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(54) Titre : PROCÉDES, DISPOSITIFS ET REACTIFS PERMETTANT DE SURVEILLER UNE CONCENTRATION DE
PACLITAXEL DANS LE PLASMA POUR UN DOSAGE DE PACLITAXEL PHARMACOCINETIQUEMENT GUIDE
(54) Title: METHODS, DEVICES, AND REAGENTS FOR MONITORING PACLITAXEL CONCENTRATION IN PLASMA
FOR PHARMACOKINETIC-GUIDED DOSING OF PACLITAXEL

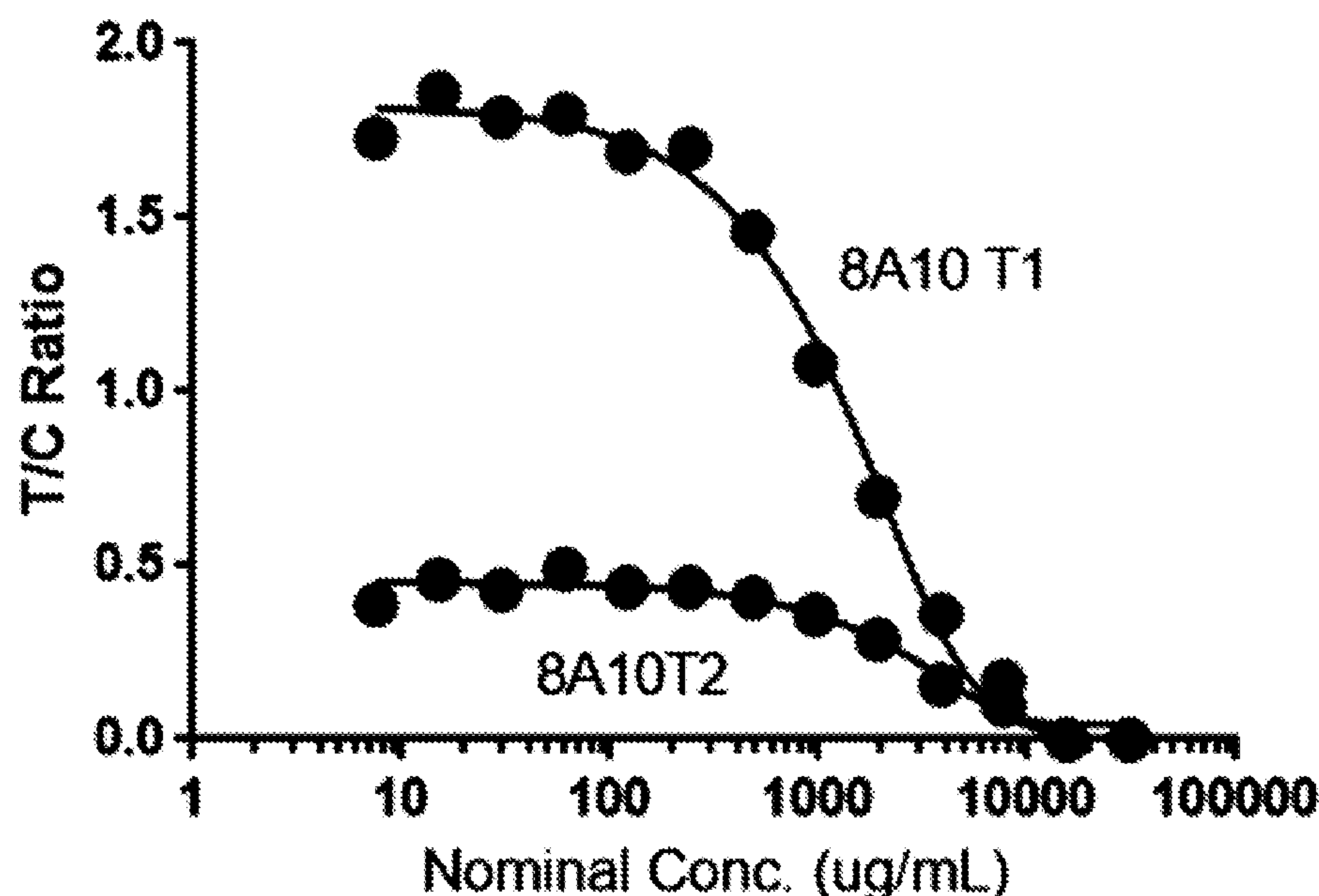


FIG. 5A

(57) Abrégé/Abstract:

Methods, devices, and compositions for assaying therapeutic agents. In one aspect, methods, devices, and compositions for assaying paclitaxel to provide therapeutic drug monitoring guided therapy of paclitaxel.

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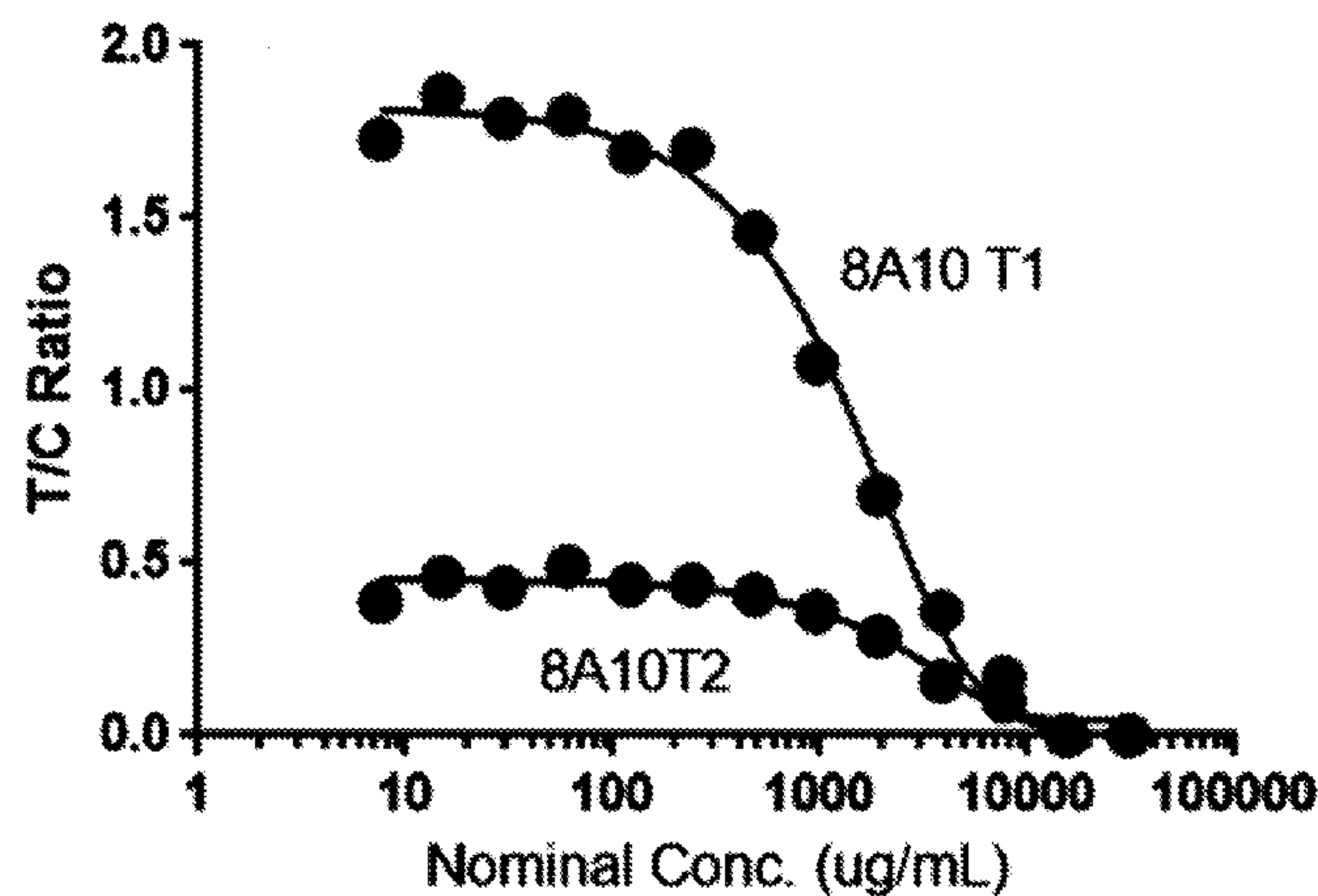
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(54) Title: METHODS, DEVICES, AND REAGENTS FOR MONITORING PACLITAXEL CONCENTRATION IN PLASMA FOR PHARMACOKINETIC-GUIDED DOSING OF PACLITAXEL

**FIG. 5A**

(57) Abstract: Methods, devices, and compositions for assaying therapeutic agents. In one aspect, methods, devices, and compositions for assaying paclitaxel to provide therapeutic drug monitoring guided therapy of paclitaxel.

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METHODS, DEVICES, AND REAGENTS FOR MONITORING
PACLITAXEL CONCENTRATION IN PLASMA FOR
PHARMACOKINETIC-GUIDED DOSING OF PACLITAXEL

5 CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Patent Application No. 61/975,386, filed April 4, 2014, and U.S. Patent Application No. 62/051,757, filed September 17, 2014, each expressly incorporated herein by reference in its entirety.

STATEMENT REGARDING SEQUENCE LISTING

10 The sequence listing associated with this application is provided in text format in lieu of a paper copy and is hereby incorporated by reference into the specification. The name of the text file containing the sequence listing is 53634_SEQ.txt. The text file is 11 KB; was created on April 6, 2015; and is being submitted via EFS-Web with the filing of the specification.

15 BACKGROUND OF THE INVENTION

Paclitaxel, originally isolated from the bark of Pacific Yew tree, has been established as one of the most effective chemotherapeutic drugs for a range of cancer types including lung, ovarian, and breast cancers. A major limitation of paclitaxel is its low solubility and the need to be formulated in toxic organic solvents, typically
20 polyoxyethylated castor oil and dehydrated ethanol mixtures (known as Taxol®). To prevent the solvent toxicity paclitaxel has been formulated with a variety of excipients as well as using nanoparticle delivery systems that can improve the solubility of hydrophobic drugs such as paclitaxel.

Abraxane®, a paclitaxel albumin bound nanoparticle formulation was approved
25 by FDA in 2005 and is currently one of the best formulations of paclitaxel for chemotherapy. Other systems have been investigated for the delivery of paclitaxel or are in development, e.g., using polymeric nanoparticles, lipid-based nanoparticle formulations, polymer conjugates, inorganic nanoparticles, carbon nanotubes, nanocrystals, or cyclodextrin nanoparticles (see, for example, Ping Ma et al., 2013,
30 J Nanomed. Nanotechnology:4:2).

Although Abraxane® is a widely used chemotherapeutic agent and practically applicable to all cancer types, the response to Abraxane®, however, can be as low as

20%. The relative insensitivity to paclitaxel found in some patients could be a contributing factor to low response rate. However, this insensitivity may not be the primary reason for the low response rate. There is up to 10-fold variations in blood concentration of paclitaxel monitored in clinical patients' samples when dosed at the various approved
5 doses (260 mg/m² for metastatic breast cancer, 125 mg/m² for pancreatic cancer, and 100 mg/m² for lung cancer (Nyman DW et al., 2005, J Clin. Oncol. 23, 7785-93). This variation suggests that the vast majority of patients are potentially dosed incorrectly with either too great a concentration of paclitaxel administration, and had to be taken off the treatment, or too low a dosage administered and providing no benefit from the treatment.
10 Even if patients are sensitive to paclitaxel, having an insufficient drug level would render them nonresponsive and the treatment ineffective. The under-dosed group is the most vulnerable patient population, as it is difficult to determine whether they are insensitive to paclitaxel or not administered sufficient paclitaxel. Full pharmacokinetic (PK) profiling is the only approach in such cases to provide guidance for proper drug dose based on the
15 individual pharmacokinetic variation.

Currently there are no available methods to perform a full PK quantitation of paclitaxel without having the patient enrolled in comprehensive clinical testing, which requires a hospital stay. Typical duration of such PK testing may be over a 48 hour period and includes repetitive blood drawing. Presently, the use of complex laboratory
20 equipment is required to analyze blood concentration of paclitaxel, including liquid chromatography/mass spectrometry (LC/MS) methods. These methods are extremely costly, currently over \$120/sample and the equipment cost is in the range exceeding \$150K-\$200K per instrument. It has also been demonstrated that a minimum of four data points collected over a period of 48 to 72 hours is needed to adequately characterize the
25 PK parameters for each particular patient. Keeping the patients in hospital for PK testing can easily push the cost to roughly \$10,000 per patient. A sufficiently powered Phase III clinical trial to demonstrate clinical efficacy for PK guided dosing would require 500 patients (250 patients for BSA dosing and 250 patients for PK guided dosing). The bioanalytical cost alone would be \$1.5M (500 points X 6 cycles of chemotherapy X 4
30 blood samplings for PK analysis X \$120/ sample analysis). The other components of trial would cost roughly \$100,000 per patient, totaling \$50M. This represents a significant barrier to obtaining meaningful clinical data necessary to guide dose adjustment for

optimum tumor response and regulatory approval of the device. The high cost of the analysis and instrumentation, therefore, has prohibitive consequences on establishing therapeutic drug monitoring (TDM) for many drugs that have a relatively narrow therapeutic range.

5 Accordingly, a need remains for a simple, effective, and inexpensive approach to monitor the pharmacokinetics of paclitaxel in a patient, thereby appropriately personalizing the therapy to the individual patient by informing any adjustment of the dosing strategy. The present invention seeks to fulfill this need and provides further related advantages.

10 SUMMARY OF THE INVENTION

The present invention provides methods, devices, and compositions for assaying therapeutic agents. In one aspect, methods, devices, and compositions for assaying paclitaxel are provided.

15 In one aspect, the invention provides a method for assaying paclitaxel in a liquid sample. In one embodiment, the methods comprises:

(a) applying a liquid sample comprising paclitaxel to a lateral flow assay device, the device comprising

(i) a sample receiving zone for receiving the liquid sample;

20 (ii) a detection reagent zone in liquid communication with the sample receiving zone and downstream in flow direction from the sample receiving zone,

wherein the detection reagent zone comprises a detection reagent deposited thereon,

wherein the detection reagent is a paclitaxel antibody, or fragment or derivative thereof that binds paclitaxel, labeled with a detectable reporting group, and

25 wherein the paclitaxel antibody, or fragment or derivative thereof, has a K_{on} from about 10^4 to about 10^7 , and a K_{off} from about 10^{-3} to about 10^{-7} ; and

(iii) a capture zone in liquid communication with the detection reagent zone and downstream in flow direction from the detection reagent zone,

30 wherein the capture zone comprises first and second capture reagents immobilized thereon,

wherein the first capture reagent is a paclitaxel material capable of binding the detection reagent (test line),

wherein the second capture reagent is an antibody capable of binding the detection reagent (control line),

wherein the first capture reagent is positioned at a first distance downstream in flow direction from the upstream end of the capture zone,

5 wherein the second capture reagent is positioned at a second distance downstream in flow direction from the upstream end of the capture zone,

wherein the second distance is greater than the first distance, and

wherein the ratio of the first distance to the second distance is from about 0.0 to about 0.4, when the K_{on} is greater than about 2.0×10^5 and the K_{off} is less than about
10 1.0×10^{-3} , and

wherein the ratio of the first distance to the second distance is from about 0.2 to about 1.0, when the K_{on} is greater than about 2.0×10^4 and the K_{off} is less than about 2.0×10^{-4} ; and

(b) allowing the sample to flow from the sample receiving zone through the
15 detection reagent zone to provide detection reagent with paclitaxel;

(c) allowing the detection reagent with paclitaxel to flow through the capture zone,

whereby the first capture reagent (test line) competes with the analyte (paclitaxel) for binding with the detection reagent, and

20 whereby the second capture reagent (control line) binds excess detection reagent; and

(d) observing the amount of detection reagent bound to the first capture reagent (test line) relative to the second capture reagent (control line).

In certain embodiments, the method further comprising determining the quantity
25 of paclitaxel in the sample by quantitating the amount of detection reagent bound to the first capture reagent. Quantitating the amount of detection reagent bound to the first capture reagent can include optical density measurement.

In one embodiment, the paclitaxel antibody is 3C6. In another embodiment, the paclitaxel antibody is 8A10. In certain embodiments, two paclitaxel antibodies, or
30 fragments or derivatives thereof that bind paclitaxel are used (e.g., 3C6 and 8A10).

In the method, the paclitaxel material is a paclitaxel antigen that competes with paclitaxel for binding to the detection reagent. In one embodiment, the paclitaxel material is a paclitaxel protein conjugate.

5 In the methods of the invention, distance between the sample receiving zone and the first capture reagent can be varied to optimize paclitaxel detection sensitivity. In certain embodiments, the distance between the sample receiving zone and the first capture reagent is minimized to optimize paclitaxel detection sensitivity.

10 In certain embodiments, the method further comprises observing the amount of excess detection reagent bound to the second capture reagent (control line). In these embodiments, the method may further comprise determining the quantity of paclitaxel in the sample by quantitating the amount of detection reagent to the second capture reagent.

15 To enhance sensitivity and dynamic range, the method may further comprise a third capture zone intermediate the first and second capture zones, wherein the third capture zone comprises a paclitaxel material capable of binding the detection reagent. In these embodiments, determining the quantity of paclitaxel can be determined by quantitating the amount of detection reagent bound to the third capture reagent. Quantitating the amount of detection reagent bound to the third capture reagent can include optical density measurement.

20 It will be appreciated that the method of the invention is applicable to other solid phase assays, including, for example, surface plasmon resonance (SPR) assays.

As noted above, more than one antibody, fragment, or derivative thereof can be used in the method. In these embodiments, the first antibody can have a relatively high K_{on} (e.g., greater than 1×10^4) and the second antibody can have a relatively low K_{off} (e.g., less than 1×10^{-3}).

25 Furthermore, in certain embodiments, the capture zone can include multiple capture positions (e.g., two or three lines, T1, T2, T3) to provide multiple readings on the same sample allowing for increase reproducibility and expanded dynamic range.

30 In another aspect, the invention provides methods for therapeutic drug monitored (TDM) guided paclitaxel therapy. In one embodiment, the invention provides a method for monitoring the efficacy of a paclitaxel therapy in a patient diagnosed with cancer, comprising:

- (a) treating a cancer patient with paclitaxel at a first point in time;

(b) determining a first concentration of paclitaxel in the patient at a first point in time, wherein determining the concentration comprises the method noted above for assaying paclitaxel;

(c) treating the patient with paclitaxel at a second point in time;

5 (d) determining a second concentration of paclitaxel drug in the patient at a second point in time, wherein determining the concentration comprises the method noted above for assaying paclitaxel; and

(e) comparing the first and second concentrations of paclitaxel in the patient to determine the efficacy of the cancer treatment.

10 In a related embodiment, the invention provides a method for PK-guided dosing of paclitaxel therapy in a patient diagnosed with cancer, comprising:

(a) treating the cancer patient with paclitaxel at a first point in time;

determining one or more pharmacokinetic parameters of paclitaxel in the patient at a first point in time, wherein determining the one or more pharmacokinetic parameters
15 comprises the method noted above for assaying paclitaxel;

(b) treating the patient with paclitaxel at a second point in time using the PK information from first dosing;

(c) determining one or more pharmacokinetic parameters of paclitaxel in the patient at a second point in time, wherein determining the one or more pharmacokinetic
20 parameters comprises the method noted above for assaying paclitaxel; and

(d) comparing one or more pharmacokinetic parameters of paclitaxel in the subject at the first point in time with the levels at the second point in time to confirm that appropriate dosing was achieved.

In certain embodiments, the pharmacokinetic parameters are selected from the
25 group consisting of time to maximum concentration (T_{max}), concentration maximum (C_{max}), area under the curve (AUC), clearance (CL), volume of distribution (V_d), apparent volume of distribution during the terminal phase (V_z), apparent volume of distribution during steady state (V_{ss}) and combinations thereof.

In further aspects, the invention provides devices and methods, similar to those
30 described above for paclitaxel, but that are useful for assaying other therapeutic agents.

In one embodiment, the invention provides a lateral flow device, comprising:

(a) a sample receiving zone for receiving a liquid sample;

(b) a detection reagent zone in liquid communication with the sample receiving zone and downstream in flow direction from the sample receiving zone,

wherein the detection reagent zone comprises one or more detection reagents deposited thereon;

5 (c) a capture zone in liquid communication with the detection reagent zone and downstream in flow direction from the detection reagent zone,

wherein the capture zone comprises one or more capture reagents immobilized thereon; and

10 (d) an absorbent zone in liquid communication with the capture zone and downstream in flow direction from the capture reagent zone.

As noted above, more than one antibody, fragment, or derivative thereof can be used in the method. In these embodiments, the first antibody can have a relatively high K_{on} (e.g., greater than 1×10^4) and the second antibody can have a relatively low K_{off} (e.g., less than 1×10^{-3}). In one embodiment, the detection reagent comprises a first
15 antibody having a first affinity for the therapeutic drug and a second antibody having a second affinity for the therapeutic drug, wherein the first affinity is greater than the second affinity. Furthermore, in certain embodiments, the capture zone can include multiple capture positions (e.g., two or three lines, T1, T2, T3) to provide multiple readings on the same sample allowing for increase reproducibility and expanded dynamic
20 range.

In the method, the detection reagent is an antibody labeled with a detectable reporting group, and the capture reagent is an antigen that competes with the therapeutic drug for binding to the detection reagent or the capture reagent is an antibody capable of binding the detection reagent. In certain embodiments, the capture reagents comprise a
25 first capture reagent that is an antigen that competes with the therapeutic drug for binding to the detection reagent, and a second capture reagent that is an antibody capable of binding the detection reagent. The first capture reagent is immobilized upstream in flow direction from the second capture reagent. The distance between the sample receiving zone and the first capture reagent can be varied to optimize detection of the therapeutic
30 drug.

In certain embodiments, the device further includes a third capture reagent immobilized intermediate the first and second capture reagents. In this embodiment, the

third capture reagent is an antigen that competes with the therapeutic drug for binding to the detection reagent.

In certain embodiments, the therapeutic agent is paclitaxel and the detection reagent comprises a monoclonal antibody selected from 8A10 and 3C6, or fragment or
5 derivative thereof, wherein the antibody, or fragment or derivative binds to paclitaxel.

In another embodiment, the invention provides a method for assaying a therapeutic drug in a sample, comprising:

- (a) applying a sample to a sample receiving zone of the device noted above;
and
- 10 (b) observing the amount of detection reagent bound to the one or more immobilized capture reagents.

The method can further include determining the quantity of therapeutic drug in the sample by quantitating the amount of detection reagent bound to the one or more immobilized capture reagents (e.g., detection reagent is bound to a capture reagent in a
15 first capture position, detection reagent is bound to a capture reagent in a second capture position, detection reagent is bound to capture reagents in first and second capture positions, detection reagent is bound to capture reagents in first, second, and third capture positions).

In further embodiments, TDM guided therapy methods are provided.

20 In one embodiment, the invention provides a method for monitoring the efficacy of a therapeutic treatment in a patient diagnosed with a disease or condition, comprising:

- (a) treating a patient with a therapeutic agent at a first point in time;
- (b) determining a first concentration of the therapeutic agent in the patient at a first point in time, wherein determining the concentration comprises the methods
25 described herein;
- (c) treating the patient with the therapeutic agent at a second point in time;
- (d) determining a second concentration of the therapeutic agent in the patient at a second point in time, wherein determining the concentration comprises the methods described herein; and
- 30 (e) comparing the first and second concentrations in the patient to determine the efficacy of the therapeutic treatment.

In another embodiment, the invention provides a method for PK-guided dosing of a therapeutic treatment in a patient diagnosed with a disease or condition, comprising:

- (a) treating the patient with a therapeutic agent at a first point in time using the PK information from first dosing;
- 5 (b) determining one or more pharmacokinetic parameters of the therapeutic agent in the patient at a first point in time, wherein determining the one or more pharmacokinetic parameters comprises assaying the therapeutic agent according to methods described herein;
- (c) treating the patient with the therapeutic agent at a second point in time;
- 10 (d) determining one or more pharmacokinetic parameters of the therapeutic agent in the patient at a second point in time, wherein determining the one or more pharmacokinetic parameters comprises assaying the therapeutic agent according to the methods described herein; and
- (e) comparing one or more pharmacokinetic parameters of the therapeutic
15 agent in the subject at the first point in time with the levels at the second point in time to confirm that appropriate dosing was achieved.

In certain embodiments, the pharmacokinetic parameters are one or more of time to maximum concentration (T_{max}), concentration maximum (C_{max}), area under the curve (AUC), clearance (CL), volume of distribution (V_d), apparent volume of distribution
20 during the terminal phase (V_z), apparent volume of distribution during steady state (V_{ss}) and combinations thereof.

The above methods are applicable to disease or condition treatments that benefit from therapeutic drug monitoring. Representative diseases or conditions include cancer, inflammation, hypertension, cardiovascular, and pain. Representative therapeutic agents
25 include paclitaxel, docetaxel, 7-epipaclitaxel, t-acetyl paclitaxel, 10-desacetyl-paclitaxel, 10-desacetyl-7-epipaclitaxel, 7-xylosylpaclitaxel, 10-desacetyl-7-glutarylpaclitaxel, 7-N,N-dimethylglycylpaclitaxel, and 7-L-alanylpaclitaxel.

In a further aspect, the invention provides paclitaxel antibodies. In one embodiment, the invention provides a monoclonal antibody selected from 8A10 and 3C6,
30 or fragment or derivative thereof, wherein the antibody, antibody fragment, or antibody derivative binds to paclitaxel. In certain embodiments, the monoclonal antibody, antibody fragment, or antibody derivative comprises one or more complementary

determining regions (CDRs) with at least 95% homology to a CDR contained in SEQ ID NOS: 2, 4, 6, or 8.

DESCRIPTION OF THE DRAWINGS

The foregoing aspects and many of the attendant advantages of this invention will become more readily appreciated as the same become better understood by reference to the following detailed description, when taken in conjunction with the accompanying drawings.

FIGURE 1 is a schematic illustration of pharmacokinetic-guided dosing concept and process using a representative system and method of the invention.

FIGURE 2A is an illustration of a representative work flow for a therapeutic drug monitoring device in accordance with the invention.

FIGURE 2B is an illustration of a representative test strip for lateral flow immunoassay in accordance with the invention.

FIGURE 2C is an illustration of a representative test strip for a paclitaxel lateral flow immunoassay in accordance with the invention.

FIGURE 3 is an image of representative test strips subject to assay conditions in accordance with the present invention in which the amount of a representative analyte (paclitaxel) was varied. In this assay configuration, the test strip includes three capture zones: Test Line 1 and Test Line 2 show capture using immobilized antigen (paclitaxel, in the form of immobilized BSA-paclitaxel) as the first capture material; Control Line shows capture using an immobilized antibody (goat anti-mouse antibody, GAM) as the second capture material. In this assay the detection reagent was an anti-paclitaxel antibody (8A10) labeled with colloidal gold.

FIGURE 4 is a graph illustrating direct binding of intact IgGs (8A10 and 3C6) of the invention to a BSA-paclitaxel antigen on an ELISA.

FIGURES 5A and 5B illustrate curves for 8A10 bound at lines T1 and T2 in a representative lateral flow assay of the invention carried out using a device as schematically illustrated in FIGURE 2C and as described in Example 3. FIGURE 5A illustrates the standard curve, i.e., the ratio of test line over control line (T/C) vs. paclitaxel concentration. The large difference in ratio for 8A10 at T1 versus T2 for the lower concentrations indicates a much higher sensitivity for this class of antibody when placed closer to the sample port, where concentration of analyte is likely to be higher.

FIGURE 5B illustrates the output intensity vs. position readout of scanned test strips as provided by a reader device.

FIGURES 6A and 6B illustrate curves for 3C6 bound at lines T1 and T2 in a representative lateral flow assay of the invention carried out using a device as schematically illustrated in FIGURE 2C and as described in Example 3. FIGURE 6A illustrates the standard curve, i.e., the ratio of test line over control line (T/C) vs. paclitaxel concentration. The relatively minor difference in ratio for 3C6 at T1 versus T2 for the lower concentrations indicates a relatively low improvement in sensitivity for this class of antibody would be gained for placing the antibody closer to the sample port, where concentration of analyte is likely to be higher. This class of antibody is characterized by location independent signal from the sample port. FIGURE 6B illustrates the output intensity vs. position readout of scanned test strips as provided by a reader device.

FIGURES 7A and 7B illustrate curves for combined 8A10 and 3C6 bound at lines T1 and T2 in a representative lateral flow assay of the invention carried out using a device as schematically illustrated in FIGURE 2C and as described in Example 3. FIGURE 7A illustrates the standard curve, i.e., the ratio of test line over control line (T/C) vs. paclitaxel concentration. The high sensitivity of T1 and T2 was obtained by combining the two classes of antibody (8A10 and 3C6) in the conjugate pad. This improved the dynamic range of the assay. FIGURE 7B illustrates the output intensity vs. position readout of scanned test strips as provided by reader device.

DETAILED DESCRIPTION OF THE INVENTION

Today there are many therapeutic agents either in clinical use or in development. The difficulty is not in finding drugs for treatment, but in tailoring treatments to fit patients. Personalized medicine without regard to pharmacokinetic (PK) variability will result in misclassification of some patients due to either too much drug exposure resulting in toxicity among patients that would have benefitted from lower amounts of administered drug or too little drug exposure in supposedly sensitive populations. Personalized medicine with therapeutic drug monitoring (TDM) removes the PK variability and allows for correct classification of the patients according to their biomarker.

The present invention provides a point-of-care (POC) therapeutic drug monitoring (TDM) methods, devices, and related compositions for pharmacokinetic (PK)-guided dosing of therapeutic drugs.

FIGURE 1 illustrates system characteristics, advantages and potential
5 connectivity of the device with the hospital information system and/or laboratory information system and enabling the data transfer to the physician, ultimately supporting the drug dosage decision. The methods and devices of the invention collect the patient's therapeutic drug (e.g., paclitaxel) PK data by taking the finger-prick blood samples over a period of 24 to 48 hours and the PK data is accessed by the physician who can analyze
10 the data and determine whether the therapeutic drug dose should be modified.

In one aspect, the invention provides methods and devices for immunoassay in general, and methods and devices for immunoassay of paclitaxel in particular. Sometimes a low level of paclitaxel in the biological sample is indicative of a need to increase dosage of paclitaxel to the subject, and a high level of paclitaxel in the biological
15 sample is indicative of a need to decrease the dosage of paclitaxel to the subject. The methods and devices of the invention provide information useful for making adjustments to the therapeutic regime for the subject.

The assay methods and devices provided herein are described in the context of compositions, methods, and devices for the detection and monitoring of paclitaxel.
20 However, it is appreciated that the format of the described compositions, methods, and devices are not so limited, and are readily applied more generally to monitoring any analyte of choice.

Representative Assay Methods and Devices

The present invention provides assay methods and devices for detecting or
25 quantifying analytes (e.g., paclitaxel) in a sample.

The methods and devices can be used to assay a biological sample, such as a sample obtained from a subject (patient) that has received a therapeutic agent (e.g., paclitaxel) for the treatment of a condition. The sample used in the assay is ultimately a liquid sample (e.g., blood, plasma, urine).

30 The methods of the invention are solid phase assays and therefore are suited for adaptation to other solid phase assay configurations. To exemplify the invention, the methods and devices are described using a lateral flow assay configuration. It will be

appreciated that other solid phase assays known in the art can be configured in accordance with the present methods and devices.

Lateral flow assay methods and devices can be used in accordance with the present invention. Depending on the format of the lateral flow assay method and device, the assay reagents can be disposed in certain configurations. In such an embodiment, one reagent will act as a "detection reagent" and another reagent will act as a "capture reagent." Within this format, the detection reagent is generally deposited on the conjugate pad at a location between the sample port and a location where the capture reagent is deposited. The detection reagent generally comprises a detectable label, whereas the capture reagent is immobilized in its location on the pad. Thus, during operation, a liquid sample introduced in the sample port can flow along the pad. The sample will come into contact with the detection reagent first, and then subsequently flow over the capture reagent.

A representative device for performing a lateral flow assay in accordance with the invention is illustrated in FIGURE 2A. Referring to FIGURE 2A, device 100 is a cassette that includes housing 110 having sample port 120, reading window 130, and test strip 200 (see FIGURE 2B). In operation, a liquid sample to be analyzed is introduced to the test strip through port 120 and is flowed along the test strip as indicated by the flow direction (from sample pad 210 to absorbent pad 240). The test results can be viewed by observing the test strip through reading window 130.

The test strip includes several zones and reagents for carrying out the assay. Referring to FIGURES 2A and 2B, representative test strip 200 includes sample pad 210, conjugate pad 220, membrane 230, and absorbent pad 240. Sample pad 210, conjugate pad 220, membrane 230, and absorbent pad 240 are in liquid communication such that liquid sample introduced to the sample pad flows through or across the conjugate pad and membrane to the absorbent pad. The size and configuration of the test strip components can be varied to suit the particular assay to be performed. For example, one or more of the component pads and membrane can overlap to facilitate optimal flow from one component to the next (sample pad 210 can overlap with conjugate pad 220, which may overlap with membrane 230, which may overlap with absorbent pad 240, as shown in FIGURE 2A). The nature of the test strip zones is not particularly critical and materials for these components are known in the art.

The operation of the representative device is described as follows. Sample pad 210 receives the liquid sample to be tested. Sample flows from sample pad to conjugate pad 220.

Conjugate pad 220 includes one or more detection reagents (e.g., antibodies
5 having an affinity for the analyte in the sample to be assayed and that are labeled to facilitate detection of the antibody in the assay).

In certain embodiments, a single detection reagent is deposited on the conjugate pad. In other embodiments, two or more detection reagents (e.g., two different antibodies, such as first and second antibodies having different affinities for the analyte to
10 be assayed, different K_{on} rates, and/or different K_{off} rates) are deposited on the conjugate pad. The first and second affinities are not the same. In one embodiment, the first K_{on} is greater than the second K_{on} . In another embodiment, the second K_{off} is greater than the first K_{off} . The description and specification of antibody affinity, K_{on} , and K_{off} rates described below in the context of the paclitaxel assay are applicable to the assay of
15 therapeutic agents in general. The amount of first and second antibody deposited can be varied and need not be the same.

The detection reagent(s) deposited on conjugate pad 220 are mobilized by the liquid sample and flow with the sample to membrane 230. When analyte is present in the sample, binding between the analyte and detection reagent begins to occur once the
20 sample contacts the detection reagents. Capture of the detection reagents, some of which may include bound analyte and some of which may not, occurs on membrane 230.

Membrane 230 includes at least two capture zones: a first capture zone for capturing detection reagent that does not include bound analyte (test line) (see 232 in FIGURES 2A, 2B, and 2C) and a second capture zone for capturing excess detection
25 reagent that does include bound analyte (control line) (see 238 in FIGURES 2A, 2B, and 2C). The first capture zone includes a first capture material (e.g., an immobilized antigen) that is effective for capturing the detection reagent that does not include bound analyte (i.e., free detection reagent). The second capture zone includes a second capture material (e.g., an immobilized antibody) that is effective for capturing the detection
30 reagent with or without bound analyte. The amount of detection reagent captured by the first and second capture materials, respectively, will depend on the amount of analyte present in the sample. The assay described above is a competitive assay in which the

analyte and first capture material compete for affinity binding to the detection reagent. The greater the amount of analyte present in the sample, the lesser the amount of detection reagent captured by the first capture material. Due to depletion of capture material, the lesser the amount of the analyte present in the sample, the more detection
5 reagent being capture by the first capture material and therefore less available for capture by the second capture material. The ratio of the intensity of the first and second capture lines give the best value for quantitation of the analyte.

In certain embodiments, the capture zone includes two or more first capture zones (e.g., 232 and 234 in FIGURES 2B and 2C) for capturing detection reagent that does not
10 include bound analyte. In certain embodiments, the capture zone includes two or more second capture zones (e.g., 236 and 238 in FIGURE 2B) for capturing detection reagent.

The illustrated approach of the lateral flow cassette can utilize any compatible reader with the appropriate sensitivity for detection of signal from the flow cassette and the ability to calibrate and quantify such a signal. Beneficial features of any reader can
15 include ease of use features, including touch screen, integrated RFID or integrated barcode reader, and the capacity to easily export results, such as to a memory card or USB stick. The reader preferably has pre-installed software facilitating an interface in a selection of languages. The reader preferably has a high memory capacity to facilitate storage of multiple (such as >1000) results and can save >100 distinct test method
20 protocols. The reader can contain connectivity to facilitate its integration into a larger system, such as through LAN or WLAN connectivity to LIS or cloud based data storage and management systems. Finally, multiple USB ports are desirable for additional connectivity capacities, such as to facilitate connection to external printers, and the like.

A representative reader is the Qiagen's Reader ESEQuant LFR (commercially
25 available from Qiagen, Germany), which has been demonstrated as a compatible effective reader for the inclusion of the lateral flow cassette described herein. This reader is a small, portable device with internal rechargeable battery allowing it to operate out in the field and serves the requirements of the point-of-care (POC) device. The lateral flow cassette is scanned using a confocal camera system embedded in the reader. On board
30 image analysis system is fully functional with the bar code reader of the lateral flow cassettes so that analysis method can be easily uploaded to the device.

Detection Reagents. In certain embodiments, the detection reagent is at least one antibody, antibody fragment, or antibody derivative, as described herein. The detection reagent is capable of binding the analyte in the sample (e.g., paclitaxel) and when the detection reagent does not bind paclitaxel in the sample, the detection reagent binds to the capture reagent.

The detection reagents include a moiety or label that can provide a detectable signal capable of reliable quantification. Suitable moieties include those known in the immunoassay art that provide colorimetric, fluorescent, chemiluminescent, enzymatic, or radiometric signals. Representative moieties include that those provide a detectable signal that is visual and may not require instrumentation to read (e.g., colored moieties or enzymes that generate colored moieties or enzymatic. Quantitation is typically achieved through instrumental analysis of the detectable signal. In one embodiment, the detection reagent is an antibody labeled with colloidal gold, which can be visually observed.

Gold colloids are generated from reduction of gold chloride with a monodisperse nature, which are of a controlled and uniform diameter, such as 40 nm monodisperse colloid. An antibody is conjugated with colloidal gold through passive absorption.

As noted above, in preferred embodiments, multiple (i.e., more than one type of) antibodies, antibody fragments, or antibody derivatives are used. In some embodiments, the multiple (distinct) antibodies, antibody fragments, or antibody derivatives are combined and deposited in the same location on the test strip (i.e., conjugate pad).

Two distinct anti-paclitaxel antibodies are described herein, 3C6 and 8A10. The 3C6 antibody is highly specific for paclitaxel, whereas the 8A10 antibody is less specific for paclitaxel and has a broader affinity to taxanes in general. Although, the two antibodies behave similarly in traditional competitive ELISA, it was surprisingly found that in solid phase lateral flow assays, the signal provided by 8A10 was improved by moving the first capture reagent (e.g., T1 location) closer to the sample port, as compared to 3C6, which was independent of location (T1 or T2). T1 being close to the sample application is exposed to higher concentration of the analyte, and T2 being further from the sample application is exposed to lower concentration of the analyte. This is a surprising finding that optimal placement of the capture line(s) is related to the K_{on} and K_{off} values of the antibodies used in the method. The availability of 3C6 allows for construction of multiple line devices wherein the high K_{on} antibody (e.g., 8A10) is

deposited as close to the sample origin as possible and the low Koff antibody (e.g., 3C6) is deposited along the pad to provide a second/third/fourth, etc., readout.

Accordingly, various modifications can be made to the lateral flow cassette device to facilitate or confer various detection properties. For example, to expand the dynamic
5 range of the device, multiple test lines (T1, T2, etc.) with the use of multiple affinity antibodies, the dynamic range and/or the reproducibility of the assay can be expanded. The description and specification of positioning capture reagents (T/C) on the test strip described below in the context of the representative paclitaxel assay is applicable to positioning of capture reagents in assay of the invention in general.

10 The preparation of representative detection reagents (e.g., paclitaxel antibody-colloidal gold conjugate) useful in the assays of the invention are described in Example 1.

Capture Reagents. The capture reagents serve to capture the detection reagent allowing for observation and quantitation of a detectable signal in the assay. As noted above, the assay methods and devices include first and second capture materials
15 immobilized at first and second capture zones, respectively.

In one embodiment, the capture reagent is an immobilized analyte (e.g., paclitaxel complex), which is an immobilized antigen when the detection reagent is an antibody, that captures detection reagent that does not include bound analyte. The immobilized analyte can be directly immobilized to the test strip. Alternatively, the immobilized
20 analyte can be immobilized via a linker or carrier material (e.g., analyte conjugated to a carrier protein, such as albumin). In such an embodiment, the capture reagent is the first capture material as described above.

In one embodiment, the capture reagent is an immobilized antibody that captures detection reagent that captures detection reagent with or without bound analyte. In
25 embodiments in which the detection reagent is a mouse monoclonal antibody, the capture reagent is an anti-mouse antibody (e.g., goat anti-mouse antibody, GAM antibody). In such an embodiment, the capture reagent is the second capture material as described above.

The preparation of representative capture reagents (e.g., BSA-paclitaxel) useful in
30 the assays of the invention are described in Example 1.

Alternative Assay Configurations. The lateral flow assay of the invention described herein is a solid phase immunoassay. It will be appreciated that the format of

the assay and device can be inverted from the format described above such that the detection reagent is the labeled antigen (e.g., BSA-paclitaxel with detectable label) and the capture reagent is the one or more antibody, antibody fragment, or antibody derivative (i.e., immobilized in the capture zone). In the operation of such a format, the sample
5 flows through/across the deposited labeled antigen and subsequently contacts the immobilized antibody, antibody fragment, or antibody derivative. At that point, the free analyte (e.g., paclitaxel) initially present in the sample competes with the labeled antigen for binding to the immobilized antibody, antibody fragment, or antibody derivative. As above, the device can include multiple, distinct antibodies, antibody fragments, or
10 antibody derivatives immobilized at the same or different locations. The capture reagent can be at the same or different locations. In all embodiments where the test strip has multiple locations where capture reagent is immobilized, an appropriate reader is used that can detect signal in those locations.

It is noted that the present devices, systems, compositions, and methods are
15 generally described herein in terms of a lateral flow assay. However, the general strategy for monitoring an antigen of choice, as described herein, does not need to be limited to lateral flow assay formats, but can be applied to other assay formats, such as other solid phase immunoassays (surface plasmon resonance assays), which are generally well-known in the art. Accordingly, notwithstanding description addressing lateral flow
20 format, the present disclosure also encompasses devices, systems, compositions, and methods that incorporate any known assay format. In some embodiments, the assay format includes immobilization of capture reagents, such as the antigen conjugate (e.g., paclitaxel conjugate) or antigen binding reagents (e.g., anti-paclitaxel antibodies, fragments, derivatives) on a substrate. The substrate can be any known appropriate
25 substrate for an assay format, such as nitrocellulose or glass. In some embodiments, the substrate is a nanostructure. In some embodiments, the substrate can comprise or consist of carbon nanostructures, such as carbon nanotubes, to which the capture reagents can be immobilized.

Representative Paclitaxel Assay. FIGURE 2C is an illustration of a representative
30 test strip for a paclitaxel lateral flow immunoassay in accordance with the invention. Referring to FIGURE 2C, representative test strip 200 includes sample pad 210, conjugate pad 220, membrane 230 with first capture zones 232 and 234 (T1 and T2) and

second capture zone 238 (C), and absorbent pad 240. As noted above with regard to FIGURES 2A and 2B, sample pad 210, conjugate pad 220, membrane 230, and absorbent pad 240 are in liquid communication such that liquid sample introduced to the sample pad flows through or across the conjugate pad and membrane to the absorbent pad; the size and configuration of the test strip components can be varied to suit the paclitaxel assay to be performed (e.g., one or more of the component pads and membrane can overlap to facilitate optimal flow from one component to the next, as shown in FIGURE 2A).

In one embodiment, the invention provides a method for assaying paclitaxel in a liquid sample, comprising

10 (a) applying a liquid sample comprising paclitaxel to a lateral flow assay device, the device having

(i) a sample receiving zone for receiving the liquid sample;

(ii) a detection reagent zone in liquid communication with the sample receiving zone and downstream in flow direction from the sample receiving zone,

15 wherein the detection reagent zone comprises a detection reagent deposited thereon,

wherein the detection reagent is a paclitaxel antibody, or fragment or derivative thereof that binds paclitaxel, labeled with a detectable reporting group; and

(iii) a capture zone in liquid communication with the detection reagent zone and downstream in flow direction from the detection reagent zone,

20 wherein the capture zone comprises first and second capture reagents immobilized thereon, the first capture reagent positioned upstream in flow direction from the second capture reagent,

25 wherein the first capture reagent is a paclitaxel material capable of binding the detection reagent, and

wherein the second capture reagent is an antibody capable of binding the detection reagent;

(b) allowing the sample to flow from the sample receiving zone through the detection reagent zone to provide a detection reagent with paclitaxel (e.g., combination of detection agent with bound paclitaxel, optionally free detection reagent, and optionally free paclitaxel);

(c) allowing the detection reagent with paclitaxel to flow through the capture zone,

whereby the first capture reagent binds free detection reagent to provide detection reagent bound to the first capture reagent, and

5 whereby the second capture reagent binds detection reagent with or without bound paclitaxel; and

(d) observing the amount of detection reagent bound to the first capture reagent relative to the second capture reagent.

10 In certain embodiments, the method further comprises determining the quantity of paclitaxel in the sample by quantitating the amount of detection reagent bound to the first capture reagent. Quantitating the amount of detection reagent bound to the first capture reagent includes optical density measurements, among others.

Suitable detectable reporting groups are described above. In one embodiment, the detectable reporting group is colloidal gold.

15 The paclitaxel antibody, or fragment or derivative thereof, useful in the present methods have a K_{on} greater than about 1×10^4 . Representative K_{on} values are greater than about 2×10^4 , 4×10^4 , 8×10^4 , 1×10^5 , 1×10^6 , and 1×10^7 . Preferred ranges are from about 1×10^4 to about 1×10^7 .

20 The paclitaxel antibody, or fragment or derivative thereof, useful in the present methods have a K_{off} less than about 1×10^{-3} . Representative K_{off} values are less than about less than about 1×10^{-3} , 1×10^{-4} , 1×10^{-5} , and 1×10^{-7} . Preferred K_{off} values range from about 1×10^{-3} to 1×10^{-7} .

In certain embodiments, the paclitaxel antibody, or fragment or derivative thereof, has a K_{on} from about 1×10^4 to about 1×10^6 and a K_{off} from about 1×10^{-3} to about
25 1×10^{-4} .

Methods for determining the K_{on} values and K_{off} values are described in Example 4.

30 In one embodiment, the antibody has a high K_{on} and low K_{off} (e.g., minimum K_{on} is 2.0×10^5 and maximum K_{off} is 1.0×10^{-3}). In this embodiment, the capture line is placed at 0.0 to 0.4 T/C. For this class, monoclonal antibody engineering would focus on keeping K_{off} constant while increasing K_{on} as much as possible. The greater the K_{on} the better is the antibody detection.

In another embodiment, the antibody has a low K_{on} and high K_{off} (e.g., minimum K_{on} is 2.0×10^4 and maximum K_{off} is 2.0×10^{-4}). In this embodiment, the capture line is placed at 0.2-1.0 T/C. For this class, monoclonal antibody engineering would focus on keeping K_{on} constant while decreasing K_{off} as much as possible. The
5 lower the off rate the better is the antibody for detection.

In certain embodiments, the paclitaxel antibody is 3C6. In other embodiments, the paclitaxel antibody is 8A10. In further embodiments, combinations of paclitaxel antibodies can be used (e.g., 3C6 and 8A10). These antibodies are described in detail below.

10 In the assay, the first capture zone includes an immobilized paclitaxel material that serves as a paclitaxel antigen that competes with paclitaxel for binding to the detection reagent. The first capture zone captures detection reagent that does not include bound paclitaxel (i.e., free detection reagent). In certain embodiments, the paclitaxel material is a paclitaxel protein conjugate. Suitable protein conjugates include serum
15 albumin conjugates, such as BSA-paclitaxel.

In the assay, the second capture zone includes an immobilized antibody capable of binding the detection reagent. In certain embodiments, the antibody is a goat anti-mouse antibody.

As noted above, the paclitaxel detection sensitivity in the assay can be optimized
20 by varying the distance between the point at which the sample is introduced to the lateral flow device (e.g., sample receiving zone) and the first capture reagent. In certain embodiments, the distance between the sample receiving zone and the first capture reagent is minimized to optimize paclitaxel detection sensitivity. In certain embodiments, the distance is less than 20 mm, less than 10 mm, less than 5 mm, less than 3 mm, less
25 than 2 mm, or less than 1 mm. In certain embodiments, the distance is from 20 to 1 mm, 10 to 1 mm, 5 to 1 mm, 3 to 1 mm, or 2 to 1 mm.

The optimization can be described as relative positioning of T (test line) and C (control line): T/C, which is defined as the distance from origin to T/distance from origin to C ratio, where the origin is defined as the upstream edge of the capture zone (upstream
30 edge of membrane 230 in FIGURES 2A-2C). T/C can be greater than about 0.0 (i.e., first capture reagent is located at upstream edge of capture zone), or about 0.01, about 0.02, about 0.04, about 0.08, about 0.10, about 0.20, about 0.40, about 0.80, or less than about

1.0 (i.e., first capture reagent is located at the downstream edge of the capture zone, with second capture reagent located intermediate the first capture reagent and the downstream edge of the capture zone). Preferably, T/C is from about 0.2 to about 0.7.

In certain embodiments, the ratio of the first distance to the second distance is
5 from about 0.0 to about 0.40. In other embodiments, the ratio of the first distance to the second distance is from about 0.20 to about 1.0.

In certain embodiments, the amount of excess detection reagent that is bound to the second capture reagent is observed and measured. In certain embodiments, determining the quantity of paclitaxel in the sample is determined by relating the final
10 capture reagent (test line) to the second capture reagent (control line).

As noted above, representative assay of the invention include more than one first capture reagents in more than one first capture zone. In certain of these embodiments, the method further includes a third capture zone (see T2, 234 in FIGURE 2C) intermediate the first (T1, 232 in FIGURE 2C) and second (C, 238 in FIGURE 2C) capture zones,
15 wherein the third capture zone comprises a paclitaxel material capable of binding the detection reagent. The paclitaxel material in the first and third zones can be the same or different. In certain of these embodiments, the quantity of paclitaxel in the sample is determined by quantitating the amount of detection reagent bound to the first and second capture reagents. Quantitating the amount of detection reagent bound to the first and
20 second capture reagents can include optical density measurement.

In certain embodiments of the method, the lateral flow device further comprises an absorbent zone in liquid communication with the capture reagent zone and downstream in flow direction from the capture reagent zone.

FIGURE 3 is an image of representative test strips subject to assay conditions in
25 which the amount of a representative analyte (paclitaxel) was varied. In these assay configurations, membrane 230 includes three capture zones: Test Line 1 and Test Line 2 show capture using immobilized antigen (paclitaxel, in the form of immobilized BSA-paclitaxel) as the first capture material; Control Line shows capture using an immobilized antibody (goat anti-mouse antibody) as the second capture material. In this assay the
30 detection reagent was an anti-paclitaxel antibody (8A10) labeled with colloidal gold.

It is noted that the methods and devices of the invention are useful for detecting levels of paclitaxel, including any formulation of paclitaxel or paclitaxel prodrug, in a

biological sample. The formulations of paclitaxel include any known agents to facilitate delivery of paclitaxel, such as polymeric nanoparticles, lipid-based nanoparticle formulations, polymer conjugates, inorganic nanoparticles, carbon nanotubes, nanocrystals, and cyclodextrin nanoparticles.

5 The description of a representative lateral flow immunoassay in accordance with the methods and devices of the invention is described in Example 3.

Paclitaxel Antibodies

In another aspect, the invention provides antibodies (e.g., monoclonal antibodies or mAbs) that bind paclitaxel. The mAbs, referred to as 8A10 and 3C6, were purified
10 from an antibody-rich harvested medium using MabSelect (GE Healthcare, Pittsburgh, PA). The mAbs were selected based on their binding to BSA-paclitaxel, which was prepared as described in J-G Leu et al., Cancer Res. (1993) 53:1388-1391.

In one aspect, the invention provides a monoclonal antibody selected from 8A10, 3C6, and fragments or derivatives thereof, wherein the antibody, antibody fragment, or
15 antibody derivative binds paclitaxel.

As used herein, the term "antibody" encompasses antibodies and antibody fragments thereof, derived from any antibody-producing mammal (e.g., mouse, rat, rabbit, camelid, and primate, including human) or synthetically or recombinantly produced, that specifically binds to a target of interest (e.g., paclitaxel) or portions
20 thereof. Exemplary antibodies include polyclonal, monoclonal, and recombinant antibodies; multispecific antibodies (e.g., bispecific antibodies); humanized antibodies; murine antibodies; chimeric, mouse-human, mouse-primate, primate-human monoclonal antibodies; and anti-idiotypic antibodies, and may be any intact molecule or fragment thereof, such as an antigen binding fragment. As described herein, monoclonal
25 antibodies are preferable because they provide for increased specificity in binding of the antigen of choice, such as a therapeutic drug (e.g., paclitaxel).

As used herein, the term "antigen binding fragment" refers to the antigen binding or variable region from or related to a full-length antibody. Illustrative examples of antibody fragments include Fab, Fab', F(ab)₂, F(ab')₂, and Fv fragments, scFv fragments,
30 diabodies, nanobodies, linear antibodies, single-chain antibody molecules, and multispecific antibodies formed from antibody fragments.

As used herein, a "single-chain Fv" or "scFv" antibody fragment comprises the V_H and V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains, which enables the scFv to form the desired structure
5 for antigen binding.

As used herein, a "chimeric antibody" is a recombinant protein that contains the variable domains and complementarity-determining regions derived from a non-human species (e.g., rodent) antibody, while the remainder of the antibody molecule is derived from a human antibody.

10 As used herein, a "humanized antibody" is a chimeric antibody that comprises a minimal sequence that conforms to specific complementarity-determining regions derived from non-human immunoglobulin that is transplanted into a human antibody framework. Humanized antibodies are typically recombinant proteins in which only the antibody complementarity-determining regions are of non-human origin.

15 As used herein, the term "derivative" indicates that the antibody or antibody fragment has been produced from a reference antibody. For example, sometimes it is desirable to modify or enhance binding characteristics of a reference antibody. Thus, the antibody can be subjected to various modifications, including mutations subjected to the encoding DNA, to alter binding properties. The resulting antibody with altered properties
20 is then referred to as a "derivative" of the reference antibody. For example, an antibody derivative can be an antibody that contains mutations resulting from affinity maturation processes that were applied to the reference antibody (or the nucleic acids encoding the reference antibody). Such mutations can result in antibodies with altered (e.g., improved) binding affinity, selectivity, and the like.

25 In some embodiments, the antibody, antibody fragment, or antibody derivative comprises one or more complementary determining regions (CDRs) with at least 95% homology to a CDR contained in SEQ ID NOS: 2, 4, 6, or 8. SEQ ID NOS: 2 and 4, described in more detail below, are the amino acid sequences of the variable region of the 8A10 light and heavy chains, respectively. SEQ ID NOS: 6 and 8, also described in more
30 detail below, are the amino acid sequences of the variable region of the 3C6 light and heavy chains, respectively. Each variable region contains three complementary determining regions (CDRs), which are indicated below. In some embodiments, the

antibody, antibody fragment, or antibody derivative comprises one, two, or all three of complementary determining regions (CDRs) with at least 95% homology to one, two, or all three of the CDRs contained in any one SEQ ID NOS: 2, 4, 6, or 8. In some embodiments, the antibody, antibody fragment, or antibody derivative has a heavy and light chain, which comprise one, two, three, four, five, or all six, of the CDR regions provided by the 8A10 variable light and heavy regions (in SEQ ID NOS: 2 and 4). In some embodiments, the antibody, antibody fragment, or antibody derivative has a heavy and light chain, which comprise one, two, three, four, five, or all six, of the CDR regions provided by the 3C6 variable light and heavy regions (in SEQ ID NOS: 6 and 8).

In some embodiments, the one or more CDRs have a sequence identity with at least 95%, 96%, 97%, 98%, 99%, or 100% homology to one or more CDRs in any one SEQ ID NOS: 2, 4, 6, or 8. As used herein, the term "percent homology" or "percent homologous," when used in connection with a polypeptide used in the practice of the present invention, is defined as the percentage of amino acid residues in a polypeptide sequence that are homologous with the amino acid sequence of a specified polypeptide after aligning the sequences to achieve the maximum percent homology. When making the comparison, no gaps are introduced into the biomarker sequences in order to achieve the best alignment. Amino acid sequence homology can be determined, for example, in the following manner. The amino acid sequence of a polypeptide is used to search a protein sequence database, such as the GenBank database using the BLASTP program. The program is used in the ungapped mode. Default filtering is used to remove sequence homologies due to regions of low complexity. The default parameters of BLASTP are utilized.

The production, processing, purification, characterization, and optimization of representative paclitaxel antibodies (3C6 and 8A10) useful in the assay methods of the invention are described in Example 2. The antibodies of the invention were generated by immunizing mice with either Taxol[®] or baccatin conjugated to KLH. The antibodies are summarized as: 3C6 anti-Taxol[®] IgG_{2a,k}, 3H5 anti-baccatin III IgG₁, 8A10 anti-taxane IgG_{2a}. Cross-reactivity profile of 3C6, 3H5, and 8A10 antibodies are shown by their IC₅₀ values towards various taxanes in Table 1.

Table 1. Paclitaxel Antibody Cross-Reactivity.

Taxane	3C6	3H5	8A10
paclitaxel (Taxol®)	10 nM	>316 nM	7 nM
10-deacetyltaxol	15 nM	>333 nM	10 nM
7-epi-10-deacetyltaxol	25 nM	>333 nM	15 nM
7-xylosyl-10-deacetyltaxol	30 nM	>286 nM	17 nM
7-epi-taxol	80 nM	>316 nM	50 nM
cephalomannine	220 nM	>325 nM	8 nM
baccatin III	>511 nM	10 nM	12 nM
baccatin V	>460 nM	10 nM	10 nM
10-deacetylbaccatin III	>551 nM	230 nM	21 nM
7-epi-10-deacetylbaccatin III	>469 nM	150 nM	27 nM
Taxotere®	>318 nM	>318 nM	10 nM
2-debenzoyl-2-(p-trifluoromethylbenzoyl)taxol	>293 nM	>293 nM	>293 nM
20-acetoxy-4-deacetyl-5-epi-20,O-secotaxol	>310 nM	>310 nM	>293 nM

In order to effectively utilize antibodies in diagnostic applications to achieve a
 5 dynamic detection range, the two key factors that need to be successfully optimized are
 the antibodies' specificity and affinity toward the targeted antigen. The 3C6 and 8A10
 monoclonal antibodies have high specificity and affinity to paclitaxel, which indicates
 that these specifically bind to a single epitope of the targeted paclitaxel antigen in a
 highly homogeneous manner. This specificity also helps eliminate cross reactivity
 10 problems in a detection assay. In conjunction with specificity, monoclonal antibodies can
 also have optimized and improved affinities toward the antigen in order to achieve a
 dynamic detection range in diagnostic tests, which can be achieved by the process of
 affinity maturation. Additionally, the mAb can be selected for rapid K_{on} to be compatible
 with the rapid flow assay.

As illustrated in FIGURE 4, both the 3C6 and 8A10 monoclonal antibodies have apparent K_d values of about 10 nM with a sensitivity limit of about 100 – 200 ng/mL.

The following examples are provided for the purpose of illustrating, not limiting, the invention.

5

EXAMPLE 1

Assay Reagents

In this example, the preparation of representative detection reagents and capture reagents useful in the assay methods and devices of the invention are described.

Detection reagents: antibody-colloidal gold conjugates. Briefly, antibodies (see
10 Example 2) were diluted to 1 mg/mL in 0.5X PBS and the following steps were taken: (1) shake or swirl gold to resuspend any settled gold then place 0.5 mL Naked Gold sol into 10 clean individual test tubes; (2) each tube was labeled with the pH value (or 1 through 10) from the provided pH charts; (3) pH charts were used to add varying amounts of buffer in microliters to each test tube, and shake to mix; (4) place each tube
15 on a low speed vortexer and add the antibody solution, and mix thoroughly (about 2 to 3 seconds), for the 20 nm gold, 14 μ L of a 2 mg/mL solution of antibody or protein is optimal; (5) a deepening purple color and/or black precipitate on some tubes indicate that the antibody or protein is below its isoelectric point, leading to cross-linking of individual gold solutions (cross-linked solutions cannot be used in immunological assays are
20 discarded; deep purple solutions are mostly inactive as well; only tubes with a slight purple color or no change in color are useful for immunological assays; (6) allow the reaction to continue for a total of 30 minutes; and (7) stop the reaction by the addition of 50 μ L of blocking solution.

Capture reagents: paclitaxel-albumin conjugates. Paclitaxel-albumin conjugates
25 (e.g., BSA-paclitaxel) were prepared as described in J-G Leu et al., Cancer Res. (1993) 53:1388-1391 was generally followed. For synthesis of 2'-hemisuccinyltaxol, the crystals are the starting material for conjugation of the taxol to carrier protein. Taxol (20 mg) and succinic anhydride (36 mg) were dried for 4 h at room temperature under vacuum over P_2O_5 and dissolved in 480 μ L of dry pyridine. After standing at room temperature
30 overnight, the pyridine was removed under vacuum and the residue was washed once with 2 mL of distilled water. Acetone (1 mL) was added, and distilled water was added dropwise to the acetone solution until a few crystals (2'-hemisuccinyltaxol) appeared.

The mixture was kept at 4°C for 3 h and the crystals were recovered by filtration and dried under vacuum. The product yield was 70%.

The dialysis step removes any unconjugated taxol. 2'-Hemisuccinyltaxol (10 mg) was dissolved in 1 mL DMSO and 300 µL acetonitrile, and 50 µL (35 mg; 0.19 mmol) of n-tributylamine was then added. The mixture was cooled to 4°C in an ice bath, and 25 µL (25 mg; 0.18 mmol) of isobutylchloroformate was added to the mixture which was kept on ice for 30 min. The solution was added dropwise into a BSA solution [25 mg, (3.73 x 10⁻⁴ mmol) in 3 mL of distilled water, pH 9.5, at 4°C]. The pH was adjusted immediately to 7.5 with 1N HCl and the mixture was kept at 4°C overnight and dialyzed against PBS at 4°C overnight.

In one exemplary embodiment, a lateral flow system was evaluated. A 0.5 mg/mL BSA-paclitaxel (Test line) and 0.5 mg/mL goat anti-mouse antibody (Control line) were striped onto the system's membrane. Paclitaxel antibody-colloidal gold conjugate was flowed through the system. The antibody-colloidal gold conjugate bound to BSA-paclitaxel immobilized on the membrane and generated a strong signal. The signal was specific to paclitaxel because a decreased signal was observed when paclitaxel was added to the spiked into the samples.

EXAMPLE 2

Paclitaxel Antibodies

In this example, the production, processing, purification, characterization, and optimization of representative paclitaxel antibodies useful in the methods and devices of the invention are described.

Antibody production and processing. Cells were grown in CCM1 (Hyclone) with 5-10% FBS and 1X Pen/Strep. Cells were split (1:4) once they reached densities of > 1 x 10⁶ cells/mL. Cells were then frozen and stored in 2 separate liquid nitrogen cryogenic tanks as backups. Cells were cultured in roller bottles until a density of 1 x 10⁶ cells/mL was reached. At that point, cultures were no longer fed and cell viability was monitored daily. Once cell viability decreased to <50%, cells were removed and the antibody-rich medium was harvested.

Affinity purification of antibody. Diafiltration was performed using PBS, pH 7.4 and the harvested antibody was concentrated 10-fold using a 50 Kd cut-off membrane. MabSelect (GE Healthcare, Pittsburgh, PA) was used for affinity purification. The

hydrophilic, high-flow agarose bead, optimized for both capacity and throughput, and the oriented coupling of the rProtein A ligand, deliver a product pool that is high in purity and yield.

Purification run program.

5 Column: XK 16/40 (16 mm i.d., 20 cm bed height).

Sample: Clarified hybridoma spent media.

Loading Buffer A: 20 mM NaH_2PO_4 , 0.15 M NaCl, pH 7.2.

Elution Buffer B: 0.1 M Na_3 -citrate, pH 3.6.

10 Antibody affinity. A plot of the results of direct binding of intact IgGs (8A10 and 3C6) to BSA-paclitaxel antigen is shown in FIGURE 4. 50 μL of BSA-paclitaxel antigen at a concentration of 3.5 $\mu\text{g}/\text{mL}$ in 1x PBS was bound to a plate and incubated at 4°C overnight. The plate was washed four times with PBS/0.05% Tween 20. The plate was blocked for 2 hours with 1% BSA in PBS/0.05% Tween 20 at room temperature. The
15 plate was washed four times with PBS/0.05% Tween 20. 50 μL of antibody sample (8A10 and 3C6) was bound (start at 300nM and titer down by 3s). The plate was washed four times with PBS/0.05% Tween 20. 50 μL of goat anti mIgG horseradish peroxidase (1:5000 dilution) was bound and then 50 μL of substrate solution TMB was added and the color allowed to develop. The reaction was stopped with 50 μL 1M HCl and the optical
20 density was read at 450nm. The results of direct binding of intact IgGs (8A10 and 3C6) to BSA-paclitaxel antigen are shown in FIGURE 4.

Antibody optimization. Antibody optimization was performed that included affinity maturation. This approach involves: (1) characterizing the antibodies by sequencing antibodies produced from hybridoma cell lines, (2) constructing antibody
25 libraries focused on CDR regions; (3) screening for beneficial mutations, and (4) combining beneficial mutations.

Step 1: Cloning and expression of anti-paclitaxel mAb as a Fab

A. Sequencing of variable regions of antibodies (8A10 and 3C6) produced from hybridoma cells.

5

i. Total RNA extraction and mRNA denaturation

Total RNA was extracted from the two hybridoma cells (8A10 and 3C6) using QIAGEN RNeasy Mini Kit. The mRNA mix (described below) was incubated at 72°C for 3 min, then cooled down to 42°C for 2 min. After cooling, the tube was spun briefly
10 for 10 seconds at 14,000 X g to collect the content at the bottom.

mRNA Mix

	RNA template (0.2 – 0.4 ug)	1-2.75 uL
	5'-RAGE primer A	1 uL
15	RNase-free water	to 3.75 uL

ii. cDNA synthesis and 5' RAGE reaction

cDNA synthesis and 5' RAGE reaction were performed as shown below.

20	<u>cDNA synthesis</u>		<u>5' RAGE reaction</u>	
	SMARTer II A oligo	1 uL	2X PCR mix	10 uL
	5X First-strand buffer	2 uL	cDNA	1 uL
	DTT (20 mM)	1 uL	10X Universal Primer mix	2 uL
	dNTP mix (10 mM)	1 uL	Reverse primer	1 uL
25	RNase inhibitor	0.25 uL	RNase-free water	6 uL
	SMARTScribe RT	1 uL		
	mRNA mix after dent	3.75 uL		
	Total	10 uL		20 uL

iii. Analysis of PCR reaction by agarose gel electrophoresis

Products of PCR amplification were electrophoresed on an agarose gel to confirm the presence of amplicons corresponding the light and heavy chain variable regions of the 8A10 and 3C6 mAbs.

5

iv. Cloning, sequencing and CDR analysis

The PCR positive bands were cloned into a vector and sequenced. Antibody sequence analysis identified one light chain and one heavy chain for 8A10 and 3C6. Initially for 3C6, only one light chain and no heavy chain (aberrant sequence) were identified. Therefore, a specific primer, designed according to the sequence obtained from the N-terminal sequencing results, was utilized to re-PCR the heavy chain. Consequently, identification of a heavy chain for 3C6 was accomplished.

8A10 Hybridoma

The 8A10 hybridoma sequencing results are set forth below.

15 The 8A10 Variable Light Chain nucleic acid sequence is SEQ ID NO: 1:

GAC ATT GTG ATG ACC CAG TCT CAA AAA TTC ATG TCC ATA ACA
CTA GGA GAG AGG GTC AGC ATC ACC TGC AAG CCC AGT CAG AAT GTG
GGT TCT GCT GTA ACC TGG TGG CAA CAG AAA CCA GGA CAA TCT CCT
AAA CTA CTG ATT TAC TCA GCT TCC AAT CGG TAT ACT GGA GTC CCT
20 GAT CGC TTC ACA GGC AGT GGA TCT GGG ACA GAT TTC ACT CTC ACC
ATT AGT AAT GTG CAG TCT GAA GAC CTG GCA GAT TAT TTC TGT CAA
CAA TAT AGC AGC TAT CCG TAC ACG TTC GGA GGG GGG ACC AAG CTG
GAA ATA AAA CG (SEQ ID NO: 1).

25 The sequences encoding the CDR regions are underlined.

The corresponding 8A10 Variable Light Chain amino acid sequence is SEQ ID NO: 2:

DIVMTQSQKFMSITLGERVSITCKKPSQNVGSAVTWWQQKPGQSPKLLIYS
ASNRYTGVDPDRFTGSGSGTDFTLTISNVQSEDLADYFCQQYSSYPYTFGGTKLEIK
30 R (SEQ ID NO: 2).

The three CDR regions are underlined (i.e., CDRL1 is KPSQNVGSAVT, CDRL2 is SASNRYT, CDRL3 is QQYSSYPYT).

The 8A10 Variable Heavy Chain nucleic acid sequence is SEQ ID NO: 3:

GAG GTC CAG CTG CAA CAA TCT GGA CCT GAA CTG GTG AAG CCT
GGG GCT TCA GTG AAG ATT TCC TGT AAG GCT TCT GGA TAC ACG TTC
ACT GAC TCC ACC ATG AAC TGG GTG AAG CAG AGC CAT GGA AAG AGC
5 CTT GAG TGG ATT GGA GAG ATT GAT CCT AAC AAT GGT GGT ACT AAC
TAC AAT CAG AAG TTC AAG GGC AAG GCC ACA TTG ACT GTA GAC AAG
TCC TCC AGC ACA GCC TAT ATG GAG CTC CGC AGC CTG ACA TCT GAG
GAC TCT GCA GTC TAT TAC TGT GCA AGA GGG GTC TGG GGC CAA GGC
ACC ACT CTC ACA GTC TCC TCA (SEQ ID NO: 3).

10 The sequences encoding the CDR regions are underlined.

The corresponding 8A10 Variable Heavy Chain amino acid sequence is SEQ ID
NO: 4:

EVQLQQSGPELVKPGASVKISCKASGYTFTDSTMNWVKQSHGKSLEWIG
EIDPNNGGTNYNQKFKGKATLTVDKSSSTAYMELRSLTSEDSAVYYCARGVWG
15 QGTTTLTVSS (SEQ ID NO: 4).

The three CDR regions are underlined (i.e., CDRH1 is GYTFTDSTMN, CDRH2
is EIDPNNGGTNYNQKFKG, and CDRH3 is GV).

3C6 Hybridoma

20 The 3C6 hybridoma sequencing results are set forth below.

The 3C6 Variable Light Chain nucleic acid sequence is SEQ ID NO: 5:

GAT GTT GTG ATG ACC CAA ACT CCA CTC TCC CTG CCT GTC AGT
CTG GGA GAT CAA GCC TCC ATC TCT TGC AGA TCT CGT CAG AGC CTT
GTA CAC AGT AAT GGA AAC ACC TAT TTA CAT TGG TAC CTG CAG AAG
25 CCA GGC CAG TCT CCA AAG CTC CTG ATC TAC AAA GTT TCC AAC CGA
TTT TCT GGG GTC CCA GAC AGG TTC AGT GGT AGT GGA TCA GGG ACA
GAA TTC ACA CTC GAG ATC AGC AGA GTG GAG GCT GAG GAT CTG GGA
GTT TAT TTC TGC TCT CAA AGT ACA CAT GTT CCT CCG ACG TTC GGT GGA
GGC ACC AAG CTG GAA ATC AAA C (SEQ ID NO: 5).

30 The sequences encoding the CDR regions are underlined.

The corresponding 3C6 Variable Light Chain amino acid sequence is SEQ ID
NO: 6:

DVVMTQTPLSLPVS LGDQASISCRRSRQSLVHSNGNTYLHWYLQKPGQSPK
LLIYKVSNRFSGVPDRFSGSGSGTEFTLEISRVEAEDLG VYFCSQSTHVPPTFGGGT
KLEIK (SEQ ID NO: 6).

5 The three CDR regions are underlined (i.e., CDRL1 is RSRQSLVHSNGNTYLH,
CDRL2 is KVSNRFS, and CDRL3 is SQSTHVPPT).

The 3C6 Variable Heavy Chain nucleic acid sequence is SEQ ID NO: 7:

GAG GTG CAG CTT CAG GAG TCG GGA CCT AGT CTC GTG AAA CCT
TCT CAG ACT CTG TCC CTC ACC TGT TCT GTC ACT GGC GAC TCC ATC ACC
AGT GGT TAC TGG AAC TGG ATC CGG AAA TTC CCA GGG AAT AGA CTT
10 GAG TAC ATG GGG TAC ATA AGC TAC AGT GGT AGC ACT TAC TAC AAT
CCG TCT CTC AAA AGT CGA ATC TCC ATC ACT CGA GAC ACA TCC AAG
AAC CAG TAC TAC CTA CAT TTG ACT TCT GTG ACT ACT GAG GAC ACA
GCC ACA TAT TAC TGT GCC CAA GGG GAT GGC GCC TAC TGG GGC CAA
GGC ACC ACT CTC ACA GTC TCC TCA (SEQ ID NO: 7).

15 The sequences encoding the CDR regions are underlined.

The corresponding 3C6 Variable Heavy Chain amino acid sequence is SEQ ID
NO: 8:

EVQLQESGPSLVKPSQTLSTCSVTGDSITSGYWNWIRKPGNRLEYMGYIS
YSGSTYYNPSLKSSRISITRDTSKNQYYLHLTSVTTEDTATYYCAQGDGAYWGQG
20 TTLTVSS (SEQ ID NO: 8).

The three CDR regions are underlined (i.e., CDRH1 is GDSITSGYWN, CDRH2
is YISYSGSTYYNPSLKS, and CDRH3 is GDGAY).

Table 2. Comparison of N-terminal sequencing results with hybridoma sequencing results for 8A10 and 3C6 mAbs.

8A10				3C6			
Residue	N-terminal sequencing data	Hybridoma sequencing data		Residue	N-terminal sequencing data	Hybridoma sequencing data	
	Calls	Variable Light Chain	Variable Heavy Chain		Calls	Variable Light Chain	Variable Heavy Chain
1	D, Q, F, (S, H, M)	D		1	D, E	D	E
2	H, I, L, (V)	I		2	V	V	V
3	V, S, Q, (T, K)	V		3	Q, (V)	V	Q
4	M, E, N, F	M	E	4	M, L	M	L
5	T, Q, V, (R)	T	V	5	Q, T	T	Q
6	Q, Q, N, Y	Q	Q	6	Q, E	Q	E
7	S, L, F, E	S	L	7	T, S	T	S
8	Q, Q, Y, D	Q	Q	8	G, P	P	G
9	K, F, N, Q	K	Q	9	L, P	L	P
10	S, D, F, L	F	S	10	S	S	S
11	M, N, L, G	M	G	11	L	L	L
12	S, P, L	S	P	12	V, P	P	V
13	I, T, E, L, (V)	I	E	13	K, V	V	K
14	(T, G)	T	L	14	S, P	S	P
15	V, K, I	L	V	15	S, L	L	S
16	K, P, G	G	K	16	Q, G	G	Q
17	E, P	E	P	17	Q, T	Q	T
18	R, (H, G)	R	G	18	Q, L	Q	L
19	V, A, (L)	V	A	19	A, S	A	S
20	S, S, H, (R, L)	S	S	20	L, S	S	L
21	I	I	V	21	T, I	I	T
22	K, T, R	T	K	22	S, (R)	S	C
23	I	C	I	23	(S)	S	S
24	S, K	K	S	24	R, V	R	V
25	P	P	C	25	T	S	T
26	K, (A), (S)	S	K	26	G, (R)	R	G
27	Q, (A)	Q	A	27	D, Q	Q	D
28	N	N	S	28	(S)	S	S
29	V, (G)	V	G	29	I, (L)	L	I
30	(Y), (T), (G)	G	Y	30	(T, V)	V	T

*Cysteine sequence cannot be determined by N-terminal sequencing
~Highlighted regions show residues of discrepancy between n-terminal seq. results and hybridoma seq. results.

5

B. Cloning of variable regions of mAb into M13 engineering vector.

The DNA coding for the VL, C kappa, and VH regions of the mAbs (i.e., 8A10 and 3C6) were amplified with sequence specific primers using PCR. The resulting PCR product was gel-purified and restriction digested for specific sites within the M13-based phage vector under the control of lacZ promoter. The double-stranded DNA coding for the VL, C kappa, and VH regions of 8A10 and 3C6 mAbs were ligated into the phage-based vector containing the gene for the constant region of the CH1 region of a human IgG1. The DNA was transformed into *E. coli* and the resulting plaques containing phage was examined in detail. Plaques were picked at random, their DNA isolated and the sequence determined. Clone 8A10_1 was determined to have the identical sequence for the VL, C kappa, and VH regions as that of mAb 8A10 (not shown). The cloning process for 3C6 can be performed according to the same protocol.

C. Demonstration of paclitaxel binding activities of the Fabs generated from the M13 engineering vector.

A Clone 8A10_1 phage-infected culture of XL1-Blue *E.coli* (gram-negative) was grown while being induced with IPTG, was harvested via centrifugation and the periplasmic contents were released by osmotic shock (periprep). The released Fab products were isolated from the culture. The Fab concentration was quantified by ELISA and antigen specific binding of the Fab was done using paclitaxel ELISA together with intact IgGs (not shown). The K_d values (approximately 10 nM for both antibodies) of the two intact IgGs were comparable to the ones previously reported (see FIGURE 3). The K_d value of Fab 8A10 was determined to be 10 nM as well, which is the same as that of intact IgG 8A10. These results suggest that the correct variable region sequence of the parental antibody 8A10 exists in the present Fab 8A10 and is correctly folded and expressed in the Fab format from the M13 engineering vector.

The Fab expressed sufficiently well in the periplasmic prep to demonstrate concentration-dependent binding to BSA-paclitaxel conjugate coated wells.

EXAMPLE 3

Representative Solid Phase Competitive Assay

In this example, a representative assay demonstrating the efficacy of a solid-phase competitive assay is described. The assay demonstrates the utility of using the anti-paclitaxel antibodies described herein in such a detection format to provide informative signals for the presence of paclitaxel in a sample. The results demonstrate that variable placement of the antibodies can enhance assay performance.

Paclitaxel lateral flow system. 1.2 mg/mL BSA-Pac (test lines, T) and 0.2 mg/ml of goat-anti-mouse antibody (control line, C) were striped onto a membrane card (high-flow plus HF180 membrane card, Millipore). Anti-paclitaxel antibody-colloidal gold conjugate was absorbed into and dried onto a conjugate pad (glass fiber pad, Millipore). Fetal bovine serum (FBS) spiked with paclitaxel (10 μ L), chased by 80 μ L of PBS Tween, was flowed in the assay.

Tandem Antibody Assay. The antibody-gold conjugates are reconstituted using distilled water and are then added to each other to make the appropriate concentrations. This tandem antibody solution is applied and then dried onto the assay conjugate pads.

Reader Output: Intensity vs Position. Readout of the results of scanning the test strips. The strips were read using Qiagen reader (Qiagen, Germany).

Paclitaxel Standard Curve. Standard curves of ratio of test line over control line vs. paclitaxel concentration were generated.

FIGURES 5A and 5B illustrate curves for 8A10 bound at lines T1 and T2. FIGURE 5A illustrates the standard curve, i.e., the ratio of test line over control line (T/C) vs. paclitaxel concentration. The large difference in ratio for 8A10 at T1 versus T2 for the lower concentrations indicates a much higher sensitivity for the antibody when placed closer to the sample port, where concentration of analyte is likely to be higher. FIGURE 5B illustrates the output intensity vs. position readout of scanned test strips as provided by a reader device.

FIGURES 6A and 6B illustrate curves for 3C6 bound at lines T1 and T2. FIGURE 6A illustrates the standard curve, i.e., the ratio of test line over control line (T/C) vs. paclitaxel concentration. The relatively minor difference in ratio for 3C6 at T1 versus T2 for the lower concentrations indicates a relatively low improvement in sensitivity would be gained for placing the antibody closer to the sample port, where concentration of analyte is likely to be higher. However, improvement in signal intensity relative to at T2 was observed. FIGURE 6B illustrates the output intensity vs. position readout of scanned test strips as provided by a reader device.

FIGURES 7A and 7B illustrate curves for combined 8A10 and 3C6 bound at lines T1 and T2. FIGURE 7A illustrates the standard curve, i.e., the ratio of test line over control line (T/C) vs. paclitaxel concentration. The assay was made more robust by combining the high sensitivity of 8A10 close to the sample port and the higher sensitivity of 3C6 at T2 that is location independent. FIGURE 7B illustrates the output intensity vs. position readout of scanned test strips as provided by reader device.

In the above analyses (and in FIGURES 5-7), the measurement of position of T1, T2, and C (Pos [mm]) in FIGURES 5B, 6B, and 7B was made from the downstream end in flow direction (e.g., sample introduced at 55 mm point, T2 at about 45 mm, T1 at about 40 mm, and C at about 35 mm) of the test strip.

EXAMPLE 4

Method for Determining Antibody Binding Properties

In this example, a method for determining antibody properties is described. The methods were carried out by Precision Antibody, Inc. (Columbia, MD) using a Biacore

Assay. This example also shows how the LFA assay of the invention can be adapted to other solid phase assays, such as Surface Plasmon Resonance (SPR) assays.

Binding experiments were performed on a Biacore 3000 instrument (GE, Pittsburgh, PA) at 25°C. Approximately 13,000 RU of anti-BSA ab (Life Technologies, A11133, lot 1637270) was directly immobilized on flow cell 2 of CM5 chip by amine coupling (EDC/NHS). 110-120 RU of BSA-paclitaxel (BSA-Ag) was captured. Flow cell 1 was treated the same way but without the ligand and used as a blank surface for reference subtraction. The unoccupied sites were blocked with 1M ethanol amine. Analytes Ab1 (8A10) and Ab2 (3C6) were flowed over the chip at variable concentrations. Binding of antigen to the antibodies was monitored in real time to obtain on (k_a) and off (k_d) rates. The equilibrium constant (K_D) was calculated from the observed k_a and k_d .

Full kinetic analysis was performed using analyte concentrations as indicated with 2-fold serial dilutions. The starting concentration was 200 nM, followed by 100, 50, 25, 12.5, and 0 nM. The 100 nM concentration was run in duplicates to confirm the reproducibility of the assay. Full kinetic analysis results are summarized in Table 3.

The assay buffer was 10 mM HEPES buffer (pH 7.4), 150 mM NaCl, 3mM EDTA, 0.05% P20 (polyoxyethylenesorbitan). The regeneration buffer was 10 mM Glycine buffer (pH 2.0). The conjugation buffer was 10 mM sodium acetate buffer (pH 5.0). The flow rate used for capturing the ligand was 1 μ L/min. The flow rate for kinetics analysis was 50 μ L/min.

Chi square (χ^2) analysis was carried out between the actual sensorgram and the sensorgram generated from the BIAAnalysis software to determine the accuracy of the analysis. A χ^2 value within 1-2 is considered significant (accurate) and below 1 is highly significant (highly accurate).

A summary of three independent SPR runs are presented in Table 4.

Table 3. Full Kinetic Analysis.

Ligand	Analyte	k_a (1/Ms)	K_d (1/s)	R_{max}	K_A (1/M)	K_D (M)	Con (nM)	χ^2
BSA-Ag (110RU)	Ab1	4.70×10^5	3.04×10^{-4}	4.01	1.55×10^9	6.45×10^{-10}	0-100	0.173
BSA-Ag (110RU)	Ab2	2.05×10^4	9.67×10^{-4}	15.6	2.12×10^7	4.72×10^{-8}	0-200	0.179

Table 4. SPR Summary.

5

Antibody	Instrument	On the Chip	On-rate	Off-rate	K_d	R_{max}	Note
8A10	Biacore 3000	Antibody	1.6×10^6	2.0×10^{-3}	1.3×10^{-9}	3.6	
	Biacore T-200	Antibody	2.4×10^5	8.7×10^{-4}	3.6×10^{-9}	8.6	
	Biacore 3000	Antigen	4.7×10^5	3.0×10^{-4}	6.5×10^{-10}	4.0	
3C6	Biacore 3000	Antibody	2.8×10^4	2.9×10^{-4}	1.0×10^{-8}	54.4	Single point
	Biacore T-200	Antibody	1.8×10^4	2.2×10^{-4}	1.2×10^{-8}	112	
	Biacore 3000	Antigen	2.1×10^4	9.7×10^{-4}	4.7×10^{-8}	15.6	

While illustrative embodiments have been illustrated and described, it will be appreciated that various changes can be made therein without departing from the spirit and scope of the invention.

CLAIMS

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A method for assaying paclitaxel in a liquid sample, comprising
 - (a) applying the liquid sample comprising paclitaxel to a lateral flow assay device, the device comprising
 - (i) a sample receiving zone for receiving the liquid sample;
 - (ii) a detection reagent zone in liquid communication with the sample receiving zone and downstream in flow direction from the sample receiving zone,

wherein the detection reagent zone comprises a detection reagent deposited thereon,

wherein the detection reagent is a paclitaxel antibody, functional fragment, or functional derivative thereof that binds paclitaxel, wherein the paclitaxel antibody, functional fragment, or functional derivative thereof is labeled with a detectable reporting group, and

wherein the paclitaxel antibody, functional fragment, or functional derivative thereof, has an association rate constant (K_{on}) from about 10^4 to about 10^7 , and a dissociation rate constant (K_{off}) from about 10^{-3} to about 10^{-7} ; and
 - (iii) a capture zone in liquid communication with the detection reagent zone and downstream in flow direction from the detection reagent zone,

wherein the capture zone comprises first, second, and third capture reagents immobilized thereon,

wherein the first capture reagent is a paclitaxel material capable of binding the detection reagent (first test line),

wherein the second capture reagent is a paclitaxel material capable of binding the detection reagent (second test line),

wherein the third capture reagent is an antibody capable of binding the detection reagent (control line),

wherein the first capture reagent is positioned at a first distance downstream in flow direction from the upstream end of the capture zone,

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wherein the second capture reagent is positioned at a second distance downstream in flow direction from the upstream end of the capture zone,

wherein the third capture reagent is positioned at a third distance downstream in flow direction from the upstream end of the capture zone,

wherein the second distance is greater than the first distance,

wherein the third distance is greater than the first and second distances, and

wherein the ratio of the first distance to the second distance is from greater than about 0.0 to about 0.4, when the detection reagent K_{on} is greater than about 2.0×10^5 and the detection reagent K_{off} is less than about 1.0×10^{-3} , or

wherein the ratio of the first distance to the second distance is from about 0.2 to about 1.0, when the K_{on} is greater than about 2.0×10^4 and the K_{off} is less than about 2.0×10^{-4} ; and

(b) allowing the sample to flow from the sample receiving zone through the detection reagent zone to provide detection reagent with paclitaxel;

(c) allowing the detection reagent with paclitaxel to flow through the capture zone,

whereby the first and second capture reagents (first and second test lines) compete with paclitaxel for binding with the detection reagent, and

whereby the third capture reagent (control line) binds excess detection reagent; and

(d) observing the amount of detection reagent bound to the first and second capture reagents (first and second test lines) relative to the third capture reagent (control line).

2. The method of Claim 1 further comprising determining the quantity of paclitaxel in the sample by quantitating the amount of detection reagent bound at control line and test lines.

3. The method of Claim 2, wherein quantitating the amount of detection reagent bound to the capture reagents comprises optical density measurement.

4. The method of Claim 1, wherein the detectable reporting group is colloidal gold.

5. The method of Claim 1, wherein the paclitaxel antibody is 3C6.
6. The method of Claim 1, wherein the paclitaxel antibody is 8A10.
7. The method of Claim 1, wherein the paclitaxel material is a paclitaxel antigen that competes with paclitaxel for binding to the detection reagent.
8. The method of Claim 1, wherein the paclitaxel material is a paclitaxel protein conjugate.
9. The method of Claim 1, wherein the antibody capable of binding the detection reagent is a goat anti-mouse antibody.
10. The method of Claim 1, wherein distance between the sample receiving zone and the first and second capture reagents is varied to optimize paclitaxel detection sensitivity.
11. The method of Claim 1, wherein distance between the sample receiving zone and the first and second capture reagents is minimized to optimize paclitaxel detection sensitivity.
12. The method of Claim 1 further comprising observing the amount of excess detection reagent bound to the third capture reagent (control line).
13. The method of Claim 1, comprising determining the quantity of paclitaxel in the sample by quantitating the amount of detection reagent bound to the third capture reagent.
14. The method of Claim 13, wherein quantitating the amount of detection reagent bound to the third capture reagent comprises optical density measurement.

15. The method of Claim 1, wherein the lateral flow device further comprises an absorbent zone in liquid communication with the capture reagent zone and downstream in flow direction from the capture reagent zone.

16. The method of Claim 1, wherein LFA can be replaced with SPR or other solid phase immunoassays.

17. The method of Claim 1, wherein first and second antibodies are used, and wherein the first has a K_{on} greater than 10^4 and the second has a K_{off} less than 10^{-3} .

18. The method of Claim 1, wherein two or three lines can be used to generate multiple readings on the same sample allowing for increase reproducibility and expanded dynamic range.

19. A method for monitoring the efficacy of a paclitaxel therapy in a patient diagnosed with cancer, comprising:

- (a) treating a cancer patient with paclitaxel at a first point in time;
- (b) determining a first concentration of paclitaxel in the patient after administering the therapeutic agent at a first point in time, wherein determining the concentration comprises the method of any one of Claims 1-18;
- (c) treating the patient with paclitaxel at a second point in time;
- (d) determining a second concentration of paclitaxel drug in the patient after administering the therapeutic agent at a second point in time, wherein determining the concentration comprises the methods of any one of Claims 1-18; and
- (e) comparing the first and second concentrations of paclitaxel in the patient to determine the efficacy of the cancer treatment.

20. A method for PK-guided dosing of paclitaxel therapy in a patient diagnosed with cancer, comprising:

- (a) treating the cancer patient with paclitaxel at a first point in time;

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(b) determining one or more pharmacokinetic parameters of paclitaxel in the patient after administering the therapeutic agent at a first point in time, wherein determining the one or more pharmacokinetic parameters comprises measuring the concentration of paclitaxel by method of any one of Claims 1-18;

(c) treating the patient with paclitaxel at a second point in time using the PK information from the first dosing;

(d) determining one or more pharmacokinetic parameters of paclitaxel in the patient after administering the therapeutic agent at a second point in time, wherein determining the one or more pharmacokinetic parameters comprises measuring the concentration of paclitaxel by the method of any one of Claims 1-18; and

(e) comparing one or more pharmacokinetic parameters of paclitaxel in the subject at the after administering the therapeutic agent first point in time with the levels after administering the therapeutic agent at the second point in time to confirm that optimal dosing was achieved.

21. The method of Claim 20, wherein the pharmacokinetic parameters are selected from the group consisting of time to maximum concentration (T_{max}), concentration maximum (C_{max}), area under the curve (AUC), clearance (CL), volume of distribution (V_d), apparent volume of distribution during the terminal phase (V_z), apparent volume of distribution during steady state (V_{ss}) and combinations thereof.

22. A lateral flow device, comprising:

(a) a sample receiving zone for receiving a liquid sample;

(b) a detection reagent zone in liquid communication with the sample receiving zone and downstream in flow direction from the sample receiving zone,

wherein the detection reagent zone comprises one or more detection reagents deposited thereon,

wherein the detection reagent is a paclitaxel antibody, functional fragment, or functional derivative thereof that binds paclitaxel, wherein the paclitaxel antibody, functional fragment, or functional derivative is labeled with a detectable reporting group, and

wherein the paclitaxel antibody, functional fragment, or functional derivative thereof, has an association constant (K_{on}) from about 10^4 to about 10^7 , and a dissociation constant (K_{off}) from about 10^{-3} to about 10^{-7} ;

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(c) a capture zone in liquid communication with the detection reagent zone and downstream in flow direction from the detection reagent zone,

wherein the capture zone comprises first, second, and third capture reagents immobilized thereon,

wherein the first capture reagent is a paclitaxel material capable of binding the detection reagent (first test line),

wherein the second capture reagent is a paclitaxel material capable of binding the detection reagent (second test line),

wherein the third capture reagent is an antibody capable of binding the detection reagent (control line),

wherein the first capture reagent is positioned at a first distance downstream in flow direction from the upstream end of the capture zone,

wherein the second capture reagent is positioned at a second distance downstream in flow direction from the upstream end of the capture zone,

wherein the third capture reagent is positioned at a third distance downstream in flow direction from the upstream end of the capture zone,

wherein the second distance is greater than the first distance,

wherein the third distance is greater than the first and second distances, and

wherein the ratio of the first distance to the second distance is from greater than about 0.0 to about 0.4, when the detection reagent K_{on} is greater than about 2.0×10^5 and the detection reagent K_{off} is less than about 1.0×10^{-3} , or

wherein the ratio of the first distance to the second distance is from about 0.2 to about 1.0, when the detection reagent K_{on} is greater than about 2.0×10^4 and the detection reagent K_{off} is less than about 2.0×10^{-4} ; and

(d) an absorbent zone in liquid communication with the capture zone and downstream in flow direction from the capture reagent zone.

23. The device of Claim 22, wherein the detection reagents comprise a first antibody having a first affinity for the therapeutic drug and a second antibody having a second affinity for the therapeutic drug, wherein the first affinity is greater than the second affinity.

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24. The device of Claim 22, wherein distance between the sample receiving zone and the first capture reagent is varied to optimize detection of the therapeutic drug.

25. The device of Claim 22, wherein the therapeutic agent is paclitaxel and the detection reagent comprises a monoclonal antibody selected from 8A10 and 3C6, or fragment or derivative thereof, wherein the antibody, or fragment or derivative binds to paclitaxel.

26. A method for assaying a therapeutic drug in a sample, comprising
(a) applying a sample to a sample receiving zone of a device of any one of Claims 22-25; and
(b) observing the amount of detection reagent bound to the one or more immobilized capture reagents.

27. The method of Claim 26 further comprising determining the quantity of therapeutic drug in the sample by quantitating the amount of detection reagent bound to the one or more immobilized capture reagents.

28. The method of Claim 26, wherein detection reagent is bound to a capture reagent in a first capture position.

29. The method of Claim 26, wherein detection reagent is bound to a capture reagent in a second capture position.

30. The method of Claim 26, wherein detection reagent is bound to capture reagents in first and second capture positions.

31. The method of Claim 26, wherein the therapeutic drug is paclitaxel.

32. The method of Claim 26, wherein the detection reagent comprises a monoclonal antibody selected from 8A10 and 3C6, or fragment or derivative thereof, wherein the antibody, or fragment or derivative binds to paclitaxel.

33. A method for monitoring the efficacy of a therapeutic treatment in a patient diagnosed with a disease or condition, comprising:

- (a) treating a patient with a therapeutic agent at a first point in time;
- (b) determining a first concentration of the therapeutic agent in the patient after administering the therapeutic agent at a first point in time, wherein determining the concentration comprises the method of any one of Claims 26-32;
- (c) treating the patient with the therapeutic agent at a second point in time;
- (d) determining a second concentration of the therapeutic agent in the patient after administering the therapeutic agent at a second point in time, wherein determining the concentration comprises the methods of any one of Claims 34-40; and
- (e) comparing the first and second concentrations in the patient to determine the efficacy of the therapeutic treatment.

34. The method of Claim 33, wherein the disease or condition treatment benefits from therapeutic drug monitoring.

35. The method of Claim 33, wherein the disease or condition is a cancer, inflammation, hypertension, cardiovascular, or pain.

36. The method of Claim 33, wherein the therapeutic agent is selected from the group consisting of paclitaxel, docetaxel, 7-epipaclitaxel, t-acetyl paclitaxel, 10-desacetyl-paclitaxel, 10-desacetyl-7-epipaclitaxel, 7-xylosylpaclitaxel, 10-desacetyl-7-glutarylpaclitaxel, 7-N,N-dimethylglycylpaclitaxel, and 7-L-alanylpaclitaxel.

37. A method for PK-guided dosing of a therapeutic treatment in a patient diagnosed with a disease or condition, comprising:

- (a) treating the patient with a therapeutic agent at a first point in time; determining one or more pharmacokinetic parameters of the therapeutic agent in the patient after administering the therapeutic agent at a first point in time, wherein determining the one or more pharmacokinetic parameters comprises assaying the therapeutic agent by the method of any one of Claims 34-40;

(b) treating the patient with the therapeutic agent at a second point in time using the PK data obtained from the first dosing;

(c) determining one or more pharmacokinetic parameters of the therapeutic agent in the patient after administering the therapeutic agent at a second point in time, wherein determining the one or more pharmacokinetic parameters comprises assaying the therapeutic agent by the method of any one of Claims 34-40; and

(d) comparing one or more pharmacokinetic parameters of the therapeutic agent drug in the subject at the first point in time with the levels at the second point in time to confirm that optimal dosing was achieved.

38. The method of Claim 37, wherein the pharmacokinetic parameters are selected from the group consisting of time to maximum concentration (T_{max}), concentration maximum (C_{max}), area under the curve (AUC), clearance (CL), volume of distribution (V_d), apparent volume of distribution during the terminal phase (V_z), apparent volume of distribution during steady state (V_{ss}) and combinations thereof.

39. The method of Claim 37, wherein the disease or condition is a cancer, inflammation, hypertension, cardiovascular, or pain.

40. The method of Claim 37, wherein the therapeutic agent is selected from the group consisting of paclitaxel, docetaxel, 7-epipaclitaxel, t-acetyl paclitaxel, 10-desacetyl-paclitaxel, 10-desacetyl-7-epipaclitaxel, 7-xylosylpaclitaxel, 10-desacetyl-7-glutarylpaclitaxel, 7-N,N-dimethylglycylpaclitaxel, and 7-L-alanylpaclitaxel.

41. A monoclonal antibody selected from 8A10 and 3C6, or antibody fragment, or antibody derivative thereof, wherein the antibody, antibody fragment, or antibody derivative comprises one or more complementary determining regions (CDRs) with at least 95% homology to a CDR contained in SEQ ID NOS: 2, 4, 6, or 8.

**IN THE UNITED STATES RECEIVING OFFICE
PATENT COOPERATION TREATY
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY**

Applicants: LEE, Yueh Jung et al.
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PACLITAXEL CONCENTRATION IN PLASMA FOR
PHARMACOKINETIC-GUIDED DOSING OF PACLITAXEL
Agent's File Reference: ATLC-1-53634
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Via EFS Upload

**RESPONSE TO WRITTEN OPINION
OF THE INTERNATIONAL SEARCHING AUTHORITY**

Applicant submits herewith claim amendments and arguments in response to the Written Opinion of the International Searching Authority mailed 08 September 2015 (08.09.2015) along with Applicants' Demand under Chapter II requesting international preliminary examination.

The Claimed Invention

Claims 1-50 are pending. Claims 1-20 are directed to a method for assaying paclitaxel; Claim 21 is directed to a method for monitoring the efficacy of paclitaxel therapy in a patient diagnosed with cancer using the method of Claims 1-20; Claims 22 and 23 are directed to a method for PK-guided dosing of paclitaxel therapy in a patient diagnosed with cancer using the method of Claims 1-20; Claims 24-33 are directed to a lateral flow device for assaying analytes, such as paclitaxel; Claims 34-40 are directed to a method for assaying a therapeutic agent using the device of Claims 24-33; Claims 41-44 are directed to a method for monitoring the efficacy of a therapeutic therapy in a patient diagnosed with a disease or condition using the method of Claims 34-40; Claims 45-48 are directed to a method for PK-guided dosing

of a therapeutic treatment in a patient diagnosed with a disease or condition using the method of Claims 34-40; and Claims 49 and 50 are directed to monoclonal antibodies.

Claims 1, 24, and 49 are the pending independent claims directed to methods for assay, lateral flow assay device, and monoclonal antibodies, respectively.

An amended claim set is attached. Claims 1 and 24 have been amended to clarify the invention. Support for the amendments can be found throughout the specification as originally filed. Claim 49 has been canceled in favor of dependent Claim 50.

Claims 2, 10-12, and 15 have been amended to conform to the amendment of Claim 1. Claims 21, 22, 41, and 45 have been amended for clarity. Claims 13, 14, 25, 27-30, and 32 have been canceled.

Claims 1-12, 15-24, 26, 31, 33-48, and 50 are now pending.

The Written Opinion

The Written Opinion has set forth the basis for rejection of the certain of the pending claims. Applicants offer the following remarks on the rejection of claims.

The Rejection of Claims 24, 25, 27-30, and 34-39 as Lacking Novelty

Claims 24, 25, 27-30, and 34-39 have been rejected as being anticipated by US 2014/0017812 (Smith et al.). Withdrawal of the rejection is requested for the following reasons.

Claims 24 is an independent claim directed to a lateral flow device. Claims 25, 27-30, and 34-39 depend from Claim 24. Claims 25 and 27-30 have been canceled.

Claims 24 is an independent claim directed to a lateral flow device and has been amended to clarify the invention. The claimed lateral flow device includes:

- (a) a sample receiving zone for receiving a liquid sample;
- (b) a detection reagent zone in liquid communication with the sample receiving zone and downstream in flow direction from the sample receiving zone,
- (c) a capture zone in liquid communication with the detection reagent zone and downstream in flow direction from the detection reagent zone, and
- (d) an absorbent zone in liquid communication with the capture zone and downstream in flow direction from the capture reagent zone.

The capture zone includes features that are neither disclosed nor taught in the cited reference.

In the capture zone, the device's detection reagents are captured thereby facilitating assay of paclitaxel. As recited in Claim 24, the detection reagent is a paclitaxel antibody, or functional fragment or functional derivative thereof having an association constant (K_{on}) from about 10^4 to about 10^7 , and a dissociation constant (K_{off}) from about 10^{-3} to about 10^{-7} .

As amended, the capture zone of the device of Claim 24 includes first, second, and third capture reagents immobilized thereon: first and second capture reagents are paclitaxel materials capable of binding the detection reagent (first and second test lines); and the third capture reagent is an antibody capable of binding the detection reagent (control line). The first capture reagent is positioned at a first distance downstream in flow direction from the upstream end of the capture zone; the second capture reagent is positioned at a second distance downstream in flow direction from the upstream end of the capture zone, and the third capture reagent is positioned at a third distance downstream in flow direction from the upstream end of the capture zone.

As described in the specification as originally filed, the position of the capture lines in the capture zone of the claimed device was surprisingly found to be a function of the detection reagent association constant (K_{on}) and the detection reagent dissociation constant (K_{off}). See specification at page 16, line 21 through page 17, line 9, and FIGURES 5A and 5B, 6A and 6B, and 7A and 7B. The optimization can be described as relative positioning of T (test line) and C (control line): T/C, which is defined as the distance from origin to T/distance from origin to C ratio, where the origin is defined as the upstream edge of the capture zone (upstream edge of membrane 230 in FIGURES 2A-2C). See specification at page 21, lines 27-30.

Claim 24 recites two options: (1) when the detection reagent K_{on} is greater than about 2.0×10^5 and the detection reagent K_{off} is less than about 1.0×10^{-3} , the ratio of the first distance to the second distance is from greater than about 0.0 to about 0.4; or (2) when the detection reagent K_{on} is greater than about 2.0×10^4 and the detection reagent K_{off} is less than about 2.0×10^{-4} , the ratio of the first distance to the second distance is from about 0.2 to about 1.0.

The recitation of the ratios of the first and second distances is not described in the cited reference. For at least this reason, the cited reference is not anticipatory and the invention as now claimed is novel in view of the reference.

Because the cited reference fails to exactly describe the invention as now claimed, the cited reference is not anticipatory. In view of the amendment of Claim 24, withdrawal of the rejection is requested.

Applicant further submits that the cited reference fails to teach, suggest, provide any motivation to make, or otherwise render obvious the invention as now claimed.

The Rejection of Claim 49 as Lacking Novelty

Claim 49 has been rejected as being anticipated by US 5,955,621 (Durzan). Claim 49 is an independent claim directed to monoclonal antibodies 8A10 and 3C6. Claim 49 has been canceled in favor of Claim 50. In view of the cancellation of Claim 49, withdrawal of the rejection is requested.

The Rejection of Claims 26, 31, 32, and 34-39 as Lacking Inventive Step

Claims 26, 31, 32, and 34-39 have been rejected as being lacking inventive step as obvious in view of US 2014/0017812 (Smith et al.). Withdrawal of the rejection is requested for the following reasons.

Claims 26, 31, 32, and 34-39 depend from Claim 24, which is an independent claim directed to a lateral flow device. Claim 32 has been canceled.

As noted above, Claim 24 has been amended to clarify the invention. The differences between the invention as now claimed and the cited reference are noted above with regard to the novelty rejection of Claim 24. As set forth above, the position of the capture lines in the capture zone of the claimed device was surprising found to be a function of the detection reagent association constant (K_{on}) and the detection reagent dissociation constant (K_{off}). The optimization is described as relative positioning of T (test line) and C (control line): the ratio T/C. Claim 24 recites two options: (1) when the detection reagent K_{on} is greater than about 2.0×10^5 and the detection reagent K_{off} is less than about 1.0×10^{-3} , the ratio of the first distance to the second distance is from greater than about 0.0 to about 0.4; or (2) when the detection reagent K_{on} is greater than about 2.0×10^4 and the detection reagent K_{off} is less than about 2.0×10^{-4} , the ratio of the first distance to the second distance is from about 0.2 to about 1.0.

The recitation of the ratios of the first and second distances in Claim 24 is neither taught nor suggested by the cited reference. The cited reference merely described a standard competitive binding lateral flow immunoassay for assaying paclitaxel. The relationship between the recited ratios and the recited association and

dissociation constants for the detection reagents (paclitaxel monoclonal antibodies) is neither taught nor suggested by the cited reference.

Because the cited reference fails to teach each and every limitation of the claimed invention, and because the cited reference fails to teach, suggest, provide any motivation to make, or otherwise render obvious the invention as now claimed, the claimed invention is non-obvious and patentable over the cited reference.

In view of the amendment of Claim 24, withdrawal of the rejection is requested.

The Rejection of Claims 1-4 and 7-20 as Lacking Inventive Step

Claims 1-4 and 7-20 have been rejected as being lacking inventive step as obvious over US 2014/0017812 (Smith et al.) in view of US 2010/0166746 (Chowdhury et al.). Withdrawal of the rejection is requested for the following reasons.

Claim 1 is an independent claim directed to a method for assaying paclitaxel. Claims 2-4 and 7-20 depend from Claim 1. Claims 13 and 14 have been canceled.

Claim 1 recites a method for assaying paclitaxel in a liquid sample that includes:

- (a) applying the liquid sample comprising paclitaxel to a lateral flow assay device,
- (b) allowing the sample to flow from the sample receiving zone through the detection reagent zone to provide detection reagent with paclitaxel;
- (c) allowing the detection reagent with paclitaxel to flow through the capture zone, and
- (d) observing the amount of detection reagent bound to the first and second capture reagents (first and second test lines) relative to the third capture reagent (control line).

Claim 1 has been amended to clarify the invention. As amended, Claim 1 has been amended to recite the lateral flow assay device of Claim 24.

Neither of the cited references teaches or suggests the invention as now claimed, particularly with regard to the positioning of capture test and control lines.

The teaching of the Smith reference with regard to the lateral flow device of Claim 24 is set forth above. The deficiencies of the teaching of the Smith reference

are not cured by the teaching of Chowdhury reference. The Chowdhury reference is directed to anti-tumor antibodies having increased K_{on} rates compared first generation anti-tumor. The Chowdhury reference simple fails to teach or remotely suggest the positioning test and control lines in a lateral flow immunoassay for paclitaxel, as recited in the claimed method.

Because the cited references, either alone or in any combination, fail to teach each and every limitation of the claimed invention, and because the cited references, either alone or in any combination, fail to teach, suggest, provide any motivation to make, or otherwise render obvious the invention as now claimed, a method for assaying paclitaxel that recites positioning test and control lines in a lateral flow immunoassay for paclitaxel, the claimed invention is non-obvious and patentable in view of the cited references.

In view of the amendment of Claim 1, withdrawal of the rejection is requested.

The Rejection of Claims 5 and 6 as Lacking Inventive Step

Claims 5 and 6 have been rejected as being lacking inventive step as obvious over US 2014/0017812 (Smith et al.) in view of US 2010/0166746 (Chowdhury et al.) and further in view of US Patent No. 5,955,621 (Durzan). Withdrawal of the rejection is requested for the following reasons.

Claims 5 and 6 are directed to monoclonal antibodies 8A10 and 3C6. Claims 5 and 6 depend from Claim 1, which has been amended. The deficiencies of the teachings of the Smith and Chowdhury references noted above with regard to Claim 1, are not cured by the teaching of the Durzan reference. The Durzan reference is directed to monoclonal antibodies, including 8A10 and 3C6, and does not teach or suggest of the features of the invention as now claimed, a method for assaying paclitaxel that recites specific positioning of test and control lines in a lateral flow immunoassay for paclitaxel.

Because the cited references, either alone or in any combination, fail to teach each and every limitation of the claimed invention, and because the cited references, either alone or in any combination, fail to teach, suggest, provide any motivation to make, or otherwise render obvious the invention as now claimed, a method for assaying paclitaxel that recites positioning test and control lines in a lateral flow immunoassay for paclitaxel, the claimed invention is non-obvious and patentable in view of the cited references.

In view of the amendment of Claim 1, withdrawal of the rejection is requested.

The Rejection of Claims 1-4 and 7-23 as Lacking Inventive Step

Claims 1-4 and 7-23 have been rejected as being lacking inventive step as obvious over US 2014/0017812 (Smith et al.) in view of US 2010/0166746 (Chowdhury et al.) and further in view of WO 2012/166795 (Trieu). Withdrawal of the rejection is requested for the following reasons.

Claim 1 is an independent claim directed to a method for assaying paclitaxel. Claims 2-4 and 7-20 depend from Claim 1. Claims 13 and 14 have been canceled.

Claim 1 has been amended to clarify the invention. As amended, Claim 1 has been amended to recite the lateral flow assay device of Claim 24.

The cited references, either alone or in any combination, fail to teach or suggest the invention as now claimed, particularly with regard to the positioning of capture test and control lines.

The deficiencies of the teachings of the Smith and Chowdhury references noted above with regard to Claim 1, are not cured by the teaching of the Trieu reference. The Trieu reference is directed to methods for therapeutic drug monitoring and dosing by point-of-care pharmacokinetic profiling and does not teach or suggest of the features of the invention as now claimed, a method for assaying paclitaxel that recites specific positioning of test and control lines in a lateral flow immunoassay for paclitaxel.

Because the cited references, either alone or in any combination, fail to teach each and every limitation of the claimed invention, and because the cited references, either alone or in any combination, fail to teach, suggest, provide any motivation to make, or otherwise render obvious the invention as now claimed, a method for assaying paclitaxel that recites positioning test and control lines in a lateral flow immunoassay for paclitaxel, the claimed invention is non-obvious and patentable in view of the cited references.

In view of the amendment of Claim 1, withdrawal of the rejection is requested.

The Rejection of Claims 5, 6, and 21-23 as Lacking Inventive Step

Claims 5, 6, and 21-23 have been rejected as being lacking inventive step as obvious over US 2014/0017812 (Smith et al.) in view of US 2010/0166746 (Chowdhury et al.), further in view of US Patent No. 5,955,621 (Durzan), and in view of WO 2012/166795 (Trieu). Withdrawal of the rejection is requested for the following reasons.

Claims 5, 6, and 21-23 depend from Claim 1, which has been amended. The deficiencies of the teachings of the Smith and Chowdhury references noted above with regard to Claim 1, are not cured by the teaching of the Durzan reference, directed to monoclonal antibodies 8A10 and 3C6, and the teaching of the Trieu reference, directed to methods for therapeutic drug monitoring and dosing by point-of-care pharmacokinetic profiling. Neither the Durzan reference nor the Trieu reference teaches or suggests the features of the invention as now claimed, a method for assaying paclitaxel that recites specific positioning of test and control lines in a lateral flow immunoassay for paclitaxel.

Because the cited references, either alone or in any combination, fail to teach each and every limitation of the claimed invention, and because the cited references, either alone or in any combination, fail to teach, suggest, provide any motivation to make, or otherwise render obvious the invention as now claimed, a method for assaying paclitaxel that recites positioning test and control lines in a lateral flow immunoassay for paclitaxel, the claimed invention is non-obvious and patentable in view of the cited references.

In view of the amendment of Claim 1, withdrawal of the rejection is requested.

Marked Up Version of the Amended Claims

In accordance with PCT Rule 66.8, please note the amendments below which show the differences between the replaced sheets (39-47) and replacement sheets 39-47.

1. (Currently amended) A method for assaying paclitaxel in a liquid sample, comprising

(a) applying ~~[[a]]~~ the liquid sample comprising paclitaxel to a lateral flow assay device, the device comprising

(i) a sample receiving zone for receiving the liquid sample;

(ii) a detection reagent zone in liquid communication with the sample receiving zone and downstream in flow direction from the sample receiving zone,

wherein the detection reagent zone comprises a detection reagent deposited thereon,

wherein the detection reagent is a paclitaxel antibody, ~~[[or]]~~ functional fragment, or functional derivative thereof that binds paclitaxel, wherein the paclitaxel

antibody, functional fragment, or functional derivative thereof is labeled with a detectable reporting group, and

wherein the paclitaxel antibody, ~~[[or]]~~ functional fragment, or functional derivative thereof, has ~~[[a K_{on}]]~~ an association rate constant (K_{on}) from about 10^4 to about 10^7 , and ~~[[a K_{off}]]~~ a dissociation rate constant (K_{off}) from about 10^{-3} to about 10^{-7} ; and

(iii) a capture zone in liquid communication with the detection reagent zone and downstream in flow direction from the detection reagent zone,

wherein the capture zone comprises first, ~~[[and]]~~ second, and third capture reagents immobilized thereon,

wherein the first capture reagent is a paclitaxel material capable of binding the detection reagent (first test line),

wherein the second capture reagent is a paclitaxel material capable of binding the detection reagent (second test line),

wherein the ~~second~~ third capture reagent is an antibody capable of binding the detection reagent (control line),

wherein the first capture reagent is positioned at a first distance downstream in flow direction from the upstream end of the capture zone,

wherein the second capture reagent is positioned at a second distance downstream in flow direction from the upstream end of the capture zone,

wherein the ~~second~~ third capture reagent is positioned at a ~~second~~ third distance downstream in flow direction from the upstream end of the capture zone,

wherein the second distance is greater than the first distance,

wherein the third distance is greater than the first and second distances, and

wherein the ratio of the first distance to the second distance is from greater than about 0.0 to about 0.4, when the detection reagent K_{on} is greater than about 2.0×10^5 and the detection reagent K_{off} is less than about 1.0×10^{-3} , ~~[[and]]~~ or

wherein the ratio of the first distance to the second distance is from about 0.2 to about 1.0, when the K_{on} is greater than about 2.0×10^4 and the K_{off} is less than about 2.0×10^{-4} ; and

(b) allowing the sample to flow from the sample receiving zone through the detection reagent zone to provide detection reagent with paclitaxel;

(c) allowing the detection reagent with paclitaxel to flow through the capture zone,

whereby the first and second capture ~~reagent~~ reagents (first and second test ~~[[line]]~~ lines) ~~competes~~ compete with ~~the analyte (paclitaxel)~~ paclitaxel for binding with the detection reagent, and

whereby the ~~second~~ third capture reagent (control line) binds excess detection reagent; and

(d) observing the amount of detection reagent bound to the first and second capture ~~reagent~~ reagents (first and second test ~~[[line]]~~ lines) relative to the ~~second~~ third capture reagent (control line).

2. (Currently amended) The method of Claim 1 further comprising determining the quantity of paclitaxel in the sample by quantitating the amount of detection reagent bound at control line and test ~~[[line]]~~ lines.

3. (Original) The method of Claim 2, wherein quantitating the amount of detection reagent bound to the capture reagents comprises optical density measurement.

4. (Original) The method of Claim 1, wherein the detectable reporting group is colloidal gold.

5. (Original) The method of Claim 1, wherein the paclitaxel antibody is 3C6.

6. (Original) The method of Claim 1, wherein the paclitaxel antibody is 8A10.

7. (Original) The method of Claim 1, wherein the paclitaxel material is a paclitaxel antigen that competes with paclitaxel for binding to the detection reagent.

8. (Original) The method of Claim 1, wherein the paclitaxel material is a paclitaxel protein conjugate.
9. (Original) The method of Claim 1, wherein the antibody capable of binding the detection reagent is a goat anti-mouse antibody.
10. (Currently amended) The method of Claim 1, wherein distance between the sample receiving zone and the first and second capture ~~reagent~~ reagents is varied to optimize paclitaxel detection sensitivity.
11. (Currently amended) The method of Claim 1, wherein distance between the sample receiving zone and the first and second capture ~~reagent~~ reagents is minimized to optimize paclitaxel detection sensitivity.
12. (Currently amended) The method of Claim 1 further comprising observing the amount of excess detection reagent bound to the ~~second~~ third capture reagent (control line).
13. **(Canceled) The method of Claim 1, further comprising determining the quantity of paclitaxel in the sample by quantitating the amount of excess detection reagent bound to the second capture reagent.**
14. **(Canceled) The method of Claim 1 further comprising a third capture zone intermediate the first and second capture zones, wherein the third capture zone comprises a paclitaxel material capable of binding the detection reagent.**
15. (Currently amended) The method of Claim ~~[[14]]~~ 1, comprising determining the quantity of paclitaxel in the sample by quantitating the amount of detection reagent bound to the third capture reagent.

16. (Original) The method of Claim 15, wherein quantitating the amount of detection reagent bound to the third capture reagent comprises optical density measurement.
17. (Original) The method of Claim 1, wherein the lateral flow device further comprises an absorbent zone in liquid communication with the capture reagent zone and downstream in flow direction from the capture reagent zone.
18. (Original) The method of Claim 1, wherein LFA can be replaced with SPR or other solid phase immunoassays.
19. (Original) The method of Claim 1, wherein first and second antibodies are used, and wherein the first has a K_{on} greater than 10^4 and the second has a K_{off} less than 10^{-3} .
20. (Original) The method of Claim 1, wherein two or three lines can be used to generate multiple readings on the same sample allowing for increase reproducibility and expanded dynamic range.
21. (Currently amended) A method for monitoring the efficacy of a paclitaxel therapy in a patient diagnosed with cancer, comprising:
- (a) treating a cancer patient with paclitaxel at a first point in time;
 - (b) determining a first concentration of paclitaxel in the patient after administering the therapeutic agent at a first point in time, wherein determining the concentration comprises the method of any one of Claims 1-20;
 - (c) treating the patient with paclitaxel at a second point in time;
 - (d) determining a second concentration of paclitaxel drug in the patient after administering the therapeutic agent at a second point in time, wherein determining the concentration comprises the methods of any one of Claims 1-20; and
 - (e) comparing the first and second concentrations of paclitaxel in the patient to determine the efficacy of the cancer treatment.

22. (Currently amended) A method for PK-guided dosing of paclitaxel therapy in a patient diagnosed with cancer, comprising:

(a) treating the cancer patient with paclitaxel at a first point in time;

(b) determining one or more pharmacokinetic parameters of paclitaxel in the patient after administering the therapeutic agent at a first point in time, wherein determining the one or more pharmacokinetic parameters comprises measuring the concentration of paclitaxel by method of any one of Claims 1-20;

[[~~(b)~~]] (c) treating the patient with paclitaxel at a second point in time using the PK information from the first dosing;

[[~~(c)~~]] (d) determining one or more pharmacokinetic parameters of paclitaxel in the patient after administering the therapeutic agent at a second point in time, wherein determining the one or more pharmacokinetic parameters comprises measuring the concentration of paclitaxel by the method of any one of Claims 1-20; and

[[~~(d)~~]] (e) comparing one or more pharmacokinetic parameters of paclitaxel in the subject at the after administering the therapeutic agent first point in time with the levels after administering the therapeutic agent at the second point in time to confirm that optimal dosing was achieved.

23. (Original) The method of Claim 22, wherein the pharmacokinetic parameters are selected from the group consisting of time to maximum concentration (T_{max}), concentration maximum (C_{max}), area under the curve (AUC), clearance (CL), volume of distribution (V_d), apparent volume of distribution during the terminal phase (V_z), apparent volume of distribution during steady state (V_{ss}) and combinations thereof.

24. (Currently amended) A lateral flow device, comprising:

(a) a sample receiving zone for receiving a liquid sample;

(b) a detection reagent zone in liquid communication with the sample receiving zone and downstream in flow direction from the sample receiving zone, wherein the detection reagent zone comprises one or more detection reagents deposited thereon.

wherein the detection reagent is a paclitaxel antibody, functional fragment, or functional derivative thereof that binds paclitaxel, wherein the paclitaxel antibody, functional fragment, or functional derivative is labeled with a detectable reporting group, and

wherein the paclitaxel antibody, functional fragment, or functional derivative thereof, has an association constant (K_{on}) from about 10^4 to about 10^7 , and a dissociation constant (K_{off}) from about 10^{-3} to about 10^{-7} ;

(c) a capture zone in liquid communication with the detection reagent zone and downstream in flow direction from the detection reagent zone,

~~wherein the capture zone comprises one or more capture reagents immobilized thereon~~

wherein the capture zone comprises first, second, and third capture reagents immobilized thereon,

wherein the first capture reagent is a paclitaxel material capable of binding the detection reagent (first test line),

wherein the second capture reagent is a paclitaxel material capable of binding the detection reagent (second test line),

wherein the third capture reagent is an antibody capable of binding the detection reagent (control line),

wherein the first capture reagent is positioned at a first distance downstream in flow direction from the upstream end of the capture zone,

wherein the second capture reagent is positioned at a second distance downstream in flow direction from the upstream end of the capture zone,

wherein the third capture reagent is positioned at a third distance downstream in flow direction from the upstream end of the capture zone,

wherein the second distance is greater than the first distance,

wherein the third distance is greater than the first and second distances, and

wherein the ratio of the first distance to the second distance is from greater than about 0.0 to about 0.4, when the detection reagent K_{on} is greater than about 2.0×10^5 and the detection reagent K_{off} is less than about 1.0×10^{-3} , or

wherein the ratio of the first distance to the second distance is from about 0.2 to about 1.0, when the detection reagent K_{on} is greater than about 2.0×10^4 and the detection reagent K_{off} is less than about 2.0×10^{-4} ; and

(d) an absorbent zone in liquid communication with the capture zone and downstream in flow direction from the capture reagent zone.

25. (Canceled) The device of Claim 24, wherein the detection reagent is an antibody labeled with a detectable reporting group.

26. (Original) The device of Claim 24, wherein the detection reagents comprise a first antibody having a first affinity for the therapeutic drug and a second antibody having a second affinity for the therapeutic drug, wherein the first affinity is greater than the second affinity.

27. (Canceled) The device of Claim 24, wherein the capture reagent is an antigen that competes with the therapeutic drug for binding to the detection reagent.

28. (Canceled) The device of Claim 24, wherein the capture reagent is an antibody capable of binding the detection reagent.

29. (Canceled) The device of Claim 24, wherein the capture reagents comprise a first capture reagent that is an antigen that competes with the therapeutic drug for binding to the detection reagent, and a second capture reagent that is an antibody capable of binding the detection reagent.

30. (Canceled) The device of Claim 24, wherein the first capture reagent is immobilized upstream in flow direction from the second capture reagent.

31. (Original) The device of Claim 30, wherein distance between the sample receiving zone and the first capture reagent is varied to optimize detection of the therapeutic drug.

32. (Canceled) The device of Claim 30 further comprising a third capture reagent immobilized intermediate the first and second capture reagents, wherein the third capture reagent is an antigen that competes with the therapeutic drug for binding to the detection reagent.

33. (Original) The device of Claim 24, wherein the therapeutic agent is paclitaxel and the detection reagent comprises a monoclonal antibody selected from 8A10 and 3C6, or fragment or derivative thereof, wherein the antibody, or fragment or derivative binds to paclitaxel.

34. (Original) A method for assaying a therapeutic drug in a sample, comprising
(a) applying a sample to a sample receiving zone of a device of any one of Claims 24-33; and
(b) observing the amount of detection reagent bound to the one or more immobilized capture reagents.

35. (Original) The method of Claim 34 further comprising determining the quantity of therapeutic drug in the sample by quantitating the amount of detection reagent bound to the one or more immobilized capture reagents.

36. (Original) The method of Claim 34, wherein detection reagent is bound to a capture reagent in a first capture position.

37. The method of Claim 34, wherein detection reagent is bound to a capture reagent in a second capture position.

38. (Original) The method of Claim 34, wherein detection reagent is bound to capture reagents in first and second capture positions.

39. (Original) The method of Claim 34, wherein the therapeutic drug is paclitaxel.

40. (Original) The method of Claim 34, wherein the detection reagent comprises a monoclonal antibody selected from 8A10 and 3C6, or fragment or derivative thereof, wherein the antibody, or fragment or derivative binds to paclitaxel.

41. (Currently amended) A method for monitoring the efficacy of a therapeutic treatment in a patient diagnosed with a disease or condition, comprising:

- (a) treating a patient with a therapeutic agent at a first point in time;
- (b) determining a first concentration of the therapeutic agent in the patient after administering the therapeutic agent at a first point in time, wherein determining the concentration comprises the method of any one of Claims 34-40;
- (c) treating the patient with the therapeutic agent at a second point in time;
- (d) determining a second concentration of the therapeutic agent in the patient after administering the therapeutic agent at a second point in time, wherein determining the concentration comprises the methods of any one of Claims 34-40; and
- (e) comparing the first and second concentrations in the patient to determine the efficacy of the therapeutic treatment.

42. (Original) The method of Claim 41, wherein the disease or condition treatment benefits from therapeutic drug monitoring.

43. (Original) The method of Claim 41, wherein the disease or condition is a cancer, inflammation, hypertension, cardiovascular, or pain.

44. The method of Claim 41, wherein the therapeutic agent is selected from the group consisting of paclitaxel, docetaxel, 7-epipaclitaxel, t-acetyl paclitaxel, 10-desacetyl-paclitaxel, 10-desacetyl-7-epipaclitaxel, 7-xylosylpaclitaxel, 10-desacetyl-7-glutarylpaclitaxel, 7-N,N-dimethylglycylpaclitaxel, and 7-L-alanylpaclitaxel.

45. (Currently amended) A method for PK-guided dosing of a therapeutic treatment in a patient diagnosed with a disease or condition, comprising:

- (a) treating the patient with a therapeutic agent at a first point in time;

determining one or more pharmacokinetic parameters of the therapeutic agent in the patient after administering the therapeutic agent at a first point in time, wherein determining the one or more pharmacokinetic parameters comprises assaying the therapeutic agent by the method of any one of Claims 34-40;

(b) treating the patient with the therapeutic agent at a second point in time using the PK data obtained from the first dosing;

(c) determining one or more pharmacokinetic parameters of the therapeutic agent in the patient after administering the therapeutic agent at a second point in time, wherein determining the one or more pharmacokinetic parameters comprises assaying the therapeutic agent by the method of any one of Claims 34-40; and

(d) comparing one or more pharmacokinetic parameters of the therapeutic agent drug in the subject at the first point in time with the levels at the second point in time to confirm that optimal dosing was achieved.

46. (Original) The method of Claim 45, wherein the pharmacokinetic parameters are selected from the group consisting of time to maximum concentration (T_{max}), concentration maximum (C_{max}), area under the curve (AUC), clearance (CL), volume of distribution (V_d), apparent volume of distribution during the terminal phase (V_z), apparent volume of distribution during steady state (V_{ss}) and combinations thereof.

47. (Original) The method of Claim 45, wherein the disease or condition is a cancer, inflammation, hypertension, cardiovascular, or pain.

48. (Original) The method of Claim 45, wherein the therapeutic agent is selected from the group consisting of paclitaxel, docetaxel, 7-epipaclitaxel, t-acetyl paclitaxel, 10-desacetyl-paclitaxel, 10-desacetyl-7-epipaclitaxel, 7-xylosylpaclitaxel, 10-desacetyl-7-glutarylpaclitaxel, 7-N,N-dimethylglycylpaclitaxel, and 7-L-alanylpaclitaxel.

49. (Canceled) A monoclonal antibody selected from 8A10 and 3C6, or fragment or derivative thereof, wherein the antibody, antibody fragment, or antibody derivative binds to paclitaxel.

50. (Currently amended) ~~[[The]]~~ A monoclonal antibody selected from 8A10 and 3C6, or antibody fragment, or antibody derivative ~~of Claim 49 thereof~~, wherein the antibody, antibody fragment, or antibody derivative comprises one or more complementary determining regions (CDRs) with at least 95% homology to a CDR contained in SEQ ID NOS: 2, 4, 6, or 8.

Conclusion

Replacement sheets 39-47 showing a clean version of the amended claims are attached hereto. These claims have been renumbered to reflect these amendments, which may not be shown in the markups above. In view of the amendment the claims, applicant believes that the pending claims, Claims 1-12, 15-24, 26, 31, 33-48 and 50, are allowable.

Respectfully submitted,

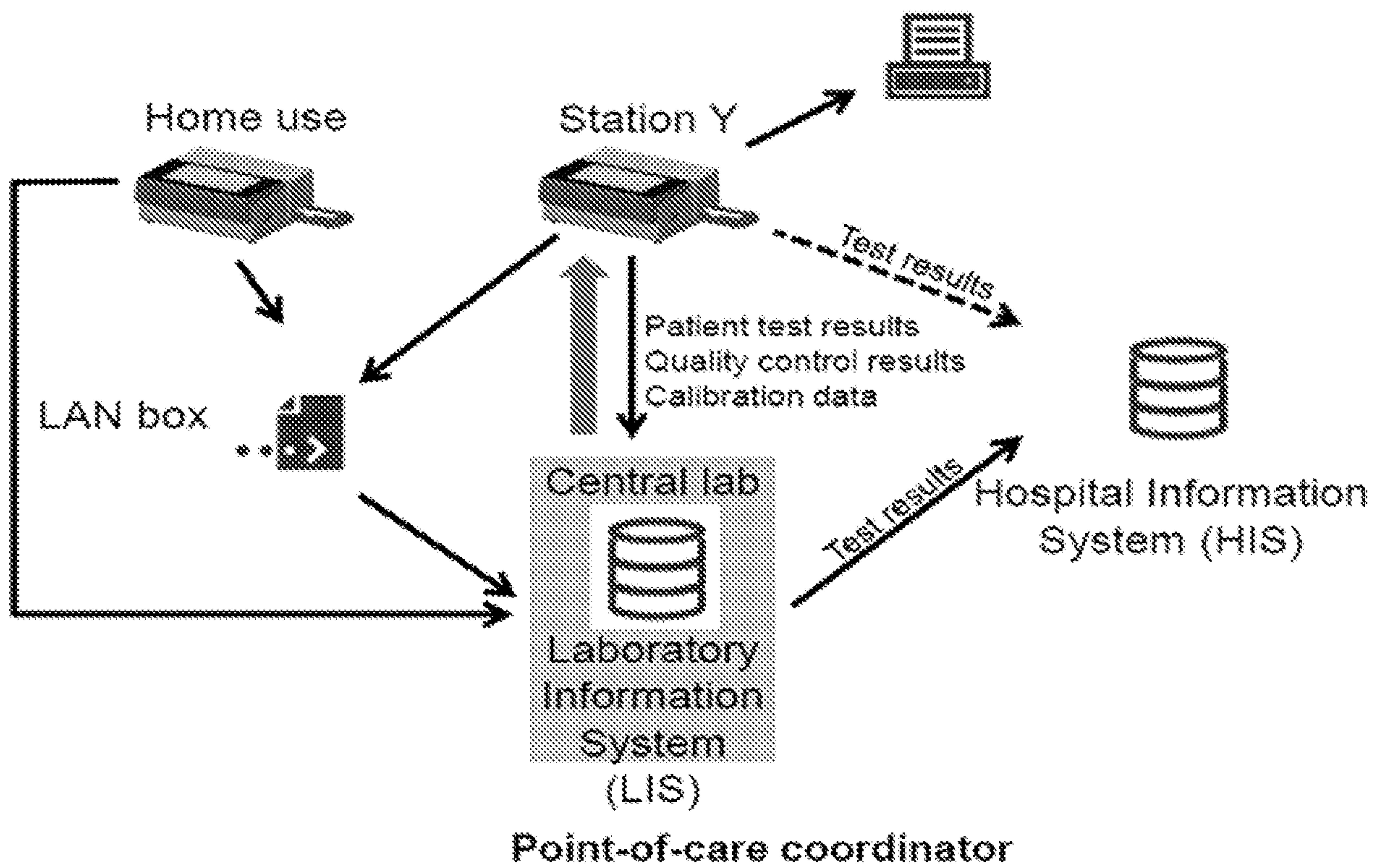
CHRISTENSEN O'CONNOR
JOHNSON KINDNESS^{PLLC}

/George E. Renzoni/

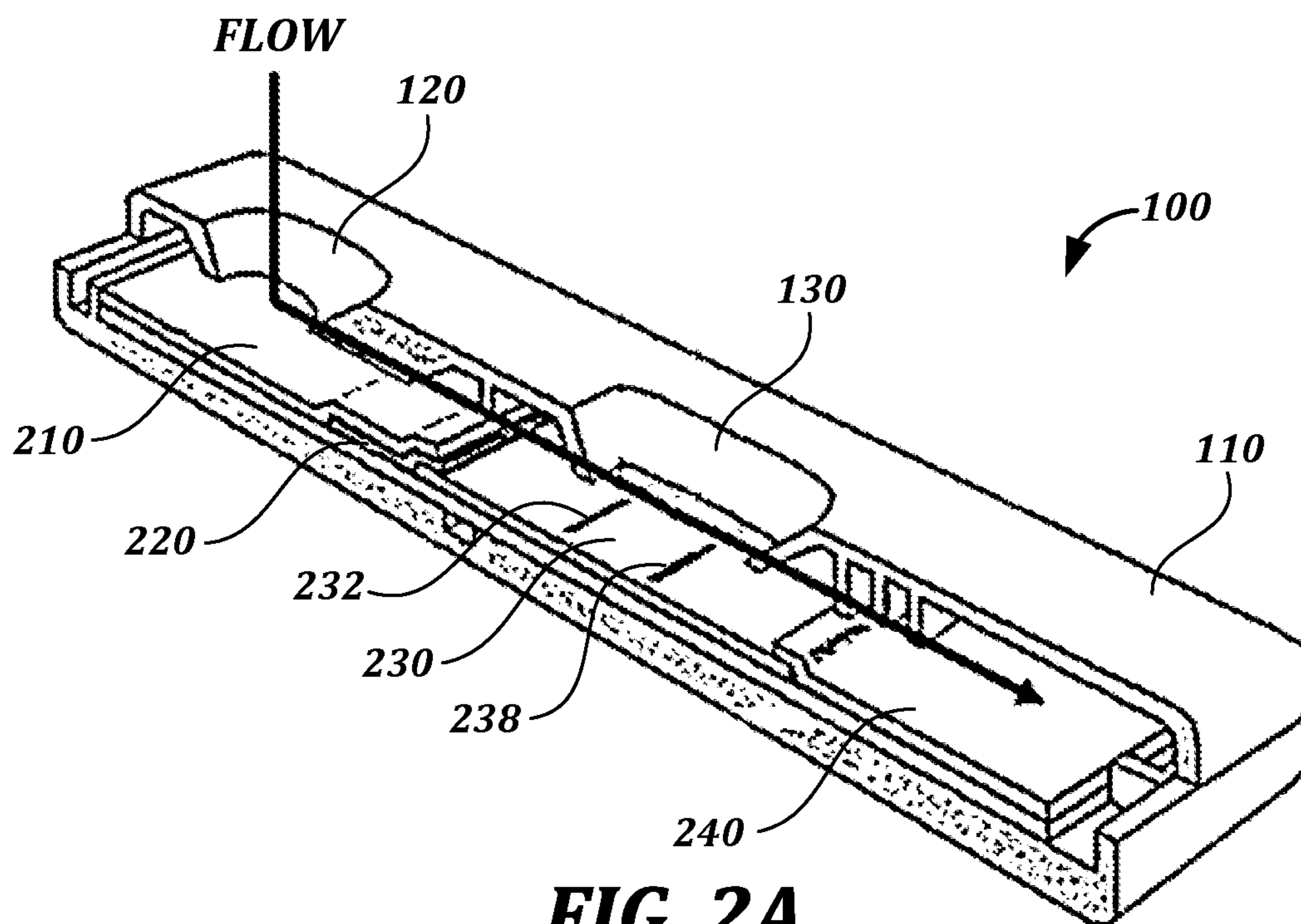
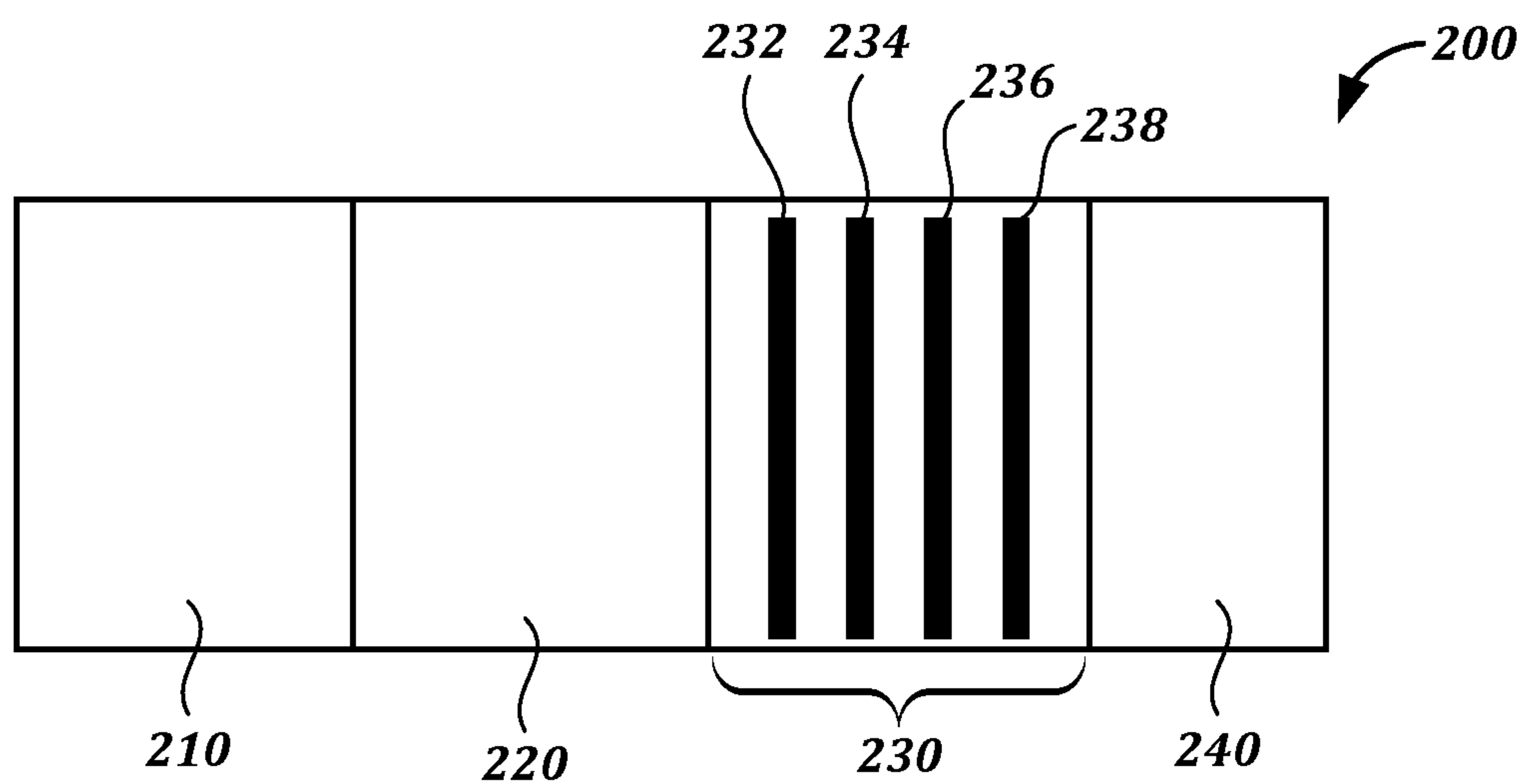
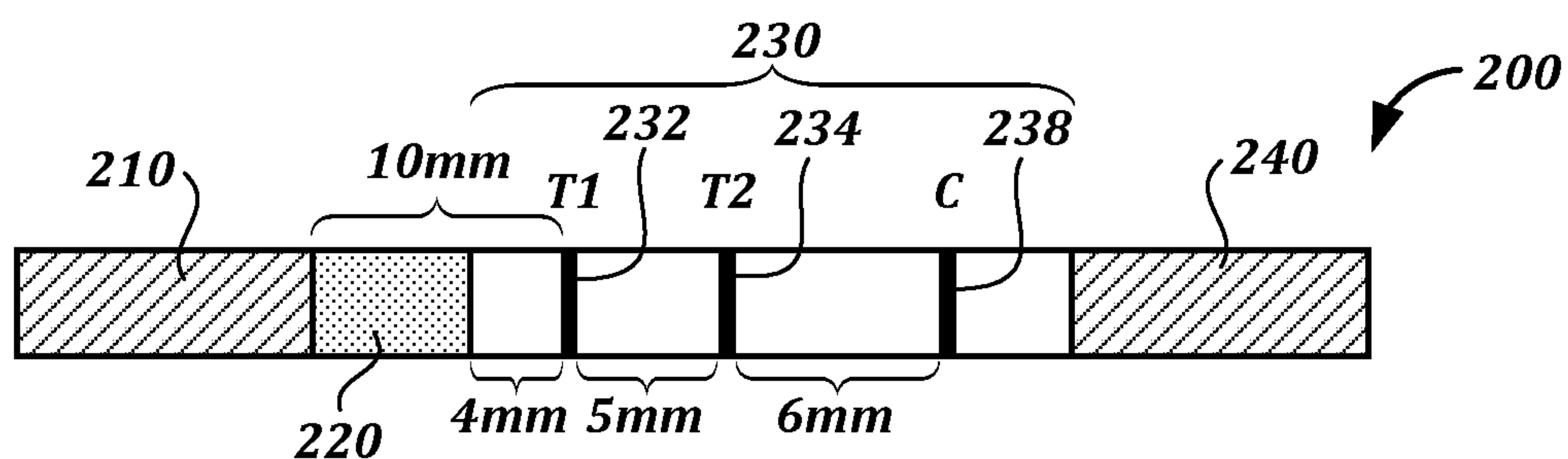
George E. Renzoni, Ph.D.
Registration No. 37,919
Direct Dial No. 206.695.1755

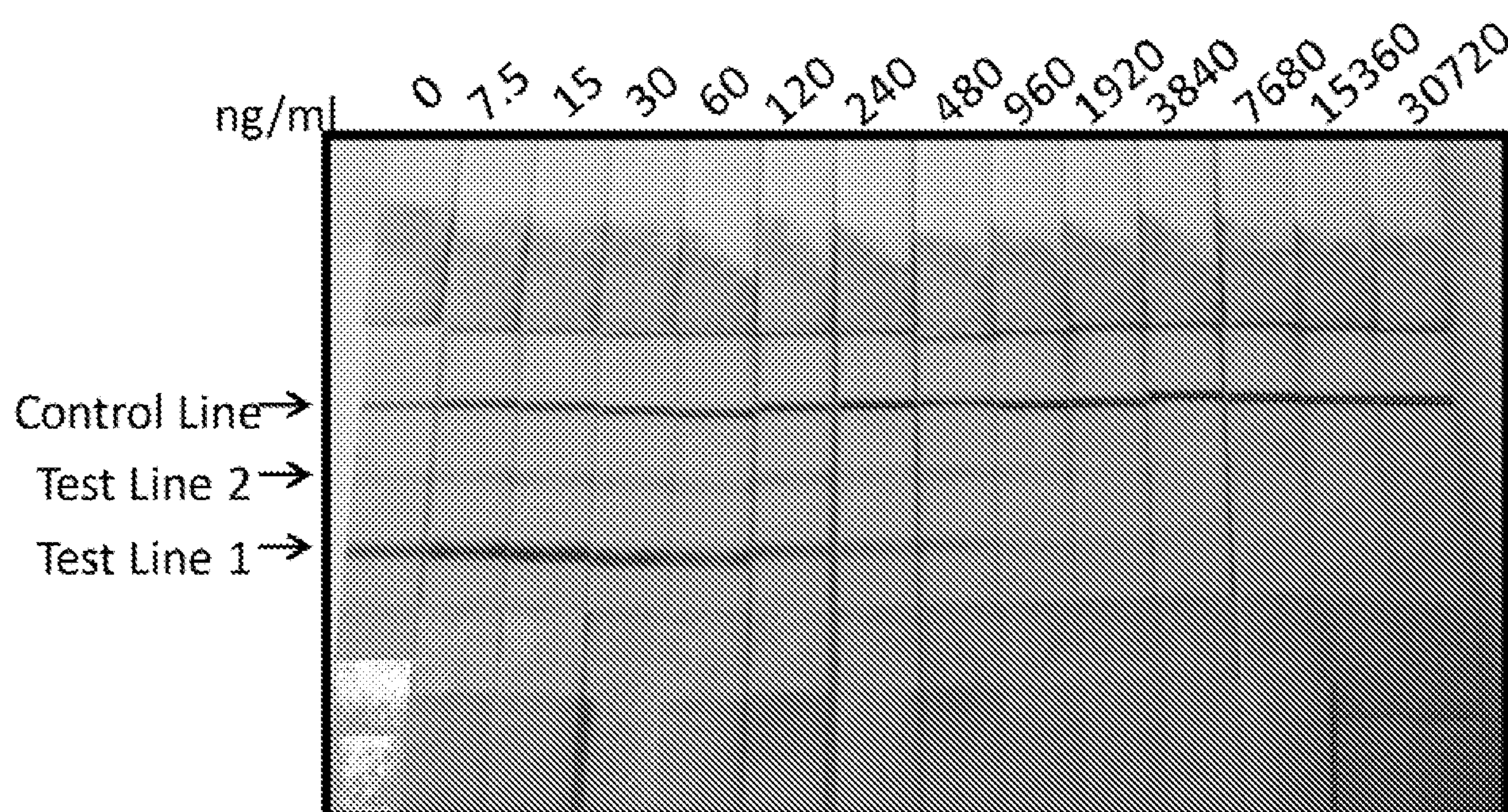
Enclosures: Replacement Sheets 39-47

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1/10**FIG. 1**

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**FIG. 2A****FIG. 2B****FIG. 2C**

3/10**FIG. 3**

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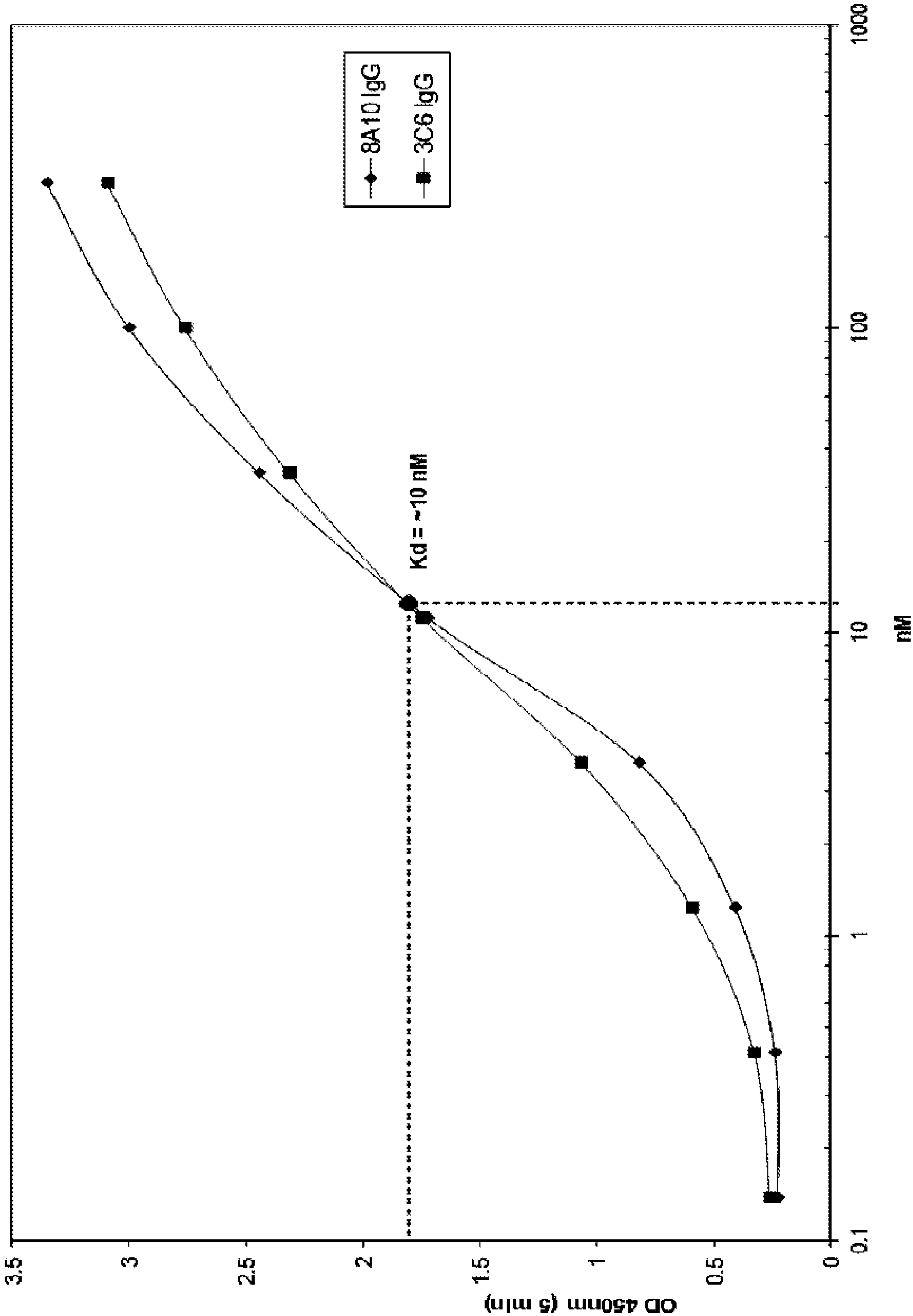
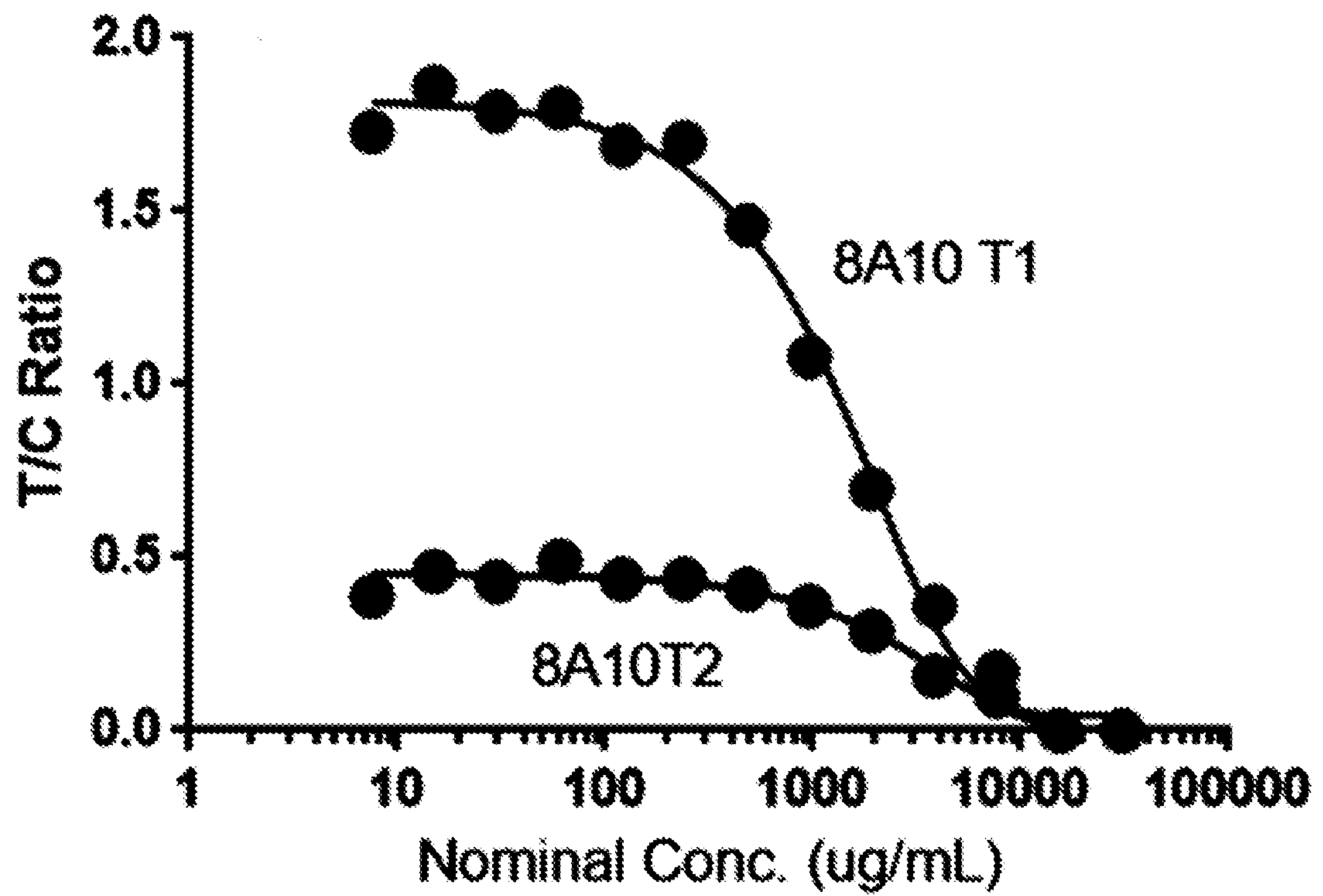


FIG. 4

5/10***FIG. 5A***

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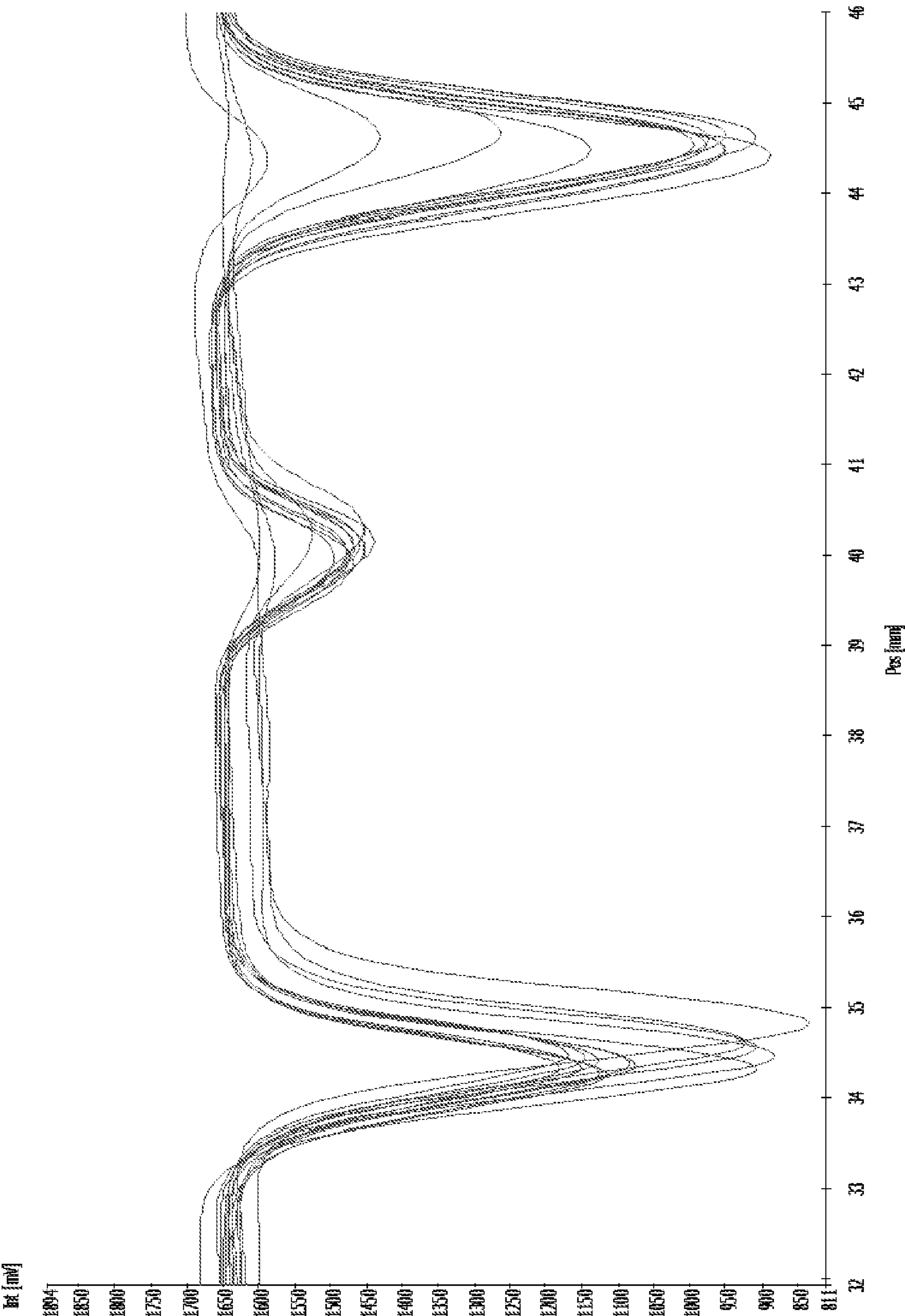
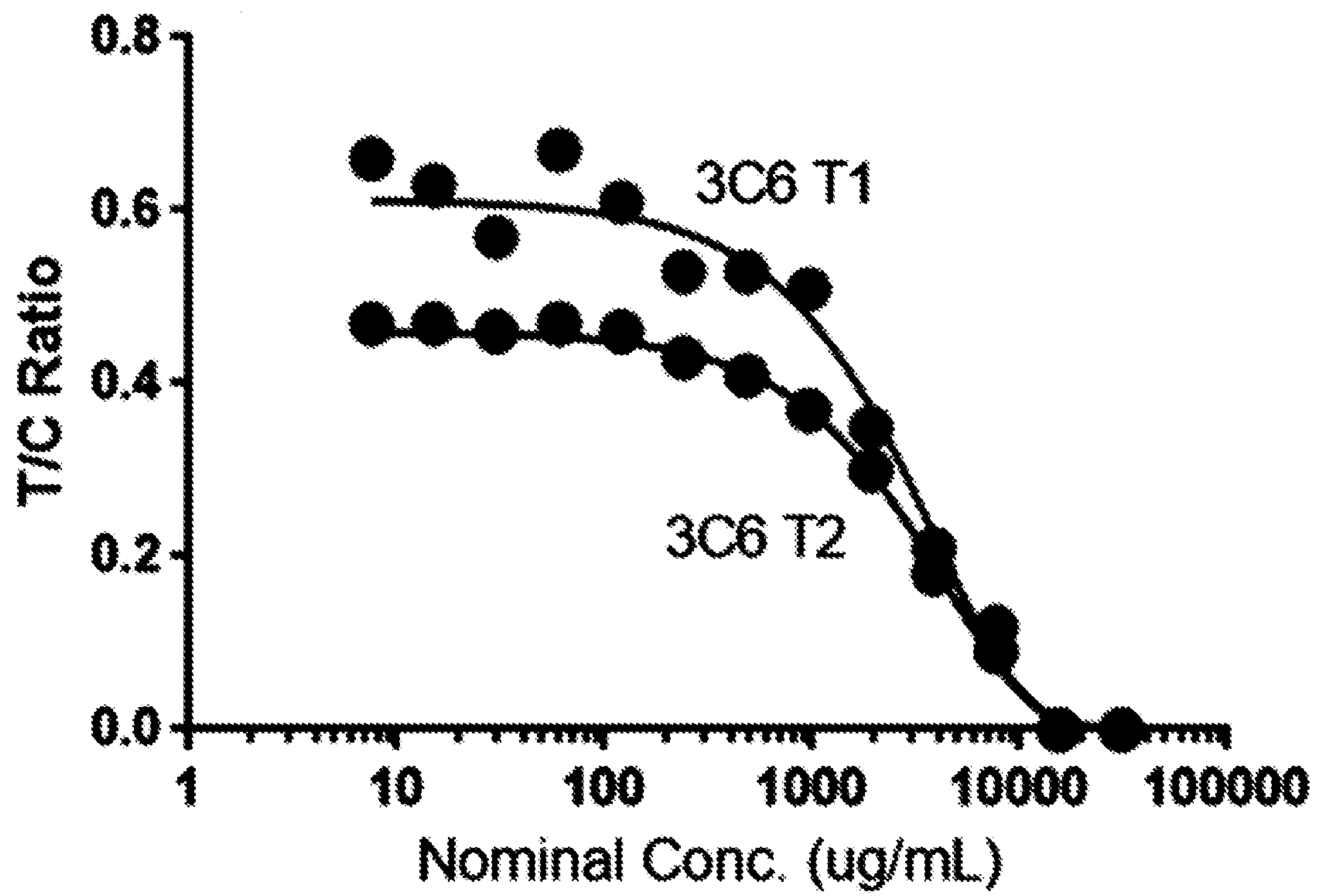


FIG. 5B

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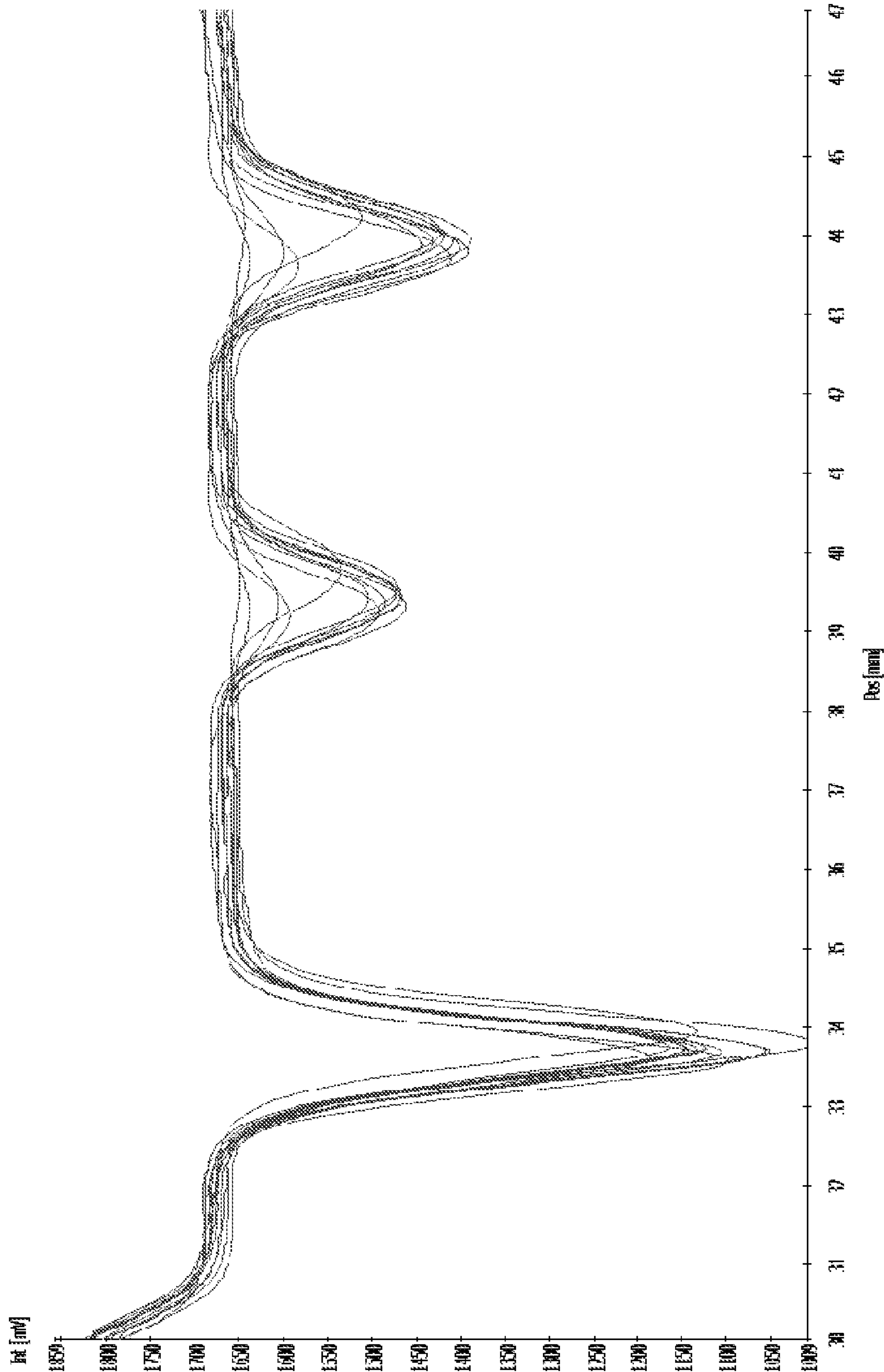
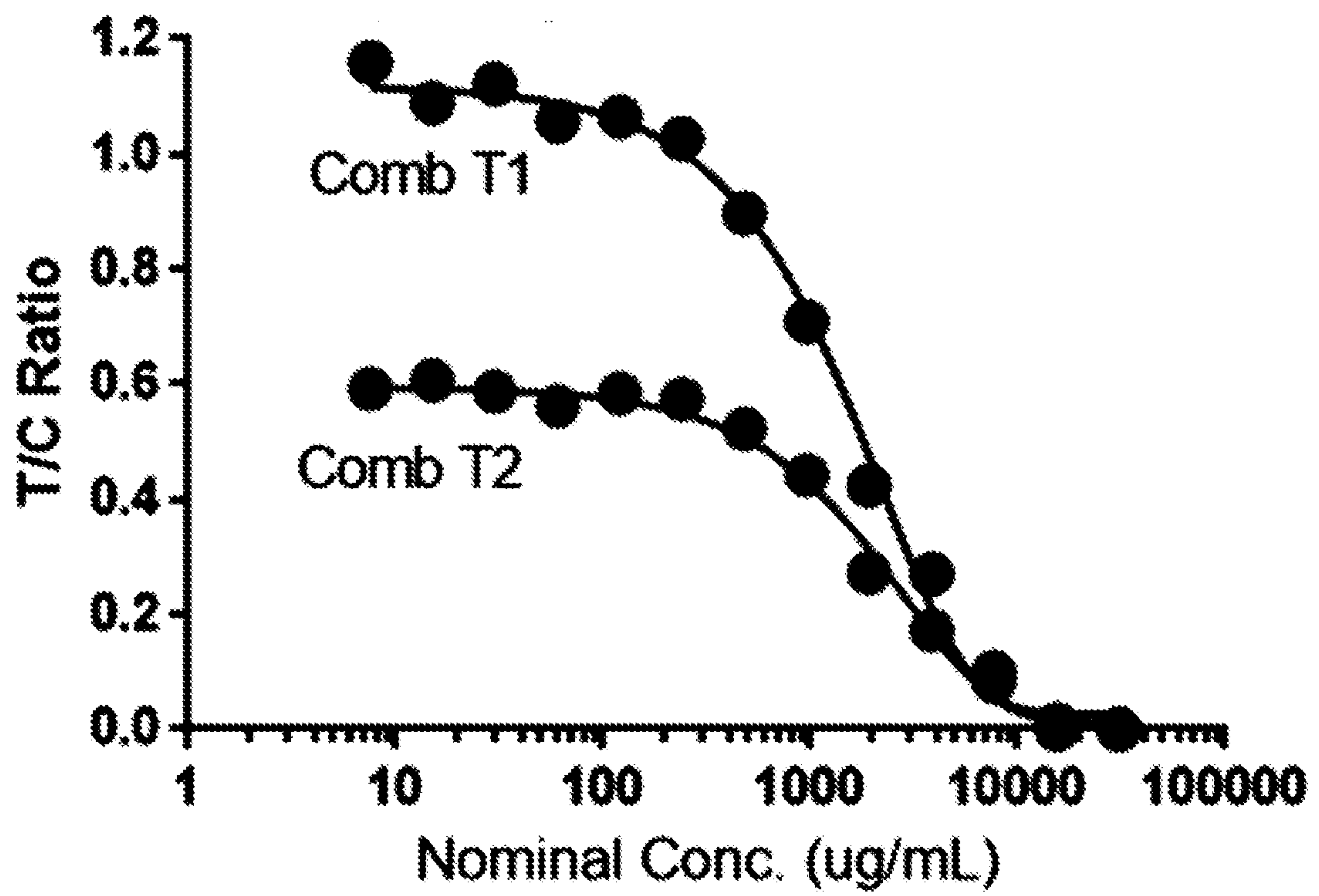


FIG. 6B

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**FIG. 7A**

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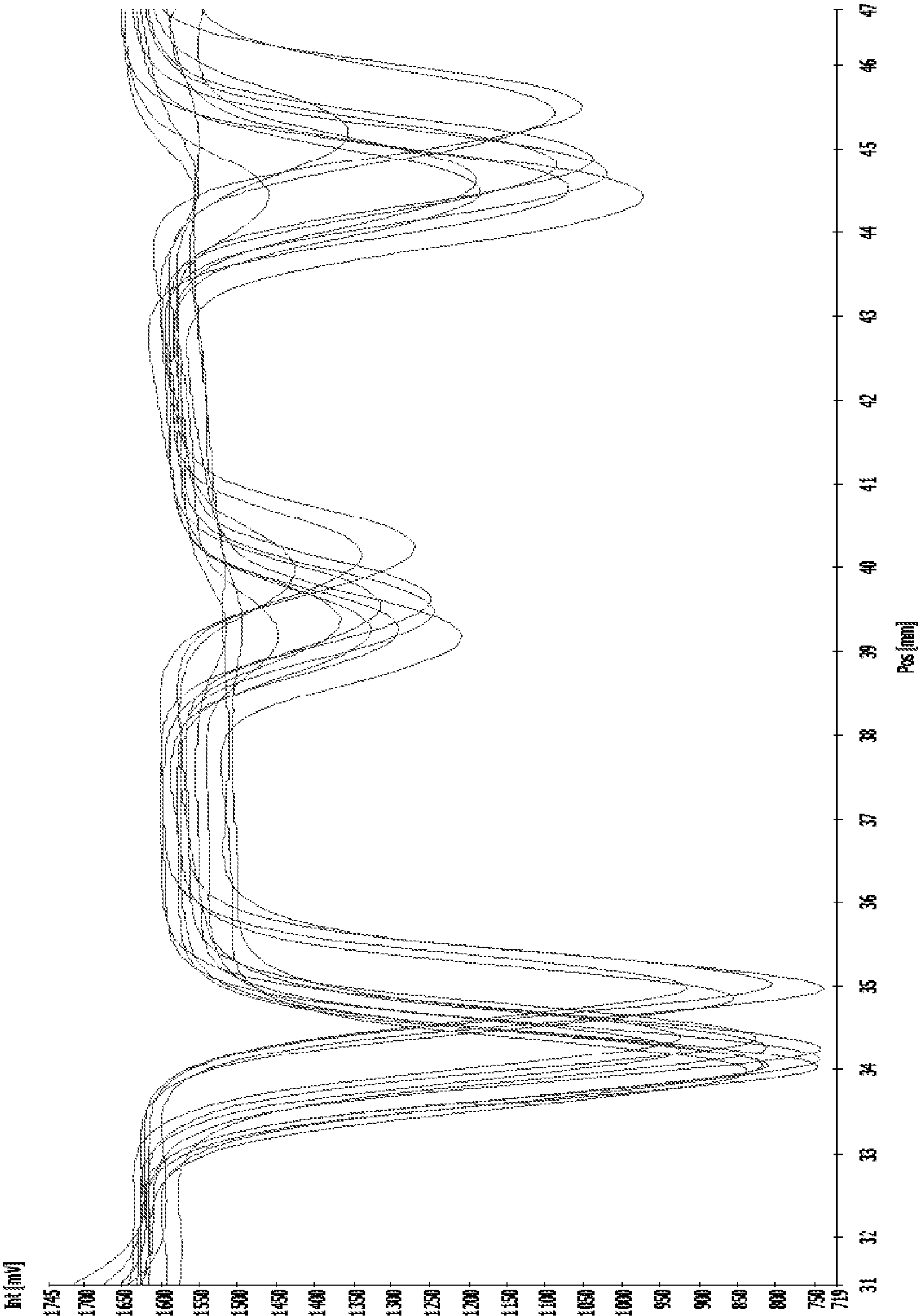


FIG. 7B

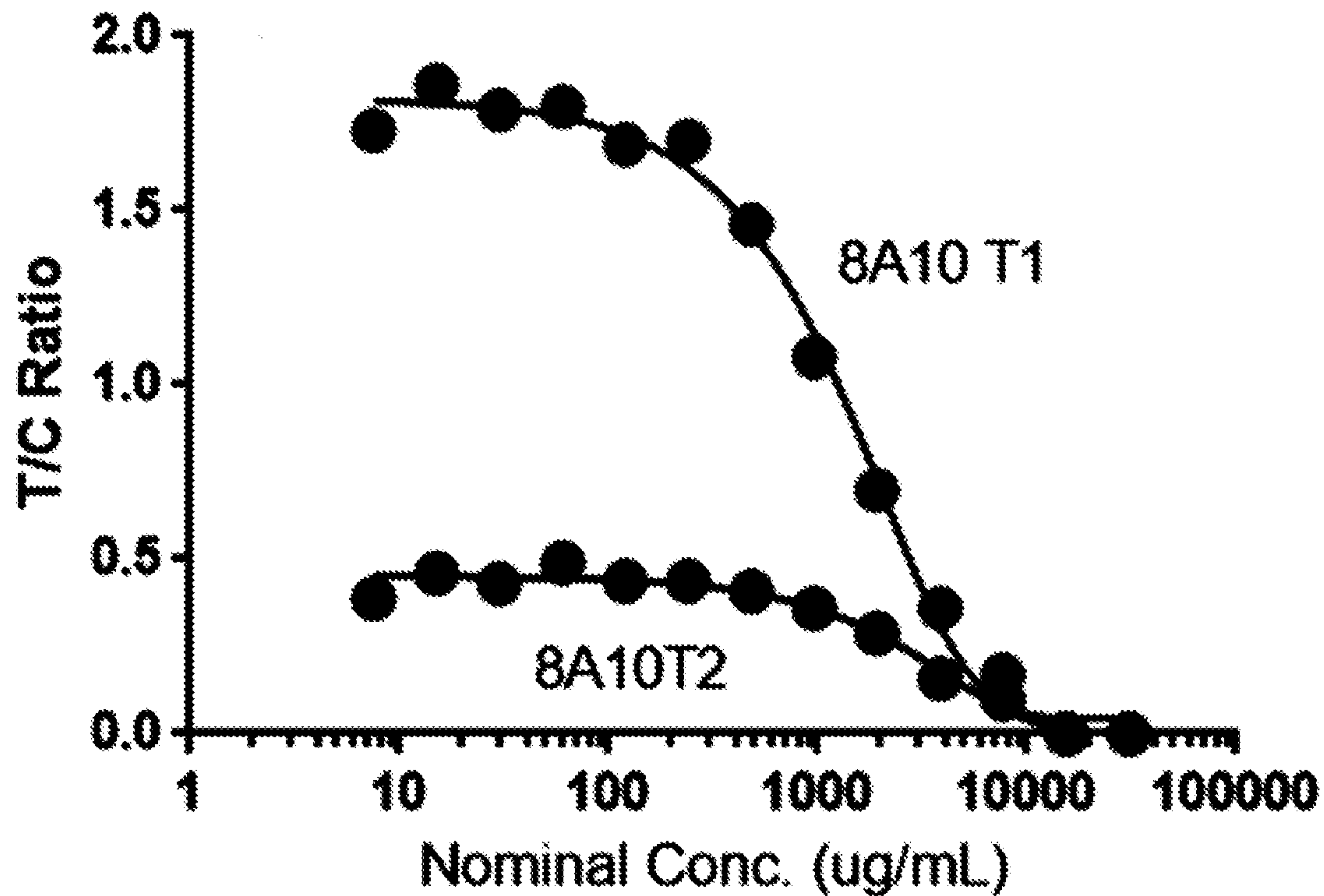


FIG. 5A