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Research paper

Development of a second generation Inhibin B ELISA

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ABSTRACT

Inhibins are heterodimeric protein hormones secreted by the granulosa cells of the ovary in the female and the Sertoli cells of the testis in the male. Published research studies have assessed Inhibin B levels in Sertoli cell function, ovarian reserve and granulosa cell tumors. A two-step sandwich-type enzymatic microplate assay to measure Inhibin B levels within 3.5 h is reported, and sample pre-treatment is not required. The assay measures Inhibin B in 50 μL of serum or Li-Hep plasma sample against Inhibin B calibrators (10–1000 pg/mL). The highly characterized antibody pair used in the assay measures 100% Inhibin B and no response was detected above the sensitivity of the assay with Inhibin A, Activin A, Activin B, Activin AB, AMH, FSH, LH or Follistatin 315 at the concentrations tested. The second generation Inhibin B assay was compared against two commercially available assays using 60 male and 60 female samples, ranging in age from 20 to 50 years. The assay showed significant positive linear correlations to Oxford Brooks Innovation (OBI) and Diagnostics Systems Laboratories (DSL) assays ($r=0.99$; $P<0.0001$; and $r=0.97$; $P<0.0001$), respectively. Method comparison to OBI and DSL resulted in the following slope and intercept (Gen II = 1.03OBI − 6.77 pg/mL and Gen II = 1.57DSL + 11.29 pg/mL), respectively. Matched serum and Li-Hep plasma samples ($n=120$) showed a correlation coefficient of >0.99 and a slope of 0.97 with zero intercept. Total imprecision calculated on three samples and two controls over 40 runs, three replicates per run, using NCCLS EP5-A guidelines was 6.8% at 19.34 pg/mL, 4.4% at 76.03 pg/mL, 4.3% at 275.3 pg/mL, 5.4% at 99.88 pg/mL, and 5.7% at 363.9 pg/mL. The LoQ for the assay at 20% CV was 4.8 pg/mL. Dilution and spiking studies showed an average recovery of 90–110%. A highly specific, sensitive, simplified and reproducible microplate Inhibin B assay has been developed to measure Inhibin B in serum and Li-Hep plasma. The performance of the assay is ideal for investigation into the physiologic role of Inhibin B in both men and women.

1. Introduction

Inhibins are dimeric polypeptide hormones which belong to the transforming growth factor β (TGF-β) super family (Evans and Groome, 2001; Robertson et al., 2004a,b). Inhibins are heterodimers consisting of a common α-subunit linked to either a βA subunit (Inhibin A) or βB-subunit (Inhibin B) by disulfide bridges (Miyamoto et al., 1985; Robertson et al., 1985; Mason et al., 1986). Inhibins are secreted by the granulosa cells of the ovary in the female and the Sertoli cells of the testis in the male. Both Inhibin A and Inhibin B are produced in females, but in males Inhibin B is the predominant circulating inhibin. The primary endocrine role of Inhibin B appears to be the regulation of gametogenesis via a negative feedback mechanism on the production of FSH by the pituitary gland. Inhibin B may also exert local paracrine actions in the gonads (Burger and Igarashi, 1988; Attardi et al., 1992; Burger et al., 1998; Knight and Glister, 2001; Chen, 1993).

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The physiologic role of Inhibin B in both men and women has been investigated for over a decade and has found application in reproductive endocrinology. In the male Inhibin B supports spermatogenesis. The serum Inhibin B level for normal males is usually <400 pg/mL (Pierik et al., 1998). However in pathological situations such as infertility, the levels of Inhibin B may be diminished and can be differentiated from normal males (Pierik et al., 1998; Anawalt et al., 1996; Ballesca et al., 2000; Pierik et al., 2003; Kumanov et al., 2006). In the female, in contrast to FSH, Inhibin B has been studied as a direct, more sensitive and earlier marker of ovarian follicle number (Roudebush et al., 2008) as it is secreted directly by the granulosa cells of the small, developing follicles of the ovary. Inhibin B also finds research applications in assisted reproductive technologies (ART). Knowledge of woman's ovarian reserve prior to and during ART is useful in assessing the level of pharmacological stimulation required in the IVF cycle. Too little stimulation can fail to produce enough oocytes for collection and over-stimulation can increase the risk of ovarian hyperstimulation syndrome (OHSS). Circulating Inhibin B finds its research application in predicting the increased risk of OHSS prior to IVF procedure (Seifer et al., 1999; Fawzy et al., 2002; Muttukrishna et al., 2005; Urbancsek et al., 2005). Inhibin B levels decline substantially during early perimenopause or transition. As circulating Inhibin B levels fall, the suppressive effect on pituitary FSH secretion declines leading to an elevation in circulating FSH. Elevated FSH levels then accelerate follicular recruitment and an overall decline in ovarian reserve as menopause approaches (Conway, 1997). The most common ovarian cancer marker, CA-125 is effective in detecting the majority of epithelial cancers, but is less effective in detecting granulosa cell tumors. Elevated inhibin levels have been observed in women with granulosa cell tumors. Combination of the two measurements is of great interest in research studies as they may detect a greater percentage of ovarian cancers (Robertson et al., 1999, 2002).

A highly specific and sensitive Inhibin B assay is needed for various research applications. This need led to the development of new generations of Inhibin B assays (Evans and Groome, 2001; Ludlow et al., 2008; Groome et al., 1996). The two commercial Inhibin B immunoassays available, from DSL (Webster, TX, USA cat # DSL10-84100) and OBI (Upper Heyford, Oxon, OX25 5HD, UG, cat # MCA 1312KZZ) both from Beckman Coulter Inc., use the same pair of monoclonal antibodies raised to a synthetic peptide. The capture antibody (C5) was raised to a peptide from the βB-subunit of inhibin and R1, the detection antibody, was raised to a peptide from the α subunit of inhibin. Both assays require a methionine (in the β-subunit) oxidation step with hydrogen peroxide to allow the C5 antibody to recognize its epitope and show approximately 0.5% cross-reactivity with Inhibin A. Additionally, the OBI assay requires a specificity enhancing step of heating samples with sodium dodecyl sulphate solution. Both the inhibin assays on the market have the limitation of being cumbersome, non-harmonized and require overnight incubation of samples for adequate sensitivity.

Our aim was to investigate the comparability of the assay results of the two commercially available Inhibin B methods versus a significantly improved second generation Inhibin B ELISA assay. The second generation Inhibin B assay uses a new monoclonal antibody to the βB-subunit of inhibin/activin (Ludlow et al., 2008) as capture. The assay is simplified in that it does not require an oxidation step (sample pre-treatment) and has improved performance with a turn around time of 3.5 h. The assay correlates well with the two commercially available assays and is traceable to the WHO 96/784 IRP.

2. Materials and methods

2.1. Coating of microtiter plates

The immunization protocol, development of monoclonal antibody to the βB-subunit of inhibin/activin and the screening procedure for the selection of the antibody to the βB-subunit have been published (Ludlow et al., 2008). The 46A/F monoclonal antibody used for coating was purified by Protein G (Millipore, Billerica, Massachusetts, USA) affinity chromatography (Harlow and Lane, 1988) at Bioserv, Inc. Ltd. UK. Microtiter plates from Greiner bio-one (Maybachstr. D-72636 Frickenhausen, Germany, cat: 705071) were coated with 100 µL/well of 10 µg/mL 46A/F in 50 mM sodium carbonate buffer, overnight at room temperature. Excess antibody was removed, and the plates were washed once using 300 µL/well of 10 mM phosphate buffer saline (PBS) with 0.1% Triton X-100 (Lab Chem Inc., Pittsburgh, PA, USA CAT# LC26280-1). The plates were blocked with 200 µL/well of 10 mM PBS containing 1% BSA , 4% sucrose, 0.05% Tween-20 (Amresco, Solon, Ohio, USA, cat # E588, 0335, 0777) and proclin (Supelco, Bellefonte, PA, USA, cat # 16823-0048) for 16–24 h at room temperature and 85% humidity. The blocking solution was aspirated and the plates were dried at 34 °C for 4–5 h. The plates were then packed in foil pouches with desiccant, labeled and stored at 2–8 °C.

2.2. Biotinylation of R1 monoclonal antibody

Monoclonal antibody R1 (IgG2a), raised to the α subunit of inhibin (Groome et al., 1990), was biotinylated with EZ-link Sulfo-NHS-LC-Biotin (Biotin, Pierce, Illinois, US, cat #21335). R1 antibody was dialyzed in 0.05 M sodium bicarbonate containing 0.15 M sodium chloride (dialysis buffer) and then diluted in 1 M carbonate buffer to give a final concentration of 2 mg/mL by reading the absorbance at 280 nm. The biotin was made up immediately prior to use by adding 5 mg to 1 mL of deionized water. 100 mmol of biotin was added per mmol of antibody and incubated at room temperature for 2 h with gentle mixing. After 2 h the reaction mixture was dialyzed against 1 L of dialysis buffer three times at 2–8 °C to remove excess biotin.

2.3. Calibration curve

Second generation Inhibin B calibrators were made in fetal bovine serum (Equitech-bio, Inc. Kerrville, TX, cat # SF B30) using recombinant Inhibin B cell culture supernatant (DSL-BCI Webster, TX, USA, clone # L 2.1.2). The calibrators are traceable to the WHO Inhibin B reference reagent preparation (NIBSC, Potters Bar, UK, cat# 96/784). The assay was calibrated to OBI Inhibin B ELISA (Upper Heyford, Oxon, OX25 5HD, UG, cat # MCA 1312KZZ). The assay uses a seven point calibration (with blank subtracted). The log of Inhibin B concentration is plotted on the X-axis, the log of matched optical density on the Y-axis, and the curve is fit using cubic regression (Opsys MR, Dynex technologies, USA, Revelation Quicklink version 4.25).

2.4. Controls

Quality control consists of fresh serum samples (PromedDx, Norton, MA, USA, www.promedDx.com) containing various levels of Inhibin B. The nominal concentrations of the control samples were established by analyzing the samples in the second generation Inhibin B ELISA.

2.5. Assay procedure

Second generation Inhibin B ELISA is an enzymatically amplified "three-step" sandwich-type immunoassay. In the first step, 50 μL of calibrators (six vials, containing concentrations of approximately 10, 30, 100, 250, 500 and 1000 pg/mL of Inhibin B in fetal bovine serum as mentioned in calibration curve), controls and unknown samples are added to microtitration wells coated with anti-activin B antibody followed by addition of 50 μL of Inhibin B Gen II assay buffer (protein-based 50 mM trizma-maleate buffer, pH 7.2) to each well. The wells were incubated with shaking on an orbital microplate shaker (Lab-line Instrument Inc. USA) for 2 h at room temperature (~25 °C) followed by five times washing (Bio-Rad model 1575, USA) with phosphate buffer saline containing a nonionic detergent (wash solution).

In the second step, 100 μL of the second generation Inhibin B antibody-biotin conjugate solution was added to each well and incubated on an orbital shaker for 1 h at room temperature followed by five times washing with a wash solution.

In the third step, the wells were incubated on an orbital shaker with 100 μL/well of horseradish peroxidase labeled streptavidin enzyme conjugate (Prozyme, Hayward, CA, USA cat # CJ30H) solution (SHRP in protein-based MES-TRIS buffer) for 30 min at room temperature followed by five times washing. After the final wash step, the wells were incubated on an orbital shaker with 100 μL/well substrate tetramethylbenzidine (Neogen Corp., Lexington, KY, USA, K-Blue Aqueous TMB Substrate, cat # 331199) for 10–12 min followed by addition of 100 μL/well stopping solution (0.2 M H_2SO_4). The degree of enzymatic turnover of the TMB was determined by dual wavelength absorbance measurement at 450 nm as primary test filter and 630 nm as primary reference filter. The absorbance measured was directly proportional to the concentration of Inhibin B in the samples. Second generation Inhibin B calibrators were used to plot a calibration curve of absorbance versus Inhibin B concentration. The Inhibin B concentrations in the samples were then interpolated from the calibration curve.

2.6. Sample type

All samples were collected under an institutional review board (IRB) approved protocol (PromedDx, Norton, MA, USA, www.promedDx.com). Matched blood samples from 120 volunteers (60 males and 60 females, between the ages of 20 and 50 years) were drawn using serum SST tubes and lithium heparin plasma. One hundred and six female (day 3 of cycle) serum samples were investigated. Twenty post-menopausal serum samples from women over 70 years of age were used in studies looking for false positives in the new assay. The results for these samples were compared using linear regression analysis using the Passing and Bablok method conversion and correlation coefficients using the Spearman rank correlation method.

2.7. Statistical methods

The data were reduced using the log–log cubic regression curve fit provided in the Revelation (version 4.25) data package included in the Dynex Opsys MR microplate ELISA reader (Dynex Technologies Ltd, Virginia, USA). Data analyses were performed using Excel 2003 (Microsoft Corp.), Analyse-it® version 1.73 (Analyse-it® Software limited, Microsoft Corp.). Descriptive data are presented as the mean and SD unless otherwise specified.

2.8. Validation procedures

The validation of the second generation Inhibin B assay was carried out following the manufacturer's (DSL-BCI, Webster, TX, USA, Inhibin B Gen II RUO, cat # A81301 and A81302) recommendations for preparation and storage of reagents, calibrators, and controls and for running the protocol.

2.8.1. Sensitivity

2.8.1.1. Limit of blank. The highest measurement that indicates Inhibin B is not present in the sample (generally expressed as the concentration at which a sample with no analyte will fall below 95% of the time). The study was designed following CLSI EP 17 guidelines (NCCLS, 2004, EP17-A). Calibrator with 0 pg/mL Inhibin B was run as a single sample over 40 inter-assay runs in two kit lots. The concentration of the sample (no analyte) observed at 95% CI was calculated.

2.8.1.2. Limit of detection. The lowest amount of Inhibin B in a sample can be detected with a 95% probability. The study was designed following CLSI EP17 guidelines (NCCLS, 2004, EP17-A). Six human serum samples with approximate Inhibin B concentration of 0.0 pg/mL, 5 pg/mL, 6 pg/mL, 9 pg/mL, 15 pg/mL and 50 pg/mL were run in duplicates with seven point calibration curve and controls. Two assay runs per day were performed over 10 days with each sample as unknown run in duplicates per run. The lowest amount of the Inhibin B in a sample that can be detected with a 95% probability was calculated. The individual SD's of the five samples over 20 runs were calculated and applied to the formula below for the best estimate of LoD.

$$LoD = LoB(\text{measured from sample with no analyte}) + 1.647 \times SD's$$

$$SD's = \sqrt{[\{(SD1 \times SD1) + (SD2 \times SD2) + (SD3 \times SD3) + (SD4 \times SD4) + (SD5 \times SD5)\} / 5]}$$

2.8.1.3. Limit of quantitation. The functional sensitivity or limit of quantification (LoQ) for second generation of Inhibin B assay was determined as the lowest concentration that can be measured with a total imprecision of 20%. The study was designed following CLSI EP17 guidelines (NCCLS, 2004, EP17-A). Eight human serum samples with Inhibin B concentration in the range of 0–308.1 pg/mL, were run with seven point calibration curve and controls. Two assay runs per day were performed over 10 days with samples run in duplicates. The average, SD and %CV was calculated and Inhibin B concentration (pg/mL) versus %CV curve was plotted. The estimated minimum dose achieved at 20% total imprecision was then interpolated.

2.8.2. Linearity of dilution

The study was designed following CLSI EP6 guidelines (NCCLS, 1986, EP-6). Four samples with Inhibin B concentration in the range of 279–1000 pg/mL were diluted in the calibrator matrix (FBS). Multiple dilutions of these samples were assayed against the calibration curve and the observed results were tabulated. Expected values were calculated by dividing the concentration from the undiluted sample by the dilution factor used. Percentage recovery was calculated by dividing the observed values by the expected values and multiplying by 100.

2.8.3. Spike recovery

Recovery was determined by spiking known amounts of Inhibin B into four human serum samples containing different levels of endogenous Inhibin B. The concentration of Inhibin B in the sample was determined before (endogenous) and after (observed concentration) the addition of exogenous Inhibin B. Expected concentration was calculated as: Expected concentration = [(Endogenous concentration × Volume of sample added) + (Spike concentration × Volume of spike added) / Volume of sample added + Volume of spike added]. The percent recovery was calculated as: %Recovery = (Observed concentration / Expected concentration) × 100.

2.8.4. Imprecision

The study was designed following CLSI EP5-A guidelines (NCCLS, 1999, EP 5-A). Precision of the assay was determined on three serum samples and the kit controls with Inhibin B concentrations of 19.3, 76.0, 275.3, 99.9, and 363.9 pg/mL, respectively. These samples and controls were run in triplicates in two assay runs per day per lot, for 10 days over two lots. Precision was expressed as percent coefficient of variation (%CV) for within run, between run and total assay ($n = 120$) variability.

2.8.5. Selectivity/specificity

Inhibin B in human serum samples, pooled male rat samples and pooled male rhesus monkey samples was assayed as unknowns to check the specificity of the assay for other species. Structurally related proteins and some of the other members of the TGF-β super family were tested in the second generation Inhibin B assay to determine their cross-reactivity: Inhibin A (DSL, Webster, USA, Cell line 1.203.2600), activin A (DSL, Webster, USA, Cell line BA83.6.2), activin B (DSL, Webster, USA, Cell line CHOB), follistatin 315 (DSL, Webster, USA, Cell line 315 M16), follicle-stimulating hormone (DSL, Webster, USA, DSL-4706), luteinizing hormone (Scripps, San Diego, CA, USA, cat # L0813) and Anti-Müllerian Hormone (DSL, Webster, USA, Cell line HEK 293). All the substances were assayed against the standard curve as unknowns at more than two times the physiological concentrations. Percent cross-reactivity was calculated as shown in the equation below.

$$\%Cross\text{-}reactivity = (Observed\,concentration / Estimated\,concentration) \times 100$$

2.8.6. Interference

Triglyceride stock (1000 mg/mL), made by mixing 1000 mg Tricaprin (Sigma, St. Louis, MO, USA, cat # T 7517), 1000 mg Tributyrin 98% (Aldrich, St. Louis, MO, USA, cat # 113026), 1000 mg Triacetin, (Aldrich, St. Louis, MO, USA cat # 240885), 1000 mg Tricaproin, (Sigma, St. Louis, MO, USA, cat # T0888) and 1000 mg Glyceryl Trioctonoate (Aldrich, St. Louis, MO, USA cat # T9126) together and hemoglobin from bovine blood (Sigma, St. Louis, MO, USA, cat # H 2500) were studied at 20 mg/mL for triglyceride and 2 mg/mL for hemoglobin, respectively. Bilirubin (Sigma, St. Louis, MO, USA, cat % B 4126), and human serum albumin, 99% fatty acid free and globulin free (Sigma, St. Louis, MO, USA, cat # A3782), were studied at 0.6 mg/mL and 60 mg/mL, respectively. Hemoglobin and triglyceride were dissolved in the kit matrix (27 mg/mL and 100 mg/mL, respectively) and spiked (15 μL and 30 μL, respectively) into samples (135 μL and 120 μL, respectively). The control sample was spiked with the equivalent amount of the same matrix, and both were assayed as unknowns. Bilirubin was dissolved in solvent (14 mg/mL) and spiked (18.75 μL) into the sample (131.25 μL), the control sample was spiked with the equivalent amount of solvent, and both were assayed as unknowns. Sixty milligrams of human serum albumin powder was spiked directly to 1 mL of the reference samples (60 mg/mL) and assayed. The % difference to control was calculated for all interfering substances using the equation below.

$$\%Difference\,to\,Reference = \frac{(Spiked\,sample\,conc. - Conc.\,of\,reference\,sample)}{Concentration\,of\,reference\,sample} \times 100$$

2.8.7. Method comparison (NCCLS, 1995, EP9-A)

The second generation Beckman Coulter Inhibin B RUO assay (Cat # A81301 and A81302) was evaluated against commercial Inhibin B assays from DSL-BCI (Cat # DSL 10-84100) and OBI (Cat # MCA 1312KZZ) in our laboratories using 60 male and 60 female serum and Li-heparin plasma samples, ranging in age from 20 to 50 years. The studies were carried out following the manufacturers' recommendations for preparation and storage of reagents, calibrators, controls and for running the protocol. The serum aliquots were kept frozen and thawed only once and analyzed within 3 h of thawing. Samples with %CV ≥ 15 were repeated and recorded. Serum/Li-heparin plasma samples were compared between second generation Inhibin B ELISA, MCA 1312KZZ and DSL-10-84100 using Passing and Bablok method conversion and Spearman rank correlation (Analyse-it®). One hundred and six day 3 female serum samples and 79 random male serum samples were run on Access 2 FSH (Beckman Coulter, Fullerton, CA, USA, cat # 33520 and 33525) and in the second generation Inhibin B ELISA. Spearman rank correlation between Inhibin B and FSH was plotted.

2.8.8. Stability studies

2.8.8.1. Sample stability/freeze–thaw stability. Blood from 20 patients were drawn by venipuncture into Vaccutainers at the vendor's site (PromedDx, Norton, MA, USA, www.PromedDx.com), 10 serums and 10 lithium heparin plasmas, per manufacturer's instructions. The specimens were shipped overnight on cool packs to BCI, Webster. The samples were aliquoted upon arrival and stored at 2–8 °C and −20 °C for seven days incorporating three freeze–thaws and then tested for stability.

3. Results

3.1. Calibration

The second generation Inhibin B assay was standardized to OBI-MCA 1312KZZ ELISA using sample value transfer. Inhibin B concentrations were assigned to 120 serum samples using OBI assay. These samples were then used to generate the Inhibin B stock value. Individual calibrators with Inhibin B concentrations of 10, 30, 100, 250, 500 and 1000 pg/mL were prepared in fetal bovine serum using the stock. The original samples were re-run on the second generation Inhibin B kit, producing a slope of 1.03 with an intercept of −6.77 pg/mL and $r = 0.99$. Experiments were performed with the serially diluted WHO NIBSC-UK-EN63QG 96/784 IRP as calibration curve and second generation Inhibin B calibrators as unknown. Calibrator traceability to WHO is defined by the factor 2.47. The calibrator's concentration from OBI and DSL kits remains almost similar when run against WHO in the second generation Inhibin B assay (OBI = 0.465 WHO + 1.95 pg/mL, Gen II = 0.414 WHO + 1.38 pg/mL, 10-84100 = 0.482 WHO + 4.76 pg/mL).

3.2. Sensitivity

3.2.1. Limit of blank

Calibrator with 0 pg/mL Inhibin B level was studied over 40 inter-assay runs on two kit lots and the highest Inhibin B concentration measured at 95% CI was reported as 0 pg/mL.

3.2.2. Limit of detection

Six serum samples in the range of 0.0–54.08 pg/mL were evaluated in duplicates against the second generation Inhibin B calibration curve over 20 inter-assay runs. The observed means of the five samples were 5.39, 5.50, 9.23, 15.21, and 54.08 pg/mL; SD's were 1.08, 1.05, 1.24, 1.24 and 2.67, respectively. The lowest amount of the Inhibin B in a sample that can be detected with a 95% probability was 2.6 pg/mL.

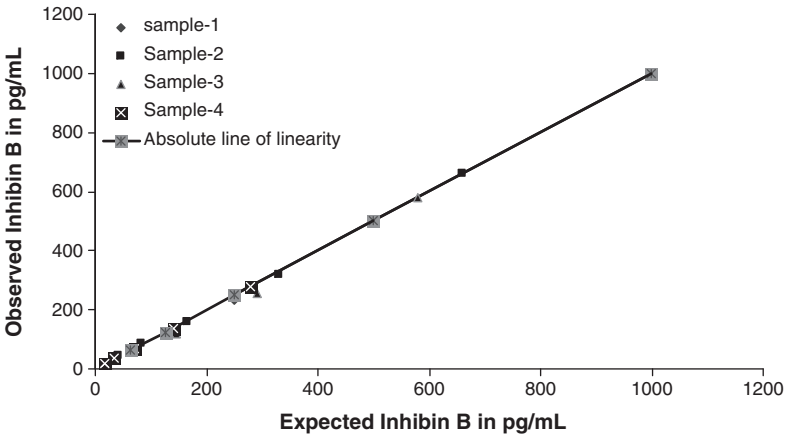

Fig. 1. Linearity of dilution of serum samples in Inhibin B Gen II assay.

3.2.3. Limit of quantitation

Eight serum samples in the range of 0–317.3 pg/mL were evaluated in duplicates against the second generation Inhibin B calibration curve over 10 days and 20 runs. The %CV of the seven samples observed were 20.09% at 5.39 pg/mL, 18.99% at 5.50 pg/mL, 13.42% at 9.23 pg/mL, 8.13% at 15.21 pg/mL, 4.93% at 54.08 pg/mL, 3.21% at 109.42 pg/mL and 2.70% at 317.32 pg/mL, respectively. The estimated minimum dose achieved at 20% total imprecision by plotting %CV with respect to Inhibin B concentration was 4.8 pg/mL.

3.3. Linearity of dilution

Serum samples in the range of 279–1000 pg/mL Inhibin B levels when diluted in the calibrator matrix showed linear results across the dynamic range of the assay (Fig. 1); and the average recovery on dilution for the samples at 999.8, 659.8, 580.2 and 279.4 pg/mL was 95, 103, 91 and 103%, respectively.

3.4. Spike recovery

Multiple volumes (8, 15 and 23 μL) of a 1000 pg/mL recombinant Inhibin B solution were added to 150 μL of four serum samples containing endogenous Inhibin B concentrations in the range of 40–57 pg/mL. The sample Inhibin B concentration before and after spiking exogenous Inhibin B was recorded. The average spiking recovery for individual samples with endogenous Inhibin B concentrations of 46.4, 57.4, 40.2 and 46.2 pg/mL, respectively spiked with exogenous Inhibin B doses of 45, 86 and 124 pg/mL was 97, 93, 98 and 105%, respectively.

3.5. Imprecision

Reproducibility of the second generation Inhibin B assay was determined using three human serum pools (QC1, QC2 and QC3) at 19.34, 76.03 and 275.30 pg/mL and two kit controls (C1 and C2) at 99.88 and 363.90 pg/mL using two reagent lots. The total imprecision calculated on QC1, QC2, QC3 and C1, C2 over 40 assays ($n=160$) were 6.8, 4.4 and 4.3% and 5.4 and 5.7%, respectively.

3.6. Selectivity/specificity

Three male rhesus monkey specimens and a pooled male rat sample were assayed in the second generation Inhibin B resulting in concentrations of 1053, 1188, 941 and 45.6 pg/mL, respectively. Inhibin A (6000 pg/mL), activin A (5750 pg/mL), activin B (6000 pg/mL), follistatin 315 (5000 pg/mL), follicle-stimulating hormone (450 mIU/mL), luteinizing hormone (10000 pg/mL) and Anti-Müllerian Hormone (280 ng/mL) were spiked individually into the calibrator matrix (Inhibin B, 0 pg/mL) and assayed as unknowns. All were undetectable and reported as zero percent cross-reactivity. Post-menopausal serum samples from 20 women with a median age of 74 years with non-detectable AMH concentrations were run in the second generation Inhibin B assay to check for false positives. Eighteen of the samples were non-detectable by the assay and two had Inhibin B concentration of ≤4.3 pg/mL.

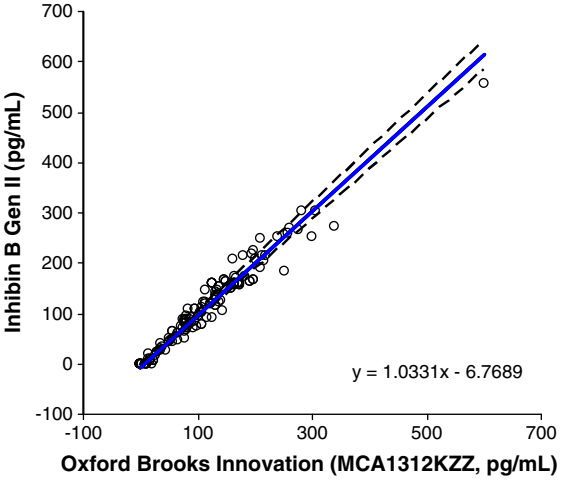

Fig. 2. Linear regression analysis between Inhibin B concentrations (pg/mL) obtained with Oxford Brooks Innovation (MCA 1312KZZ) and second generation Inhibin B ELISA for 120 serum samples. Linear regression analysis results were as follows: $r=0.99$; $p<0.0001$; $y=1.03x-6.77$.

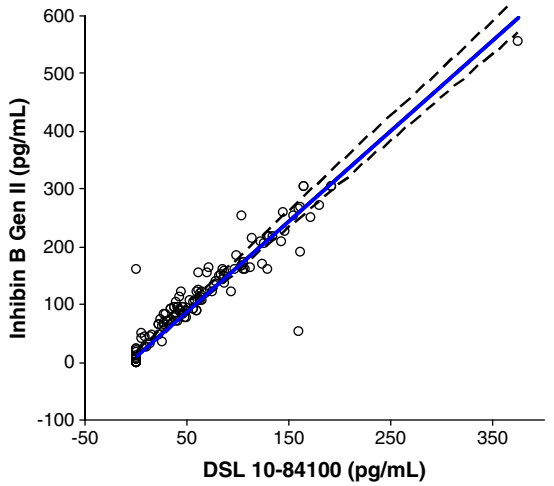

Fig. 3. Linear regression analysis between Inhibin B concentrations (pg/mL) obtained with DSL10-84100 and second generation Inhibin B ELISA for 120 serum samples. Linear regression analysis results were as follows: $r=0.97$; $p<0.0001$; $y=1.57x+11.29$.

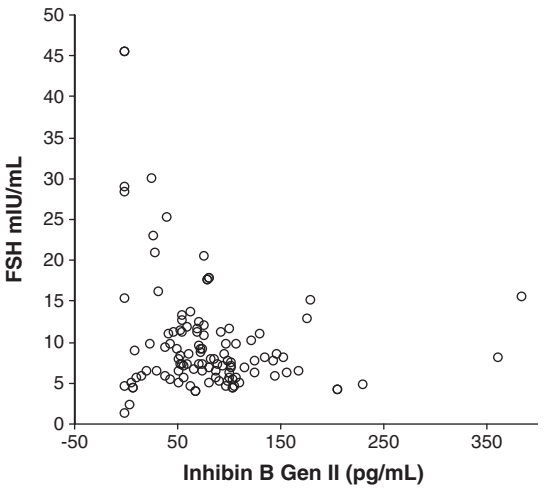

Fig. 5. Spearman rank correlation analysis between FSH concentrations (mIU/mL) and Inhibin B concentration (pg/mL) on one hundred and six female day three serum samples using Beckman Coulter FSH and Inhibin B Gen II ELISA. Correlation observed was as follows: $r=-0.4$; $p<0.1$.

3.7. Interference

The % dose difference to reference, when samples were spiked with hemoglobin from bovine blood (2 mg/mL), triglycerides (20 mg/mL), bilirubin (0.6 mg/mL), and human serum albumin, (60 mg/mL) individually was at −8.5, 2.3, 6.2 and 6.4%, respectively.

3.8. Method comparison

The second generation Inhibin B assay was compared to two commercially available assays using 60 male and 60 female samples, ranging in age from 20 to 50 years. The assay showed significant positive linear correlations to OBI-MCA 1312KZZ and DSL10-84100 assays (Inhibin B Gen II = 1.03 OBI − 6.77 pg/mL, $r=0.99$; $P<0.0001$; and Inhibin B Gen II = 1.57 DSL + 11.29 pg/mL, $r=0.97$; $P<0.0001$), respectively as shown in Figs. 2 and 3. Linear regression analysis of 120 matched serum and lithium heparin plasma specimens in the second generation Inhibin B assay yielded as: Serum = 0.97 lithium heparin plasma with a correlation coefficient of $r=0.99$ and $P<0.0001$ as shown in Fig. 4. One hundred and six female (day 3) serum and 79 male serum samples were assayed for FSH on Beckman Coulter's Access 2 instrument and Inhibin B on second generation Inhibin B microplate assay, respectively. The correlation coefficient observed between FSH and Inhibin B for female day 3 specimens (Fig. 5) and male samples were $r=-0.4$ and $r=-0.55$, respectively.

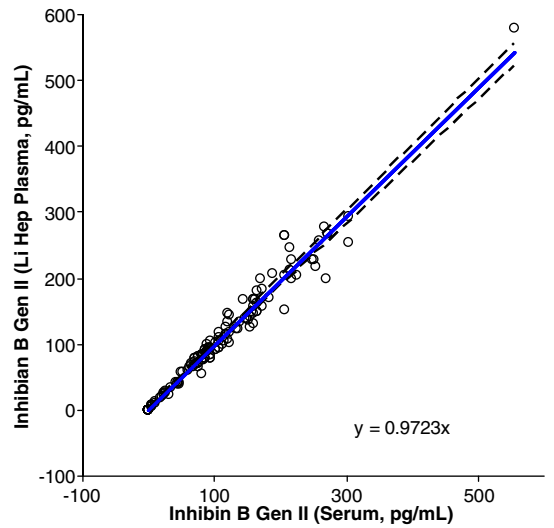

Fig. 4. Linear regression analysis between Inhibin B concentrations (pg/mL) obtained with 120 matched serum and lithium heparin plasma specimens using second generation Inhibin B ELISA. Linear regression analysis results were as follows: $r=0.99$; $p<0.0001$; $y=0.97x$.

3.9. Sample stability/freeze–thaw stability

Aliquots of 10 serum and 10 lithium heparin plasma samples were stored at 2–8 °C and at −20 °C for 1, 4 and 7 days and then analyzed for Inhibin B concentration using comparative descriptive analysis (Analyse-it®). The observed median Inhibin B values for 10 serum and 10 lithium heparin plasma fresh specimens (day 0) and aliquots stored at −20 °C for 1, 4 and 7 days were 61.27, 65.64, 57.71, and 61.24 pg/mL and 72.52, 63.82, 67.19, and 71.80 pg/mL, respectively. The change in median between fresh samples and those stored for seven days at −20 °C was <2%. The observed median Inhibin B values for 10 serum and 10 lithium heparin plasma samples stored at 2–8 °C on days 0, 1, 4 and 7 were 61.27, 57.94, 51.41, and 59.27 pg/mL and 72.52, 62.82, 70.89, and 72.73 pg/mL respectively. The average variation between fresh samples and those stored for seven days is <4%. The individual sample variations are shown in Fig. 6. The results show that the serum/Li-heparin plasma specimens are stable up to 7 days at 2–8 °C. Freeze–thaw studies were conducted on 10 aliquots of serum and 10 aliquots of lithium heparin plasma samples as described previously. The median Inhibin B values for the 10

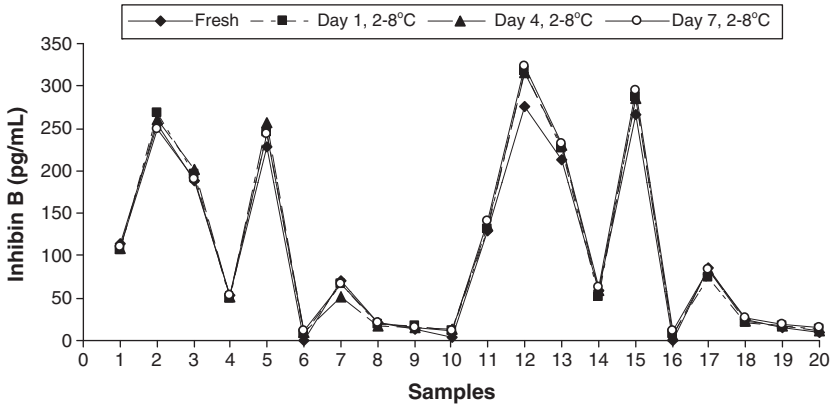

Fig. 6. Change in Inhibin B concentrations on individual serum and plasma sample studied for stability at 2–8 °C for seven days. Serum samples are labeled as samples 1–10 and plasma samples are labeled as samples 11–20.

serum and 10 lithium heparin plasma samples for fresh and after three freeze–thaw cycles were 61.27, 65.64, 61.02, and 60.39 pg/mL and 72.52, 63.82, 73.40, and 73.72 pg/mL respectively. The individual sample variation is shown in Fig. 7. The results demonstrate that the fresh versus frozen serum/Li-heparin plasma specimens were within 10% and are fairly stable up to three freeze–thaw cycles.

4. Discussion

The Inhibin B assays from OBI and DSL have been highly cited for the measurement of Inhibin B in plasma and serum. Both the assays have provided significant clinical information related to various normal and disease states. The OBI and DSL Inhibin B assays use the monoclonal antibodies C5 and R1 developed by Groome and coworkers. Both C5 and R1 were raised to the synthetic peptides made from the βB- and α-subunits of inhibin, respectively (Ludlow et al., 2008). Both assays require a methionine (in the β-subunit) oxidation step with hydrogen peroxide to allow the C5 antibody to recognize its epitope (Groome et al., 1996). The OBI assay also uses SDS detergent and heat pre-treatment of the sample. From an immunoassay view point, the need for sample oxidation creates unnecessary complexity. For example, the boiling step in the OBI assay may lead to the sample becoming gelatinized in the presence of SDS. The oxidation of different recombinant Inhibin B calibrator preparations with respect to the serum/plasma specimens may vary. Groome and coworkers (Ludlow et al., 2008) successfully raised a monoclonal antibody (46A/F) against activin B that does not require the oxidation of the βB-subunit for binding. The second generation Inhibin B assay uses this 46A/F and the biotinylated R1 monoclonal antibodies as capture and detection, respectively. The second generation assay uses a very simple procedure that is extremely robust and specific, with no sample pre-treatment requirement.

Challenges can arise when different methods produce different results for the same analyte. In an effort to minimize the variation between the measurements of Inhibin B methods, the second generation Inhibin B assay was standardized to the highly cited OBI assay. The calibrators used in the second generation assay (recombinant Inhibin B in FBS) are traceable to the WHO Inhibin B reference reagent (96/784) using a factor of 2.47. The WHO preparation is composed of a mixture of inhibin forms immunopurified from human follicular fluid. The 'Factor' with respect to WHO will vary between methods for the same recombinant calibrator materials due to the unavailability of the pure WHO preparation.

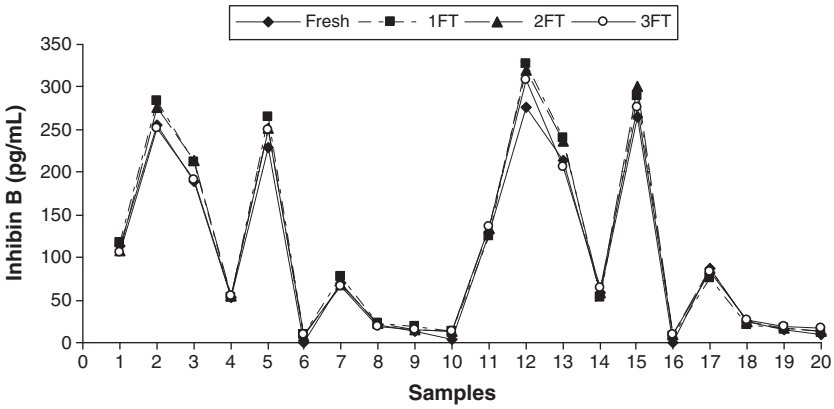

Fig. 7. Change in Inhibin B concentrations on individual serum and plasma sample studied for freeze–thaw stability for up to four thaw cycles. Serum samples are labeled as samples 1–10 and plasma samples are labeled as samples 11–20.

The OBI and DSL assays have a detection limit of approximately 7 pg/mL after overnight incubation. In contrast, the second generation Inhibin B assay has a limit of detection of 2.6 pg/mL and a limit of quantitation of 4.8 pg/mL that are achieved in <3 h. The cross-reactivities of Inhibin A, activin A, activin B, follistatin 315, follicle-stimulating hormone, luteinizing hormone and Anti-Müllerian hormone in the second generation assay were undetectable. Human anti-species antibodies have been reported to be found anywhere from 1 to 80% of a population (Kricka, 1999). These antibodies can cause interference in immunoassay by generating false positive results. To investigate potential false positive results in the Inhibin B assay using post-menopausal serum samples has a great value. Heterophilic blockers have been used in the assay to minimize the false positives and were tested on post-menopausal serum samples with non-detectable AMH concentrations. Inhibin B concentration of ≤4.3 pg/mL in the Gen II assay in post-menopausal serum samples supports the finding that the optimized formulation minimizes false positives results. Interference due to hemoglobin, triglyceride, bilirubin and human serum albumin was within 10% of the reference Inhibin B measurement when the interferents were spiked with at least twice the physiological concentrations. The assay also finds application in the animal research area as the assay is highly specific, sensitive and detects human, monkey and rat species. Our collaborators are studying Inhibin B levels in male Sprague–Dawley and male Wistar Hanover rat serum and plasma specimens; this study will be published separately. The specificities for monkey, equine and bovine species are also under investigation. Human serum/plasma samples when serially diluted and run against the recombinant calibrators in FBS showed good recovery across the entire dynamic range of the assay. Similarly, a good recovery was observed ($100 \pm 7\%$) when exogenous rec-Inhibin B was spiked into human samples. The total imprecision of the second generation Inhibin B assay was <7%. The second generation Inhibin B assay reagents have shown good open vial stability for 18 days, stability during shipping at ambient conditions and up to four freeze–thaw cycles for the calibrators and controls. Inhibin B in human serum and Li-heparin plasma is stable at least up to 7 days at 2–8 °C and did not show significant difference between fresh versus frozen and four freeze–thaw cycles. The performance of the second generation Inhibin B assay and the stability of the analyte are highly reproducible and compatible with complete automation or even large scale manual sample analysis.

The second generation Inhibin B assay gave significant positive linear correlations to OBI and DSL assays ($r = 0.99$; $P < 0.0001$ and $r = 0.97$; $P < 0.0001$). Inhibin B measurement in the second generation assay was standardized to OBI assay and the second generation assay had a positive bias of 3%. The same when compared to DSL assay gave results that were apparently 1.57 times greater than those obtained with DSL assay, however the calibrator concentration remains almost similar when run in second generation Inhibin B assay. A number of possible reasons may account for this apparent difference. The DSL capture antibody undergoes acidification followed by neutralization before coating to the solid surface in order to improve the immunoreactivity. Second generation Inhibin B assay may have been able to measure more of the available Inhibin B in the sample, as the assay does not depend on acidification and oxidation steps for the capture antibody to recognize the βB-subunit. The oxidation step uses a known amount of hydrogen peroxide added to the sample. Although the amount of Inhibin B can vary considerably between samples, it is difficult to know the portion of Inhibin B that has been oxidized. Two discrepant samples between DSL and second generation assay were measured in OBI assay. The second generation measurements were in agreement with OBI results. The same samples when re-tested and diluted in DSL assay showed consistent results, but did not recover on dilution. A total of 12 repeats in DSL assay and 35 repeats in OBI assay were performed for samples with CV>15% and were incorporated in method comparison. No repeat run was required on the second generation assay. Serum and lithium heparin plasma can be used as sample type in the second generation assay as they matched and correlated well (slope of 0.97, $r = 0.99$). The negative correlation of Inhibin B and FSH was obtained using the second generation assay. The fact that the second generation assay does not contain an oxidation step is an added advantage as it minimizes the variation that can possibly occur due to this treatment. This has considerably reduced operator error and number of repeats due to gel formation or bad duplicates.

In summary, we have described the development of second generation Inhibin B ELISA for the measurement of Inhibin B in serum and Li-heparin plasma. The assay is a significant improvement over the current methodologies, allowing for automation and/or timely manual determination of Inhibin B levels. The antibody pair used in the assay is highly specific and has shown a parallel relationship to OBI boiling sample pre-treatment assay. The performance of the assay is ideal for investigation into the physiological role of Inhibin B in both men and women.

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