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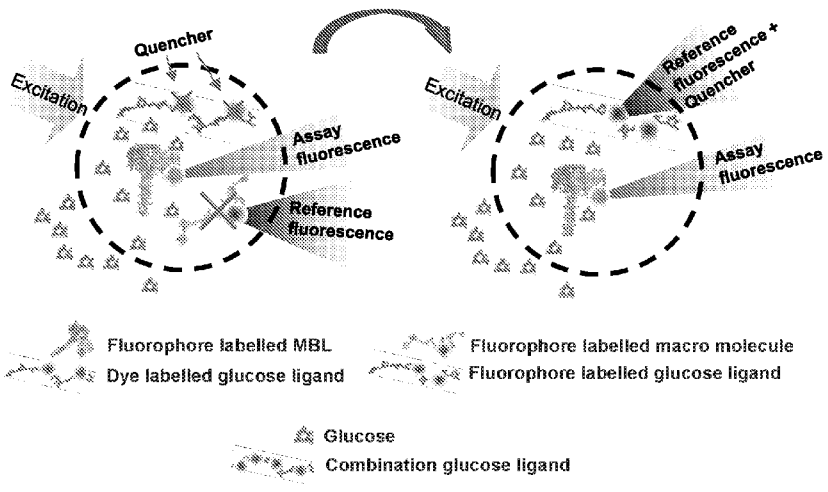


FIG. 1A

(57) Abstract: The invention is directed to a competitive glucose binding affinity assay comprising a glucose receptor (typically mannan binding lectin) labeled with an assay fluorophore and a modified glucose analog (typically dextran) labeled with a reference fluorophore. In certain embodiments, the glucose analog is dextran and is coupled to both a reference fluorophore and a quencher dye (e.g. hexamethoxy crystalviolet-1). Optionally the reference fluorophore is blue shifted relative to the assay fluorophore.

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USING A BLUE-SHIFTED REFERENCE DYE IN AN OPTICAL GLUCOSE ASSAY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under Section 120 from U.S. Patent Application Serial No. 15/581,540, filed April 28, 2017, the contents of which are incorporated herein by reference.

TECHNICAL FIELD

The invention relates to optical analyte assays, and in particular, fluorescent competitive binding assays for sensing glucose.

BACKGROUND OF THE INVENTION

Maintaining normal glucose levels *in vivo* is a crucial way that diabetic patients can avoid the long term problems associated with diabetes such as retinopathy, circulatory problems and other sequelae. For this reason, diabetic patients regularly monitor their blood glucose levels to, for example, optimize insulin dosing. In this context, a variety systems and methods have been developed for monitoring blood glucose levels. One strategy detects glucose levels using fluorescent compounds, for example with a competitive binding assay where glucose and fluorophore labeled glucose ligands/analogs compete for the binding site of glucose receptors and the resulting change in fluorescence is translated to a glucose concentration.

In certain competitive glucose binding assays, dextran is used as a displaceable glucose ligand. In such assays, dextran can be labeled with a lipophilic (and cationic) dyes such as hexamethoxy crystal violet-1 (HMCV1). However, the presence of a large number (e.g. greater than 10) of lipophilic dye molecules coupled to the flexible poly-(1,6)-glucose backbone of dextran can cause such labeled dextran molecules to adopt less water soluble conformations, which can result in the precipitation of these molecules. Moreover, such dye-induced conformal changes can

drive the labeled dextran into a more lipophilic state, which can cause adverse changes on the binding ability of dextran to the glucose receptor as well as affecting its Förster Resonance Energy Transfer (FRET) efficiency. In addition, in conventional systems, dyes can be shielded intra-molecularly on the dextran, a phenomenon which can result in the calibration of the assay changing over time, thereby introducing instability into the assay.

Reference dyes are also used in certain fluorescence assays in order to track of variations in the experimental setup, e.g. light source fluctuations, changes in the optical path (coupling light into light guides, mechanical perturbations like bending, temperature variations, etc.). Traditionally, optical or fluorescence-based sensing systems have utilized reference fluorophores that are red-shifted relative to the assaying fluorophore. However, by exciting fluorophores using light of a lower wavelength with more energy than needed, there is an increased risk that the electronic transition in the fluorescent molecule will occur from the electronic ground state (S0) to the second excited state (S2) and not to the first excited electronic state (S1). Molecules in S2 are much more likely to decompose than the same molecule in S1. Hence, faster photobleaching occurs if the fluorophores are excited to S2 instead of only S1.

Accordingly, there is a need in the art for optical glucose assays that utilize agents and materials selected to enhance assay stability. The invention disclosed herein meets this need for example, by using assays designed to include multi-labeled glucose analogs/ligands (e.g. dextran coupled/labelled with agents that promote hydrophilicity) and/or blue-shifted reference fluorophores. As discussed below, glucose assays designed to include the multi-labeled glucose analogs and/or blue-shifted reference fluorophores exhibit improvements in material properties such as assay stability.

SUMMARY OF THE INVENTION

The invention provides optimized materials and methods for use in glucose assays. As discussed in detail below, elements in certain fluorescent glucose assays can be selected and/or modified in order to, for example, optimize the hydrophilic-hydrophobic balance of the constellation of elements used to form the assay complex. In doing so, improved assays are generated, ones where fewer undesirable conformal changes to assay elements occur over time. Illustrative modifications to glucose assay complexes include those where dextran is modified by coupling this molecule with agents selected to prevent dextran from becoming unduly negatively or positively charged. Such modifications produce assays that are more stable than their unmodified equivalents. Additionally, in embodiments of where a reference fluorophore is selected for its hydrophilic/hydrophobic profile and/or to be blue-shifted relative to an assay or indicator fluorophore (instead of the typical red-shifted reference fluorophores that can become photo-labile due to low wavelength excitation), the stability of the reference fluorophore and the assay complex as a whole can be increased.

Embodiments of the invention comprise competitive glucose binding affinity assays, and methods for making and using these assays. Typically, the assay comprises a glucose receptor labeled with an assay fluorophore and a glucose analog labeled with both a reference fluorophore and a quencher dye. In embodiments of the invention, the glucose receptor can be selected from the group consisting of mannan binding lectin (MBL), Concanavalin A, glucose galactose binding protein, an antibody, and Boronic acid. In typical embodiments, the glucose receptor is mannan binding lectin. In an illustrative embodiment, the glucose analog is dextran, the assay fluorophore and reference fluorophore are Alexa Fluors™, and the quencher dye is hexamethoxy crystalviolet-1 (HMCV1). In some embodiments, the assay fluorophore and reference fluorophore are individually selected from the group consisting of Alexa Fluor™ 647 (AF647) and Alexa Fluor™ 700 (AF700), and the assay fluorophore and reference fluorophore are different. Typically, in such embodiments,

one or more elements of the complex (e.g. dextran) is coupled to or labelled with an agent selected to have a charge and/or hydrophilicity/hydrophobicity profile that contributes to the stability of the agent within the complex and consequently the glucose sensing complex as a whole. Embodiments of the invention also include methods for making and using these improved glucose assays.

In other embodiments of the invention, the assay comprises a glucose receptor labeled with an assay fluorophore and a glucose analog labeled with a reference fluorophore, wherein the reference fluorophore is blue shifted relative to the assay fluorophore. In some embodiments, the glucose analog is further labeled with a quencher dye (e.g. hexamethoxy crystalviolet-1, HMCV1). The glucose receptor may be selected from the group consisting of mannan binding lectin (MBL), Concanavalin A, glucose galactose binding protein, an antibody, and Boronic acid. In one instance, the glucose receptor is mannan binding lectin. Typically, the glucose analog is dextran and the assay fluorophore and reference fluorophore are Alexa Fluors™. Typically, the fluorophore and quencher dye form a Förster Resonance Energy Transfer (FRET) pair. The fluorophore and/or quencher dye is also typically water soluble. In a typical embodiment, the assay fluorophore and reference fluorophore are individually selected from the group consisting of Alexa Fluor 594 (AF594), Alexa Fluor 647 (AF647), and Alexa Fluor 700 (AF700), wherein the reference fluorophore has a shorter wavelength than the assay fluorophore.

Other objects, features and advantages of the present invention will become apparent to those skilled in the art from the following detailed description. It is to be understood, however, that the detailed description and specific examples, while indicating some embodiments of the present invention, are given by way of illustration and not limitation. Many changes and modifications within the scope of the present invention may be made without departing from the spirit thereof, and the invention includes all such modifications.

BRIEF DESCRIPTION OF THE DRAWINGS

Referring now to the drawings in which like reference numbers represent corresponding parts throughout:

Figures 1A-C illustrate a PreciSense™ Optical with a fluorescing ligand, in accordance with one or more embodiments of the invention. Figure 1A illustrates the quencher being changed from a dye to a fluorophore and omitting the reference fluorophore. Figure 1B is a graph illustrating the changes in absorbance and emission spectra. Figure 1C is a graph illustrating glucose concentration measurements and sensor dose response loss (DR loss).

Figures 2A-C illustrate a PreciSense™ Optical with a combination ligand, in accordance with one or more embodiments of the invention. Figure 2A illustrates the reference fluorophore labeled onto the same ligand as the dye quencher, thereby eliminating the need for a reference carrier. Figure 2B is a graph illustrating the changes in absorbance and emission spectra. Figure 2C is a graph illustrating glucose concentration measurements and sensor dose response loss (DR loss).

Figure 3 is a graph illustrating ORS SITS data for an AF594-MBL/AF647-dex assay, in accordance with one or more embodiments of the invention.

Figure 4 is a graph illustrating ORS SITS data for an AF594-MBL/AF647-HMCV1-dex assay, in accordance with one or more embodiments of the invention. The same is used for Rat trial 76 and 77 as shown in Figures 5-7 below.

Figure 5 is a graph illustrating data from Rat study 76: Rat #1, ORS sensor #2, in accordance with one or more embodiments of the invention. Sensor calibrated during low-high transition, first clamp.

Figures 6A-D illustrate data from Rat study 76: Rat #1, ORS sensor #2 for clamp 1 (Figure 6A), clamp 2 (Figure 6B), and clamp 3 (Figure 6C), in accordance with one or more embodiments of the invention. Figure 6D is a picture of an explant.

Figures 7A-B illustrate data from Rat study 77, in accordance with one or more embodiments of the invention. Figure 7A illustrates the baselines of assay and

reference fluorophores. Note the stable assay and reference baselines. Figure 7B illustrates glucose concentration measurements from Rat study 77.

Figure 8 is a graph illustrating data from an AF594/AF647 assay, in accordance with one or more embodiments of the invention.

Figure 9 is a graph illustrating data from AF594MBL/AF647-Dex and AF594/AF647-HMCV1-Dex assays: optimum ratio between AF594 and AF647 fluorescence, in accordance with one or more embodiments of the invention.

Figure 10 is a graph illustrating data from filter configuration for AF594-MBL/647-Dex and AF594-MBL/AF647-HMCV1-Dex assays, in accordance with one or more embodiments of the invention.

Figure 11 illustrates the filter configuration for an AF594/647 assay, in accordance with one or more embodiments of the invention.

Figures 12A-C provide examples of degree of labeling (DOL) determination for multi-labeled dextran (MLD), in accordance with one or more embodiments of the invention. Figure 12A is a graph illustrating the normalized absorbance spectra for HMCV1 and AF647. Figure 12B is a graph illustrating the absorbance spectra of conjugates #478 (HMCV1 X5 and AF647 X15) and #479 (HMCV1 X15 and AF647 X5). Figure 12C is a graph illustrating conjugate 479 (HMCV1 X15 and AF647 X5) spectra and linear combination of the HMCV1 and AF647 abs spectra. The DOL of MLD conjugates carrying HMCV1 and AF647 include: DOL #472 (X10/X10) = 5.7 / 4.1; DOL #478 (X5/X15) = 3.6 / 9.4; and DOL #479 (X15/X5) = 8.1 / 2.1.

Figure 13 is a graph illustrating single-labeled dextran performance, in accordance with one or more embodiments of the invention. The graph shows Dose Response (DR) development for five groups of sensors all build using a single labeled dextran in the glucose responding assay. DR is calculated as the difference between the normalized intensity at 400mg/dL glucose and 40mg/dL glucose relative to the normalized intensity at 40mg/dL glucose. The DR loss relative to the start DR is between 3% to 6% per day. Dextrans labeled with HMCV1 only exhibits large Dose Response (DR) loss.

Figure 14 is a graph illustrating multi-labeled dextran performance HMCV1-AF647-Dextran, in accordance with one or more embodiments of the invention.

Figure 15 is a graph illustrating multi-labeled dextran performance HMCV1-AF700-Dextran, in accordance with one or more embodiments of the invention.

Figure 16 is a graph illustrating multi-labeled dextran performance HMCV1-HMCV3-Dextran, in accordance with one or more embodiments of the invention.

Figure 17 is a graph illustrating multi-labeled dextran performance HMCV1- Dextran succinylated, in accordance with one or more embodiments of the invention.

Figure 18 is a graph illustrating normalized absorption and emission spectra of different dyes and fluorophores in a capsule sensor, in accordance with one or more embodiments of the invention.

Figure 19 is a table illustrating that a combination ligand with different DOL values, their DR, and intensity levels in capsule sensors, in accordance with one or more embodiments of the invention.

DETAILED DESCRIPTION OF THE INVENTION

Unless otherwise defined, all terms of art, notations and other scientific terms or terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this invention pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art. Many of the techniques and procedures described or referenced herein are well understood and commonly employed using conventional methodology by those skilled in the art. All publications, patents, and patent applications cited herein are

hereby incorporated by reference in their entirety for all purposes. In the description of a typical embodiment, reference may be made to the accompanying drawings which form a part hereof, and in which is shown by way of illustration a specific embodiment in which the invention may be practiced. It is to be understood that other embodiments may be utilized and structural changes may be made without departing from the scope of the present invention.

Blood glucose is commonly monitored by diabetic patients with the use of commercially available calorimetric test strips or electrochemical biosensors (e.g. enzyme electrodes), both of which require the regular use of a lancet-type instrument to withdraw a suitable amount of blood each time a measurement is made. This imposes a considerable burden on the diabetic patient, both in financial terms and in terms of pain and discomfort, particularly in the long-term diabetic who has to make regular use of a lancet to draw blood from the fingertips. Thus, there have been a number of proposals for glucose measurement techniques that do not require blood to be withdrawn from the patient. It has been observed that the concentration of analytes in subcutaneous fluid correlates with the concentration of said analytes in the blood, and consequently there have been several reports of the use of glucose monitoring devices which are sited in a subcutaneous location. The use of competitive binding assays for glucose which can be remotely interrogated is of particular interest.

A typical method of assaying a competitive binding is to use a proximity-based signal generating/modulating moiety pair (see, e.g. U.S. Pat. No. 6,232,120), which is typically an energy transfer donor-acceptor pair (comprising an energy donor moiety and an energy acceptor moiety). The energy donor moiety is photoluminescent (usually fluorescent). In such methods, an energy transfer donor-acceptor pair is brought into contact with the sample (such as subcutaneous fluid) to be analyzed. The sample is then illuminated and the resultant emission detected. Either the energy donor moiety or the energy acceptor moiety of the donor-acceptor pair is bound to a receptor carrier, while the other part of the donor-acceptor pair

(bound to a ligand carrier) and any analyte present compete for binding sites on the receptor carrier. Energy transfer occurs between the donors and the acceptors when they are brought together, which produces a detectable lifetime change (reduction) of the fluorescence of the energy donor moiety. Also, a proportion of the fluorescent signal emitted by the energy donor moiety is quenched. The lifetime change is reduced or even eliminated by the competitive binding of the analyte. Thus, by measuring the apparent luminescence lifetime, for example, by phase-modulation fluorometry or time-resolved fluorometry (see Lakowicz, Principles of Fluorescence Spectroscopy, Plenum Press, 1983, Chapter 3), the amount of analyte in the sample can be determined.

In addition to the lifetime of the excited state, the intensity of the emitted fluorescence also correlates to the glucose concentration. In contrast to a lifetime measurement, the measured intensity of the emitted fluorescence is affected by the intensity of the light source and the coupling between the assay and the optical system. Therefore, the intensity measurement requires an internal reference fluorophore to be incorporated into the assay. The reference fluorophore must differ from the assay fluorophore in a way that the emitted fluorescence from the assay and that from the reference may be separated from one another, e.g., by having different absorption spectra or emission spectra. The reference fluorophore may be, e.g., Alexa Fluor™ 594 (AF594) labeled onto Human Serum Albumin (HSA) or another macro molecule, which largely does not bind to the glucose receptor. Alexa Fluor™ 700 (AF700) may be excited simultaneously with the AF594 as their absorption spectra spectrally overlap. The emission spectrum from AF594 is slightly blue-shifted with respect to AF700, which makes it possible to detect their respective fluorescence emissions in separate wavelength regions. As they are excited simultaneously by the same light source, any changes in the intensity of the light source will scale fluorescence from AF594 and AF700 equally. As such, any effect originating from changes in the intensity of the light source may be canceled out. The excitation, as well as the detection, of the emitted fluorescence for the assay and the reference follow the same

optical path from the optical system to the assay. As such, the detected signal from the reference serves as a measure for the optical coupling between the optical interrogating system and the assay. Any effect originating from changes in the optical coupling such as alignment may be canceled out.

A special property of fluorescence known as Förster resonance energy transfer (FRET) occurs when the energy of the excited electron of one fluorophore (i.e. donor), passes onto a nearby acceptor dye, either a quencher (non-emitting chromophore) or another fluorophore, which has an excitation spectrum that overlaps with the emission spectrum of the donor. Energy transfer occurs without the appearance of a photon and is the result of long-range dipole-dipole interactions between the donor and acceptor. An important characteristic of FRET is that it occurs over distances comparable to the dimensions of biological macromolecules. The distance at which FRET is 50% efficient, called the Förster distance, is typically in the range of 20-60 Å. Förster distances ranging from 20 to 90 Å are convenient for competitive binding studies. WO91/09312 describes a subcutaneous method and device that employs an affinity assay based on glucose (incorporating an energy transfer donor-acceptor pair) that is interrogated remotely by optical means. Examples WO1997/19188, WO2000/02048, WO2003/006992 and WO2002/30275 each describe glucose sensing by energy transfer, which produce an optical signal that can be read remotely.

The invention disclosed herein is generally directed towards optical or fluorescence-based assays and analyte sensing compositions. In illustrative embodiments of the invention. the assays, compositions, systems, and methods of the invention are described with reference to glucose as the analyte whose level/concentration is to be determined. However, this is by way of illustration and not limitation, as the principles, devices, systems, and methods of the present invention may be used for sensing and/or determining the level of a variety of other physiological parameters, agents, characteristics, and/or compositions.

As described herein, embodiments of the invention provide sensors designed to include compositions disposed in specific areas of the sensor in order to provide the

sensors with enhanced functional and/or material properties. The disclosure further provides methods for making and using such sensors. Embodiments of the invention described herein such as those discussed in the paragraph immediately below can be adapted to and implemented with a wide variety of elements in sensors having sensing complexes that generate an optical signal that can be correlated with the concentration of an analyte such as glucose. A number of these sensors and elements are disclosed, for example in U.S. Patent Nos. 6,6761,527, 7,228,159, 7,884,338, 7,567,347, 8,305,580 and 8,691,517, and U.S. Patent Application Publication Nos. 2008/0188723, 2009/0221891, 2009/018708, 2009/0131773, 2013/0060105 and 2014/0200336 the contents of each of which are incorporated herein by reference.

The invention disclosed herein have a number of embodiments. An illustrative embodiment is a glucose sensing complex comprising mannan binging ligand coupled to an assay fluorophore, a reference fluorophore (e.g. Alexa Fluor 647 (AF647) or Alexa Fluor 700 (AF700)); dextran that functions in the assay as a glucose analog and is coupled to the reference fluorophore, an agent that enhances the hydrophilicity of the dextran, and a quenching agent (e.g. hexamethoxy crystal violet-1 (HMCV1)). In this embodiment, the assay fluorophore and quenching agent form a Förster Resonance Energy Transfer (FRET) pair. Typically, the fluorophores and/or quencher dye are water soluble. Optionally, the Degree of Labeling (DOL) of the fluorophore is at least 4.1 and the DOL of the quencher dye is at least 5.7. In some embodiments of the invention, the agent that enhances the hydrophilicity of the dextran is the quenching agent. Optionally, the dextran is modified by an anhydride compound (e.g. so as to couple the dextran to a moiety that modulates the hydrophilicity of the dextran). Typically, the dextran comprises less than 1500 glucose units. In some embodiments of the invention, the glucose sensing assay complex exhibits a sensor dose response (DR) loss of less than 2.5% per day.

Embodiments of the invention can comprise a competitive glucose binding affinity assay comprising a glucose receptor labeled with an assay fluorophore, and a glucose analog labeled with both a reference fluorophore (e.g. Alexa Fluor 647

(AF647) or Alexa Fluor 700 (AF700)) and a quencher dye. In certain embodiments of the invention, the glucose receptor is selected from the group consisting of mannan binding lectin (MBL), Concanavalin A, glucose galactose binding protein, an antibody, and Boronic acid, the glucose analog is dextran, the assay fluorophore and reference fluorophore are Alexa Fluors, and the quencher dye is a hexamethoxy crystal violet compound. In typical embodiments, the dextran is coupled to or modified by a compound that enhances its hydrophilicity.

Another embodiment of the method of sensing glucose in a solution (e.g. interstitial fluid or blood) comprising contacting the solution with a glucose sensing complex comprising mannan binging ligand coupled to an assay fluorophore, a reference fluorophore, dextran selected to function in the assay as a glucose analog and which is further coupled to the reference fluorophore. This complex further comprises an agent that enhances the hydrophilicity of the dextran, and a quenching agent. Optionally, the agent that enhances the hydrophilicity of the dextran is the quenching agent. In this embodiment, the assay fluorophore and quenching agent form a Förster Resonance Energy Transfer (FRET) pair. The method includes observing the glucose sensing complex for signals indicative of the presence of glucose, and then correlating observed signals with the concentration of glucose.

Yet another embodiment of the invention is a glucose sensing complex comprising a glucose binding agent (e.g. mannan binding lectin) coupled to an assay fluorophore and a glucose analog coupled to a reference fluorophore. In this embodiment, the reference fluorophore selected for use in this complex is blue shifted relative to the assay fluorophore. Typically, the reference fluorophore has a lower wavelength than the assay fluorophore. Optionally, the assay fluorophore exhibits a wavelength that is at least 50 nanometers greater than the wavelength of the reference fluorophore. Optionally, the glucose analog is further coupled to a quenching agent (e.g. a hexamethoxy crystal violet compound). In certain embodiments, the glucose receptor is selected from the group consisting of mannan binding lectin (MBL), Concanavalin A, glucose galactose binding protein, an antibody, and Boronic acid;

and/or the glucose analog is dextran (e.g. dextran having a molecular weight between 90kDa-110kDa). In certain embodiments, the assay fluorophore and reference fluorophore are individually selected from the group consisting of Alexa Fluor 594 (AF594), Alexa Fluor 647 (AF647), and Alexa Fluor 700 (AF700). Typically, the reference fluorophore and quencher agent are water soluble, form a Förster Resonance Energy Transfer (FRET) pair.

Another embodiment of the invention is a method of making a glucose sensing complex comprising forming a glucose sensing complex by coupling a glucose binding agent (e.g. mannan binding lectin (MBL)) to an assay fluorophore and coupling a glucose analog (e.g. dextran) to a reference fluorophore. In this embodiment, the reference fluorophore and the assay fluorophore are selected so that reference fluorophore emits light at a wavelength that is blue shifted relative to light emitted by the assay fluorophore. Typically, the assay fluorophore exhibits a wavelength that is at least 50 nanometers greater than the wavelength of the reference fluorophore. Typically, the assay fluorophore exhibits a wavelength that is not more than 100 nanometers from the wavelength of the reference fluorophore. In certain instances, the glucose analog is further treated with succinic acid anhydride. Typically, the dextran is approximately 100kDa. In certain embodiments, the composition exhibits a sensor dose response (DR) loss of less than 2.5% per day. In one instance, the Degree of Labeling (DOL) of the fluorophore is at least 4.1 and the DOL of the quencher dye is at least 5.7.

Another embodiment of the invention is a method of sensing glucose in a solution (e.g. interstitial fluid or blood) comprising contacting the solution with a glucose sensing complex having a glucose binding agent coupled to an assay fluorophore, and a glucose analog coupled to a reference fluorophore, where the reference fluorophore is selected to be blue shifted relative to the assay fluorophore. This method comprises observing the glucose sensing complex for signals indicative of the presence of glucose, and correlating observed signals with the concentration of glucose. Optionally the method comprises exciting the reference fluorophore and the

assay fluorophore with two different light sources. Typically, the assay fluorophore exhibits a wavelength that is not more than 100 nanometers from the wavelength of the reference fluorophore and the assay fluorophore exhibits a wavelength that is at least 50 nanometers greater than the wavelength of the reference fluorophore.

There are a variety of permutations of the invention. As described herein, the analyte receptor is typically a lectin, which includes any carbohydrate-binding protein. In typical embodiments, the glucose receptor is a fluorophore labeled mannan binding lectin (MBL, also called mannose/mannan binding protein, Sheriff et al., Structural Biology, 1:789-794 (1994); Dumestre-Perard et al., Molecular Immunology, 39:465-473 (2002)). Typically, the lectin provides a stable signal in the assay for at least 10 days, more typically for at least 14 days. It is particularly preferable that a stable signal is provided when the sensor is implanted in the human body. Surprisingly, MBL has been found to be stable in a glucose assay for at least 17 days.

Other analyte receptors or analyte binding moieties may be used instead in the assays and sensor systems described herein. For example, the analyte receptor may be a human lectin derived from the human body, including human pulmonary surfactant protein A (SP-A, Allen, et al., Infection and Immunity, 67:4563-4569 (1999)), human pulmonary surfactant protein D (SP-D, Persson et al., The Journal of Biological Chemistry, 265:5755-5760 (1990)) or CL-43 (a human serum protein). Alternatively, the lectin may be a recombinantly manufactured lectin or a humanized animal lectin, for example a humanized bovine lectin. The lectin may alternatively be an animal lectin, bird lectin, fish lectin, vertebrate lectin, invertebrate lectin (e.g. insect lectin) or plant lectin. Suitable animal lectins include conglutinin, collectin-43 (e.g. bovine CL-43), pulmonary surfactant proteins (lung collectins), PC-lectin (US 2003/0216300, US 2004/0265898), CTL-1 (US 2010/179528), Keratinocyte membrane lectins (Parfuemerie und Kosmetik 74, 164-80), CD94 (Eur J Immunol 25, 2433-7), P35 (synonym: human L-ficolin, a group of lectins) (Immunol Lett 67, 109-12), ERGIC-53 (synonym: MR60) (Mol Biol Cell, 7, 483-93), HIP/PAP (Eur J Biochem 267, 1665-71), CLECSF8 (Eur J Immunol 34, 210-20), DCL (group of

lectins) (Appl no 00231996/US), and GLUT family proteins, especially GLUT1, GLUT4 and GLUT11 (PNAS 97, 1125-30). Further suitable animal lectins are set out in Appendices A, B, and C of "Handbook of Animal Lectins: Properties and Biomedical Applications", David C. Kilpatrick, Wiley 2000. Suitable plant lectins or phytohemoagglutinins (PHA's) include concanavalin A (Con A) and those derived from pisum sativum (pea), lathyrus odoratus (sweet pea), lens culinaris (lentil), narcissus pseudonarcissus (daffodil), vicia faba (fava bean), and vicia sativa (garden vetch). The analyte receptor may also be a periplasmic glucose/galactose-binding receptor, antibody raised against glucose-like molecules or boronic acid.

As described herein, the analyte analog can comprise a plurality of carbohydrate or carbohydrate mimetic moieties which bind to the binding sites of the analyte receptor. The analyte analogue should have a molecular weight high enough to prevent escape from the sensor but low enough that precipitation does not occur when the analyte analog binds to the analyte receptor. The analyte analog may have a weight in the range of 25 to 250 kDa and more typically between 90 to 120 kDa. In typical embodiments where glucose is the analyte, dextran is used as the displaceable glucose analog/ligand. Dextran is a flexible macromolecule consisting of up to 1500 glucose units. In certain instances, dextran consists of approximately 600 glucose units (~100 kDa), or consists of between 500-700 glucose units.

Other analyte analogs and ligands may be used instead in the illustrative assays and sensor systems described herein. The analyte analog may be a synthetic polymer which bears different carbohydrate or carbohydrate mimetic moieties of different affinity for MBL and similar lectins. Alternatively, the analyte analog may be a carbohydrate-protein conjugate or a carbohydrate-dendrimer conjugate. Examples of suitable carbohydrates for use in such conjugates are monosaccharides and oligosaccharides. Suitable monosaccharides are optionally derivatized tetroses, pentoses, hexoses, heptoses or higher homologous aldoses or ketoses, for example optionally derivatised D-glucose, D-mannose, N-acetyl-D-glucosamine, L-fucose, D-fructose, D-tagatose or D-sorbitol. Suitable oligomers may be linear or branched

homooligomers or mixed oligomers, for example containing from 2 to 50 carbohydrate units.

As described herein, fluorophores or fluorochromes are chemical compounds that are able to absorb light energy of a specific wavelength and re-emit light energy at a longer wavelength. Fluorophores can also be used to quench the fluorescence of other fluorescent dyes or to relay their fluorescence at even longer wavelengths (FRET). Typically, Alexa Fluor™ (AF) 594, 647 and/or 700 are used as reference and assay fluorophores for respectively labeling the glucose analog and glucose receptor. Those skilled in the art understand that other fluorophores suitable for optical glucose assays may also be used instead, for example, coumarin, rhodamine, xanthene, cyanine, and Alexa Fluor dyes that cover other excitation and emission wavelengths (e.g. AF350, AF405, AF488, AF532, AF546, AF555, AF568, AF594, AF680, AF750).

An energy acceptor which does not emit fluorescence is referred to as a quenching moiety. The HMCV dyes described in WO05/059037 are suitable energy acceptor moieties for use in the invention. These dyes are stabilized carbenium ions. In typical embodiments, hexamethoxy crystal violet-1 (HMCV1) is used as the quencher/acceptor dye. Alternatively, QSY 21 may be used as an energy acceptor moiety with AF594 as an energy donor moiety.

The binding assay generating the optical signal should typically be reversible such that a continuous monitoring of fluctuating levels of analyte can be achieved. This reversibility is a particular advantage of the use of a binding assay format in which the components of the assay are not consumed. Typically, the sensor is suitable for the detection or measurement of glucose in body fluid, for example subcutaneous fluid. It is desirable for the sensor to be suitable for use in vivo. Typically, the assay is capable of measuring blood glucose for concentrations over at least part of the range of 0 to 35 mM glucose, typically over the range of 2 to 10 mM glucose. Suitable detection techniques include FRET, fluorescence energy transfer, fluorescence polarization, fluorescence quenching, phosphorescence, luminescence

enhancement, luminescence quenching, diffraction or plasmon resonance. Typically, the sensor of the invention incorporates an assay which generates an optical readout using the technique of FRET.

As discussed above, there is a need in the art for optical or fluorescence-based assays with enhanced stability and that require a lower calibration frequency of the optical sensor. In one aspect of the invention, an analyte sensing composition with significantly improved stability and solubility is provided. The analyte sensing composition comprises an analyte analog labeled with both a fluorophore and a quencher dye. In typical embodiments, the analyte sensing composition is a glucose sensing composition comprising a multi-labeled glucose analog (e.g. dextran) labeled with both a fluorophore (e.g. Alexa Fluor™ 647, Alexa Fluor™ 700) and a quencher dye (e.g. hexamethoxy crystal violet-1, HMCV1).

In another aspect of the invention, a competitive analyte binding affinity assay based on the analyte sensing composition is provided. The competitive analyte binding affinity assay comprises an analyte receptor labeled with an assay fluorophore and an analyte analog labeled with both a reference fluorophore and a quencher dye. In certain instances, the reference fluorophore is blue-shifted relative to the assay or indicator fluorophore, which improves the stability of the assay, and more specifically the reference fluorophore. In typical embodiments, the competitive analyte binding affinity assay is a competitive glucose binding affinity assay comprising a glucose receptor/lectin (e.g. mannan binding lectin, MBL) labeled with an assay fluorophore (Alexa Fluor™ 647, AF647) and a multi-labeled glucose analog (e.g. dextran) labeled with both a reference fluorophore (e.g. Alexa Fluor™ 594, AF594) and a quencher dye (e.g. HMCV1).

In embodiments of the invention, the binding between the MBL and glucose-like molecules (e.g., dextran) is reversible. When no glucose is present, MBL and dextran will predominantly be bound together. When glucose is added to the assay, it will compete off a part of the dextran population, such that the assay enters a new equilibrium state. The equilibrium state at all times corresponds to the glucose

concentration. In order to determine this equilibrium state, MBL is labeled with a fluorophore (e.g. AF647, AF700) and the dextran is multi-labeled with a quencher dye (e.g. HMCV1) and a reference fluorophore (e.g. AF594). The donor assay fluorophore and the acceptor quencher dye together form a Förster Resonance Energy Transfer (FRET) pair— i.e., the emission spectrum of the assay fluorophore and the absorption spectrum of the quencher dye overlap. It is noted that the fluorophore and dye are typically water soluble, as they are to function in an aqueous environment.

Multi-Labeled Analyte Analogs

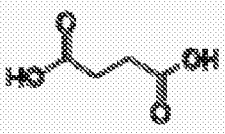
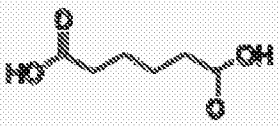
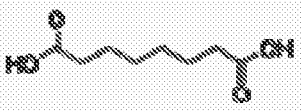
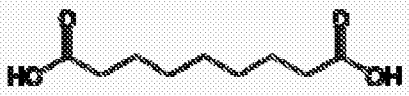
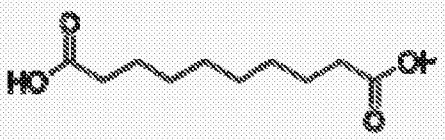
Embodiments of the invention include glucose assay complexes comprising dextran as a glucose analog. Dextran is a flexible macromolecule made up of up to 1500 glucose units. In certain typical instances, dextran is made up of approximately 600 glucose units (~100 kDa). Dextran may be used as a displaceable analyte analog/ligand in an optical mannan binding lectin (MBL)-based glucose responding competitive assay. In order to function in the Förster Resonance Energy Transfer (FRET) assay, dextran is typically (heavily) labeled with a lipophilic (and cationic) dye such as hexamethoxy crystal violet-1 (HMCV1). The presence of a large number (greater than 10) of lipophilic dyes on the flexible poly-(1,6)-glucose backbone of dextran can cause the HMCV1-labeled dextran to fold to less soluble conformations and hence create precipitations. Dye-induced conformal changes turn the HMCV1-labeled dextran into a more lipophilic state which causes various problems, including adverse changes on the binding ability of dextran to the glucose receptor as well as affecting its Förster Resonance Energy Transfer (FRET) efficiency. An inner filter effect also occurs due to the dyes being shielded intra-molecularly on the dextran, which causes the calibration of the assay to change, i.e. during this process the assay behaves unstably.

In one aspect of the invention, dextran is co-substituted with a typical quencher (e.g. HMCV1) together with dyes or other components having a more hydrophilic character, which allows for a better hydrophilic-hydrophobic balance

where less conformal changes to the glucose ligand/analog over time occurs. The co-substitution can also comprise of positive and negative charged substitutes which prevent dextran from becoming fully negative or positive. Illustrative experiments have shown that these factors allow for a significantly more stable assay for the optical sensor.

In one embodiment, as shown in FIG. 1A, the quencher is changed from a dye to a fluorophore and the reference fluorophore is omitted. This improves the stability of the assay by improving the solubility of the ligand. It also reduces complexity of the assay as the AF647-labeled dextran acts as both an acceptor and a reference at the same time. Additionally, this improves the photo stability of the assay by exciting the reference fluorophore. In this context, blue shifting the reference fluorophore towards the excitation source will prevent fluorophore excitation to the second excited state, a phenomenon which can lead to dye degradation. By shifting the dye towards the light source excitation wave length we can obtain less photo bleaching (UV light bleaches visible dyes more than visible light). Blue shifted reference dyes useful in embodiments of the invention (depending on, for example, the excitation filter and the wavelength width of the light source) include Alexa Fluor (AF) 546, AF555, AF568, AF594 and Cy3, Cy3B and Cy3.5. These are the blue shifted reference dyes compatible with the AF647 donor and HMCV1 acceptor FRET system.

A variety of other agents can be coupled to dextran to improve its conformation and/or hydrophilic-hydrophobic balance, typically to enhance hydrophilicity. Illustrative agents include cyclic anhydrides (e.g. Phthalic anhydride), Tartraic anhydride derivates (e.g. O,O-Diacetyl-L-tartaric anhydride), and the agents shown in Table 1 below:

n	Common name	IUPAC name	Structure	pK_a1	pK_a2
2	Succinic acid	butanedioic acid		4.21	5.41
3	Glutaric acid	pentanedioic acid		4.34	5.41
4	Adipic acid	hexanedioic acid		4.41	5.41
5	Pimelic acid	heptanedioic acid		4.50	5.43
6	Suberic acid	octanedioic acid		4.526	5.498
7	Azelaic acid	nonanedioic acid		4.550	5.498
8	Sebacic acid	decanedioic acid			

In another embodiment, as shown in FIG. 2A, the reference fluorophore is labeled/coupled onto the same ligand as the dye quencher, thereby eliminating the

need for a reference carrier. This improves the stability of the assay by improving the solubility of the ligand. This has shown even better stability than the fluorescing ligand. It also reduces the complexity of the assay as the AF647-labeled dextran acts as acceptor and reference at the same time. Additionally, this improves the photo stability of the assay by exciting the reference "where it absorbs more". It also enables further dose response optimization.

It has been found that when using a ligand labeled with only a fluorophore (e.g. AF647-100Dex(d)), increasing (AF647-100Dex(d)) concentration in the assay results in higher reference (REF) signal (risk of saturating REF) and more bleed-over into the assay (ASY) channel, which reduces sensor dose response (DR). There is a limit to (dex) concentration and the degree of labeling (DOL) of the pure AF647 ligand due to the above issues. High DOL HMCV1-dex has problems with solubility and AF647-dex appears more soluble in Tris or water. Adding HCMV1 to an AF647 ligand lowers the REF signal and bleed-over without reducing the quenching ability. Adding AF647 to an HMCV1 ligand increases solubility. Thus, specific embodiments of the invention provide adding AF647 to the HMCV1-dextran, thereby forming an AF647-HMCV1-Dex ligand with improved solubility, which improves assay stability. AF647 may be substituted with AF700. The combination ligand as well as just a HMCV1-Dex ligand may be slightly succinylated to further improve assay stability.

In another aspect of the invention, a method is provided for making the multi-labeled/combination glucose analog/ligand. In typical embodiments, the combination ligand is a dextran that carries both a quencher dye and a fluorophore, for example both HMCV1 and AF647. The two dyes are stained simultaneously to the dextran. In one instance, 10X of HMCV1-SE and 10X of AF647-SE are added to 100Dex(d). This is typically followed with purification, dialysis or passing through a small gel permeation chromatography (GPC) column.

The degree of labeling (DOL) of the individual dyes on a multi-labeled dextran (MLD) is determined by UV-Vis spectroscopy. The DOL is varied for both dyes to get a best fit. The resulting spectrum $\tilde{A}(\lambda)$ from the two dyes on the dextran is

a linear combination of the spectra of the individual dyes $\bar{\bar{\varepsilon}}_{Dye1}(\lambda)$ and $\bar{\bar{\varepsilon}}_{Dye2}(\lambda)$ and the respective dye concentration (Dyex). The dye concentration is determined by solving the equation below for all λ in the three recorded spectra (using HMCV1 and AF647 as example).

$$\bar{\bar{A}}(\lambda) = \bar{\bar{\varepsilon}}_{HMCV1}(\lambda)\times[HMCV1]\times d + \bar{\bar{\varepsilon}}_{AF647}(\lambda)\times[AF647]\times d$$

$$\bar{\bar{A}}(\lambda) = \bar{\bar{\varepsilon}}_{HMCV1}(\lambda)\times[Dex]\times DOL_{HMCV1}\times d + \bar{\bar{\varepsilon}}_{AF647}(\lambda)\times[Dex]\times DOL_{AF647}\times d$$

$$\varepsilon_{HMCV1}(\lambda_{max}) = 42000\ M^{-1}cm^{-1}$$

$$\varepsilon_{AF647}(\lambda_{max}) = 270000\ M^{-1}cm^{-1}$$

As a performance evaluation of the sensors, a sensor dose response (DR) is calculated using the following equation:

$$DR = \frac{I_{400} - I_{40}}{I_{40}} \times 100\%$$

where I_{400} and I_{40} are the normalized intensities at 400mg/dL and 40 mg/dL glucose. The loss in sensor dose response (DR loss) is calculated using the following equation:

$$\text{Relative Loss per day (Diff)} = \frac{DR_{Start} - DR_{end}}{DR_{Start}\times(t_{start} - t_{end})} \times 100\%$$

or from a linear regression of the DR vs. time (Excel function slope):

$$\text{Relative Loss per day (Slope)} = (-1) * \frac{Slope(DR(time);Time)}{DR_{Start}} \times 100\%$$

The relative DR loss is used as a key parameter in evaluating the quality of the sensor. Historically, the term was DR loss and hence a negative development in the DR turns out to be a positive loss, hence the (-1) multiplication in the "slope" formula. Baseline drift is evaluated but drifts much less. In illustrative experiments, as shown in FIGS. 13-17, single-labeled dextran exhibits relative DR loss between 3% to 6% per day, whereas the multi-labeled-dextran surprisingly only drifts between 0.5% and 2.5%.

Blue-Shifted Reference Dye

Embodiments of the invention include group different fluorescent dyes (e.g. a reference fluorophore and an assay fluorophore) that are selected to for use together due to their wave length profiles. Reference dyes in (intensity) fluorescence assays are needed in order to keep track of variations in the experimental setup e.g. light source fluctuations, changes in the optical path (coupling light into light guides, mechanical perturbations like bending, temperature variations, etc.). Traditionally optical or fluorescence based sensing systems have chosen references fluorophore that are red-shifted relative to the assaying fluorophore. Exciting fluorophores using light of a lower wavelength with more energy than needed there is an increased risk that the electronic transition in the fluorescent molecule will occur from the electronic ground state (S0) to the second excited state (S2) and not to the first excited electronic state (S1). Molecules in S2 are much more likely to decompose than the same molecule in S1, hence if excited to S2 faster photo-bleaching is obtained than if only S1 got occupied. Since a reference dye has to be very stable, the use of a reference fluorophore red-shifted relative to the assay fluorophore can be a suboptimal choice.

Instead of the usual red-shifted reference fluorophore that could appear photo-labile due to low wavelength excitation, certain embodiments of the invention use a reference fluorophore blue-shifted relative to the assay or indicator fluorophore instead. In other words, the reference fluorophore has a shorter wavelength/increased frequency than the assay fluorophore. In visible light, the reference fluorophore is closer to the blue end of the spectrum whereas the assay fluorophore is closer to the red end. This improves the stability of the reference fluorophore, which is a key property in providing accurate assay measurements. In one or more embodiments, a competitive glucose binding affinity assay comprises a glucose receptor/lectin (e.g. mannan binding lectin) labeled with an assay fluorophore (Alexa Fluor™ 647) and a multi-labeled glucose analog (e.g. dextran) labeled with both a reference fluorophore (e.g. Alexa Fluor™ 594) and a quencher dye (e.g. hexamethoxy crystal violet-1), wherein the reference fluorophore is blue-shifted relative to the assay fluorophore.

As is known in the art, with such systems, spectra and available light sources dictate the selection of the fluorophores and the design of the optical set-up. There are a variety of semiconductor light sources (LED's) useful with embodiments of the invention such as those found in the MIGHTEX SYSTEMS LED Wavelength Portfolio. In addition, in embodiments of the invention, continuum light sources (white light sources with laser characteristics) can be filtered to select a particular wavelength range for excitation to provide an arbitrary wavelength(range)

Typically, for a red-shifted reference the concentration of the reference can in principle be increased to reduce the effect of the assay fluorophore "red" tail bleed over and must be separated approximately 50nm to avoid reference fluorophore "blue" tail bleed over to the assay fluorophore and thereby reduce dose response. For a blue-shifted reference the fluorophore must typically be blue-shifted approximately 50nm to avoid reference assay "blue" tail bleed over to the reference fluorophore and thereby make the reference insensitive to assay fluorophore fluorescence level. Reference fluorophore concentrations can in this case be reduced in order to avoid dose response reduction by reference "red" tail bleed into the assay fluorophore emission. For most fluorophore pairs, it is typically difficult to separate fluorophores more than 100nm and still be able to excite the most red-shifted fluorophore simultaneously with the blue-shifted. One way to circumvent this is to excite with two different light sources, and increase the complexity of the optical system as it is required to monitor the output of the light sources to compensate for changes in output.

Nomenclature

As described herein and in the figures, the different dextran conjugates have the following general nomenclature:

Dye1-Dye2-XXXDex(Y)succ(a/b/Xc)

As described herein and in the figures, the different MBL conjugates have the following general nomenclature:

Dye1-zMBL(a)

Dye1 and **Dye2** are abbreviations of the dye names;

XXX is the Mw of the dextran in kDa;

Y is the Ion Exchange Chromatography (IEX) fraction of the amino-dextran;

Z is either "r" for recombinant MBL, "p" for plasma MBL, "UHP" for Ultra Highly Purified MBL;

succ denotes if the dextran is treated with succinic acid anhydride in molar excess "c". If succ is not stated the dextran is stained with dye(s) only; and

(a/b/Xc) denotes the Degree Of Labeling (DOL) of the dyes and excess of succinic acid anhydride used. "a" is the DOL of **Dye1**, "**b**" is the **DOL or Dye2** and "**Xc**" the **molar excess of succinic** acid anhydride used. DOL is defined as number of dyes per dextran i.e. a dimensionless number.

Illustrative examples are as follows:

HMCV1-AF647-100Dex(d)(5.2/3.9)

This conjugate is a 100 kDa Dextran IEX peak-d labeled with HMCV1 and AF647 and having respectively DOLs of 5.2 and 3.9.

HMCV1-100Dex(c)succ(12.1/X10)

This conjugate is a 100 kDa Dextran IEX peak-c labeled with HMCV1 and succinic acid anhydride having DOL of 12.1 and 10 times molar excess of succinic acid anhydride used for modification.

AF647-rMBL(0.51)

This conjugate is recombinant MBL labeled with AF647 having DOL of 0.51.

Assays are described as MBL and ref-conjugates (when needed) from the same nomenclature and the concentrations of the individual conjugates in square brackets (PP;DD;RR) where PP is the concentration of MBL (rMBL) in μM, DD is the ligand dextran concentration (Dex) in μM and RR is the reference dextran concentration in μM.

Further aspects and embodiments of the invention are disclosed in the following examples.

EXAMPLES

Example 1: Illustrative Multi-Labeled Dextrans Generated

HMCV1-AF647-Dextran

Dextran carrying both HMCV1 quencher dye and AF647 fluorophore. Both HMCV1 and AF647 acts as quencher to the AF594 donor in the glucose sensing assay. AF647 also functions as reference in the system. The emitted fluorescence from the direct excitation from the light source is much larger than the glucose dependent fluorescence origin from the FRET in the system.

HMCV1-AF700-Dextran

Dextran carrying both HMCV1 quencher dye and AF700 fluorophore. HMCV1 acts as quencher to the AF594 donor in the glucose sensing assay. AF700 functions as reference in the system.

HMCV1-HMCV3-Dextran

Dextran carrying both HMCV1 quencher dye and HMCV3 quencher dye. HMCV3 is a negatively charged version of HMCV1 (that is positively charged). Both HMCV1 and HMCV3 acts a quencher to the AF594 donor in the glucose sensing assay. *A* reference is needed in the system e.g. heavily succinylated AF700-Dextran.

HMCV1-Dextran-succinlylated

Dextran carrying HMCV1 quencher dye and further treated low excess succinic acid anhydride. HMCV1 acts as quencher to the AF594 donor in the glucose sensing assay. The low degree of succinylation prevents the dextran from fast obtaining a lipophilic structure. A reference is needed in the system e.g. heavily succinylated AF700-Dextran.

Example 2: Experiment With Blue-Shifted Reference Fluorophore

In the glucose responding assay, the assay has traditionally been built from two or three conjugates. Option 1 (three ligands) using red-shifted reference: AF594-rMBL, HMCV1-100Dex(d), AF700-100Dex(a)succ; AF700 on the non-binding Dex(a)succ acts as a reference. Option 2 (two ligands) using red-shifted reference: AF594-rMBL, HMCV1-AF647-100Dex(d); AF647 on the binding Dex(d) will act a reference since the fluorescence from direct excitation is much stronger than the fluorescence originating from the FRET. Changing the system to a blue-shifted reference becomes: AF647-rMBL and HMCV1-AF594-100Dex(d), resulting in better stability and lower calibration frequency of the optical sensor.

The assay was been built but unfortunately the AF594-DOL of the HMCV1-AF594-100Dex(d)(6.0/6.3) was far too large (SITS6017). This resulted in full saturation in both the REF and ASY channel during testing. New assays were made, but not tested (closing of site). Option 1: AF647-rMBL(0.24)/HMCV1-100Dex(d)succ(17.1/X5)/AF594-100Dex(a)succ(2.0); (10:40:1) and (10:40:0.5). Option 2: AF647-rMBL(0.24)/HMCV1-70Dex(d)succ(13.8/X5)/AF594-100Dex(a)succ(2.0) (10:40:1) and (10:80:1). Option 3: AF647-rMBL(0.24)/HMCV1-HMCV3-100Dex(d)succ(14.2)/AF594-100Dex(a)succ(2.0) (10:40:1) and (10:40:0.1); AF647-rMBL(0.24)/HMCV1-HMCV3-100Dex(d)succ(18.6)/AF594-100Dex(a)succ(2.0) (10:40:1) and (10:40:0.1).

CONCLUSION

This concludes the description of the typical embodiment of the present invention. The foregoing description of one or more embodiments of the invention has been presented for the purposes of illustration and description. It is not intended to be exhaustive or to limit the invention to the precise form disclosed. Many modifications and variations are possible in light of the above teaching.

CLAIMS:

1. A glucose sensing complex comprising:

a glucose binding agent coupled to an assay fluorophore; and

a glucose analog coupled to a reference fluorophore;

wherein the reference fluorophore is blue shifted relative to the assay fluorophore.

2. The glucose sensing complex of claim 1 wherein the glucose analog is further coupled to a quenching agent.

3. The glucose sensing complex of claim 2 wherein the quenching agent is hexamethoxy crystalviolet-1 (HMCV1).

4. The glucose sensing complex of claim 1 wherein:

the glucose receptor is selected from the group consisting of mannan binding lectin (MBL), Concanavalin A, glucose galactose binding protein, an antibody, and Boronic acid; and/or

the glucose analog is dextran.

5. The glucose sensing complex of claim 4 wherein the glucose receptor is mannan binding lectin.

6. The glucose sensing complex of claim 4 wherein the assay fluorophore and reference fluorophore are individually selected from the group consisting of Alexa Fluor 594 (AF594), Alexa Fluor 647 (AF647), and Alexa Fluor 700 (AF700), and wherein the reference fluorophore has a lower wavelength than the assay fluorophore.

7. The glucose sensing complex of claim 2 wherein the reference fluorophore and quencher dye form a Förster Resonance Energy Transfer (FRET) pair.

8. The glucose sensing complex of claim 2 wherein the fluorophores and/or quencher dye are water soluble.

9. The glucose sensing complex of claim 1 wherein the assay fluorophore exhibits a wavelength that is at least 50 nanometers greater than the wavelength of the reference fluorophore.

10. The glucose sensing complex of claim 4 wherein the dextran is approximately 100kDa.

11. A method of making a glucose sensing complex comprising forming a glucose sensing complex by:

coupling a glucose binding agent to an assay fluorophore; and

coupling a glucose analog to a reference fluorophore;

wherein the reference fluorophore and the assay fluorophore are selected so that reference fluorophore emits light at a wavelength that is blue shifted relative to light emitted by the assay fluorophore.

.

12. The method of claim 11, wherein the assay fluorophore exhibits a wavelength that is at least 50 nanometers greater than the wavelength of the reference fluorophore.

13. The method of claim 11, wherein the assay fluorophore exhibits a wavelength that is not more than 100 nanometers from the wavelength of the reference fluorophore

14. The method of claim 11, wherein the glucose receptor comprises mannan binding lectin (MBL) and the glucose analog is dextran.

15. A method of sensing glucose in a solution comprising:

(a) contacting the solution with a glucose sensing complex comprising:

a glucose binding agent coupled to an assay fluorophore; and

a glucose analog coupled to a reference fluorophore;

wherein the reference fluorophore is blue shifted relative to the assay fluorophore;

(b) observing the glucose sensing complex for signals indicative of the presence of glucose; and

(c) correlating observed signals with the concentration of glucose.

16. The method of claim 15, wherein the solution comprises interstitial fluid or blood.

17. The method of claim 15, wherein the glucose sensing complex is disposed *in vivo*.

18. The method of claim 15, wherein the sensing comprises exciting the reference fluorophore and the assay fluorophore with two different light sources.

19. The method of claim 15, wherein the assay fluorophore exhibits a wavelength that is not more than 100 nanometers from the wavelength of the reference fluorophore.

20. The method of claim 15, wherein the assay fluorophore exhibits a wavelength that is at least 50 nanometers greater than the wavelength of the reference fluorophore.

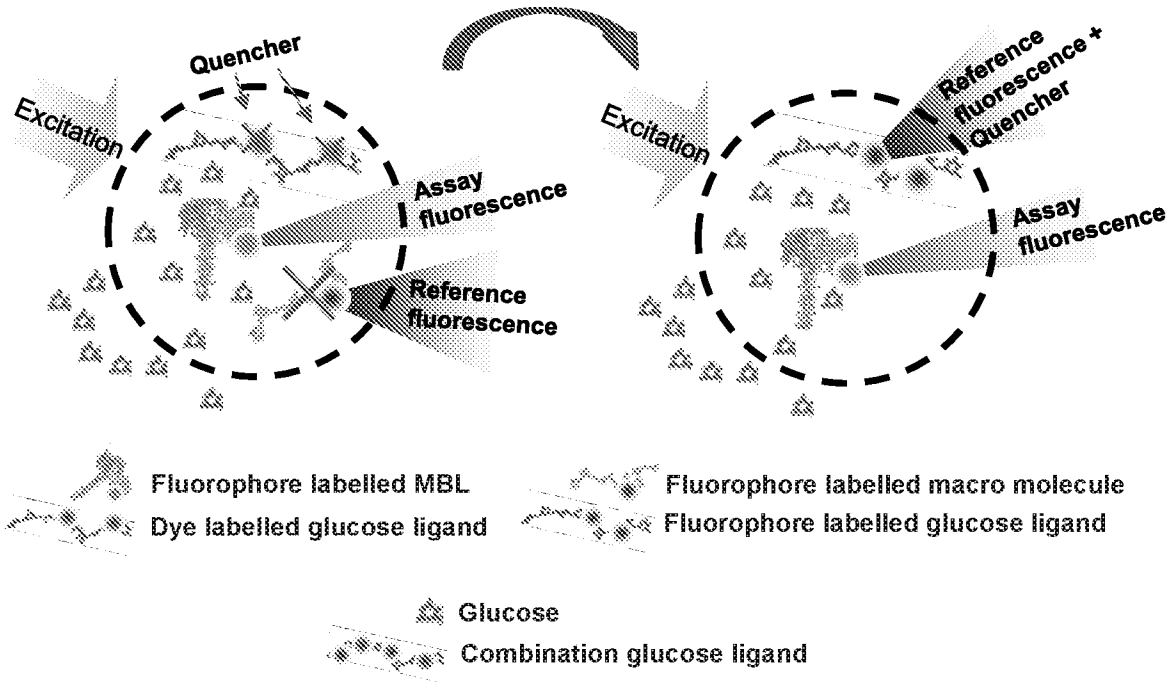

Quencher
Excitation
Assay fluorescence
Reference fluorescence
Excitation
Reference fluorescence + Quencher
Assay fluorescence
Fluorophore labelled MBL
Dye labelled glucose ligand
Fluorophore labelled macro molecule
Fluorophore labelled glucose ligand
Glucose
Combination glucose ligand

FIG. 1A

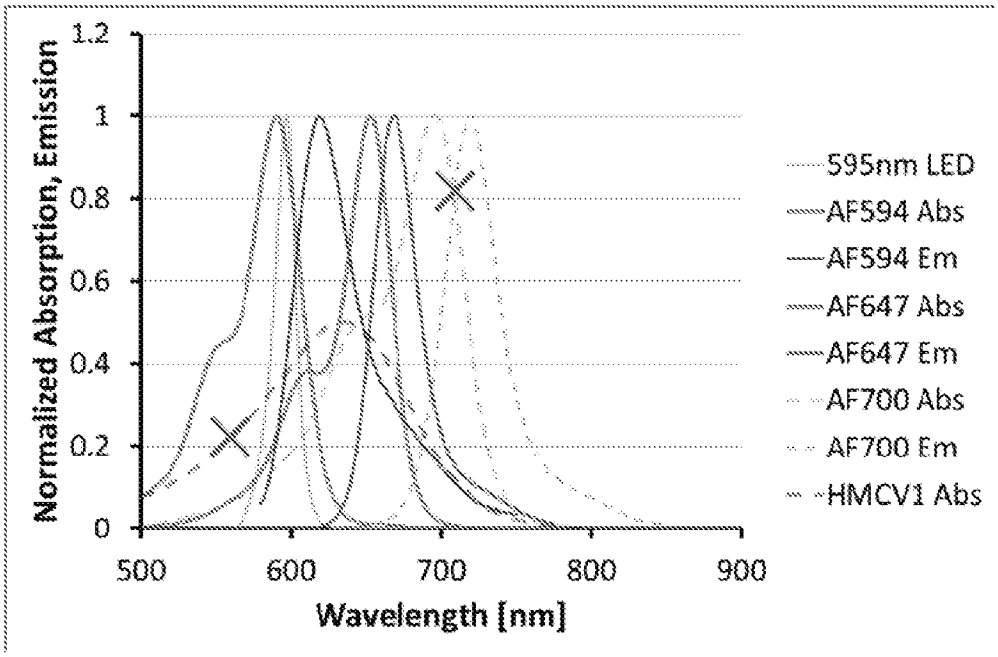

Normalized Absorption, Emission
1.2
1
0.8
0.6
0.4
0.2
0
500
600
700
800
900
Wavelength [nm]
595nm LED
AF594 Abs
AF594 Em
AF647 Abs
AF647 Em
AF700 Abs
AF700 Em
HMCV1 Abs

FIG. 1B

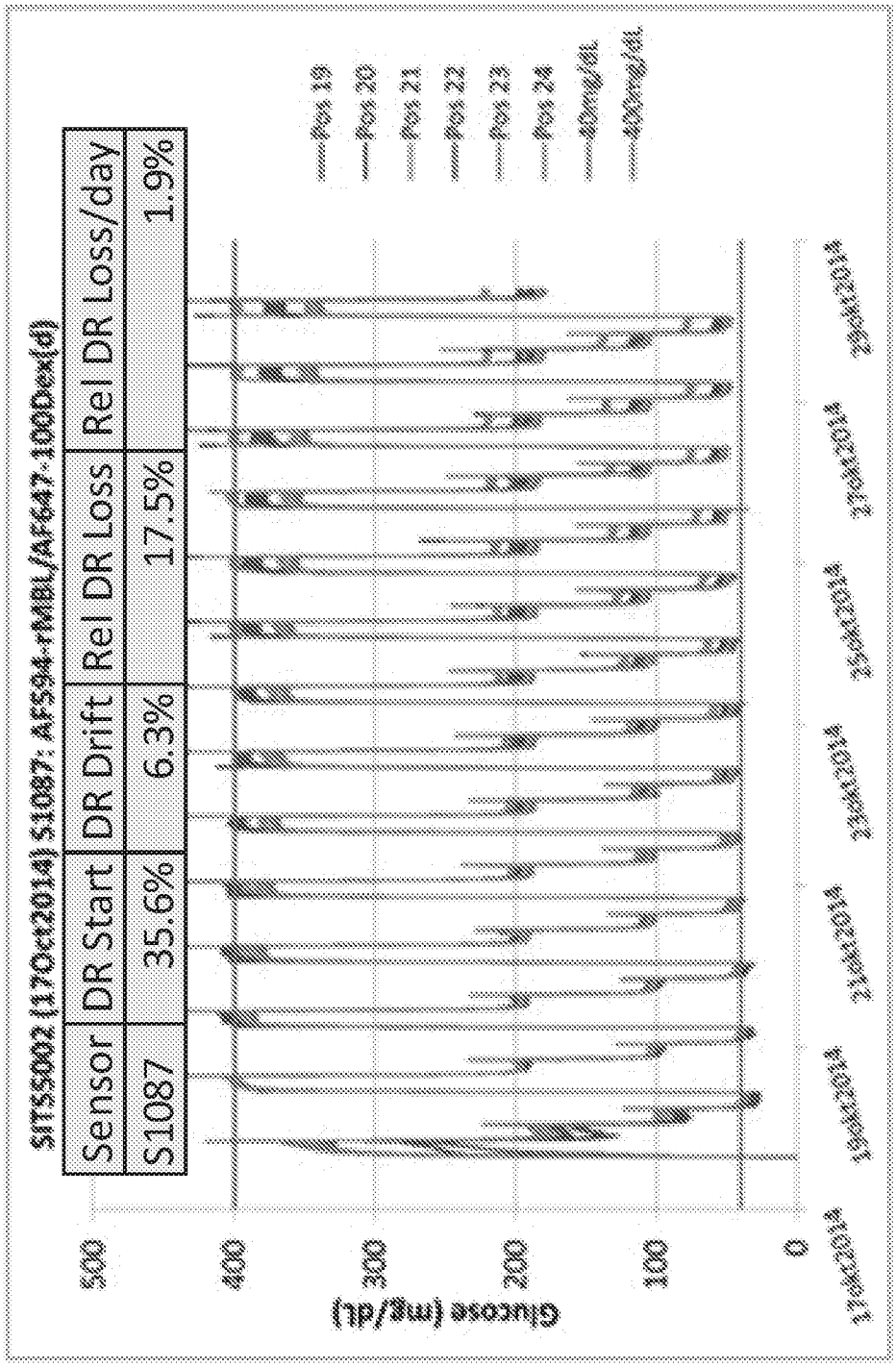

SITS5002 (17Oct2014) S1087: AF594-rMBL/AF647-100Dex(d)
Sensor
DR Start
DR Drift
Rel DR Loss
Rel DR Loss/day
S1087
35.6%
6.3%
17.5%
1.9%
Fos 19
Fos 20
Fos 21
Fos 22
Fos 23
Fos 24
40mg/dL
400mg/dL
Glucose (mg/dL)
500
400
300
200
100
0
17Oct2014
19Oct2014
21Oct2014
23Oct2014
25Oct2014
27Oct2014
29Oct2014

FIG. 1C

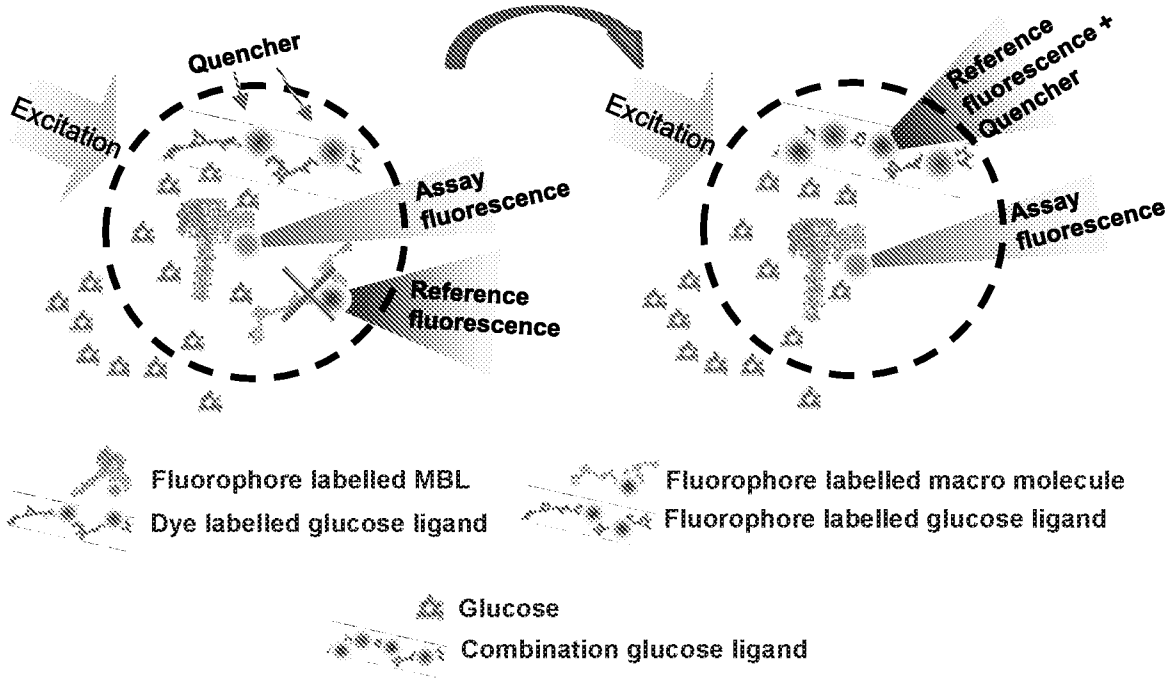

Quencher
Excitation
Assay fluorescence
Reference fluorescence
Excitation
Reference fluorescence + Quencher
Assay fluorescence
Fluorophore labelled MBL
Dye labelled glucose ligand
Fluorophore labelled macro molecule
Fluorophore labelled glucose ligand
Glucose
Combination glucose ligand

FIG. 2A

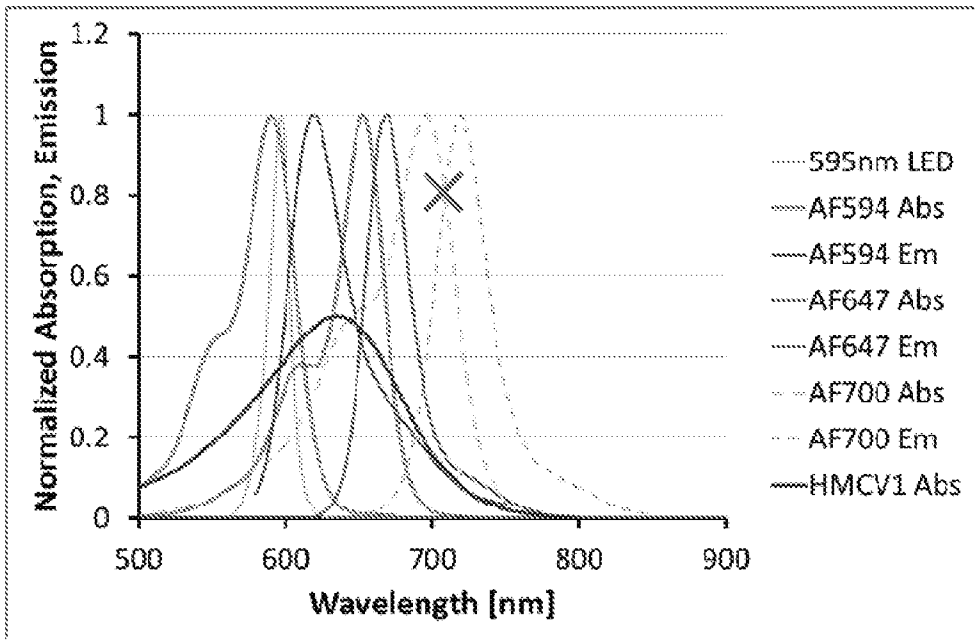

Normalized Absorption, Emission
1.2
1
0.8
0.6
0.4
0.2
0
500
600
700
800
900
Wavelength [nm]
595nm LED
AF594 Abs
AF594 Em
AF647 Abs
AF647 Em
AF700 Abs
AF700 Em
HMCV1 Abs

FIG. 2B

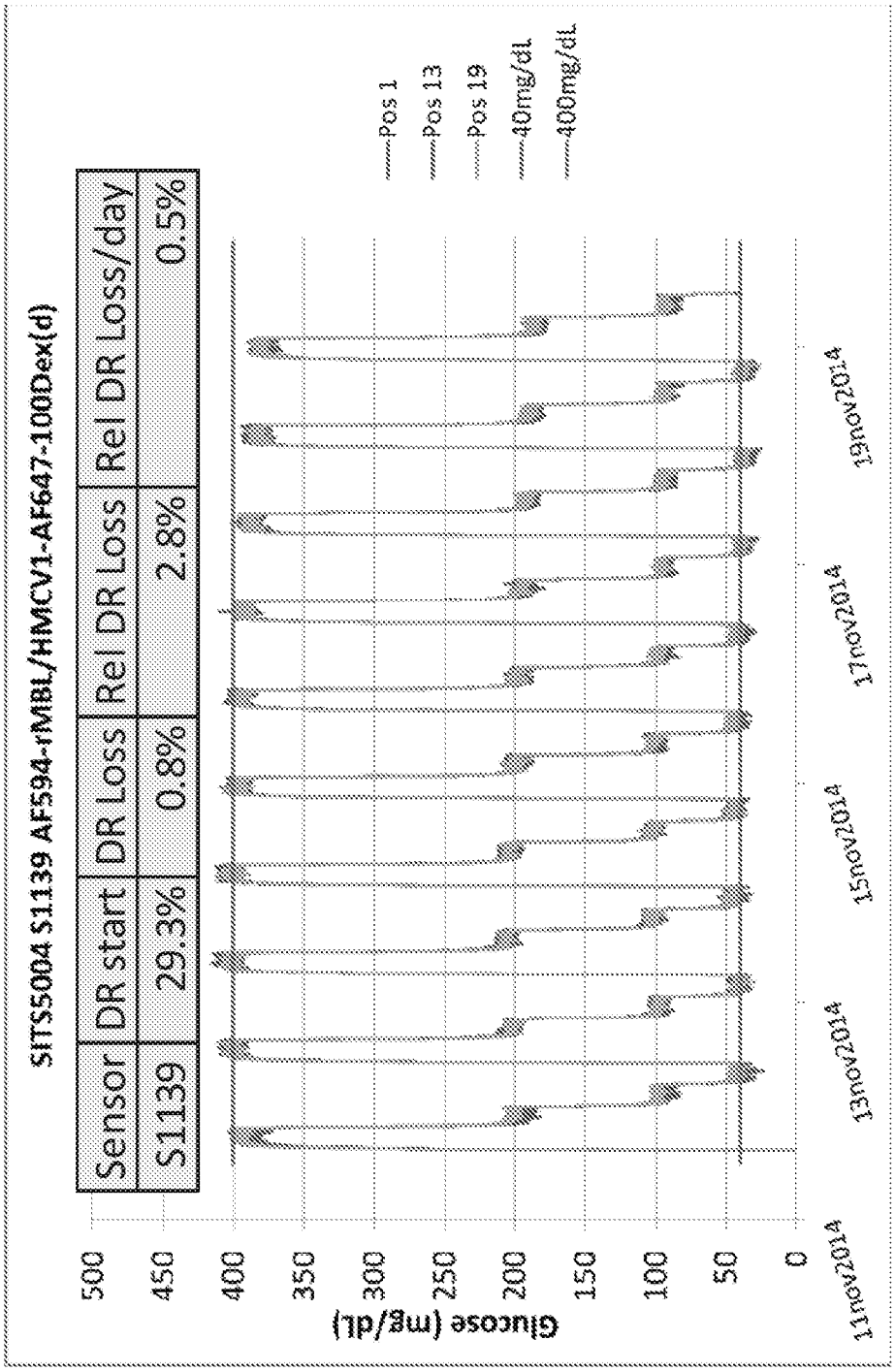

SITS004 S1139 AF594-rMBL/HMCV1-AF647-100Dex(d)
Sensor
DR start
DR Loss
Rel DR Loss
Rel DR Loss/day
S1139
29.3%
0.8%
2.8%
0.5%
Glucose (mg/dL)
500
450
400
350
300
250
200
150
100
50
0
11nov2014
13nov2014
15nov2014
17nov2014
19nov2014
Pos 1
Pos 13
Pos 19
40mg/dl
400mg/dl

FIG. 2C

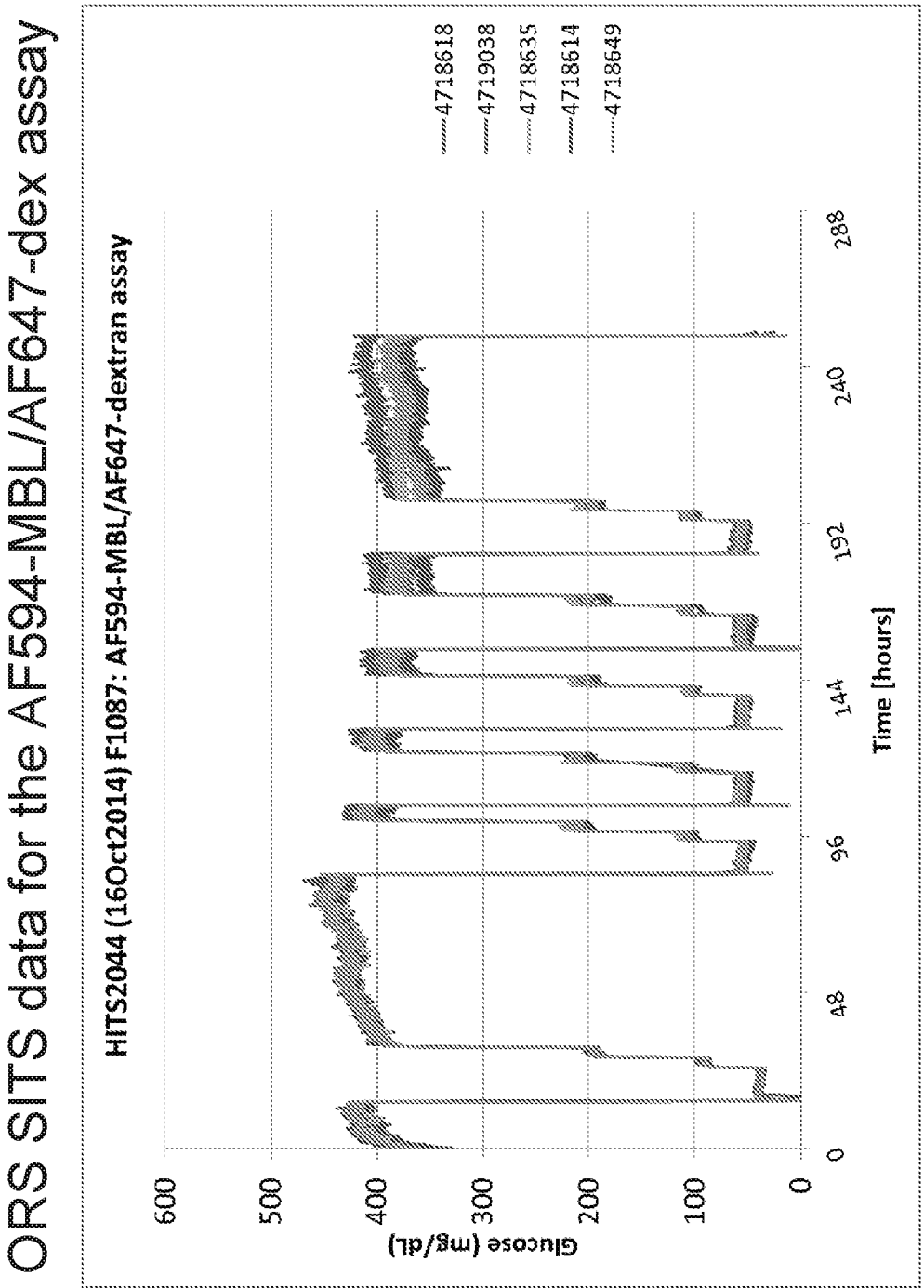

ORS SITS data for the AF594-MBL/AF647-dex assay
HITS2044 (16Oct2014) F1087: AF594-MBL/AF647-dextran assay
Glucose (mg/dL)
600
500
400
300
200
100
0
0
48
96
144
192
240
288
Time [hours]
4718618
4719038
4718635
4718614
4718649

FIG. 3

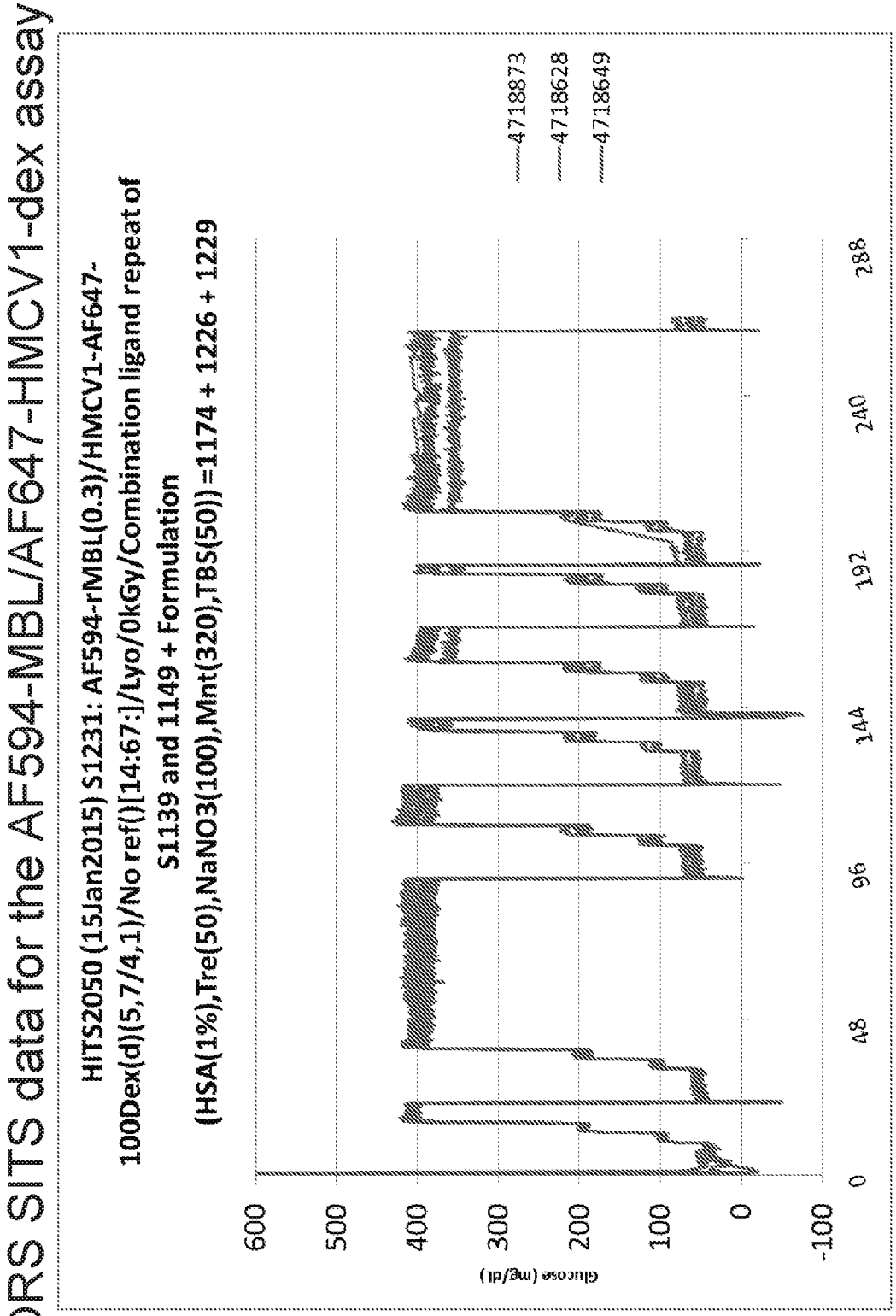

ORS SITS data for the AF594-MBL/AF647-HMCV1-dex assay
HITS2050 (15Jan2015) S1231: AF594-rMBL(0.3)/HMCV1-AF647-100Dex(d)(5,7/4,1)/No ref()[14:67:]/Lyo/0kGy/Combination ligand repeat of S1139 and 1149 + Formulation (HSA(1%),Tre(50),NaNO3(100),Mnt(320),TBS(50))=1174 + 1226 + 1229
4718873
4718628
4718649
Glucose (mg/dL)
600
500
400
300
200
100
0
-100
0
48
96
144
192
240
288

FIG. 4

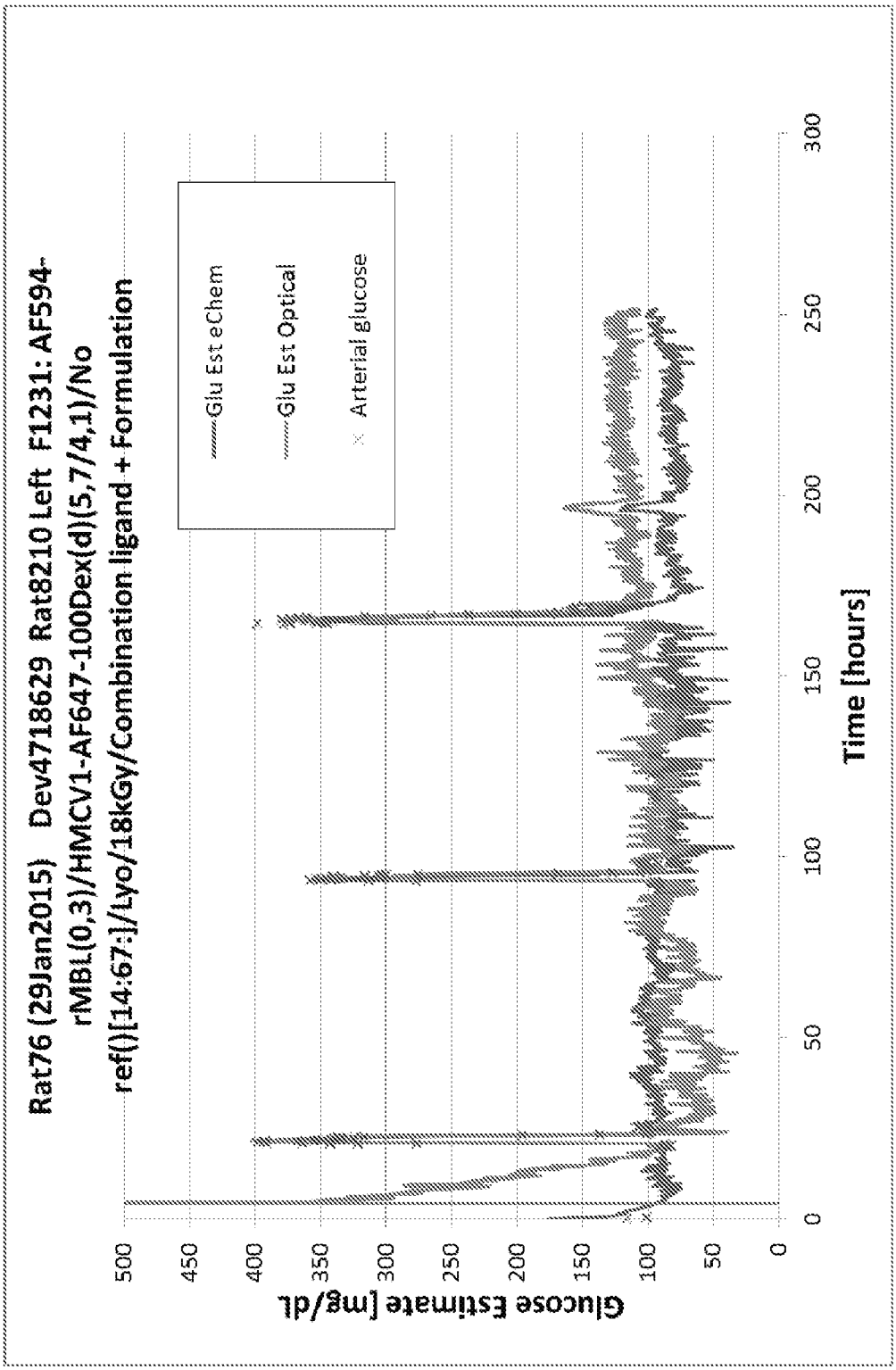

Rat study 76: Rat #1, ORS sensor #2
Rat76 (29Jan2015) Dev4718629 Rat8210 Left F1231: AF594-rMBL(0,3)/HMCV1-AF647-100Dex(d)(5,7/4,1)/No ref()[14:67:]/Lyo/18kGy/Combination ligand + Formulation
Glu Est eChem
Glu Est Optical
Arterial glucose
Glucose Estimate [mg/dL]
Time [hours]
0
50
100
150
200
250
300
350
400
450
500

FIG. 5

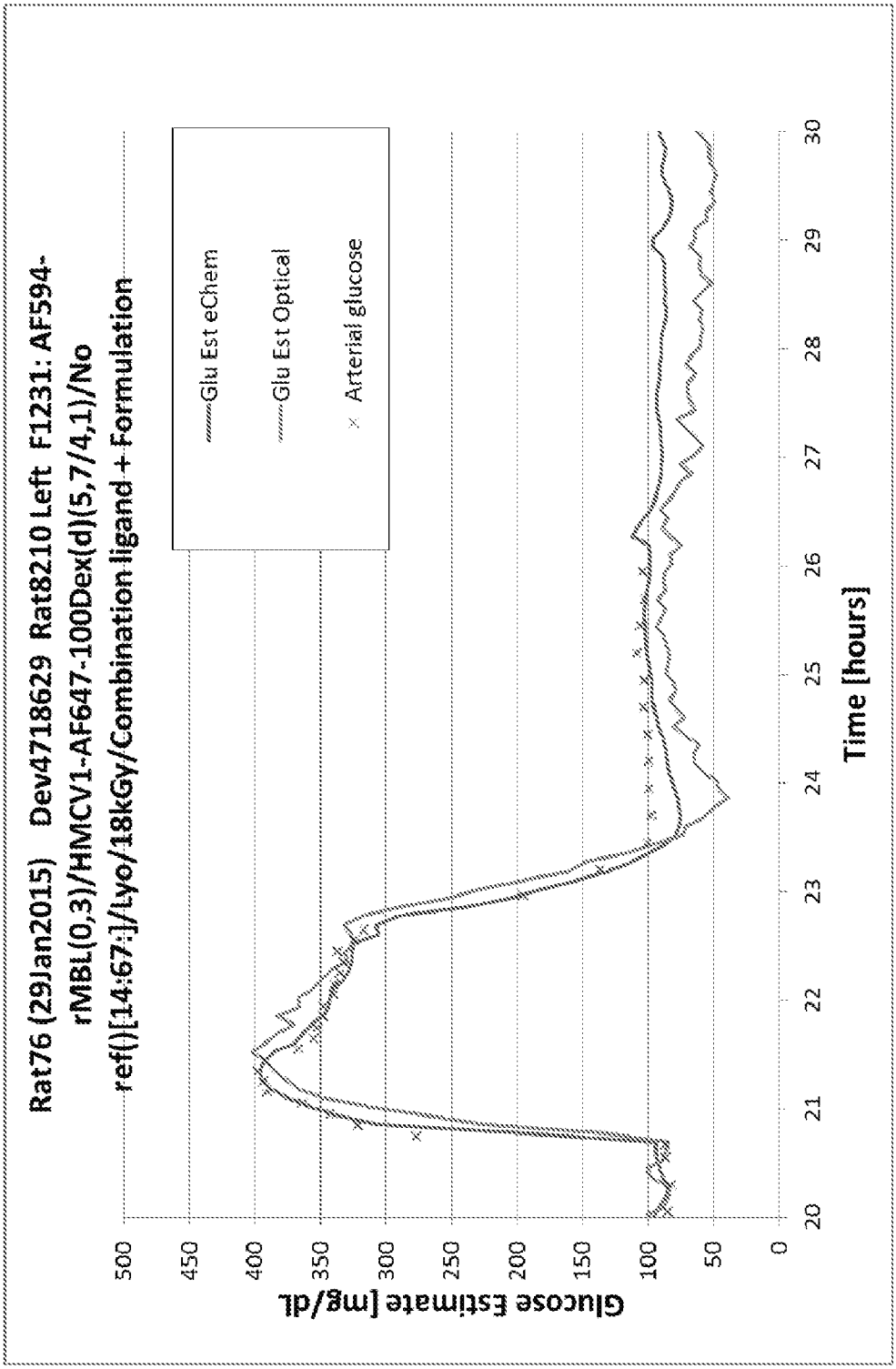

Rat study 76:Rat #1, ORS sensor #2 – Clamp 1
Rat76 (29Jan2015) Dev4718629 Rat8210 Left F1231: AF594-rMBL(0,3)/HMCV1-AF647-100Dex(d)(5,7/4,1)/No ref()[14:67:]/Lyo/18kGy/Combination ligand + Formulation
Glu Est eChem
Glu Est Optical
Arterial glucose
Glucose Estimate [mg/dL]
500
450
400
350
300
250
200
150
100
50
0
20
21
22
23
24
25
26
27
28
29
30
Time [hours]

FIG. 6A

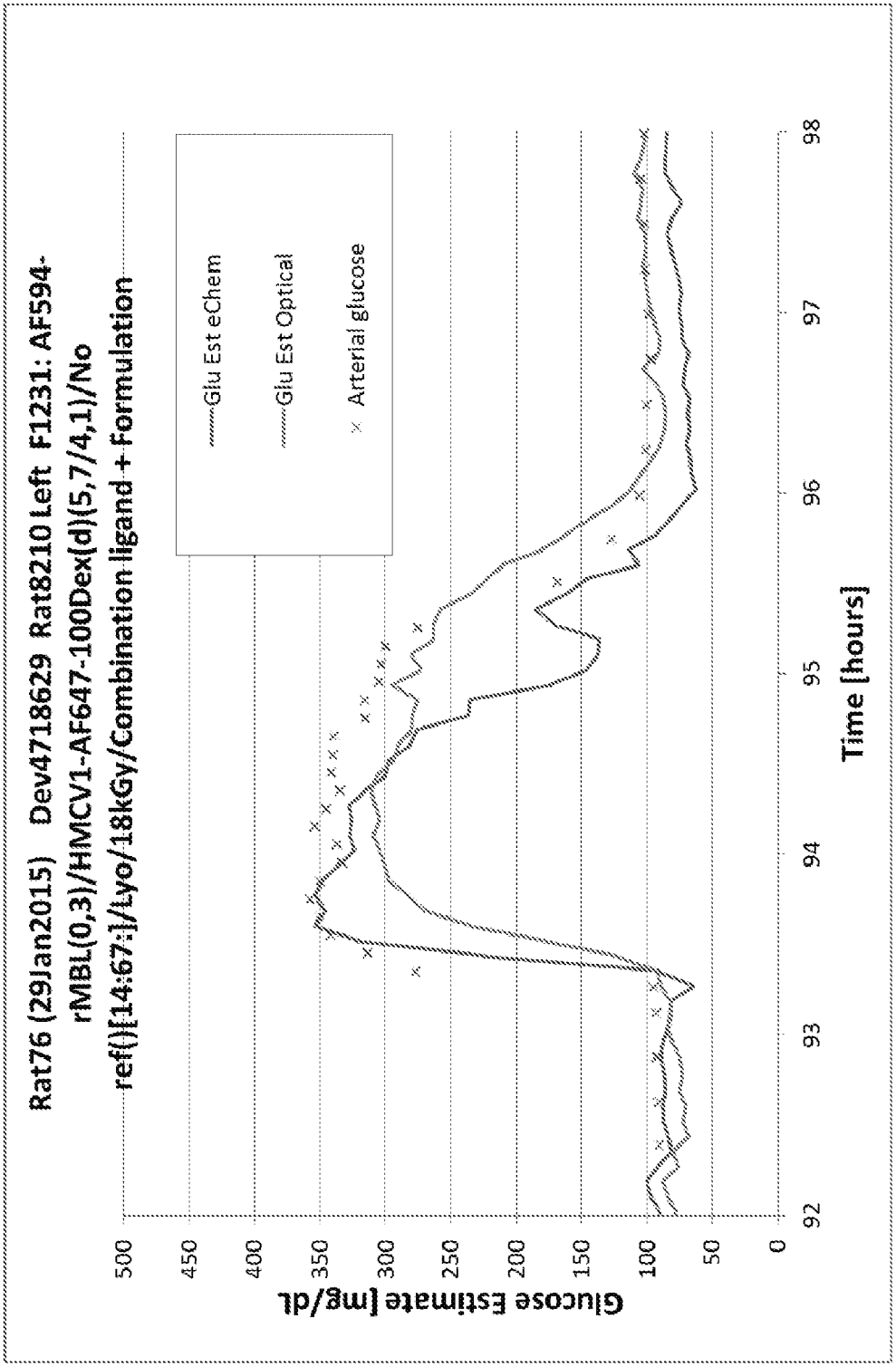

Rat study 76:Rat #1, ORS sensor #2 – Clamp 2
Rat76 (29Jan2015) Dev4718629 Rat8210 Left F1231: AF594-rMBL(0,3)/HMCV1-AF647-100Dex(d)(5,7/4,1)/No ref()[14:67:]/Lyo/18kGy/Combination ligand + Formulation
Glu Est eChem
Glu Est Optical
Arterial glucose
Glucose Estimate [mg/dL]
500
450
400
350
300
250
200
150
100
50
0
92
93
94
95
96
97
98
Time [hours]

FIG. 6B

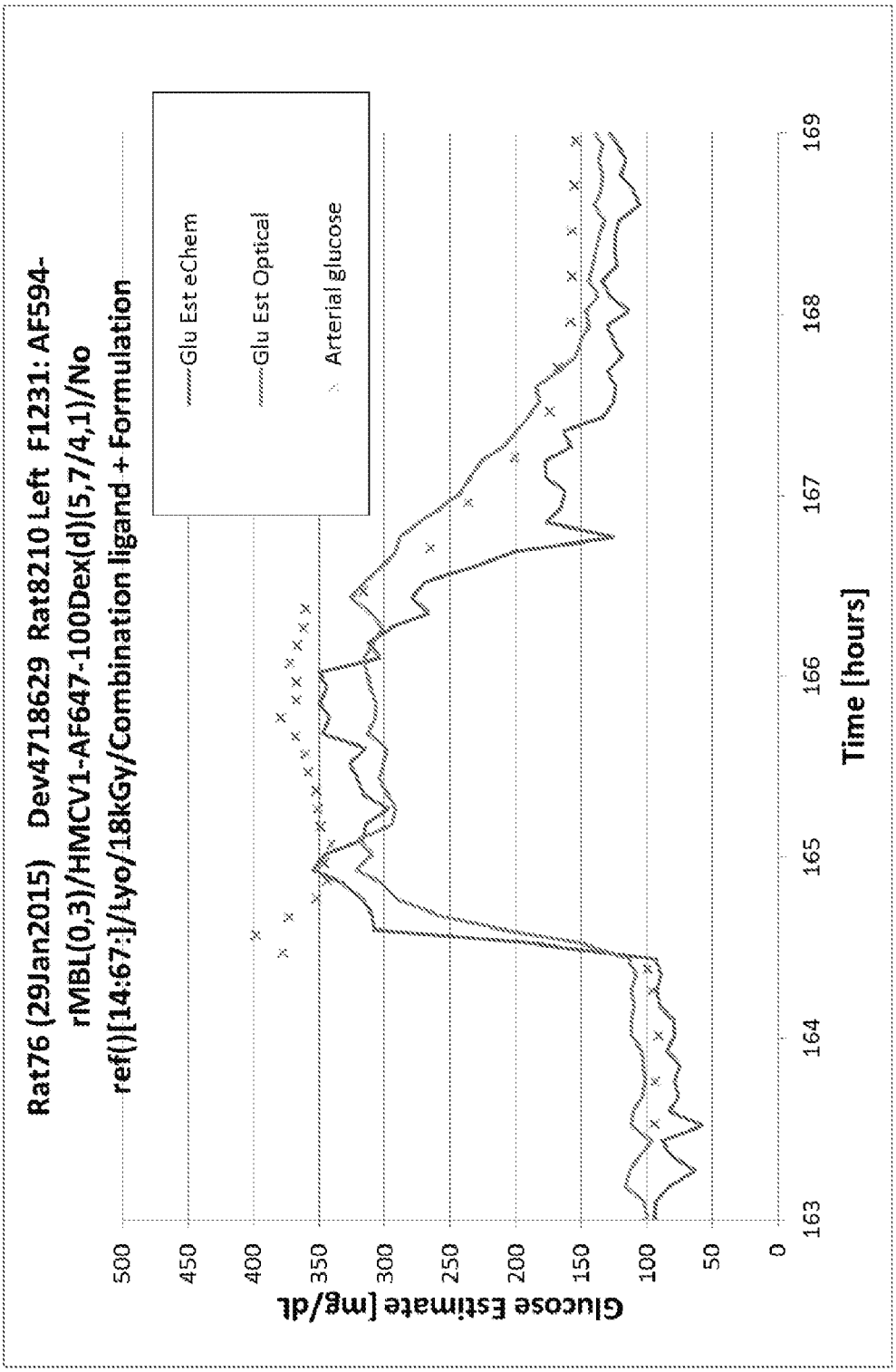

Rat study 76:Rat #1, ORS sensor #2 – Clamp 3
Rat76 (29Jan2015) Dev4718629 Rat8210 Left F1231: AF594-rMBL(0,3)/HMCV1-AF647-100Dex(d)(5,7/4,1)/No ref()[14:67:]/Lyo/18kGy/Combination ligand + Formulation
Glu Est eChem
Glu Est Optical
Arterial glucose
Glucose Estimate [mg/dl
500
450
400
350
300
250
200
150
100
50
0
163
164
165
166
167
168
169
Time [hours]

FIG. 6C

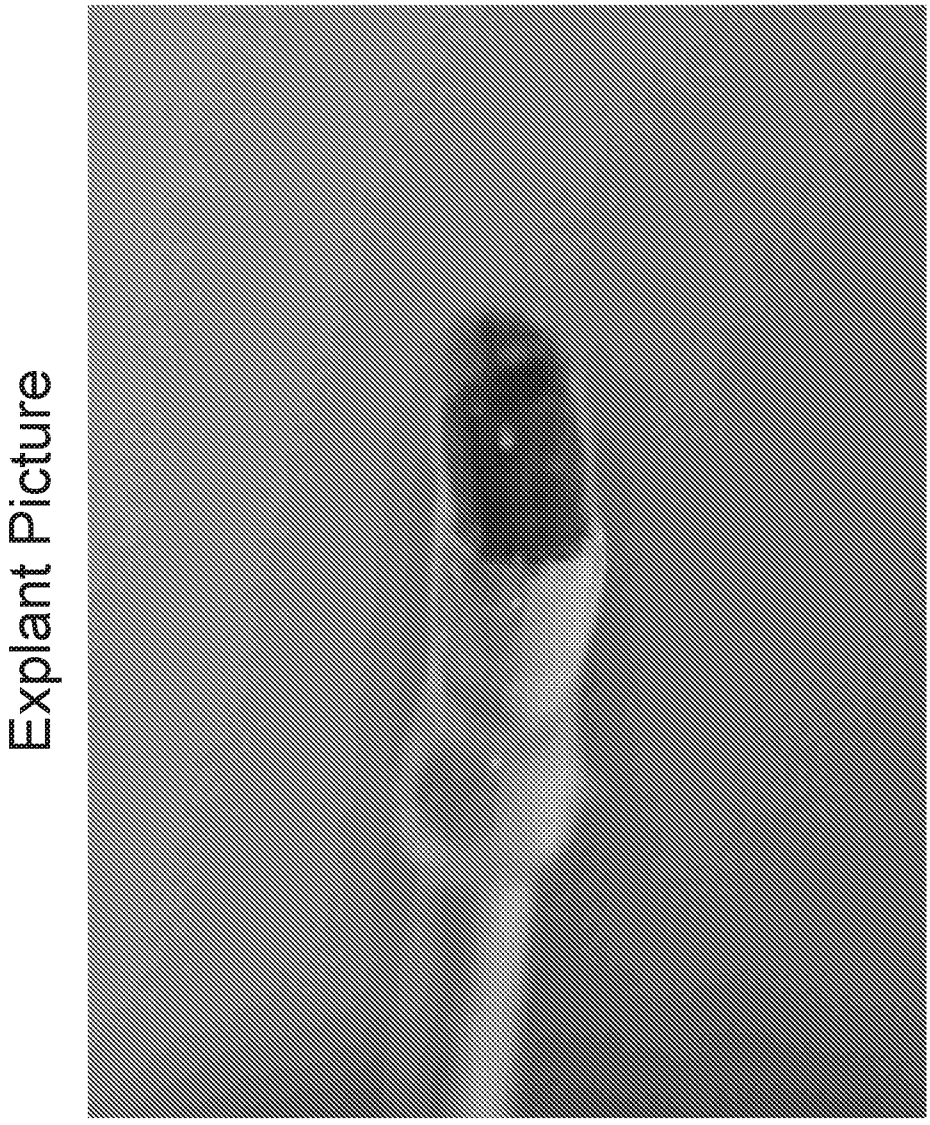


FIG. 6D

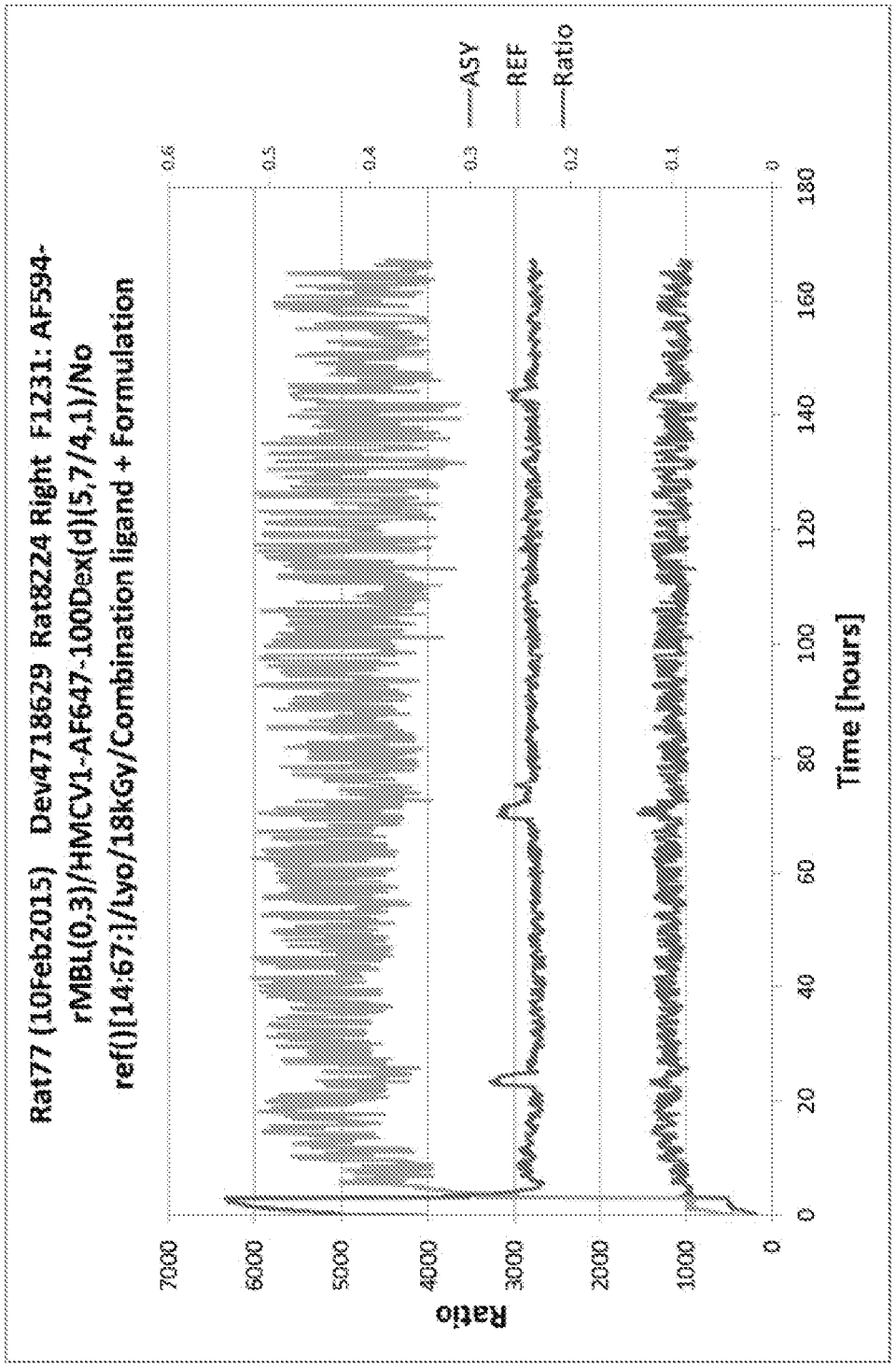

Data from Rat study 77
(Note the stable Assay and Reference baselines)
Rat77 (10Feb2015) Dev4718629 Rat8224 Right F1231: AF594-rMBL(0,3)/HMCV1-AF647-100Dex(d)[5,7/4,1)/No ref()[14:67:]/Lyo/18kGy/Combination ligand + Formulation
Ratio
Time [hours]
ASY
REF
Ratio
0
1000
2000
3000
4000
5000
6000
7000
0
20
40
60
80
100
120
140
160
180
0
0.1
0.2
0.3
0.4
0.5
0.6

FIG. 7A

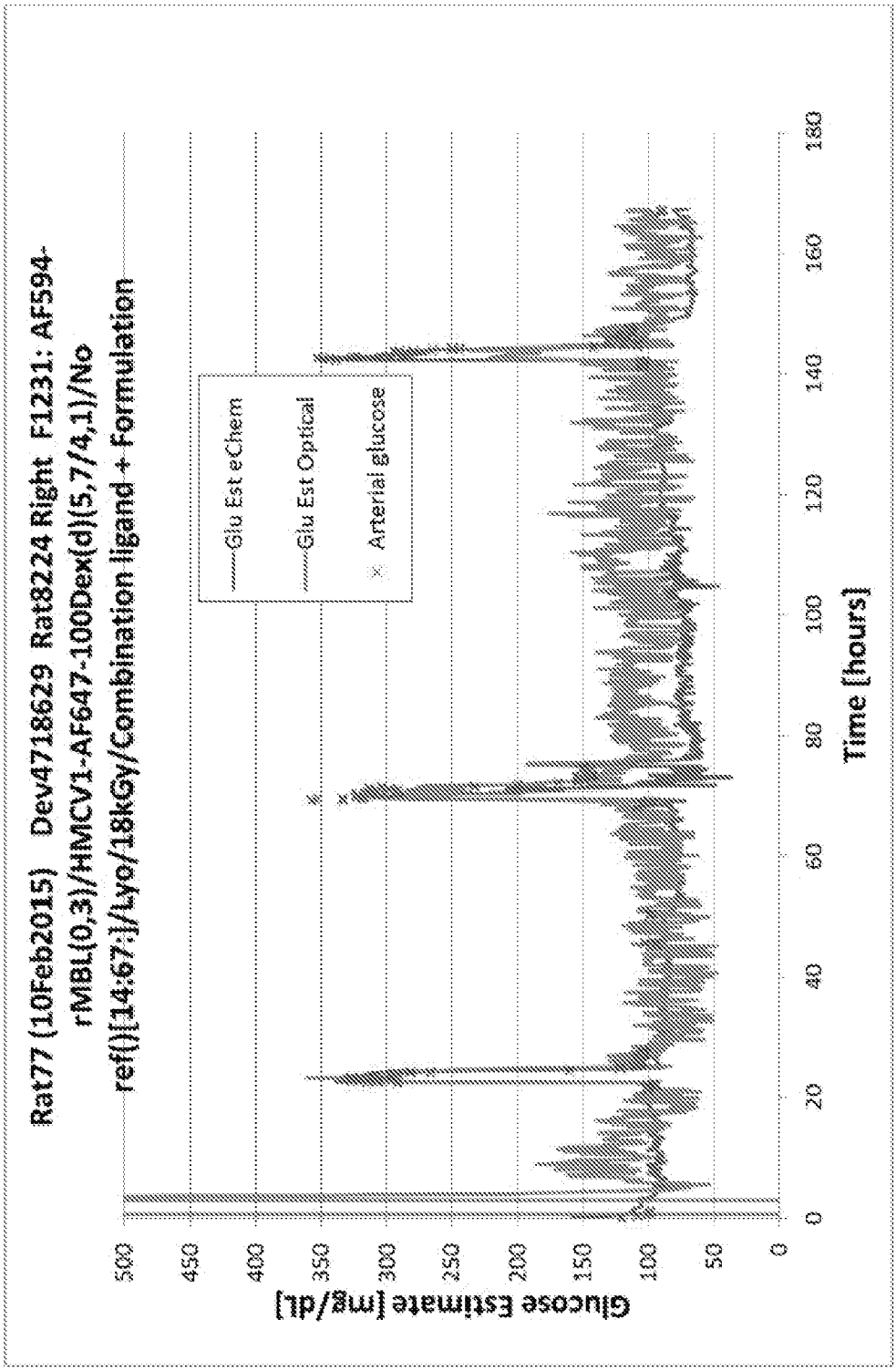

Rat77 (10Feb2015) Dev4718629 Rat8224 Right F1231: AF594-rMBL(0,3)/HMCV1-AF647-100Dex(d)(5,7/4,1)/No ref()[14:67:]/Lyo/18kGy/Combination ligand + Formulation
Glu Est eChem
Glu Est Optical
Arterial glucose
Glucose Estimate [mg/dL]
Time [hours]
0
50
100
150
200
250
300
350
400
450
500
0
20
40
60
80
100
120
140
160
180

FIG. 7B

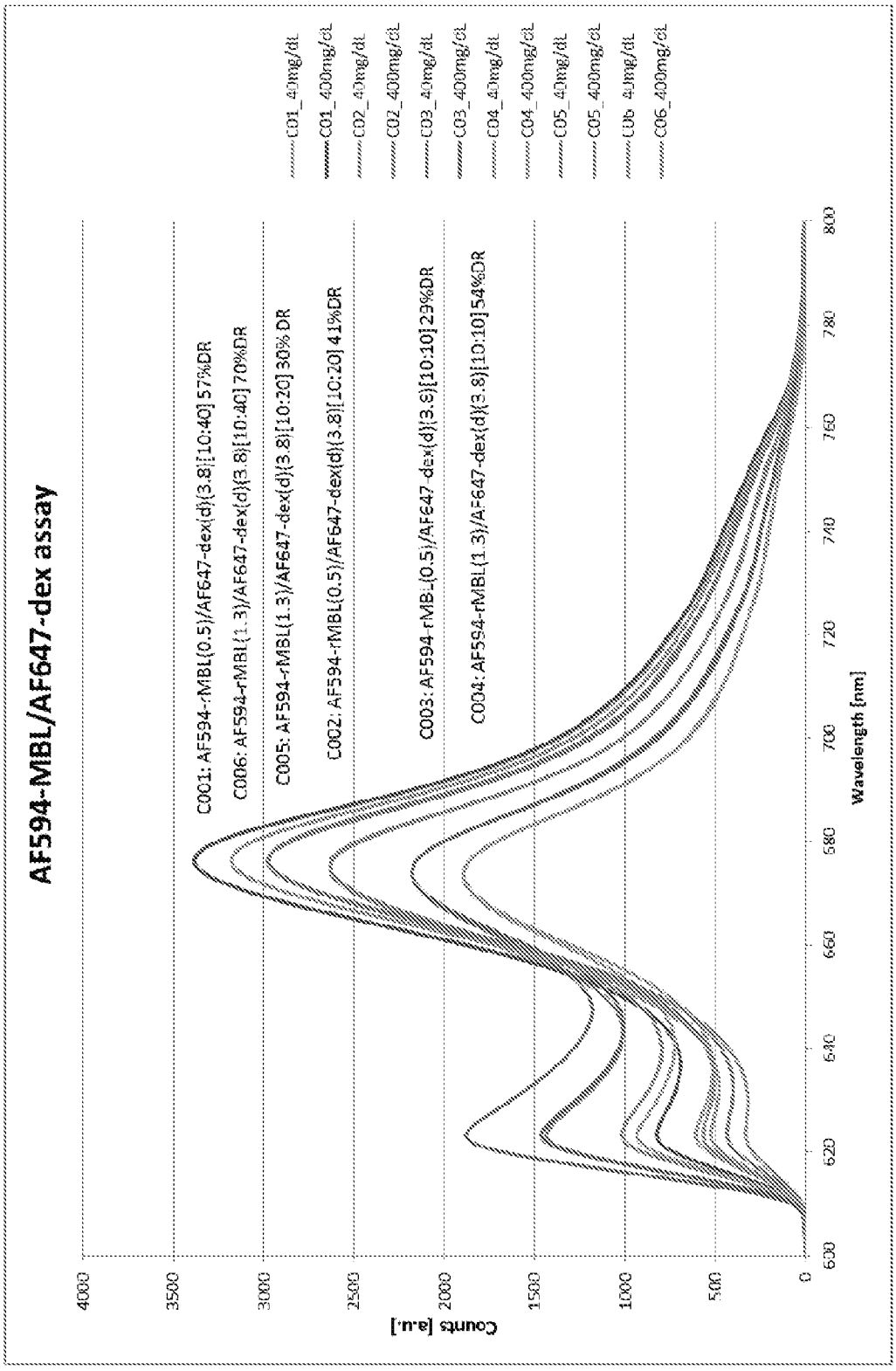

AF594/AF647 assay
AF594-MBL/AF647-dex assay
C001: AF594-rMBL(0.5)/AF647-dex(d)(3.8)[10:40] 57%DR
C006: AF594-rMBL(1.3)/AF647-dex(d)(3.8)[10:40] 70%DR
C005: AF594-rMBL(1.3)/AF647-dex(d)(3.8)[10:20] 30% DR
C002: AF594-rMBL(0.5)/AF647-dex(d)(3.8)[10:20] 41%DR
C003: AF594-rMBL(0.5)/AF647-dex(d)(3.6)[10:10] 29%DR
C004: AF594-rMBL(1.3)/AF647-dex(d)(3.8)[10:10] 54%DR
C01_40mg/dL
C01_400mg/dL
C02_40mg/dL
C02_400mg/dL
C03_40mg/dL
C03_400mg/dL
C04_40mg/dL
C04_400mg/dL
C05_40mg/dL
C05_400mg/dL
C06_40mg/dL
C06_400mg/dL
Counts [a.u.]
Wavelength [nm]
0
500
1000
1500
2000
2500
3000
3500
4000
600
620
640
660
680
700
720
740
760
780
800

FIG. 8

AF594MBL/AF647-Dex and AF594/AF647-HMCV1-Dex assays:
Optimum ratio between AF594 and AF647 fluorescence

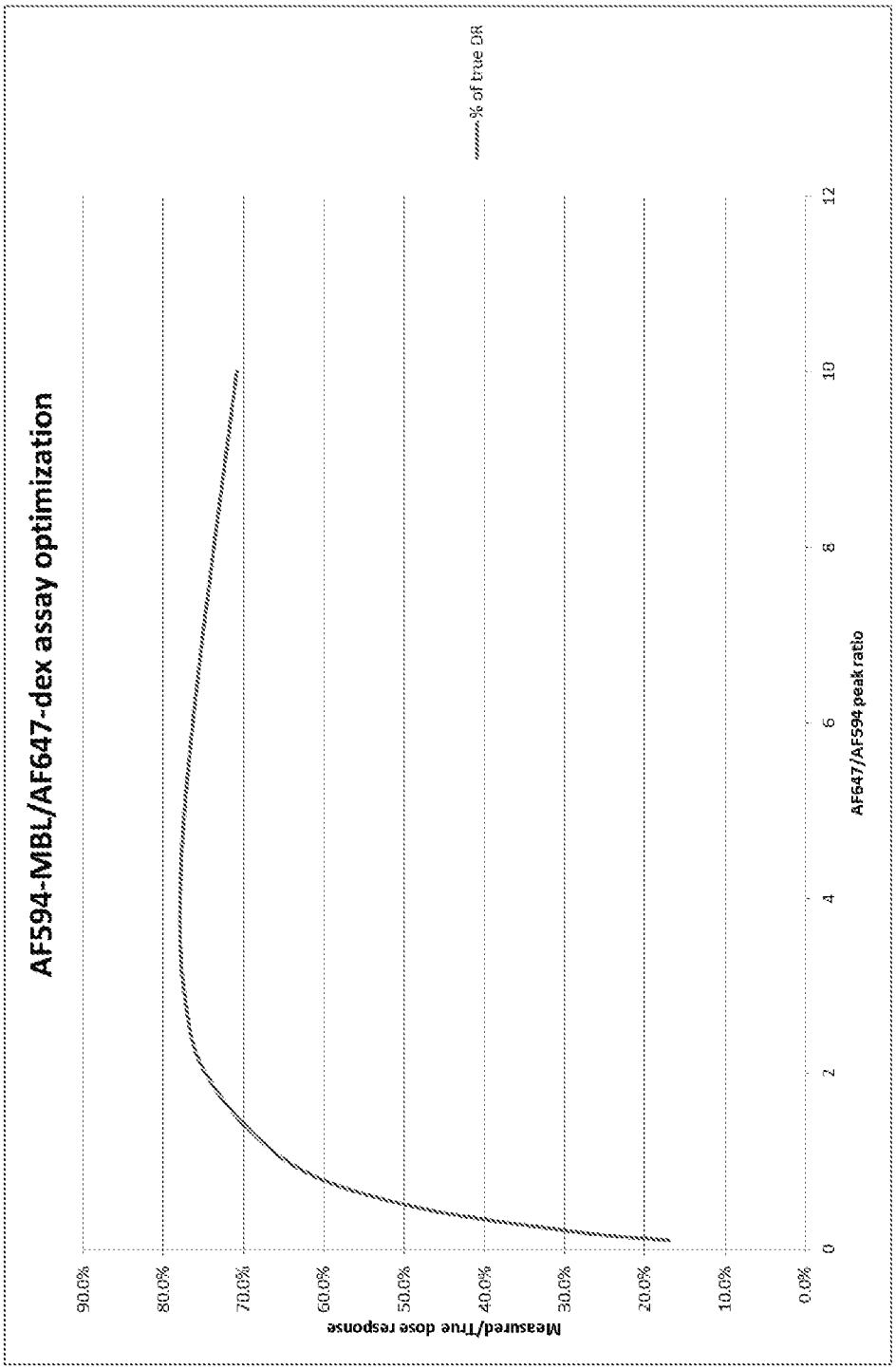
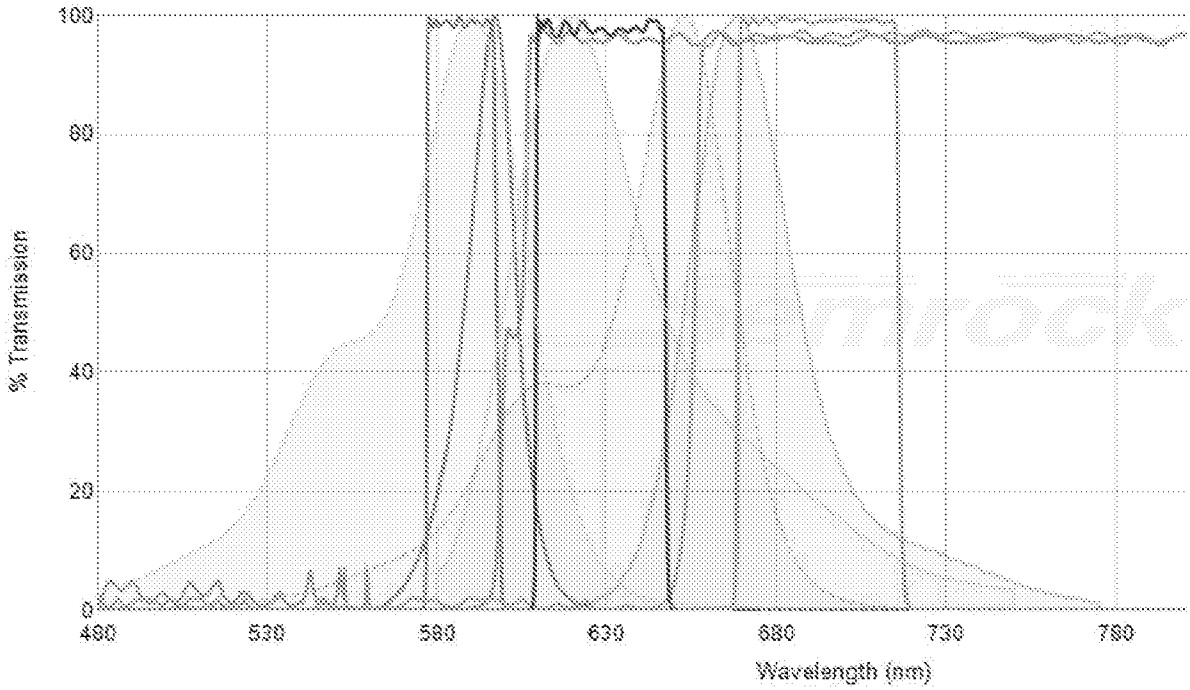


FIG. 9

Filter configuration for AF594-MBL/647-Dex and AF594-MBL/AF647-HMCV1-Dex assays



595 LED Single Wavelength (CoolLED)

FF02-586/15

DI01-R594

DI02-R635

FF01-628/32

FF01-692/40

Alexa Fluor 594

Alexa Fluor 594 - Abs

Alexa Fluor 594 - Em

Alexa Fluor 647

Alexa Fluor 647 - Abs

Alexa Fluor 647 - Em

FIG. 10

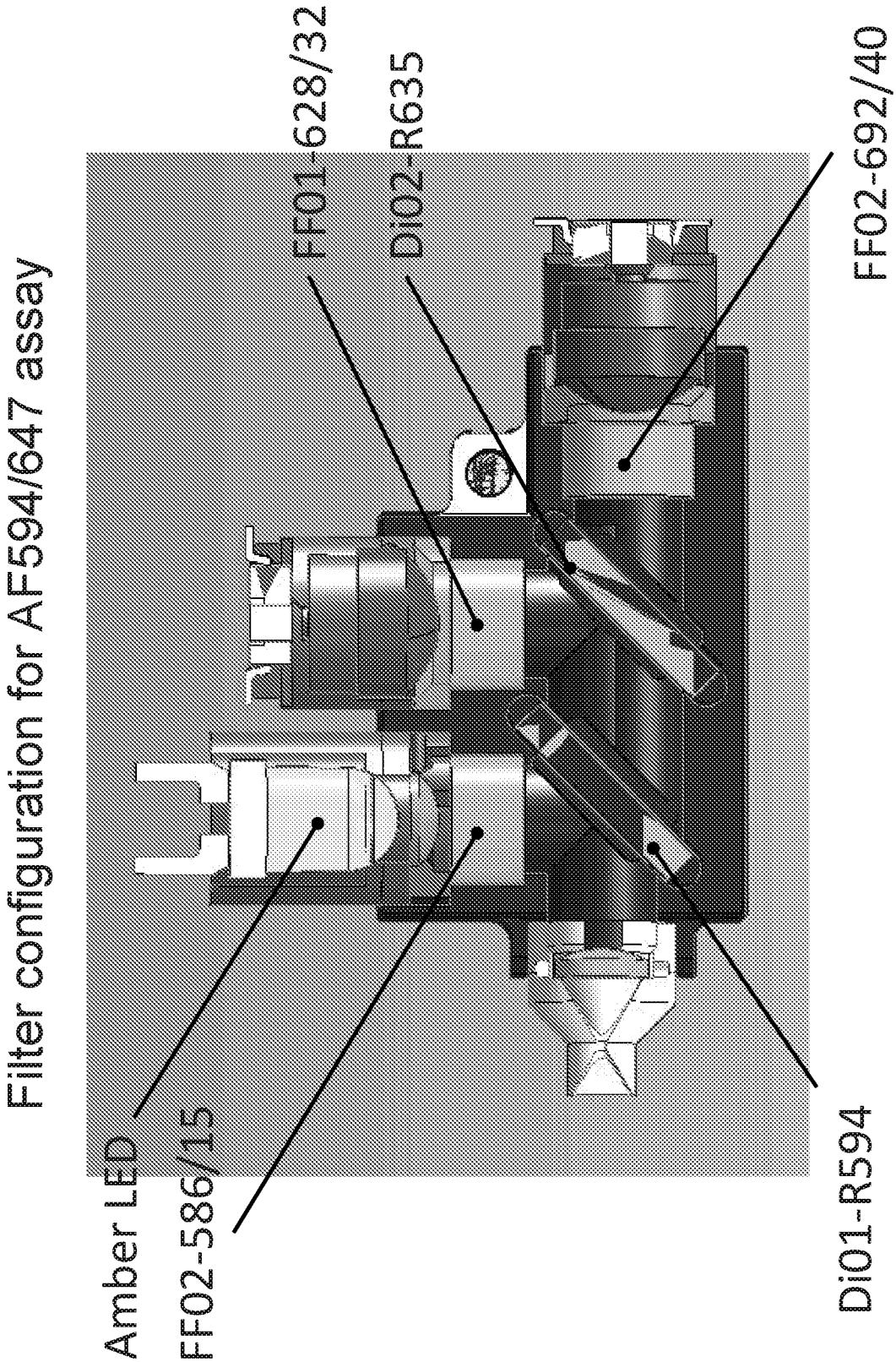

Filter configuration for AF594/647 assay
Amber LED
FF02-586/15
FF01-628/32
Di02-R635
FF02-692/40
Di01-R594

FIG. 11

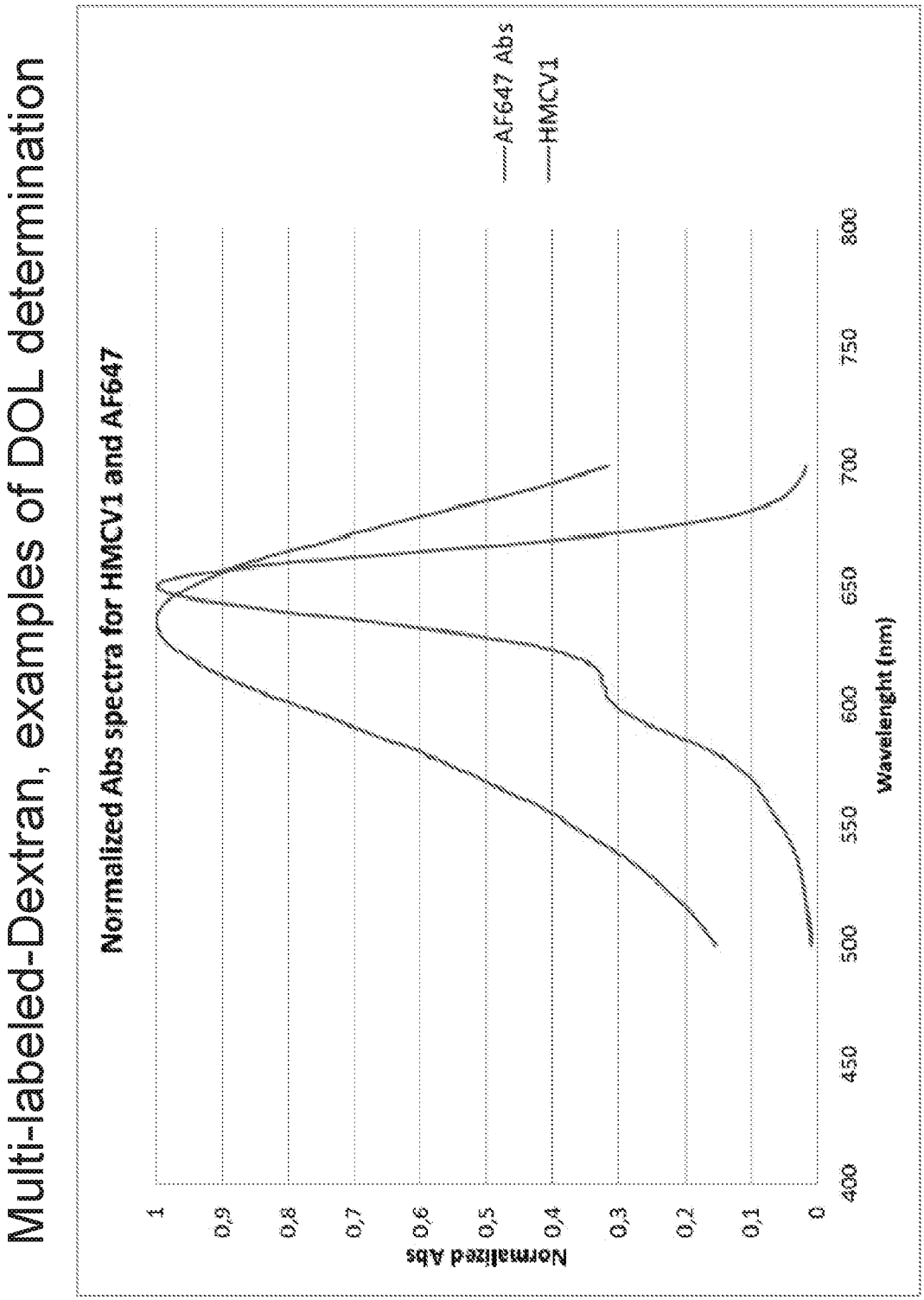

Multi-labeled-Dextran, examples of DOL determination
Normalized Abs spectra for HMCV1 and AF647
AF647 Abs
HMCV1
Normalized Abs
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
400
450
500
550
600
650
700
750
800
Wavelenght (nm)

FIG. 12A

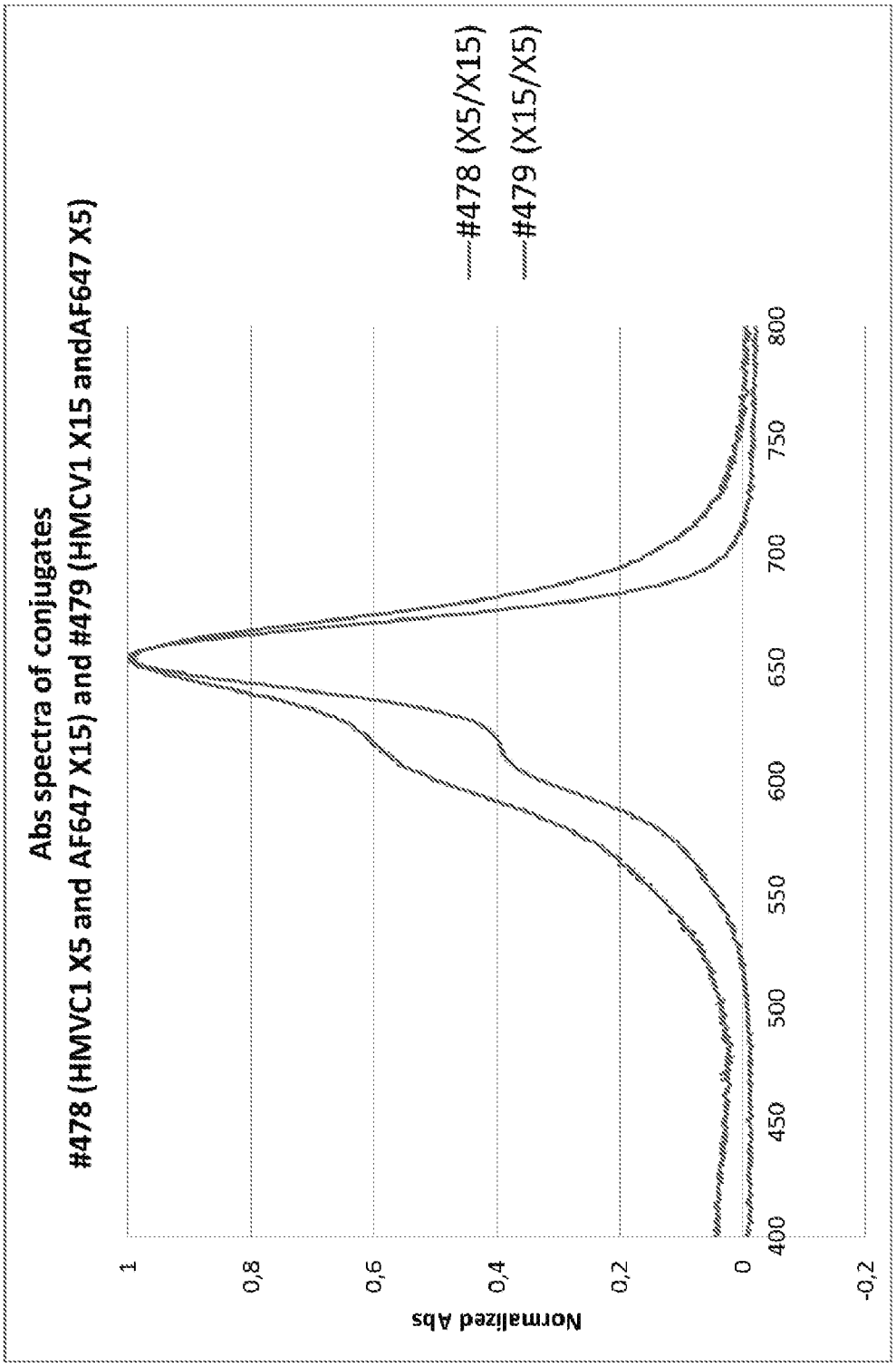

Abs spectra of conjugates
#478 (HMVC1 X5 and AF647 X15) and #479 (HMCV1 X15 andAF647 X5)
#478 (X5/X15)
#479 (X15/X5)
Normalized Abs
1
0,8
0,6
0,4
0,2
0
-0,2
400
450
500
550
600
650
700
750
800

FIG. 12B

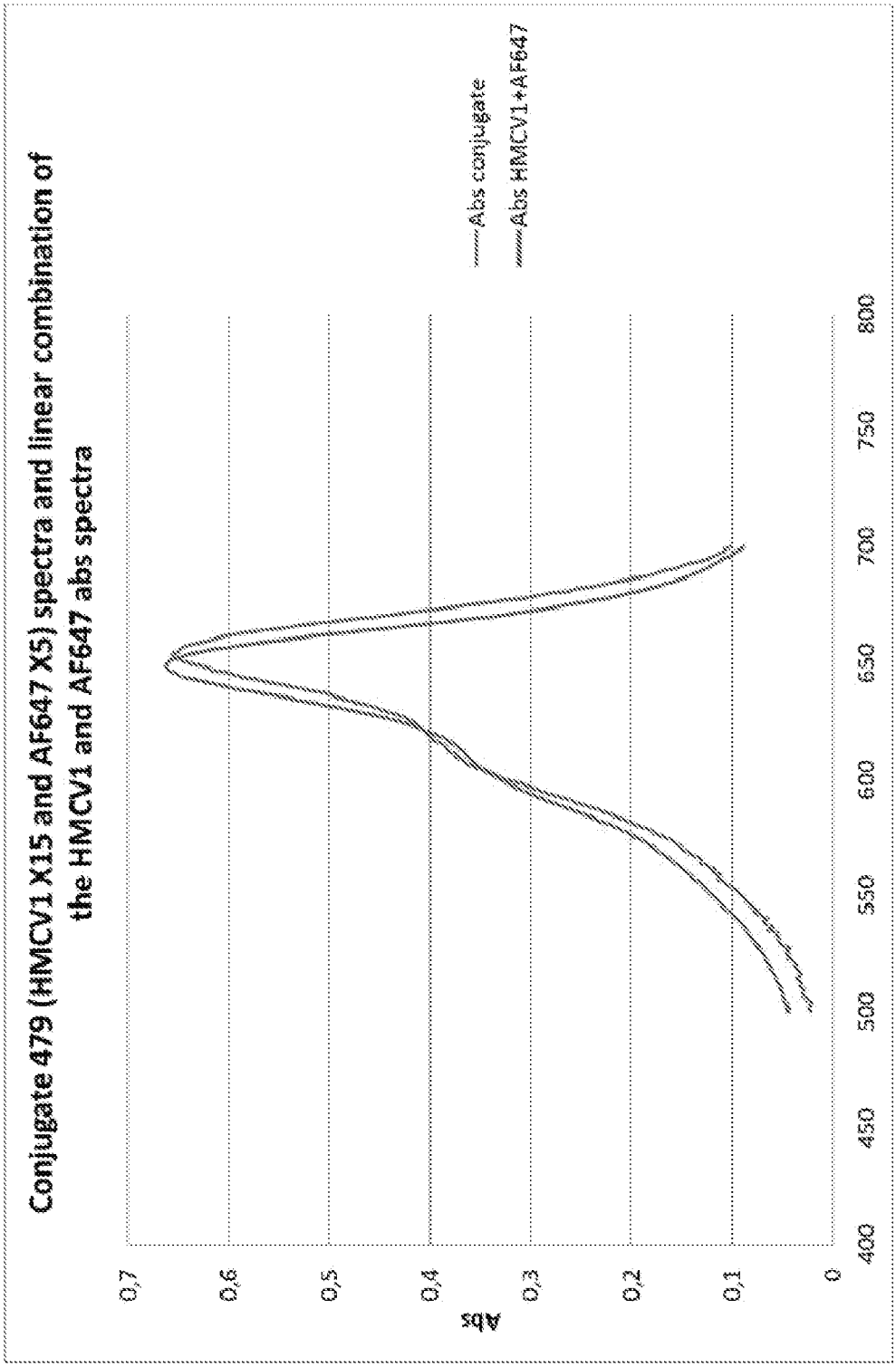
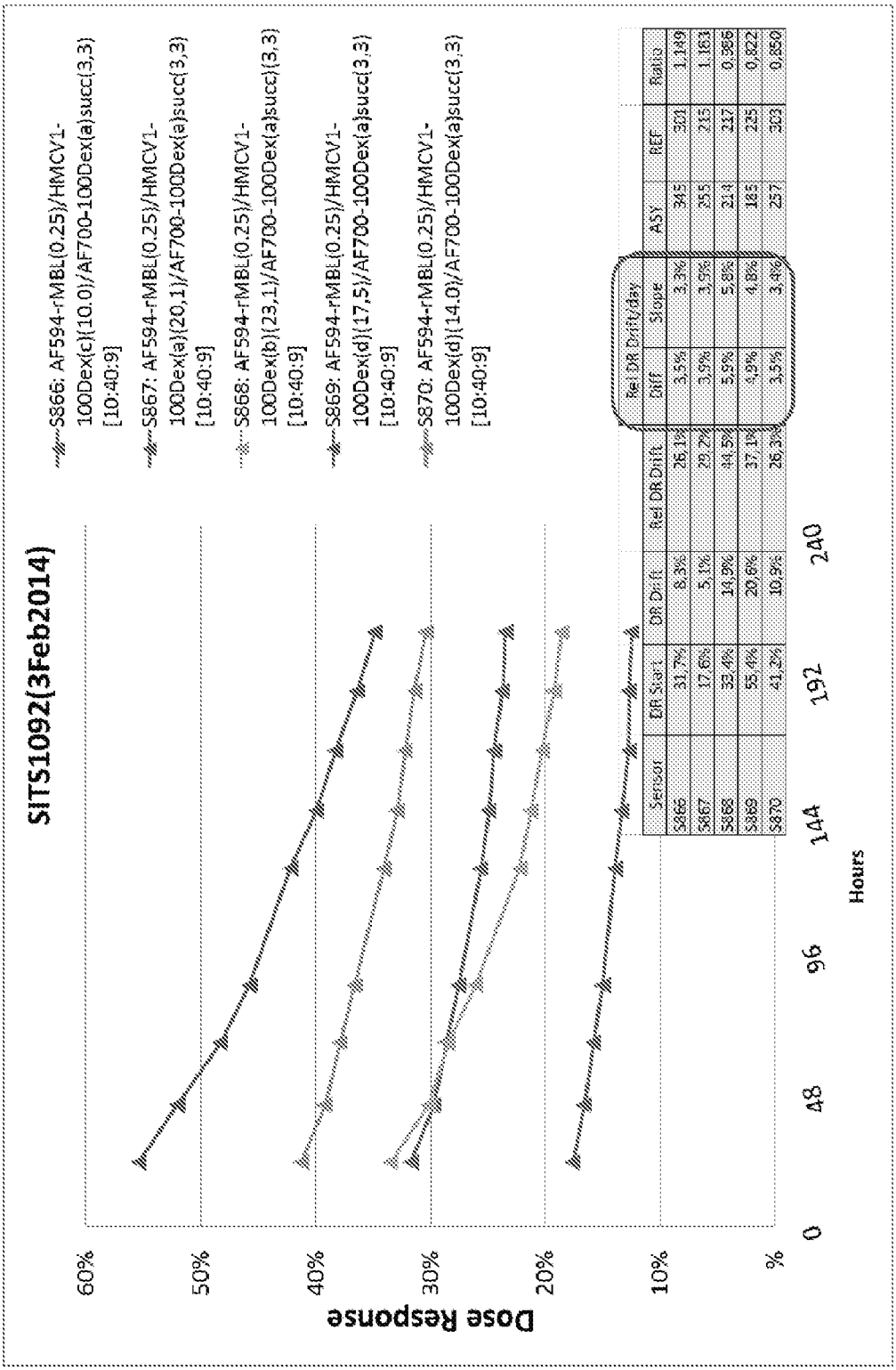
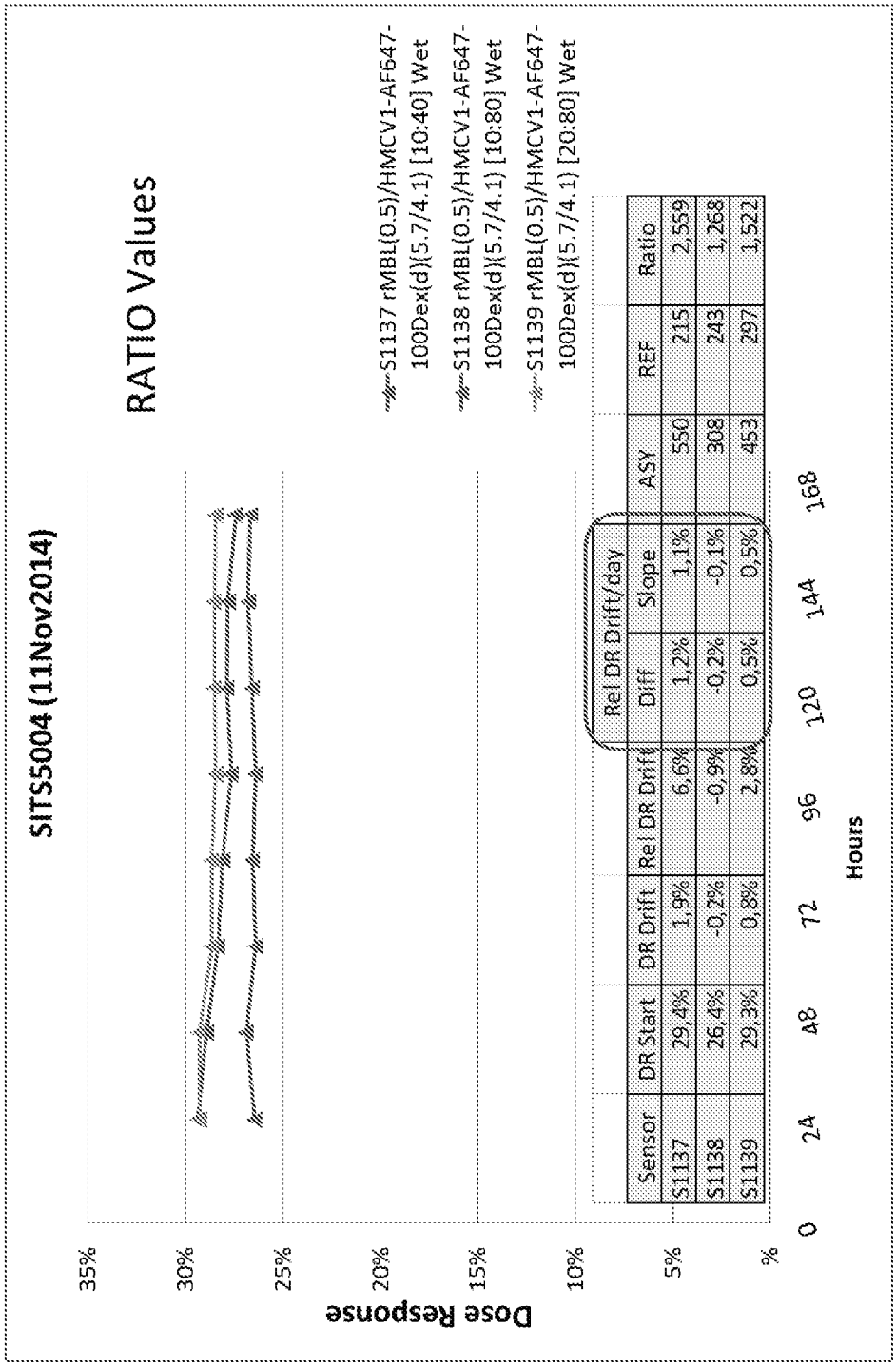

Conjugate 479 (HMCV1 X15 and AF647 X5) spectra and linear combination of the HMCV1 and AF647 abs spectra
Abs conjugate
Abs HMCV1+AF647
Abs
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
400
450
500
550
600
650
700
750
800

FIG. 12C



Sensor	DR Start	DR Drift	Rel DR Drift	Rel DR Drift/day		ASY	REF	Ratio
				Diff	Slope			
S866	31,7%	8,3%	26,1%	3,5%	3,3%	345	301	1,149
S867	17,6%	5,1%	29,2%	3,9%	3,9%	255	215	1,183
S868	33,4%	14,9%	44,5%	5,9%	5,8%	214	217	0,986
S869	55,4%	20,6%	37,1%	4,9%	4,8%	185	225	0,822
S870	41,2%	10,9%	26,3%	3,5%	3,4%	257	303	0,850

FIG. 13



Sensor	DR Start	DR Drift	Rel DR Drift	Rel DR Drift/day Diff	Slope	ASY	REF	Ratio
S1137	29,4%	1,9%	6,6%	1,2%	1,1%	550	215	2,559
S1138	26,4%	-0,2%	-0,9%	-0,2%	-0,1%	308	243	1,268
S1139	29,3%	0,8%	2,8%	0,5%	0,5%	453	297	1,522

FIG. 14

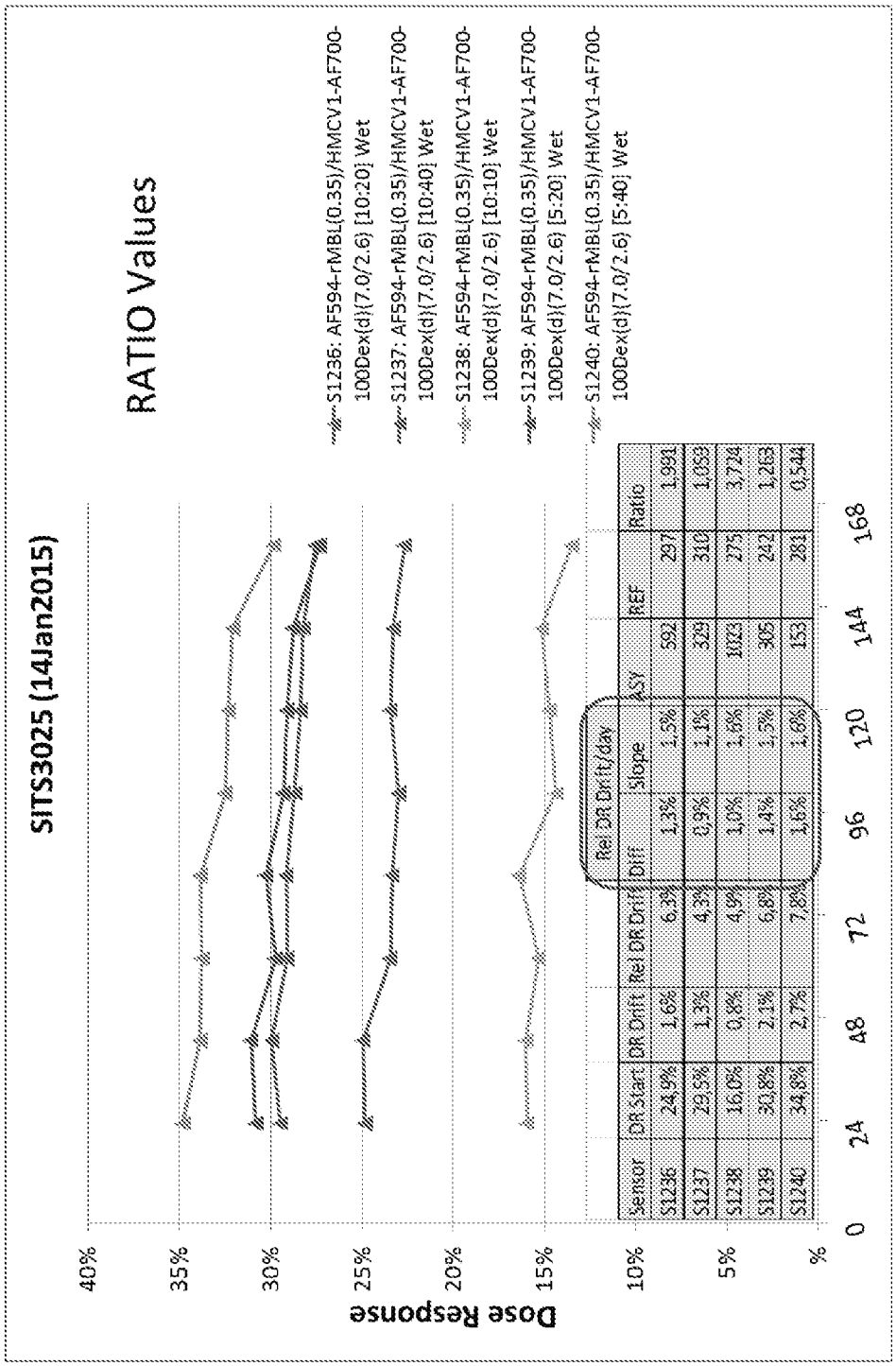
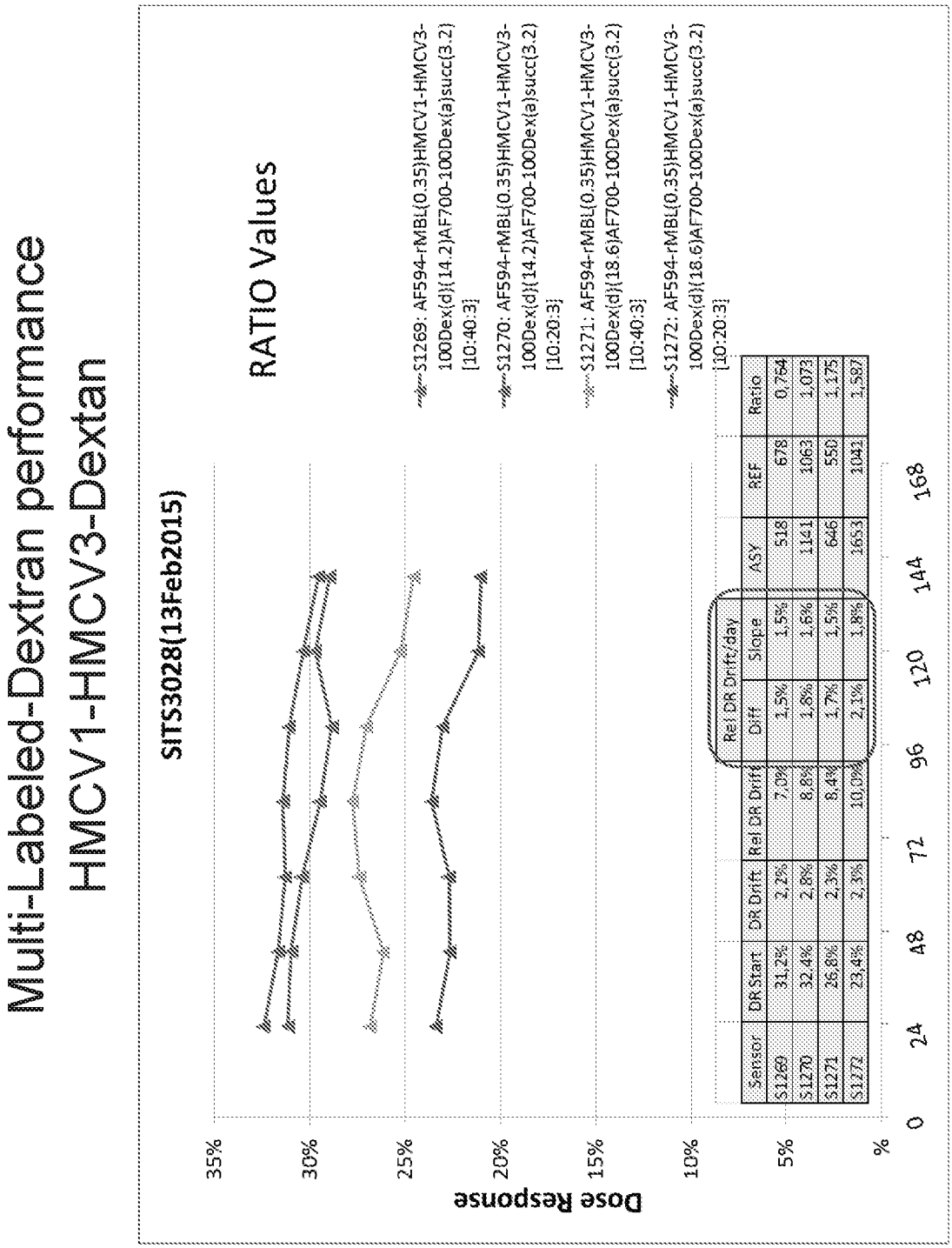
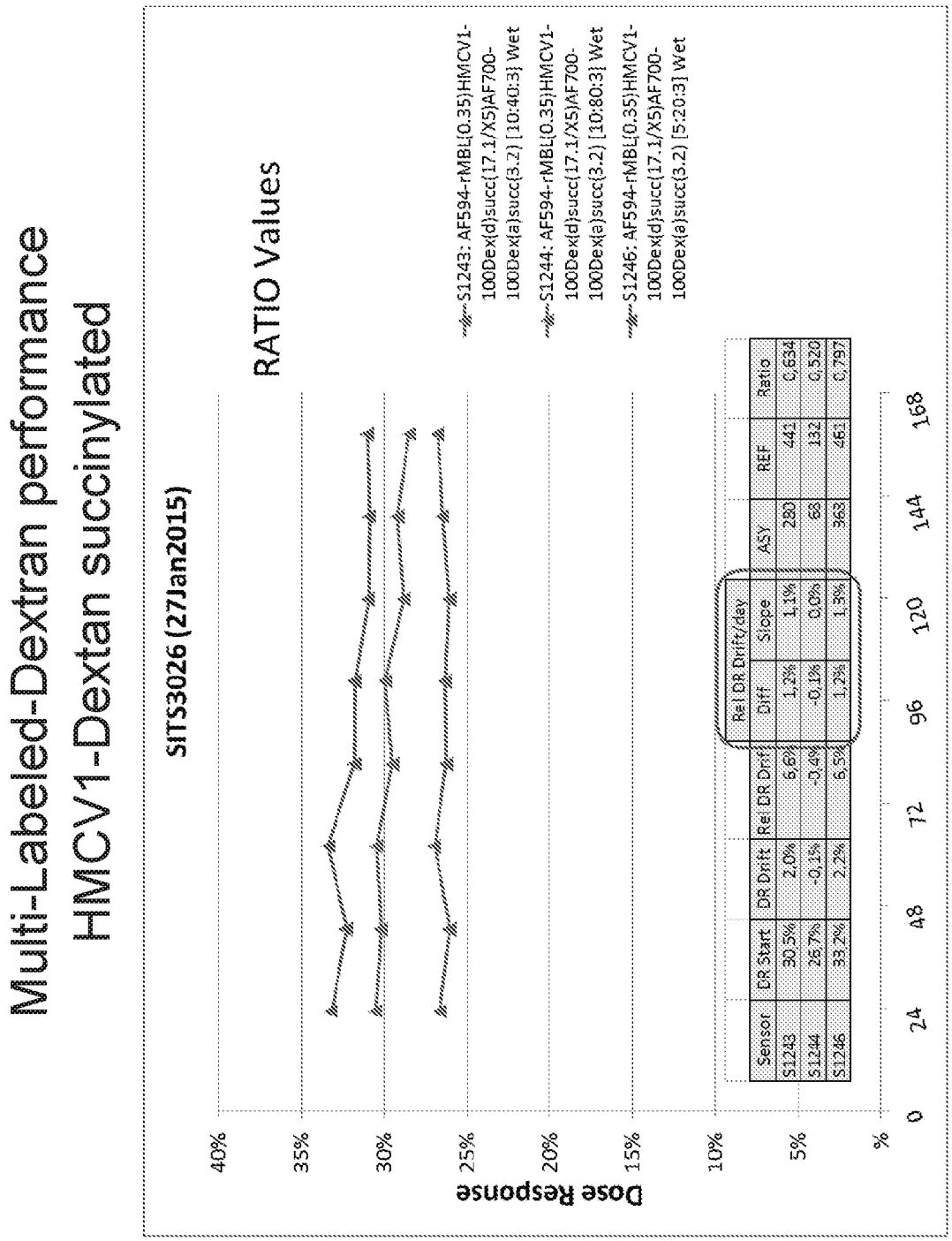

Multi-Labeled-Dextran performance
HMCV1-AF700-Dextan
SITS3025 (14Jan2015)
RATIO Values
Dose Response
40%
35%
30%
25%
20%
15%
10%
5%
%
0
24
48
72
96
120
144
168
S1236: AF594-rMBL(0.35)/HMCV1-AF700-100Dex(d)(7.0/2.6) [10:20] Wet
S1237: AF594-rMBL(0.35)/HMCV1-AF700-100Dex(d)(7.0/2.6) [10:40] Wet
S1238: AF594-rMBL(0.35)/HMCV1-AF700-100Dex(d)(7.0/2.6) [10:10] Wet
S1239: AF594-rMBL(0.35)/HMCV1-AF700-100Dex(d)(7.0/2.6) [5:20] Wet
S1240: AF594-rMBL(0.35)/HMCV1-AF700-100Dex(d)(7.0/2.6) [5:40] Wet
Rel DR Drift/day
Sensor | DR Start | DR Drift | Rel DR Drift | Diff | Slope | ASY | REF | Ratio
S1236 | 24.9% | 1.6% | 6.3% | 1.3% | 1.5% | 592 | 297 | 1.991
S1237 | 29.5% | 1.3% | 4.3% | 0.9% | 1.1% | 329 | 310 | 1.059
S1238 | 16.0% | 0.8% | 4.9% | 1.0% | 1.6% | 1023 | 275 | 3.724
S1239 | 30.8% | 2.1% | 6.8% | 1.4% | 1.5% | 305 | 242 | 1.263
S1240 | 34.8% | 2.7% | 7.8% | 1.6% | 1.6% | 153 | 281 | 0.544

FIG. 15



Sensor	DR Start	DR Drift	Rel DR Drift	Rel DR Drift/day Diff	Slope	ASY	REF	Ratio
S1269	31,2%	2,2%	7,0%	1,5%	1,5%	518	678	0,764
S1270	32,4%	2,8%	8,8%	1,8%	1,6%	1141	1063	1,073
S1271	26,8%	2,3%	8,4%	1,7%	1,5%	646	550	1,175
S1272	23,4%	2,3%	10,0%	2,1%	1,8%	1653	1041	1,587

FIG. 16



Sensor	DR Start	DR Drift	Rel DR Drift	Rel DR Drift/day		ASY	REF	Ratio
				Diff	Slope			
S1243	30.5%	2.0%	6.6%	1.2%	1.1%	280	441	0.634
S1244	26.7%	-0.1%	-0.4%	-0.1%	0.0%	68	132	0.520
S1246	33.2%	2.2%	6.5%	1.2%	1.3%	363	461	0.787

FIG. 17

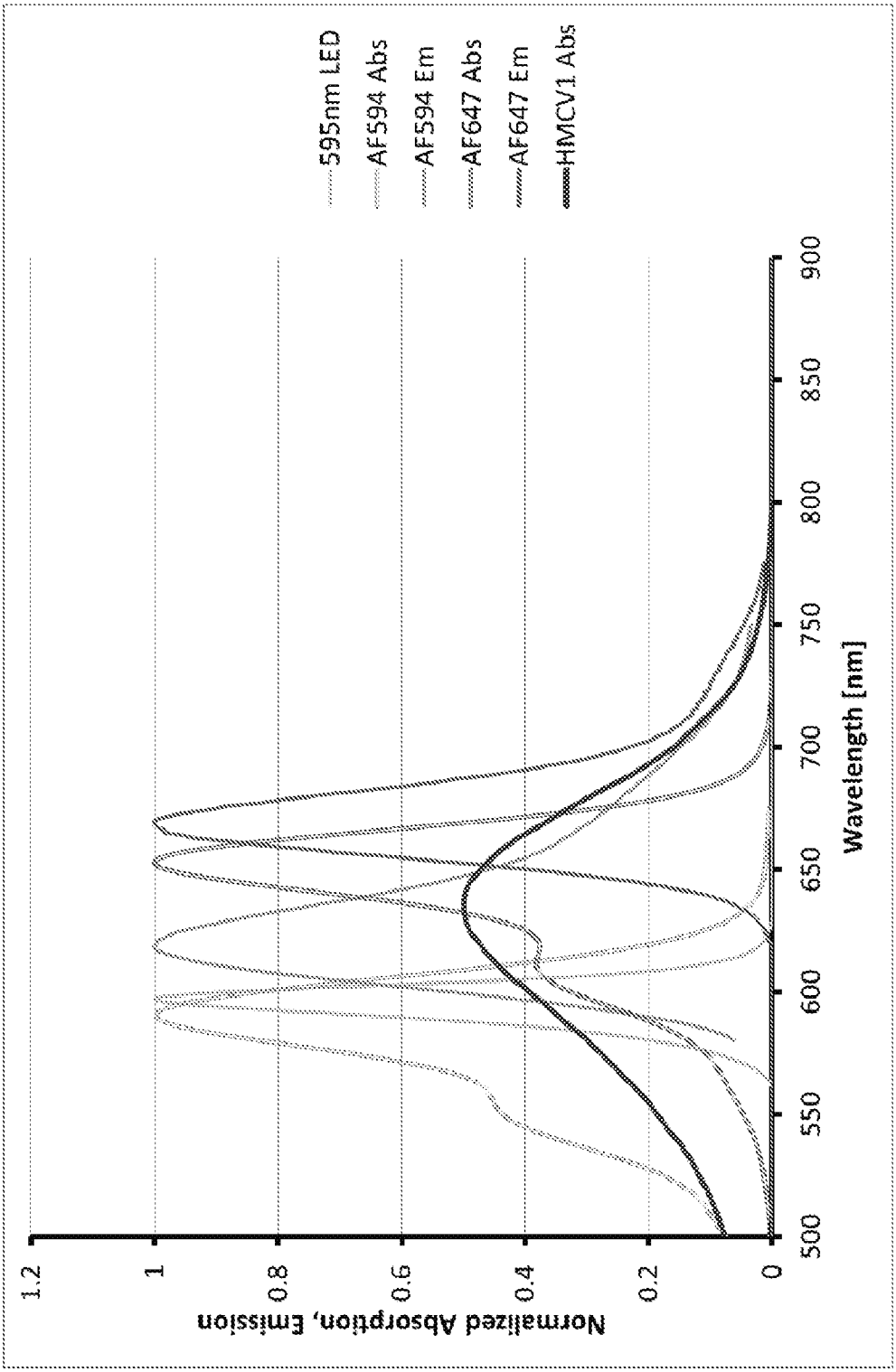

595nm LED
AF594 Abs
AF594 Em
AF647 Abs
AF647 Em
HMCV1 Abs
Normalized Absorption, Emission
Wavelength [nm]
0
0.2
0.4
0.6
0.8
1
1.2
500
550
600
650
700
750
800
850
900

FIG. 18

HMCV1 X10 and AF647 X10 HMCV1 X5 and AF647 X15 HMCV1 X15 and AF647 X5

	5.7/4.1				3.6/9.4				8.1/2.1			
	ASY	REF	Ratio	DR	ASY	REF	Ratio	DR	ASY	REF	Ratio	DR
10:20					1200	225	5,33	15,4%	1000	300	3,33	11,0%
10:40	550	215	2,56	29,4%	400	450	0,89	34,1%	525	225	2,33	18,3%
10:80	308	243	1,27	25,4%								
15:71	453	297	1,52	29,3%								

FIG. 19

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/029823

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/542 C08B37/02 G01N33/66
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N C08B

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, COMPENDEX, EMBASE, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 485 703 B1 (COTE GERARD L [US] ET AL) 26 November 2002 (2002-11-26) the whole document in particular: claims 1,7-11 column 8, line 52 - column 9, line 2 example 4 -----	1-10, 12-20
A	US 2013/060105 A1 (SHAH RAJIV [US] ET AL) 7 March 2013 (2013-03-07) cited in the application the whole document in particular: claims 1,16-21-27 paragraph [0063] - paragraphs [0076], [0081] ----- -/--	1-20

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
27 June 2018	10/07/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Tuynman, Antonin

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

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