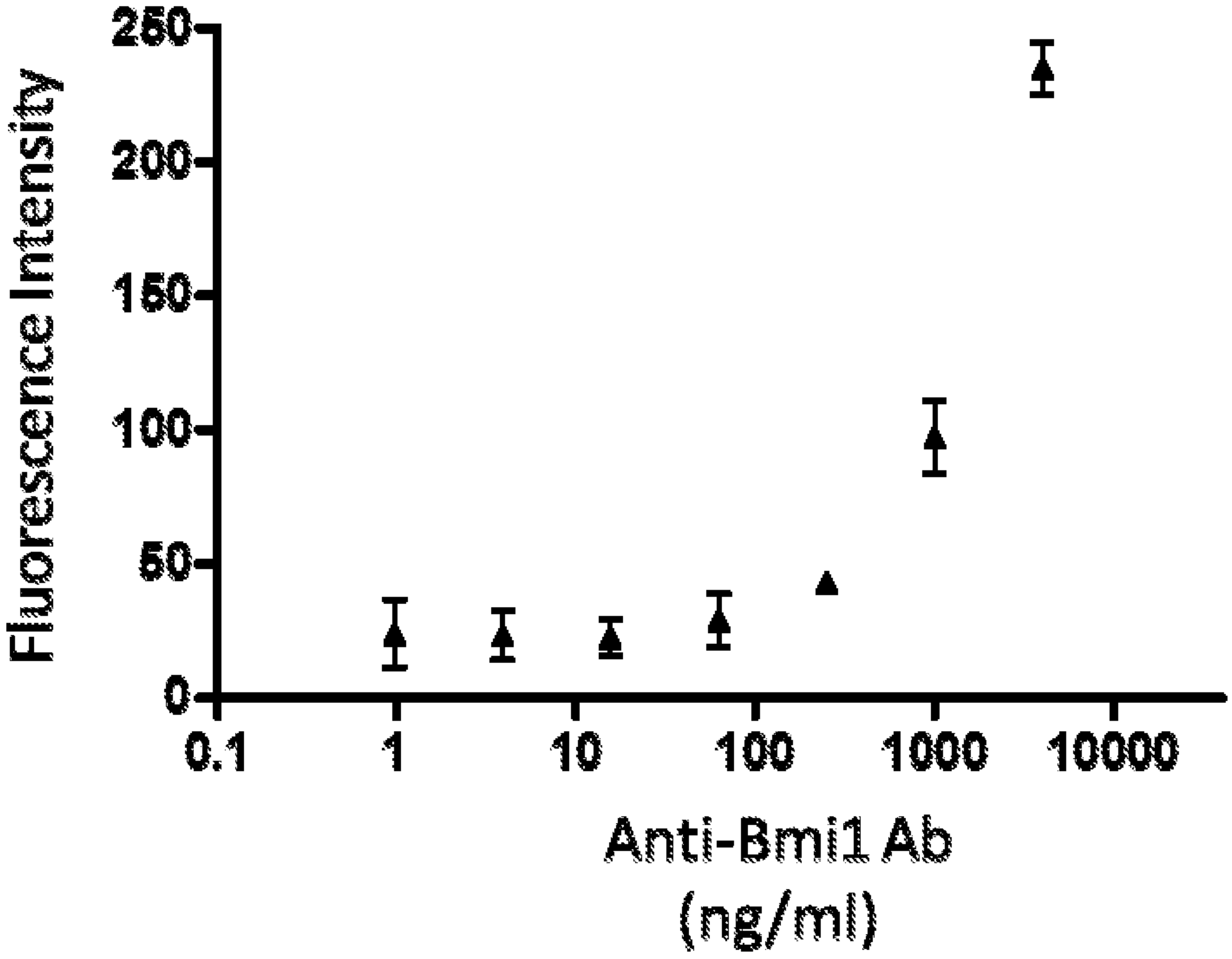

Fluorescence Intensity
250
200
150
100
50
0
0.1
1
10
100
1000
10000
Anti-Bmi1 Ab
(ng/ml)

FIG. 12

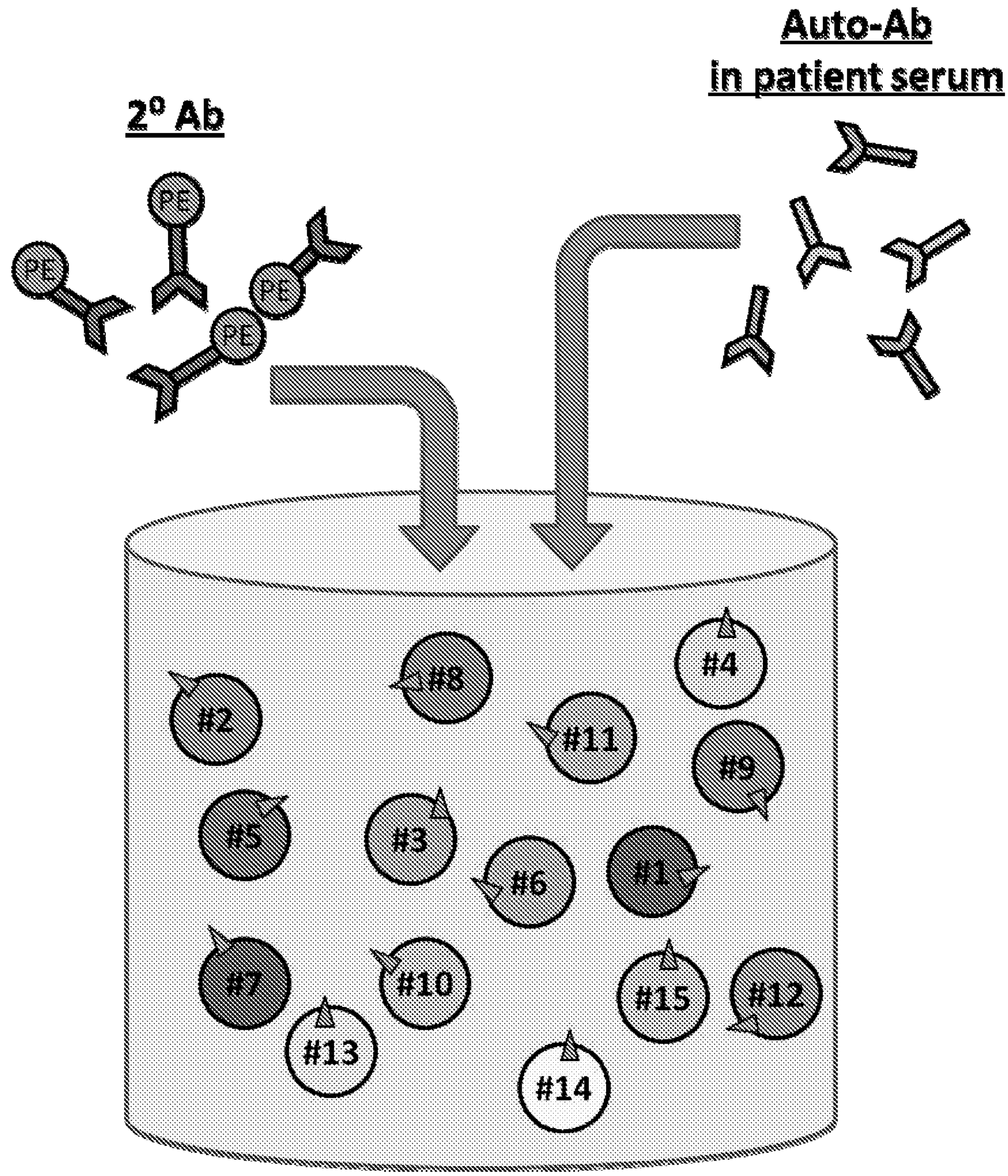
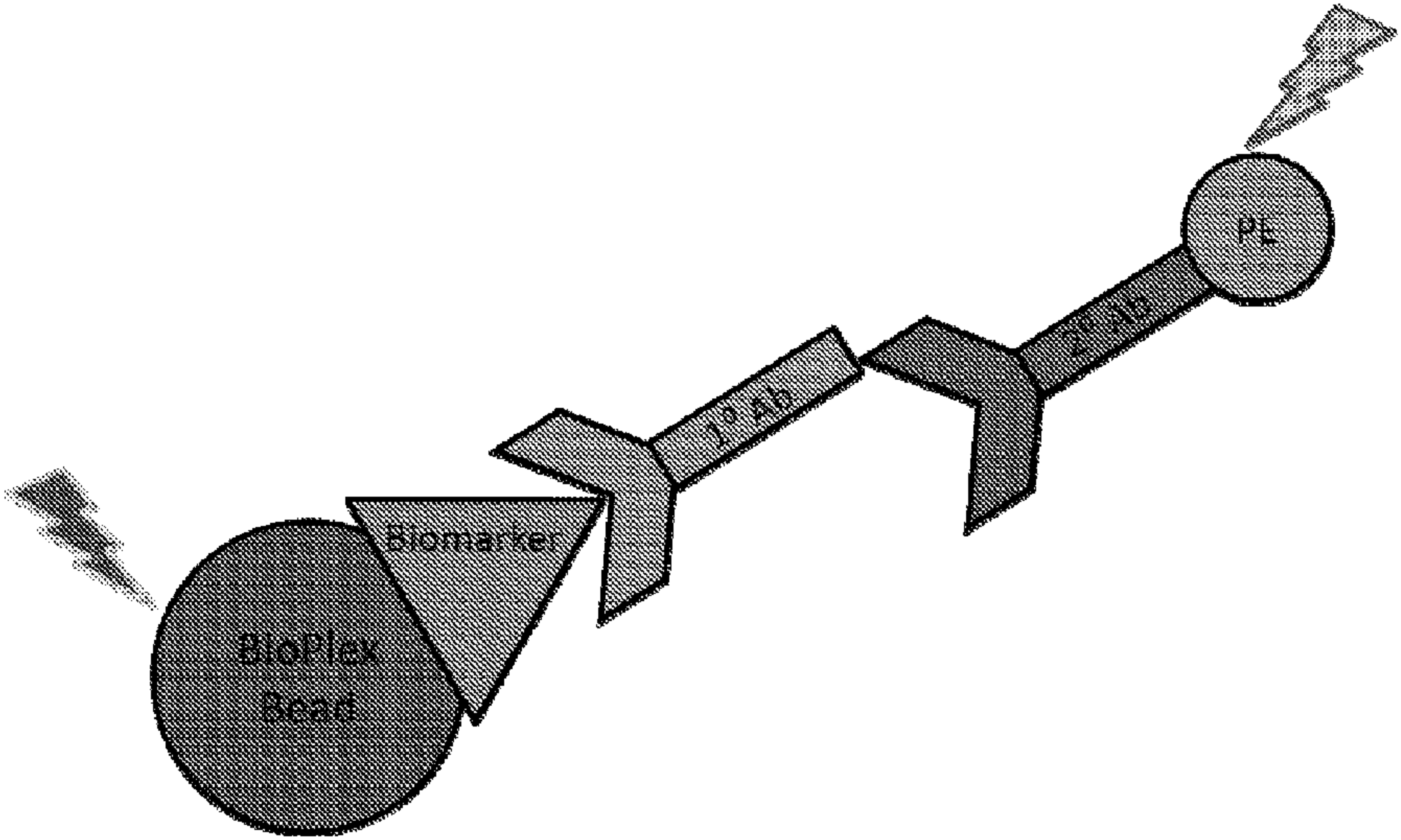

Auto-Ab
in patient serum
2° Ab
PE
PE
PE
PE
#8
#4
#2
#11
#9
#5
#3
#6
#1
#7
#10
#15
#12
#13
#14

FIG. 13


PE
2° Ab
1° Ab
Biomarker
BioPlex
Bead