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(54) Title: METHODS FOR DETECTING HYPERGLYCOSYLATED HUMAN CHORIONIC GONADOTROPIN (HCG-H)

(57) Abstract: Provided herein are methods for the detection of hCG-H at low concentrations and to diagnose pregnancy.



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## **METHODS FOR DETECTING HYPERGLYCOSYLATED HUMAN CHORIONIC GONADOTROPIN (HCG-H)**

### **CROSS REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims the benefit under 35 U.S.C. § 119(e) of United States Provisional Application Serial Number 61/581,609, filed December 29, 2011, which is incorporated by reference in its entirety.

### **FIELD OF THE INVENTION**

[0002] The present invention relates to methods of detecting hyperglycosylated human chorionic gonadotropin at low concentrations and use of such methods for the accelerated diagnosis of pregnancy.

### **BACKGROUND OF THE INVENTION**

[0003] The following discussion of the background of the invention is merely provided to aid the reader in understanding the invention and is not admitted to describe or constitute prior art to the present invention.

[0004] In 1997, a unique molecule was isolated from the urine of women with invasive trophoblastic disease. Initially called Invasive Trophoblast Antigen (ITA), the name was changed to hyperglycosylated human chorionic gonadotropin (hCG-H) when structural analysis revealed that ITA was actually a hyperglycosylated isoform of human chorionic gonadotropin (hCG), sharing an identical 92 amino acid alpha chain and a 145 amino acid beta chain. Both hCG and hCG-H are glycoproteins, and it is the difference in the extent and complexity of sugar residues that differentiates the two isoforms with hCG-H having more extensive and more complex carbohydrate moieties. More particularly, hyperglycosylated hCG has a molecular weight of 41,000 compared with 36,700 for regular hCG. The size difference of hCG-H is due to four hexasaccharide O-linked sugar structures instead of four trisaccharide structures and four triantennary N-linked sugar structures compared with monoantennary and biantennary oligosaccharides. Consistent with the extremely early production of hCG-H is the finding that hCG-H is only produced by the stem cytotrophoblast cells, whereas regular hCG is only produced by the mature or differentiated cells, the syncytiotrophoblast cells.

[0005] Hyperglycosylated hCG is the primary hCG isoform present during the initial three weeks of pregnancy. Many antibodies raised to hCG have significant cross reactivity with hCG-H. However, a monoclonal antibody, B152, was developed that has >99% specificity for hCG-H compared to hCG. Early in pregnancy hCG-H is the predominant isoform of hCG. Hyperglycosylated hCG is synthesized primarily by cytotrophoblasts, whereas hCG is produced by syncytiotrophoblasts. The role of hCG-H in early pregnancy appears to be facilitation of the invasion of the pre-embryo into the uterine wall during implantation.

[0006] The standard test for pregnancy using hCG-H is an automated immunochemiluminometric assay (ICMA) that uses two monoclonal antibodies: the hCG-H-specific B152 antibody described above and an hCG  $\beta$ -subunit-specific antibody (B207). B152 is biotinylated with long-chain NHS-biotin, and B207 is conjugated with acridinium ester. One form of this test is widely-known and marketed as the Nichols Institute Diagnostics Advantage® assay. Generally, this assay is a sandwich ELISA in which the B152 antibody is used to specifically capture sample hCG-H which is subsequently detected using the B207 antibody with a chemiluminescent detection means. In one exemplary embodiment of this type of assay, patient sample (e.g., urine or serum) is mixed and incubated with streptavidin-coated magnetic particles (e.g., Dynal M-270) and biotinylated B152 antibody. The sample hCG-H binds to the B152 capture antibody, which in turn is bound to the magnetic particles via the streptavidin-biotin binding pair members. The magnetic particles are washed and the B207 detection antibody, conjugated to an acridinium ester, is then added. The B207 antibody binds to an epitope common to hCG and hCG-H. The magnetic particles (including the B152-hCGH-B207 complex) are transferred to a luminometer. Hydrogen peroxide and sodium hydroxide are added to initiate the chemiluminescence reaction and the amount of sample hCG-H is proportional to the chemiluminescent signal produced (e.g., measured as relative light units (RLU)). The limit of detection using these standard chemiluminescent/sandwich ELISA methods, as described above, is about 0.1  $\mu\text{g/L}$  (or 100 picograms/mL). For a more complete discussion of the Nichols Advantage® assay, see U.S. Patent Nos. 6,627,457, 7,439,026, and 7,897,362, each of which is hereby incorporated by reference in their entirety.

## SUMMARY OF THE INVENTION

[0007] The present invention is based on the development of an hCG-H detection method that is significantly more sensitive than conventional methods. The use of these methods

allows for the positive identification of embryo implantation (or non-implantation) (i.e., pregnancy) at earlier times than can be achieved using conventional methods. This is particularly important in the context of embryo transfer procedures (i.e., in vitro fertilization and transplantation) wherein individuals seek to definitively confirm successful implantation (pregnancy) at the earliest possible time point. In one embodiment, the inventive method detects hCG-H using an electrochemiluminescence (ECL) method.

[0008] In one aspect, the present invention provides a method of detecting implantation of a human embryo comprising measuring the level of hyperglycosylated hCG (hCG-H) in a biological sample obtained from a female human, comparing the level of hCG-H to a diagnostic cutoff value of between 25 pg/ml and 1,000 pg/ml, and identifying that embryo implantation has occurred in the female human when the level of sample hCG-H is greater than or equal to the diagnostic cutoff value, and identifying that embryo implantation has not occurred when the level of sample hCG-H is less than the diagnostic cutoff value. In some embodiments, the diagnostic cutoff value may be between 25 pg/ml and 500 pg/ml, or, in further embodiments, between 25 pg/ml and 200 pg/ml. In some embodiments, the biological sample may be urine, which, in still further embodiments, may be obtained 10 days or less, 8 days or less, or 6 days or less following the day of suspected embryo transplantation.

[0009] In further embodiments, the biological sample may be serum. The serum may be obtained 8 days or less following the day of suspected embryo transplantation. In still further embodiments, the diagnostic cutoff value may be 75-200 pg/ml, or, in some embodiments, between 75-100 pg/ml. In some embodiments, the female human may have received an embryo transfer.

[0010] In some embodiments, the level of hCG-H may be determined using an electrochemiluminescence immunoassay comprising a capture antibody and a detection antibody. Such an electrochemiluminescence immunoassay may comprise at least one capture antibody immobilized on an electrode. In some embodiments, the detection antibody may comprise a detectable label, which, in further embodiments, may be the MSD sulfo-Tag reagent. The capture or detection antibody may be B152. In some embodiments, the one or more capture and/or detection antibodies are selected from the group consisting of B207, clone 820, and clone 827.

[0011] In another aspect, a method is provided for detecting implantation of a human embryo comprising measuring the level of hyperglycosylated hCG (hCG-H) in a biological sample obtained from a female human eight days or less following the day of suspected embryo implantation, comparing the level of hCG-H to a diagnostic cutoff value, and identifying that embryo implantation has occurred in the female human when the level of sample hCG-H is greater than or equal to the diagnostic cutoff value, and identifying that embryo implantation has not occurred when the level of sample hCG-H is less than the diagnostic cutoff value. In some embodiments, the biological sample obtained from a female human six days or less following the day of suspected embryo implantation. In further embodiments, the biological sample is selected from the group consisting of serum and urine. The female human may, in still further embodiments, have received an embryo transfer. In some embodiments, the level of hCG-H may be determined using an electrochemiluminescence immunoassay comprising a capture antibody and a detection antibody. Such an immunoassay may comprise at least one capture antibody immobilized on an electrode. In some embodiments, the detection antibody may comprise a detectable label, which may, in further embodiments, be the MSD sulfo-Tag reagent. In still further embodiments, the capture antibody may be B152, and the one or more hCG-H-reactive antibodies are selected from the group consisting of B207, clone 820, and clone 827.

[0012] In another aspect, a method is provided for quantifying hCG-H in a sample, said method comprising measuring the level of hyperglycosylated hCG (hCG-H) in a biological sample obtained from a female human eight days or less following the day of suspected embryo implantation, comparing the level of hCG-H to a diagnostic cutoff value, and identifying that embryo implantation has occurred in the female human when the level of sample hCG-H is greater than or equal to the diagnostic cutoff value, and identifying that embryo implantation has not occurred when the level of sample hCG-H is less than the diagnostic cutoff value.

[0013] In another aspect, a method is provided for distinguishing between an ongoing pregnancy and a biochemical pregnancy comprising: (i) measuring the level of hyperglycosylated hCG (hCG-H) in a biological sample obtained from a female human following an embryo transfer procedure, (ii) comparing the level of hCG-H to a diagnostic cutoff value of between 25 pg/ml and 1,000 pg/ml, and (iii) identifying ongoing pregnancy in the female human when the level of sample hCG-H is greater than or equal to the diagnostic

cutoff value, and identifying a biochemical pregnancy when the level of sample hCG-H is less than the diagnostic cutoff value. In some embodiments, the diagnostic cutoff value is between 25 pg/ml and 500 pg/ml, between 200 pg/ml and 400 pg/ml, or is about 300 pg/ml. In other embodiments, the biological sample is serum, plasma, or urine. In some embodiments, the biological sample is obtained about 8 days or less, or about 6 days or less following the embryo transfer procedure.

[0014] In some embodiments of any of the foregoing aspects, the day of suspected embryo implantation is the day of embryo transfer. Embryo transfers may include, for example, blastocyst transfer and cleavage stage embryo transfer.

[0015] As used herein, the term "implantation" refers to the event after fertilization of a human ovum wherein the resultant human embryo, at this stage a blastocyte, adheres to the uterine wall.

[0016] As used herein, the term "ongoing pregnancy" refers to a pregnancy which is characterized by a gestational sac and/or heart beat, or otherwise has anatomical, developmental, and/or physiological indicia of normalcy at appropriate gestational ages.

[0017] As used herein, the term "biochemical pregnancy" refers to a condition in which certain biochemical, physiological, or hormonal markers indicate a pregnancy but wherein successful implantation of the embryo in the uterine wall has failed. A biochemical pregnancy is distinct from an ectopic pregnancy in which the embryo becomes implanted in a location other than the uterine wall.

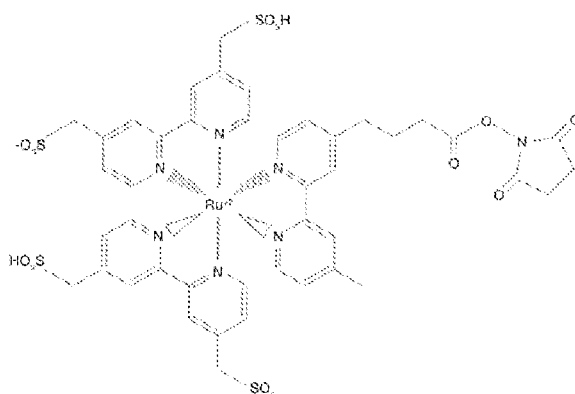
[0018] As used herein, the term "electrochemiluminescent" technology refers to the chemiluminescent reaction of species that are generated electrochemically and comprise a capture antibody, a protein of interest, and a label that emits light when electrochemically oxidized.

[0019] As used herein, the term "hyperglycosylated hCG" or "hCG-H" refers to a glycosylated variant of the hormone human chorionic gonadotropin having a molecular weight of 41,000 and four hexasaccharide O-linked sugar structures and four-triantennary N-linked sugar structures. hCG-H is also known as "invasive trophoblast antigen (ITA)."

[0020] As used herein, the term "B152" refers to a monoclonal antibody specific for the  $\beta$ -subunit C-terminal peptide and the O-linked oligosaccharide of hCG-H and produced by the hybridoma deposited with the American Type Culture Collection (ATCC) having ATCC Accession Number RB-12467.

[0021] As used herein, the term "B207" refers to a monoclonal antibody reactive with the beta subunit of hCG, and cross reactive with the beta subunit of hCG-H. The hybridoma producing the B207 monoclonal antibody was deposited with the ATCC, having Accession Number PTA 1626.

[0022] As used herein, "MSD sulfo-Tag reagent" refers to an amine-reactive, N-hydroxysuccinimide ester which readily couples to primary amine groups under mildly basic conditions to form a stable amide bond, and which has the structure:



[0023] As used herein, the "limit of detection" or "LOD" is the point at which the measured value is larger than the uncertainty associated with it. The LOD is the point at which a value is beyond the uncertainty associated with its measurement and is defined as four standard deviations from the zero concentration.

[0024] As used herein, the term "lower limit of quantification", "limit of quantitation" or "LOQ" refers to the point where measurements become quantitatively meaningful. The analyte response at this LOQ is identifiable, discrete and reproducible and is calculated as the mean background signal (i.e., signal obtained in the absence of the measured analyte) plus 10 standard deviations (SD).

[0025] As used herein, a "capture antibody" is defined as an antibody, preferably a monoclonal antibody, which does not comprise a detectable label. Preferably, the capture

antibody specifically binds to the analyte of interest (e.g., hCG-H) over closely-related, but non-target molecules (e.g., hCG). In certain embodiments of the present inventions, the capture antibody is immobilized to a solid substrate.

[0026] As used herein, a "detection antibody" is defined as an antibody, preferably a monoclonal antibody, which comprises a detectable label and is typically the second antibody that binds the analyte of interest in a sandwich-style immunoassay. Preferably, the detection antibody specifically binds to the analyte of interest (e.g., hCG-H) over closely-related, but non-target molecules (e.g., hCG); however, a high degree of binding specificity is not required provided that a highly specific capture antibody is used in the assay.

### BRIEF DESCRIPTION OF THE FIGURES

[0027] Figure 1A is a scatter plot of the serum hCG-H levels from blastocyst transfer patients for non-pregnancies ( $\Delta$ ), failed pregnancies (+), and continuing pregnancies (o). Figure 1B is a scatter plot of the serum hCG-H levels from embryo transfer patients for non-pregnancies ( $\Delta$ ) and successful pregnancies (o).

[0028] Figure 2A is a line graph showing the kinetics of the rise of hCG-H in serum from blastocyst transfer patients for non-pregnancies ( $\Delta$ ), failed pregnancies (+), and continuing pregnancies (o). Figure 2B is a line graph showing the kinetics of the rise of hCG-H in serum from embryo transfer patients for non-pregnancies ( $\Delta$ ) and successful pregnancies (o).

[0029] Figure 3A is a line graph showing the kinetics of the rise of hCG-H in urine from blastocyst transfer patients for non-pregnancies ( $\Delta$ ), failed pregnancies (+), and continuing pregnancies (o). Figure 3B is a line graph showing the kinetics of the rise of hCG-H in urine from embryo transfer patients for non-pregnancies ( $\Delta$ ) and successful pregnancies (o).

### DETAILED DESCRIPTION

[0030] The present invention is directed to methods for measuring levels of hCG-H and, therefore, for early diagnosis of pregnancy.

[0031] Elevated urine or serum hCG levels are a common marker of pregnancy. Normally or prior to implantation of a human embryo, hCG-H levels are undetectable in a biological sample. However, hCG-H levels rise quickly, usually doubling approximately every 24-72 hours, following embryo implantation. Current methods for early pregnancy detection utilize

threshold values from about 25 mIU/mL to about 100 mIU/mL (IU = International Units; 25 mIU/mL corresponds to approximately 1.79 ng/mL of hCG) as indicating pregnancy (i.e., embryo implantation). It is believed that hCG-H levels increase before the increase in hCG levels. Thus, measuring the concentration of hCG-H in a biological sample provides a marker to detect pregnancy before hCG levels increase. Because hCG-H levels are negligible prior to pregnancy and rise rapidly upon implantation, early pregnancy diagnosis is dependent upon the sensitivity (i.e., limit of detection) of the analytical method, wherein more sensitive hCG-H detection methods permit earlier pregnancy determinations. Current analytical methods (e.g., Nichols Advantage®) are sensitive enough to permit accurate pregnancy determinations by about 10-14 days after implantation. Patient hCG-H, although present, is typically too low for reliable detection and/or quantitation using existing techniques prior to that time. Thus, there is a need for hCG-H analytical techniques with improved sensitivity (i.e., lower limits of detection) which may be used for the earlier diagnosis of pregnancy than possible using existing techniques. Such an analytical method may be useful for patients seeking information about pregnancy status following natural conception or in cases of embryo transfer (e.g., following in vitro fertilization procedures).

**[0032]** Biological samples useful for practicing the methods of the invention include, but are not limited to, whole blood, serum, plasma, urine, and amniotic fluid. In addition, the samples may include tissue samples, such as, for example, tissue from the placenta, vagina, or uterus of a pregnant woman. Samples may be obtained from pregnant women by any conventional method known to those skilled in the art. For example, serum samples may be obtained by withdrawing a volume of blood from the pregnant woman using conventional intravenous/phlebotomy techniques. Amniotic samples can be obtained by withdrawing amniotic fluid from pregnant women using a needle and syringe. Preferably, a urine sample comprises the first morning urine, although samples of later urine voids may be used.

**[0033]** Screening the biological sample for hCG-H may be performed by contacting the sample with antibodies that specifically bind hCG-H. In one embodiment of the invention, "sandwich" type immunoassays are utilized to measure hCG-H in a sample in which the hCG-H is "sandwiched" between a capture antibody and a detection antibody; the latter being detectably-labeled. The methods of the invention may utilize a capture antibody that specifically binds to the hCG-H, or may be generic to multiple forms of hCG, including hCG, hCG-H, and/or nicked hCG, provided that the capture antibody is hCG-H-specific. In one

embodiment, both the capture antibody and the detection antibody specifically bind hCG-H. The capture antibody may be coupled to a solid substrate or solid phase. Examples of suitable substrates include, but are not limited to, wells of microtiter plates or cuvettes, and nitrocellulose or nylon membranes. In one embodiment of the invention, the capture antibodies are coupled to integrated carbon ink electrodes in wells of microtiter plates or cuvettes. Other methods of coupling the capture antibody to the solid phase of the assays are known to those skilled in the art.

[0034] In one embodiment of the invention, one of the antibodies (preferably the capture antibody) is the B152 monoclonal antibody. The B152 antibody specifically binds hCG-H as described in WO 98/10282, Prenatal Screening for Down's Syndrome Using Hyperglycosylated Gonadotropin; WO 99/41584, Methods for Predicting Pregnancy Outcome in a Subject by hCG Assay; WO 00/70094, Methods for Predicting Pregnancy Outcome in a Subject by hCG Assay; O'Connor et al., (1998) Differential Urinary Gonadotrophin Profiles in Early Pregnancy and Early Pregnancy Loss, Prenatal Diagnosis, 18:1232-1240; Cole et al, (1999) Hyperglycosylated Human Chorionic Gonadotropin (Invasive Trophoblast Antigen) Immunoassay: A New Basis for Gestational Down Syndrome Screening, Clinical Chemistry, 45:2109-21 19; Cole et al, (1999) Urinary Screening Tests for Fetal Down Syndrome: II. Hyperglycosylated hCG, Prenatal Diagnosis, 19:351-359; and Shahabi et al., (1999) Serum Hyperglycosylated hCG: a Potential Screening Test for Fetal Down Syndrome, Prenatal Diagnosis, 19:488-490.

[0035] As disclosed herein, B152 may be attached to a solid substrate comprising electrodes. Monoclonal antibody B152 specifically binds hCG-H. The hybridoma producing the B152 monoclonal antibody was deposited on Feb. 3, 1998 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive Rockville, Md. 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. The hybridoma was accorded ATCC Accession Number HB-12467. The B152 antibody was raised against C5 hCG, as disclosed in WO 98/10282, Prenatal Screening for Down's Syndrome Using Hyperglycosylated Gonadotropin; Cole et al., (1998) Hyperglycosylated hCG, a Potential Alternative to hCG in Down Syndrome Screening, Prenatal Diagnosis, 18:926-933; Cole et al, (1999) Hyperglycosylated Human Chorionic Gonadotropin (Invasive Trophoblast Antigen) Immunoassay: A New Basis for Gestational Down Syndrome Screening, 45:2109-21 19.

[0036] Another antibody used to practice the methods of the invention is the publicly available monoclonal antibody clone 820 available from Biodesign International, Saco, Me. (Catalog Number E45550M). The Clone 820 monoclonal antibody specifically binds to intact hCG (cross reactivity is 100%). The cross reactivity with beta hCG is less than 1.0%, with alpha-hCG is less than 1.0%, with luteinizing hormone is less than 0.1%, with thyroid stimulating hormone is less than 0.1%, and with follicle stimulating hormone is less than 1.0%. However, Clone 820 may also specifically bind hCG-H, because the hCG-H standards were reactive with the Clone 820. Clone 820 was produced in mouse, and is an IgG1 isotype. The hybridoma was prepared by fusing myeloma cells with spleen cells from Balb/c mice. Purified Clone 820 is stored in liquid format at a concentration of 5.64 mg/mL in 0.015 M potassium phosphate buffer, 0.15M NaCl, at a pH 7.2. The preservative is 0.1 % sodium azide.

[0037] Another antibody used to practice the methods of the invention is the publicly available monoclonal antibody clone 827 available from Biodesign International, Saco, Me. (Catalog Number E45575M). The Clone 827 monoclonal antibody specifically binds to beta-hCG (cross reactivity is 100%). The cross reactivity with intact hCG is 0.5%, with alpha-hCG is less than 0.1%, with luteinizing hormone is less than 0.1%, with thyroid stimulating hormone is less than 0.1%, and with follicle stimulating hormone is less than 0.1%. However, as described in Example 3, *infra*, Clone 827 may also specifically bind hCG-H, because the hCG-H standards were reactive with the Clone 827. Clone 827 was produced in mouse, and is an IgG1 isotype. The hybridoma was prepared by fusing myeloma cells with spleen cells from Balb/c mice. Purified Clone 827 is stored in liquid format at a concentration of 4.44 mg/mL in 0.015 M potassium phosphate buffer, 0.15M NaCl, at a pH of 7.2. The preservative is 0.1 % sodium azide.

[0038] In certain embodiments of the invention, the one antibody (e.g., a detection antibody) is a monoclonal antibody that recognizes the beta subunit of hCG or the beta subunit of hCG-H. One example is the B207 monoclonal antibody that binds to the beta subunit of hCG, but is cross reactive with the beta subunit of hCG-H. The hybridoma producing the B207 monoclonal antibody was deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md. 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure at Accession Number PTA 1626. The

B207 mAb was developed and described in Krichevsky et al., (1994) The Development of a Panel of Monoclonal Antibodies to Human Luteinizing Hormone and its Application to Immunological Mapping and Two-Site Assays, *Endocrine*, 2:51 1-520; WO 99/41584, Methods for Predicting Pregnancy Outcome in a Subject by hCG Assay; and WO 00/70094, Methods for Predicting Pregnancy Outcome in a Subject by hCG Assay; O'Connor et al, (1998) Differential 45 Urinary Gonadotrophin Profiles in Early Pregnancy and Early Pregnancy Loss, *Prenatal Diagnosis*, 18:1232-1240.

**[0039]** When used in a sandwich immunoassay, the detection antibody is coupled to a label, as described herein. The concentration of detection antibody used in practicing the methods of the invention is predetermined and optimized by conducting experiments to determine amounts of detection antibodies that are needed to provide a detectable signal. It will be understood by persons skilled in the art that a sufficient concentration of detection antibody is provided to ensure binding of the detection antibody to all, or essentially all, of the test antigen molecules. In other words, it is preferable to use as much detection antibody as possible without increasing non-specific binding of the detection antibody in the assay to improve the signal-to-noise ratio of the device of the invention.

**[0040]** In certain embodiments of the invention, the sandwich immunoassays are electrochemiluminescent assays. The range of sensitivity of hCG-H concentration of the assays disclosed herein is from about 4.580 to about 50,000 pg/ml.

**[0041]** In such embodiments wherein electrochemiluminescent assays are used, any known protocol or methodology may be used. However, in a preferred embodiment, the Meso Scale system (Meso Scale Diagnostics) may be used. The Meso Scale system uses a 96-well plate that is prepared as follows, or any other device or method as disclosed in U.S. Publication Nos. 2004-0022677 and 2005-0052646, which are hereby incorporated by reference in their entirety: Multi-layer plate bottoms are prepared by screen printing electrodes and electrical contacts on 0.007" thick Mylar polyester sheet. The Mylar sheet is first cut with a CO<sub>2</sub> laser so to form conductive through-holes (i.e., holes that are subsequently made conductive by filling with conductive ink) as well as to form alignment holes that were used to align the plate bottom with the plate top. Electrical contacts are formed on the bottom of the Mylar sheet by screen printing an appropriately patterned silver ink layer (Acheson 479ss) and a carbon ink overlayer (Acheson 407c). The carbon ink layer is dimensioned slightly larger (0.01 inches) than the silver ink layer to prevent exposure of the edge of the silver film.

Working and counter electrodes are formed on the top of the Mylar film in a similar fashion except that three layers of carbon ink were used to ensure that no silver remained exposed. The conductive throughholes fill with conductive ink during these screen-printing steps. A dielectric ink is subsequently printed over the electrode layers so as to define the active exposed surface area of the working electrode. Typically, nine plate bottoms are simultaneously printed on an 18"x12" Mylar sheet. Typical registrational tolerances during the screen printing steps are  $\pm 0.007$ -0.008 inches on the top side of the substrate and  $\pm 0.010$  inches on the bottom side. The separation between the printed counter and working electrode strips is kept at  $>0.010$  inches to prevent the formation of short circuits. The working electrodes are conditioned for use in assays by treating the patterned plate bottoms for 5 min. with an oxygen plasma (2000 W, 200 mtorr) in a plasma chamber (Series B, Advanced Plasma Systems, St. Petersburg, Fla.) modified with large area flat electrodes.

[0042] Multi-well assay plates are assembled using the plate bottoms described above and injection molded plate tops. The dimensions of the plate tops meets industry standards as established by the Society of Biomolecular Screening. The plate tops EW either made of black plastic (polystyrene loaded with black pigment) or white plastic (polystyrene loaded with titanium dioxide). The bottom surfaces of the plate tops ew contacted with die-cut double sided tape (1 mil PET coated on each side with 2 mil of acrylic pressure sensitive adhesive) so as to allow for sealing of the plate tops to the plate bottoms. The tape is cut to form holes that are slightly oversized relative to the holes in the plate tops. The plate bottoms are fixed (using the laser cut alignment holes) onto alignment pins on an X-Y table. The plate bottoms are optically aligned to the platetops and then sealed together using a pneumatic press (400 pounds, 10 s). Alignment is carried out sufficiently accurately so that the exposed working electrodes were centered within the wells ( $\pm 0.020$  inches for 96-well plates and  $\pm 0.015$  inches for 384 well plates). These tolerances ensure that the exposed regions of the working electrodes were within the wells and that there were exposed counter electrode surfaces on both sides of the working electrode.

[0043] ECL measurements may be taken as follows: ECL was induced from multi-well assay plates and measured using one of two instrumental configurations. Plates that are sectioned into 12 columnar sectors of 8 wells are read on an instrument designed to make electrical contact to single columnar sectors. The sector in electrical contact with the instrument is aligned with an array of 8 photodiodes that are used to measure the

ECL emitted from each well. A translation table is used to translate the plate under the array of photodiodes so as to allow all 12 sectors to be read. Plates that are sectioned into 6 square sectors are read on an instrument designed to make electrical contact to individual square sectors. The sector in electrical contact with the instrument is aligned with a telecentric lens (having a front element with a diameter of 4.1") coupled to a cooled CCD camera (VersArray: 1300F, Princeton Instruments) that is used to image ECL emitted from the sector. The camera employs a CCD chip with dimensions of roughly 2.6 cm x 2.6 cm and having a 1340x1300 array of pixels. The pixel size is 0.02 mm x 0.022 mm. An optical band pass filter in the optical path is used to select for light matching the emission profile of ruthenium-tris-bipyridine. A translation table is used to translate the plate under the telecentric lens so as to allow all 6 sectors to be read. Image analysis software is used to identify wells or assay domains within wells and to quantitate ECL from specific wells or domains. ECL from plates having screen printed carbon working electrodes was induced using a linear voltage scan from 2.5 V to 5.5 V over 3 seconds. ECL from plates having fibril-EVA composite electrodes was induced using a linear voltage scan from 2 V to 5 V over 3 seconds. ECL is reported as the total integrated light signal measured over the period of the voltage scan (after correcting for background light levels and detector offset).

## EXAMPLES

[0044] The present methods, thus generally described, will be understood more readily by reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present methods and kits.

### [0045] **EXAMPLE 1: Use of Electrochemiluminescence Assay in Detection of Low Levels of hCG-H**

[0046] To conduct a sandwich immunoassay on an MSD Multi-Array™ 96-well electrode-coated plate (Meso Scale Discovery), B152 capture antibodies were first immobilized on the working electrode surface via direct adsorption. 5 µL of B152 diluted with a 1% triton X-100 solution (5 mL buffer and 75 µL of 1% Triton X-100) were prepared for each well, and was applied to the center of each working electrode surface on a standard plate. The plate was allowed to stand overnight at room temperature for complete antibody adsorption to surface.

[0047] The detection antibody, here B207, was then conjugated to MSD Sulfo-Tag NHS-Ester. A 1-2 mg/mL solution of B207 was prepared in preservative-free PBS, pH 7.9. The

B207 was then conjugated with Sulfo-Tag NHS-Ester at the conjugation temperature of 23°C. The amount of MSD Sulfo-Tag NHS-Ester stock solution required to the conjugation reaction was calculated using the following formula:

$$\frac{1000 \times (\text{Protein Concentration (mg/mL)} / \text{Protein MW}) \times \text{Challenge ratio} \times \text{Volume of protein in solution (}\mu\text{L)}}{\text{nmol Sulfo-Tag reagent required}}$$

[0048] 150  $\mu\text{L}$  of a blocking solution (3% MSD Blocker A and IX base buffer) was added to each coated well and incubated 1 hour, after which it was washed. 25  $\mu\text{L}$  serum samples were then added to each well and incubated 1-2 hours, followed by another wash. 25  $\mu\text{L}$  of the MSD Sulfo-Tag labeled B207 was then added to each well, incubated 1 hour, and washed. Finally, 150  $\mu\text{L}$  of read buffer was added and each plate was analyzed with a SECTOR instrument.

**[0049] EXAMPLE 2: Detection of hCG-H in Urine or Blood Serum Facilitates Early Diagnosis of Pregnancy**

[0050] A prospective, blinded, clinical trial of patients undergoing routine IVF in was performed in 4 centers (The Center for Assisted Reproduction in Bedford TX, HRC Fertility Center in Los Angeles CA, the University of Colorado in Denver CO, and the University of California San Francisco Center for Reproductive Medicine in San Francisco CA) in order to determine how soon after ET an IVF pregnancy could be detected.

[0051] Contributing centers offered participation to all couples presenting for routine IVF-ET. Couples using donor oocytes or embryos were excluded from the study as were couples using frozen embryos. Participating patients had blood drawn collected on the day of ET, and at days 4, 6, 8, and 12 thereafter. A first morning urine was obtained on days 0, 4, 6, 8, 10, and 12. The samples were identified by case number and transported to the laboratory where ECG assays and analysis were performed as described in Example 1 above within 2 weeks of sample acquisition. The laboratory was blinded. Centers performed routine pregnancy testing and ultrasound confirmations according to their usual protocols. A successful pregnancy was defined as a positive pregnancy test using standard practices of the center and an ultrasound demonstrating a gestational sac and/or heart beat. A failed or biochemical pregnancy was defined as a positive pregnancy test with no gestational sac or heartbeat. A non-pregnancy was defined as a negative pregnancy test. After all data had been obtained, the code was broken and data analyzed.

[0052] There were 58 enrolled patients. Two patients withdrew prior to completing the study leaving 56 completed cycles. Not all women provided samples at all time points. The mean patient age was 34.8 years with a range of 23 - 44 years. There were 6 Asians, 4 African Americans, 6 Hispanics and 40 Caucasians. A single patient received 5,000 IU of hCG for ovulation induction, another received 6,500 IU while all others were given 10,000 IU. The mean weight of the patients was 158 lb with a range of 102 lb - 247 lb. Blastocyst transfer was performed in 70% of cycles and earlier stage embryos transferred in 30% of cases. The average number of embryos transferred was 1.3 with a range of 1 - 2. The overall ongoing pregnancy rate was with 44%. There was no significant difference between the pregnancy rate following blastocyst transfers (17 of 39, 44%) versus earlier stage embryo transfer (8 of 17, 47%). There were 12 singleton and 13 twin pregnancies. There were no triplets or higher order pregnancies. There were 6 biochemical pregnancies in the series, all of them following blastocyst transfer.

[0053] Figure 1A is a scatter plot of the serum hCG-H levels following blastocyst transfer for the 3 outcome categories: ongoing pregnancies, failed pregnancies and non-pregnancies. On the day of transfer all but 2 patients had some measureable hCG-H, presumably due to the cross-reactivity of the B152 antibody with the exogenously administered hCG. By day 6, serum and urine hCG-H levels began to rise in ongoing pregnancies whereas there was a decline in hCG-H levels in non-pregnancies, presumably due to the metabolism of injected hCG for ovulation induction. Figure 1B is the scatter plot for cleavage stage embryo transfers. The trends are similar but the variation is much greater.

[0054] Table 1 summarizes the performance of a single serum or urine hCG-H value in detecting ongoing pregnancies following IVF-ET at various times following ET. For cycles involving blastocyst transfer, a serum hCG-H > 75 pg/ml or a urine hCG-H > 25 pg/ml has a sensitivity and specificity of 100% in diagnosing pregnancy. Even as early as day 4, a single serum hCG-H value of > 25 pg/ml had an 82% sensitivity and 87% specificity.

Cycles involving cleavage stage embryo transfer are more variable. There were 2 ultimately ongoing pregnancies with delayed hCG-H rises until after 8 days. All other ongoing pregnancies were detected at days 6 and 8.

**TABLE 1: Sensitivities and Specificities for Pregnancy (both successful and failed) for a single urine or blood hCG-H level**

		Non-pregnant	Successful Pregnancy	Sensitivity	Specificity
<b>Serum hCG-H</b>					
Day 4: > 25 pg/ml	All Blastocyst Cleavage Stage	9 / 24 2 / 15 7 / 9	18 / 25 14 / 17 4 / 8	72% 82% 50%	72% 87% 22%
Day 6: >75 pg/ml	All Blastocyst Cleavage stage	0 / 19 0 / 10 0 / 9	19 / 21 13 / 13 6 / 8	90% 100% 75%	100% 100% 100%
Day 8: > 175 pg/ml	All Blastocyst Cleavage Stage	0 / 24 0 / 15 0 / 9	24 / 25 17 / 17 7 / 8	96% 100% 88%	100% 100% 100%
Day 12: >175 pg/ml	All	23 / 23	24 / 24	100%	100%
<b>Urine hCG-H</b>					
Day 4: > 15 pg/ml	All Blastocyst Early Stage	7 / 24 2 / 15 5 / 9	18 / 25 11 / 17 7 / 8	71% 65% 88%	72% 87% 44%
Day 6: > 25 pg/ml	All Blastocyst Cleavage Stage	0 / 24 0 / 15 0 / 9	19 / 25 17 / 17 8 / 2	76% 100% 75%	100% 100% 100%
Day 8: > 25 pg/ml	All	0 / 24	25/25	100%	100%
Day 10: > 200 pg/ml	All	0 / 24	25/25	100%	100%
Day 12: > 200 pg/ml	All	0 / 24	25/25	100%	100%

[0055] Figures 2A and 2B represent the kinetics of the rise in hCG-H in serum for blastocyst and cleavage stage embryo transfers respectively during the first 12 days following blastocyst transfer. The Y axis is a log scale to accommodate the large increases in hCG-H we observed during early pregnancy. As noted above, all patients had a low level of detectable hCG-H on the day of transfer. In non-pregnancies, the level of hCG-H decreased steadily thereafter. In ongoing and biochemical pregnancies the hCG-H levels did not fall during the initial 4 days post-ET, indicating probable synthesis of hCG-H by the pre-implantation transferred embryos. By day 6, hCG-H levels had risen appreciably above day 0 levels. By day 12 hCG-H levels were approximately 100 fold higher than day zero levels.

[0056] Figure 3A and 3B demonstrate the kinetics of urine hCG-H values during the post ET period. Although the curves show similar trends, the difference in urine hCG-H values between ongoing and biochemical pregnancies is not nearly as pronounced as for serum and urine hCG-H values cannot discriminate between them.

[0057] A commonly used calculation in monitoring early IVF-ET pregnancies is the approximate doubling of hCG-H levels every 48 hours in the period beginning approximately 12 days following ET. We examined our data for the rate of increase for various 48 hr time periods for both singleton and twin pregnancies. For the interval between day 4 and day 6, serum hCG-H levels increased on average 18 fold in singleton pregnancies and 13 fold in twin pregnancies. The increases were 4 fold for singletons and 5 fold for twins in the interval of day 6 to day 8. Therefore the rate of rise of hCG-H is not helpful in differentiating singleton from twin pregnancies. Of note, between days 6 and 8 hCG-H rises much more steeply than the approximate doubling of hCG in the second week following ET.

[0058] There were 6 failed pregnancies in our series, all following blastocyst transfers. The hCG-H levels are lower biochemical pregnancies than in ongoing pregnancies (Figure 1A, Figure 2A). Table 2 summarizes these data. A serum hCG-H level of <300 pg/ml was able to identify all of the failed pregnancies at day 6 and a level of <6,000 pg/ml was able to identify all of the biochemical pregnancies at day 12 (see table 2). A single ongoing pregnancy was below the cut-off value at day 6 and 2 pregnancies were below the cut-off value at day 12. It is important to note that all these data are for blastocyst transfers only. There were no cases of biochemical pregnancies for cleavage stage embryo transfers so no conclusions can be made. Urine hCG-H levels did discriminate between continuing and biochemical pregnancies.

**TABLE 2: Discrimination Between Successful and Failed Pregnancies using serum hCG-H for blastocyst transfers**

Day post ET	Successful	Failed	Sensitivity	Specificity
6 day hCG-H $\geq$ 300 pg/ml	12 / 13	0 / 6	100%	92%
12 day hCG-H $\geq$ 6,000 pg/ml	14 / 16	0 / 6	100%	88%

[0059] In sum, the present study presents results from a blinded prospective trial measuring serum and urine hCG-H levels in the 12 days following ET. It was determined that following a blastocyst transfer, a single hCG-H level measured in urine or serum is sufficient to differentiate pregnant from non-pregnant patients with 100% accuracy at 6 days post ET. Serum hCG-H levels at 6 days and 12 days post ET can distinguish biochemical versus continuing pregnancies with a sensitivity of 100% and specificities of 92% and 88%, at days 6 and 12 respectively.

[0060] If used clinically, a patient who has an hCG-H level above 300 pg/ml at 6 days following a blastocyst transfer can be told that she probably will have an ongoing pregnancy. A patient with a level between 75 pg/ml and 300 pg/ml can be told she likely has a biochemical pregnancy with a small chance it will become a continuing pregnancy. A repeat hCG-H level could be drawn at 12 days post ET for these patients. Any patient with an hCG-H level below 75 pg/ml can be told she is almost certainly not pregnant. Similarly, at 12 days post blastocyst transfer, a level of > 6,000 pg/ml is indicative of a continuing pregnancy, levels between 175 pg/ml and 6,000 pg/ml indicate a probable biochemical pregnancy, and levels below 175 pg/ml demonstrate a non-pregnancy.

[0061] The contents of the articles, patents, and patent applications, and all other documents and electronically available information mentioned or cited herein, are hereby incorporated by reference in their entirety to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference. Applicants reserve the right to physically incorporate into this application any and all materials and information from any such articles, patents, patent applications, or other physical and electronic documents.

[0062] The inventions illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those

skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[0063] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0064] Other embodiments are within the following claims. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

## WHAT IS CLAIMED IS:

1. A method of detecting implantation of a human embryo comprising  
  
measuring the level of hyperglycosylated hCG (hCG-H) in a biological sample  
obtained from a female human,  
  
comparing the level of hCG-H to a diagnostic cutoff value of between 25 pg/ml  
and 1,000 pg/ml, and  
  
identifying that embryo implantation has occurred in the female human when the  
level of sample hCG-H is greater than or equal to the diagnostic cutoff value,  
and identifying that embryo implantation has not occurred when the level of  
sample hCG-H is less than the diagnostic cutoff value.
2. The method of claim 1, wherein the diagnostic cutoff value is between 25 pg/ml  
and 500 pg/ml.
3. The method of claim 1, wherein the diagnostic cutoff value is between 25 pg/ml  
and 200 pg/ml.
4. The method of claim 1, wherein the biological sample is urine.
5. The method of claim 4, wherein the urine is obtained 10 days or less following the  
day of suspected embryo transplantation.
6. The method of claim 4, wherein the urine is obtained 8 days or less following the  
day of suspected embryo transplantation.
7. The method of claim 4, wherein the urine is obtained 6 days or less following the  
day of suspected embryo transplantation.
8. The method of claim 6, wherein the diagnostic cutoff value is 25-100 pg/ml.
9. The method of claim 1, wherein the biological sample is serum.
10. The method of claim 9, wherein the serum is obtained 8 days or less following  
the day of suspected embryo transplantation.

11. The method of claim 10, wherein the diagnostic cutoff value is 75-200 pg/ml.
12. The method of claim 9, wherein the urine is obtained 6 days or less following the day of suspected embryo transplantation.
13. The method of claim 12, wherein the diagnostic cutoff value is 75-100 pg/ml.
14. The method of claim 1, wherein the female human received an embryo transfer.
15. The method of claim 1, wherein the level of hCG-H is determined using an electrochemiluminescence immunoassay comprising a capture antibody and a detection antibody.
16. The method of claim 15, wherein the electrochemiluminescence immunoassay comprises at least one capture antibody immobilized on an electrode.
17. The method of claim 15, wherein the detection antibody comprises a detectable label.
18. The method of claim 17, wherein the detectable label is the MSD sulfo-Tag reagent.
19. The method of claim 15, wherein the capture antibody is B152.
20. The method of claim 15, wherein the one or more hCG-H-reactive antibodies are selected from the group consisting of B207, clone 820, and clone 827.
21. A method of detecting implantation of a human embryo comprising  
  
measuring the level of hyperglycosylated hCG (hCG-H) in a biological sample  
obtained from a female human eight days or less following the day of  
suspected embryo implantation,  
  
comparing the level of hCG-H to a diagnostic cutoff value, and  
  
identifying that embryo implantation has occurred in the female human when the  
level of sample hCG-H is greater than or equal to the diagnostic cutoff value,  
and identifying that embryo implantation has not occurred when the level of  
sample hCG-H is less than the diagnostic cutoff value.

22. The method of claim 21, wherein the biological sample obtained from a female human six days or less following the day of suspected embryo implantation.
23. The method of claim 21, wherein the biological sample is selected from the group consisting of serum and urine.
24. The method of claim 21, wherein the female human received an embryo transfer.
25. The method of claim 21, wherein the level of hCG-H is determined using an electrochemiluminescence immunoassay comprising a capture antibody and a detection antibody.
26. The method of claim 25, wherein the electrochemiluminescence immunoassay comprises at least one capture antibody immobilized on an electrode.
27. The method of claim 25, wherein the detection antibody comprises a detectable label.
28. The method of claim 27, wherein the detectable label is the MSD sulfo-Tag reagent.
29. The method of claim 25, wherein the capture antibody is B152.
30. The method of claim 25, wherein the one or more hCG-H-reactive antibodies are selected from the group consisting of B207, clone 820, and clone 827.
31. A method for distinguishing between an ongoing pregnancy and a biochemical pregnancy comprising
- measuring the level of hyperglycosylated hCG (hCG-H) in a biological sample obtained from a female human following an embryo transfer procedure,
- comparing the level of hCG-H to a diagnostic cutoff value of between 25 pg/ml and 1,000 pg/ml, and
- identifying ongoing pregnancy in the female human when the level of sample hCG-H is greater than or equal to the diagnostic cutoff value, and identifying a biochemical pregnancy when the level of sample hCG-H is less than the diagnostic cutoff value.

32. The method of claim 31, wherein the diagnostic cutoff value is between 25 pg/ml and 500 pg/ml.

33. The method of claim 31, wherein the diagnostic cutoff value is between 200 pg/ml and 400 pg/ml.

34. The method of claim 31, wherein the diagnostic cutoff value is about 300 pg/ml.

35. The method of claim 31, wherein the biological sample is serum.

36. The method of claim 35, wherein the serum is obtained about 8 days or less following the embryo transfer procedure.

37. The method of claim 35, wherein the serum is obtained about 6 days or less following the embryo transfer procedure.

38. The method of claim 31, wherein the level of hCG-H is determined using an electrochemiluminescence immunoassay comprising a capture antibody and a detection antibody.

39. The method of claim 38, wherein the electrochemiluminescence immunoassay comprises at least one capture antibody immobilized on an electrode.

40. The method of claim 38, wherein the detection antibody comprises a detectable label.

41. The method of claim 40, wherein the detectable label is the MSD sulfo-Tag reagent.

42. The method of claim 38, wherein the capture antibody is B152.

43. The method of claim 38, wherein the one or more hCG-H-reactive antibodies are selected from the group consisting of B207, clone 820, and clone 827.

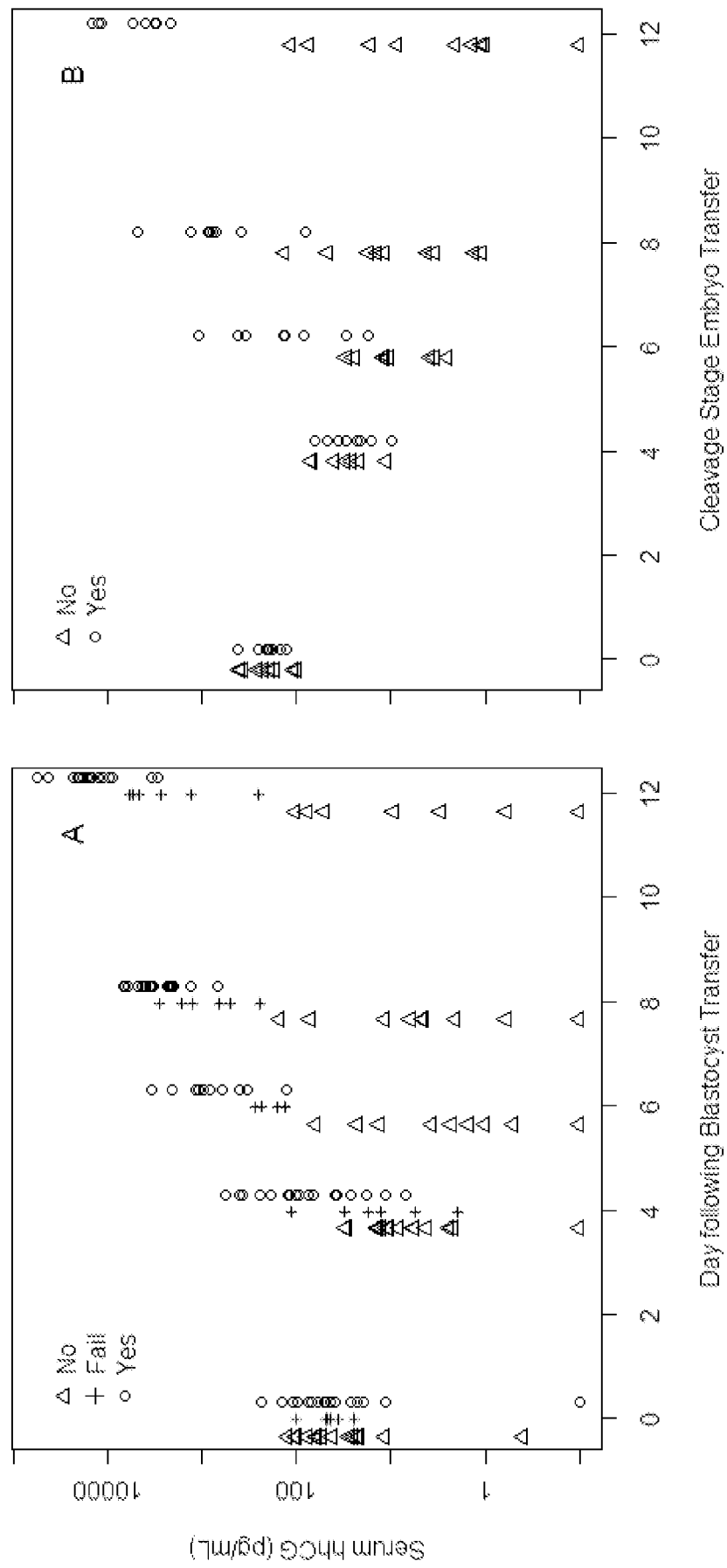
**FIGURE 1**

FIGURE 2

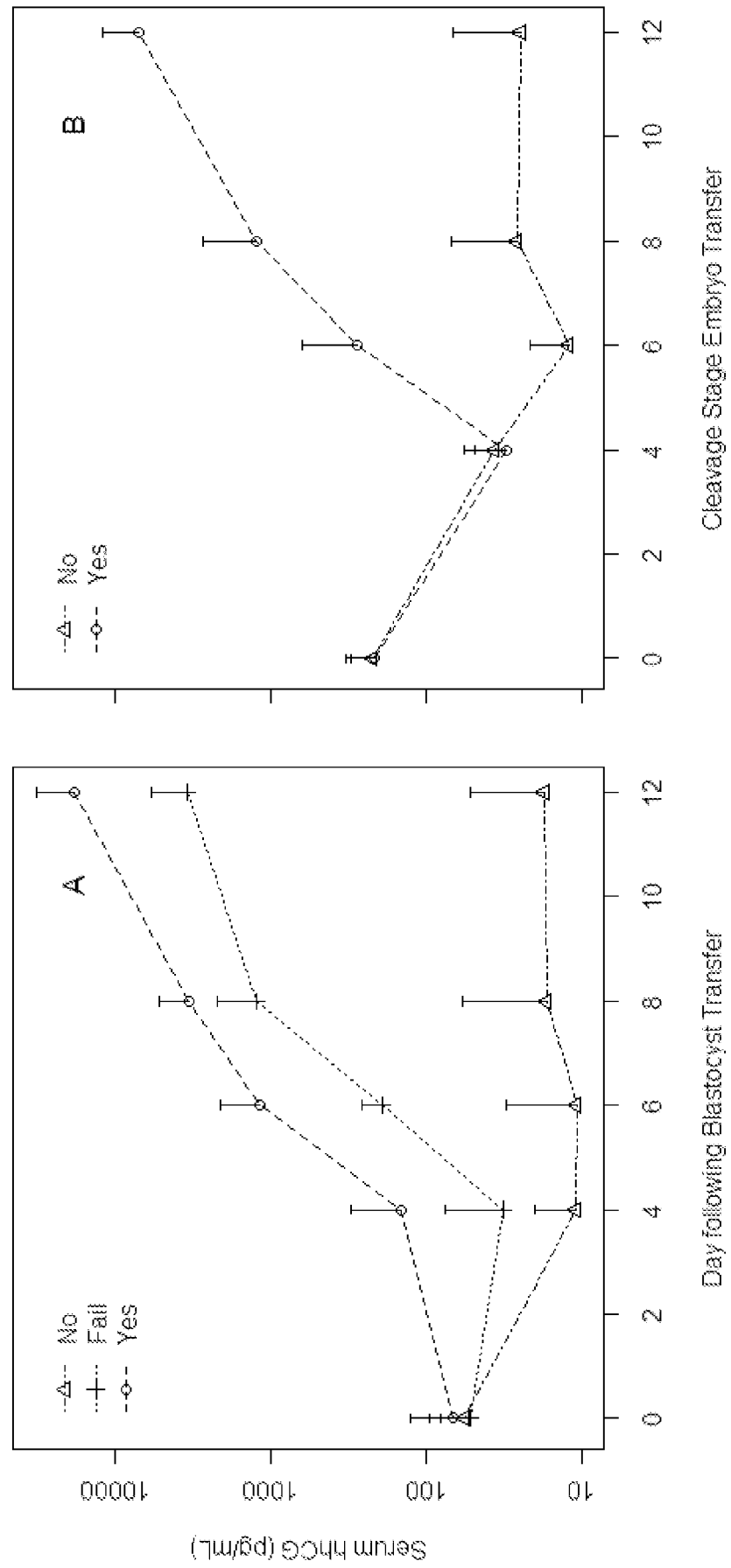
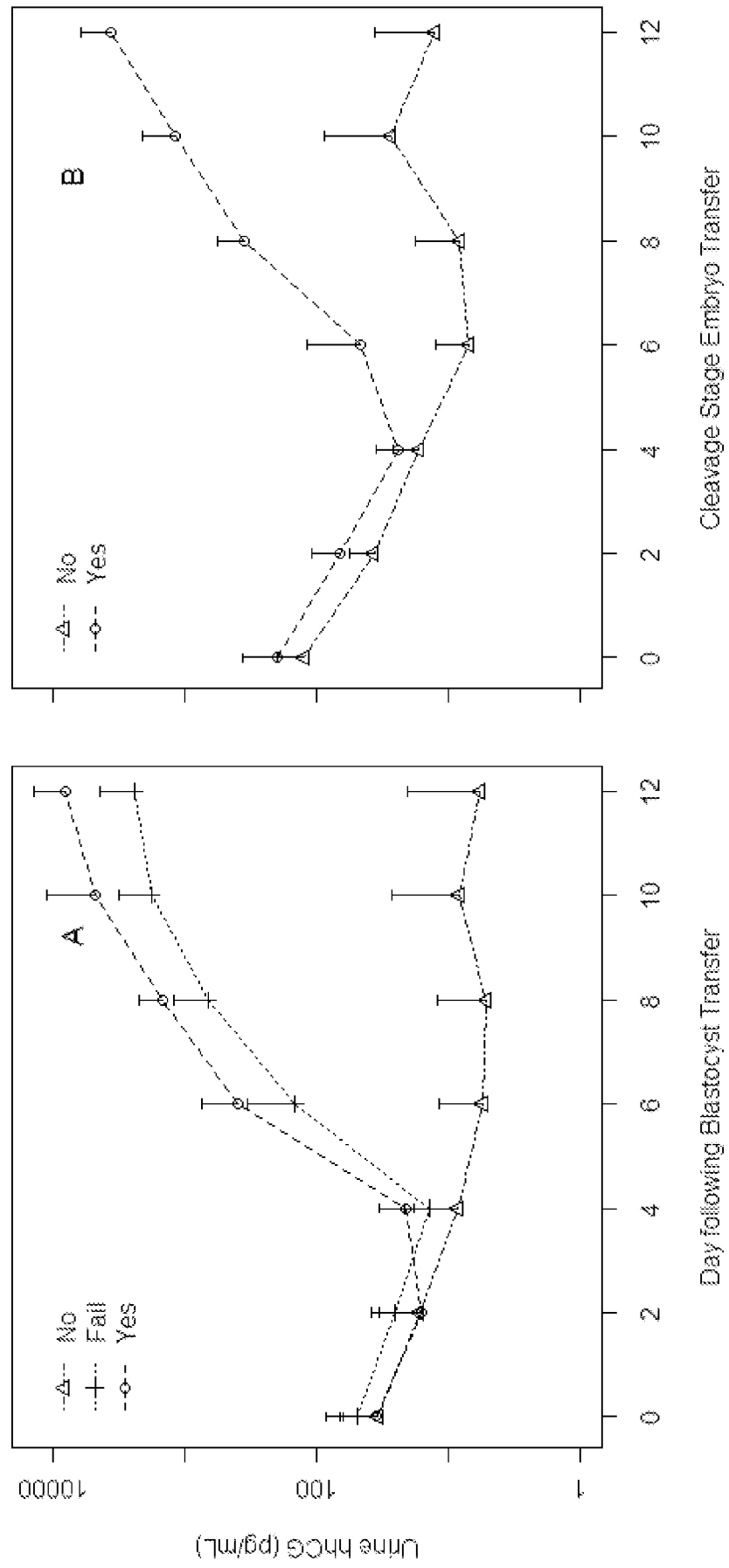


FIGURE 3



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/71749

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G01 N 33/53 (2013.01)

USPC - 435/7.1 ; 436/87

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)

USPC: 435/7.1; 436/87

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

USPC: 435/7.1; 436/86, 87

(keyword limited; terms below)

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

PatBase; Google; PubMed

Search terms: hCG-H, hyperglycosylated hCG, invasive trophoblast antigen, implantation, pg/ml, ng/ml, electrochemiluminescence, immobilized, electrode, MDS sulfo-Tag, biochemical pregnancy

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2005/0148096 A1 (COLE et al.) 7 July 2005 (07.07.2005) para [0002], [0004], [0008], [0011], [0013], [0019], [0033], [0048], [0073]-[0074]	1-15, 17, 19-25, 27, 29-30 ----- 16, 18, 26, 28, 31-43
Y	US 2005/0142033 A1 (GLEZER et al.) 30 June 2005 (30.06.2005) para [0012], [0015], [0167], [0899]	16, 26, 39
Y	US 2007/0009526 A1 (BENSON et al.) 11 January 2007 (11.01.2007) para [0254]	18, 28, 41
Y	US 2011/0201122 A1 (NAZARETH et al.) 18 August 2011 (18.08.2011) para [0010]	31-43
Y	COLE et al. Hyperglycosylated hCG. Placenta. October 2007, Vol. 28, No. 10, pages 977-986	1-43
Y	GUIBOURENCHÉ et al. Hyperglycosylated hCG is a marker of early human trophoblast invasion. J. Clin. Endocrinol. Metab. October 2010, Vol. 95, No. 10, pages E240-E244	1-43
Y, P	STROM et al. The sensitivity and specificity of hyperglycosylated hCG (hhCG) levels to reliably diagnose clinical IVF pregnancies at 6 days following embryo transfer. J. Assist. Reprod. Genet. 24 April 2012 (24.04.2012), Vol. 29, No. 7, pages 609-614	1-43

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

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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

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

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

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Date of the actual completion of the international search

14 February 2013 (14.02.2013)

Date of mailing of the international search report

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Name and mailing address of the ISA/US

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