Development of a two-site solid-phase immunochemiluminescent assay for measurement of dimeric inhibin-A in human serum and other biological fluids

Daniel S. McConnell,1* Vasanth Padmanabhan,1 Tamara B. Pollak,1 Nigel P. Groome,2 James J. Ireland,3 and A. Rees Midgley, Jr.1

Inhibin is a heterodimeric glycoprotein that inhibits the secretion of follitropin from the pituitary and has been isolated in two distinct forms composed of a common \( \alpha \) subunit and either a \( \beta_A \) or \( \beta_B \) subunit. Utilizing paired monoclonal antibodies specific to the \( \alpha \) and \( \beta_A \) subunit, we have developed an immunochemiluminescent assay for dimeric inhibin-A. The assay is capable of quantifying free and bound inhibin-A in human serum and follicular fluid. The limit of detection is 10 ng/L. Related proteins exhibit little cross-reactivity or interference. Recovery is excellent. Whereas samples from men and postmenopausal women are near the detection limit of the assay, inhibin-A is higher in the luteal than the follicular phase of normally cycling women, 20-fold higher during in vitro fertilization treatment, and ~200-fold greater in pregnancy. The assay measures inhibin-A in follicular fluid from a variety of other species.

INDEXING TERMS: activin • follistatin • follitropin • acridinium ester tracer

1 Reproductive Sciences Program, University of Michigan, Ann Arbor, MI 48109-0404.
2 School of Biological and Molecular Sciences, Oxford Brooks University, Oxford, UK OX3 0BP.
3 Department of Animal Science, Molecular Reproductive Endocrinology Laboratory, Michigan State University, East Lansing, MI 48824-1225.

*Address correspondence to this author at: Reproductive Sciences Program, University of Michigan, 300 N. Ingalls Bldg., Rm 1110, Ann Arbor, MI 48109-0404. Fax 313-936-8620; e-mail DanMcC@umich.edu.


Received January 24, 1996; accepted April 2, 1996.

Inhibin is a heterodimeric protein implicated in the regulation of follitropin (FSH) [1–5]. Comprising an \( \alpha \) and either a \( \beta_A \) (inhibin-A) or a \( \beta_B \) (inhibin-B) subunit, inhibin is present in the circulation and in follicular fluid in a variety of molecular forms [6, 7]. The amino acid sequences for both subunits are highly conserved with distant species such as goldfish and Xenopus [8], showing a remarkable degree of conservation of 94% and 96% homology, respectively. Consequently, raising antibodies and developing highly specific assays has proven to be a formidable problem. Compounding this difficulty, the two \( \beta \) inhibin subunits show remarkable similarity to a family of growth regulator proteins that, in addition to inhibin and activin, includes transforming growth factor \( \beta \), mullerian inhibiting substance (MIS), and fly decapetide gene complex [9–11].

Existing RIAs have included radiolabeled inhibin or inhibin \( \alpha \) fragments as tracer with an anti-\( \alpha \) subunit antibody [1–5, 12, 13]. Because of high concentrations of free \( \alpha \) subunit, interpretations based on these assays have been equivocal because the assay detects the biologically inactive free \( \alpha \) subunit as well as the biologically active, intact dimer. Needed are specific assays with low limits of detection that can be used to measure circulating, dimeric inhibin concentrations. Previously an ELISA was developed [14] that addressed these problems and enabled estimation of bioactive, dimeric inhibin concentrations.

We report here the combination of chemiluminescent detec-

*Nonstandard abbreviations: FSH, follitropin; MIS, mullerian inhibiting substance; rc-hINH, recombinant human inhibin-A; rc-hACT, recombinant human activin-A; rc-hFS-288, recombinant human follistatin 288; NHS, N-hydroxysuccinimide; \( \alpha_2 \)-M, human \( \alpha_2 \)-macroglobulin; TGF\( \beta \), transforming growth factor \( \beta \); GnRH, gonadotropin-releasing hormone; BSA, bovine serum albumin; PMP, superparamagnetic particles; anti-\( \alpha \)-INH-DMAF, anti-\( \alpha \)-inhibin-conjugated acridinium ester; anti-\( \beta_A \)-INH-PMP, anti-\( \beta_A \)-inhibin-conjugated PMP; RLU, relative light units; MLA-II, Magic® Lite Analyzer-II; PBS, phosphate-buffered saline; SPICA, solid-phase immunochemiluminescent assay; bINH, bovine \( \alpha \) subunit; hTSH, human thyrotropin; hCG, human chorionic gonadotropin; hLH, human lutein; and IVF, in vitro fertilization.
tion of an acridinium tracer and superparamagnetic particle (PMP) separation in the development of a two-site assay specific for the \( \alpha \beta \) inhibin dimer and reveal its utility in detecting inhibin-A from several species. The method involves a pair of monoclonal antibodies, one specific for the \( \alpha \) subunit and the other specific for the \( \beta \) subunit [15, 16] of the mature form of human inhibin-A, and combines them with a chemiluminescent detection technique. We chose monoclonal antibodies because of their inherent specificity, and chemiluminescence for the enhanced sensitivity that this technique has the potential to provide. Chemiluminescence methods can be readily automated and also offer the advantage of eliminating radioactive waste generation inherent in RIA technology. Through the use of two monoclonal antibodies, one directed against the \( \alpha \) subunit and the other against the \( \beta \) subunit, we are able to detect only dimeric inhibin-A with no \( \alpha \) subunit interference.

Materials and Methods
Recombinant human 32-kDa inhibin-A (rc-hINH) and activin-A (rc-hACT), kindly provided by Jennie Mather, were gifts from Genentech (San Francisco, CA). Recombinant human follistatin (rc-FS-288) was prepared by Nicholas Ling and generously provided through the National Hormone Distribution Program of the NIH. Horse serum-based diluent used in inhibin calibrators, PMP, the N-hydroxysuccinimide (NHS)-activated dimethyl acridinium ester (DMAE) used for labeling antibodies, and the magnetic rack for particle separation (Corning Magic\textsuperscript{\copyright} Rack, cat. no. 472231) were obtained from Ciba Corning Diagnostics (Walpole, MA). Human \( \alpha \)-macroglobulin (\( \alpha \)-M), transforming growth factor beta (TGF\( \beta \)), and gonadotropin-releasing hormone (GnRH) were obtained from Sigma (St. Louis, MO). Bovine serum albumin (BSA) and all other reagents used in preparation of buffers were obtained from Aldrich (Milwaukee, WI). All animals used in this study were housed and treated in accordance with regulations of the University Committee on Use and Care of Animals. Human volunteers and human serum samples were handled according to the University guidelines of safe laboratory practices.

Assay buffers were prepared by using reverse osmosis and MilliQ (Millipore, Bedford, MA) filtered water (ddH\( _2 \)O). Two different buffers are involved in this assay that differ only in the final adjustment of pH: 0.01 mol/L sodium phosphate, 12 g/L NaCl, 0.01 mol/L EDTA, 5 mL/L Tween 20, and 10 g/L BSA, pH 7.0 (assay buffer 1) and pH 5.0 (assay buffer 2).

The monoclonal anti-inhibin antibodies (clone R1, anti-inhibin \( \alpha \); clone E4, anti-inhibin \( \beta \)) have been described previously [15, 16].

ASSAY FORMAT
The assay format consists of an \( \alpha \)-inhibin monoclonal detection antibody conjugated to an acridinium ester (\( \alpha \)INH-DMAE) and an \( \beta \)-inhibin monoclonal capture antibody conjugated to PMP (\( \beta \)-INH-PMP) to effect separation. Briefly, a sample is incubated with \( \beta \)-INH-PMP, washed to remove unbound reactants, incubated with \( \alpha \)INH-DMAE, washed again, and then treated with acid and then peroxide anion to initiate light emission [17]. Calibration curves are generated on the basis of increasing relative light units (RLU) determined in a Magic\textsuperscript{\textregistered} Lite Analyzer-II (MLA-II) obtained from Ciba Corning Diagnostics.

PREPARATION OF ANTI-\( \alpha \)-INH-DMAE
An immunoglobulin-rich fraction of anti-\( \alpha \)-inhibin, R1, was purified by affinity chromatography on Protein A (Pharmacia Protein A CL-4B, Uppsala, Sweden) and conjugated to NHS-activated DMAE [18–20] by the method of Weeks et al. [21]. The resulting labeled antibody was diluted to 50 mL in assay buffer 1. The working concentration for the assay was a 1:250 dilution of this concentrate, which gave \(~700\,000\) RLU in 50 \( \mu L \).

PREPARATION OF ANTI-\( \beta \)-INH-PMP
An immunoglobulin-rich fraction of anti-\( \beta \)-inhibin, E4, was purified by affinity chromatography on Protein A and conjugated to PMP by the method of Groman et al. [22]. The particles were washed and suspended in 25 mL of assay buffer 1. The working concentration was a 1:10 dilution of this concentrate, which provided 1 \( \mu g \) of antibody coupled to 11.6 \( \mu g \) of PMP per tube.

ASSAY METHODOLOGY
Inhibin calibrator was prepared from rc-hINH in assay buffer 1 at a concentration of 100 mg/mL. Assay calibrators were prepared from this stock by dilution in horse serum diluent (Ciba Corning Diagnostics MultiDiluent). The horse serum was used to minimize assay matrix effects by calibrating the protein concentration in each assay tube. Other, similar products (Diagnostic Products Corp., for example) are readily available and may be substituted in the assay. Using 12 \( \times \) 75 mm glass test tubes, calibrator or sample (100 \( \mu L \) of serum, follicular fluid, or bovine inhibin) was added, followed by 25 \( \mu L \) of 5% H\( _2 \)O\(_2\). The mixture was incubated for 30 min at room temperature to completely oxidize the methionine residues in inhibin [23]. After this incubation, 200 \( \mu L \) of assay buffer 2 was added to bring the total volume to 325 \( \mu L \). This was followed by the addition of 25 \( \mu L \) of anti-\( \beta \)-INH-PMP to each tube. The tubes were shaken by hand and incubated on an orbital shaker overnight at room temperature. After 24 h, the particles were magnetically separated and washed sequentially with three 1.0-mL aliquots of assay buffer 1. To each tube was added 400 \( \mu L \) of assay buffer 1, followed by 100 \( \mu L \) of anti-\( \alpha \)-INH-DMAE. The tubes were shaken by hand and incubated overnight at room temperature. After 24 h, the tubes were again shaken by hand to resuspend the particles. Separation of the bound antibody complex was performed magnetically, the supernatant was decanted, and the particles washed with a further two 1.0-mL aliquots of assay buffer 1, followed by 1.0 mL of ddH\( _2 \)O. The tubes were briefly vortex-mixed, 100 \( \mu L \) of ddH\( _2 \)O was added, and the tubes were read in an MLA-II by injecting 300 \( \mu L \) of 0.1 mol/L HNO\(_3\) and 0.5% H\( _2 \)O\(_2\), followed by 300 \( \mu L \) of a solution containing the surfactant Arquad at 0.5% (Azko Chemical Co., Chicago, IL) and 0.25 mol/L NaOH, to initiate light emission. The MLA-II was programmed to read for a 2-s integral with data output RLU's.
STATISTICAL ANALYSIS

Calibration curve parameters were calculated via a four-parameter logistical function by using AssayZap version 2.32 (BioSoft, Cambridge, UK). Parallelism of assay curves was evaluated by analysis of variance (F-test). Assay curves were judged to be parallel if the P-value for the test of the sum of squares associated with parallelism was >0.05. Inhibin concentrations in different physiological states were compared by using the Student's t-test.

**Results**

**ASSAY OPTIMIZATION**

**Matrix effects.** Comparison of dose–response relations of rc-hINH–A (calibrator) in various matrices, including inhibin assay buffer, phosphate-buffered saline (PBS), gel PBS, horse serum, NHS, and serum from postmenopausal women, revealed striking differences in sensitivity of the calibration curves (data not shown). Addition of human serum to the inhibin assay buffer interfered with and reduced light output. To eliminate this effect of serum, the calibration curve was prepared in a high-protein horse-serum diluent (Ciba Corning MultiDiluent), which closely mimics the protein concentration of human serum.

**Configuration.** To determine how the order of reagent addition and antibody configuration would affect assay results, all four possible assay configurations were explored: (a) anti-β,1-INH-PMP/anti-α-INH-DMAE, (b) anti-α-INH-DMAE/anti-β,1-INH-PMP, (c) anti-α-INH-PMP/anti-β,1-INH-DMAE, and (d) anti-β,1-INH-DMAE/anti-α-INH-PMP. Option a above, which placed the anti-β,1 antibody on the solid phase (PMP) followed by washing away all unbound components and subsequent addition of the anti-α antibody, proved to be the best configuration. This approach eliminated interference of free α-inhibin and provided the lowest limits of detection. Solid-phase capture also allowed us to incorporate a wash step after addition of the first antibody. This helped to eliminate saturation of the capture antibody, which resulted in a net decrease in signal (hook effect) and consequently reduced the need to assay samples at multiple dilutions.

**ASSAY CHARACTERISTICS**

**Calibration curve.** A log dose–response curve was linear from 100 to 10,000 ng/L ($R^2 = 0.997$), with a minimum detection limit, defined as the concentration that exceeded by 2 SD the mean RLU's of tubes containing no dimeric inhibin-A, of 10 ng/L (1 pg/tube) (Fig. 1). The assay ED$_{50}$ (the effective dose at which 50% binding occurs) was 42 μg/L and the intrassay CV was 4.3% (n = 10) and 3.3% (n = 10) at mean doses of 570 and 3353 ng/L, respectively. The interassay CV was 10.5% (n = 8). Oxidation of methionine residues of the inhibin dimer has been reported to increase the sensitivity (minimum detectable concentration) of the inhibin-A IRMA [23]. Although this reduced the limit of detection by two- to threefold in the solid-phase immunochemiluminescent assay (SPICA) (inset, Fig. 1), the 10-fold gain reported in the IRMA was not achieved. To take advantage of the gain, although minimal, this step was incorporated as a routine modification.

**pH effects.** To determine if any of the binding steps might be affected by hydrogen ion concentration and to optimize the sensitivity, both assay steps were evaluated at a pH of 2.0, 5.0, 7.0, and 10.0. The pH for the β,1 capture was optimal at pH 5.0 and exhibited a limit of detection of 10.0 ng/L, but at a pH of 7.0 (inset, Fig. 1), 2.0, or 10.0 the detection limit was severely attenuated. The second step of the assay, in which the anti-α antibody was allowed to react, was less sensitive to pH, with changes in pH having almost no effect. Thus, the first step was run at pH 5.0 and the second step at pH 7.0.

**Assay cross-reactivity.** Virtually no cross-reaction was observed with rc-hFS-288 (10 mg/L), α$_2$-M (10 g/L), rc-hACT$_A$ (1 mg/L), 29-kDa βINH (bovine α subunit; 1 mg/L), TGFβ (2.5 mg/L), electroluted BSA (1 mg/L), human thyrotropin (hTSH; 5 mg/L), hFSH (1 mg/L), GnRH (1 mg/L), human chorionic gonadotropin (hCG; 10 mg/L), or human lutropin (hLH; 10 mg/L), indicating a high specificity of the SPICA (Fig. 2). rc-hMIS (2 mg/L) tested in subsequent assays also showed no cross-reactivity (data not shown).

**Assay interference.** To study the effects of potential interfering substances on binding of inhibin-A to the antibodies in the assay, we added a variety of peptides and proteins to two doses (50 and 500 ng/L) of inhibin calibrators. As shown in Fig. 3A, BSA (1 mg/L), inhibin α subunit (29 kDa βINH, 1 mg/L), TGFβ (2.5 mg/L), hFSH (1 mg/L), GnRH (1 mg/L), hCG (10
mg/L), hLH (10 mg/L), and hTSH (5 mg/L) did not inhibit the binding of dimeric inhibin-A to antibody. Preincubation of the inhibin-A calibrator for 0 or 3 h at room temperature (Fig. 3B) with α2M (1 g/L), rc-hACT (1.0 mg/L), rc-hFS-288 (1.0 mg/L), and rc-hMIS (1 mg/L) did not interfere with binding of the rc-hINH calibrator.

**Parallelism.** When analyzed as described in Materials and Methods, logit-log-transformed dose–response curves of a variety of preparations containing dimeric inhibin-A were parallel to the rc-hINH calibrator (Fig. 4). These samples included human third-trimester pregnancy serum, human third-trimester pregnancy urine, and serum samples from women (obtained at 5 and 7 days after FSH treatment) during in vitro fertilization (IVF) treatment (P = 0.56 for human pregnancy serum, P = 0.68 for human pregnancy urine, P = 0.50 and 0.70 for IVF serum).

**Serum recovery.** Sera were supplemented with 10, 25, 100, and 1000 ng/L rc-hINH-A before measurement in the SPICA. Assay estimates minus concentrations measured in the absence of the added inhibin were used to calculate recovery. Fig. 5 shows the recoveries in serum pools from human males and females. Recoveries were parallel to the line representing 100% (P = 0.59 for the female pool and 0.32 for the male pool). Recovery was also estimated with 11 human serum samples spanning various physiological states (normal male as well as female follicular phase, luteal phase, and postmenopausal). Recoveries at 10 and 100 pg averaged 99% and 111%, respectively.

**INHIBIN-A CONCENTRATIONS IN VARIOUS PHYSIOLOGICAL STATES**

**Human serum samples.** Fig. 6 summarizes the concentrations of inhibin-A measured by the SPICA in sera obtained from volunteers, which included healthy men, postmenopausal women, normally cycling women (ages 24–35) during the follicular (day 3–5) or midluteal (day 12–14) phases of the cycle, pregnant women (third trimester), and women stimulated with FSH for IVF treatment (day 5–6 postgonadotropin treatment).
The highest concentrations of inhibin-A were found in sera from pregnant women. Serum from healthy men, postmenopausal women, and women during the follicular phase showed dimeric inhibin-A concentrations near the limit of detection (dashed line). Compared with the follicular phase of normally cycling women, women in the midluteal phase (two- to threefold; \( P = 0.002 \)), undergoing FSH stimulation for IVF treatment (20-fold; \( P = 0.01 \)), or pregnant (240-fold; \( P = 0.056 \)) showed much higher concentrations of inhibin-A.

**Cross-species samples.** The SPICA is capable of detecting dimeric inhibin-A in follicular fluids of porcine, bovine, and human species. These results are summarized in Fig. 7. Serial dilutions of these follicular fluids resulted in dose-response curves that were parallel to the rc-hINH-A calibration curve over the range of 10–1000 ng/L (\( P = 0.75 \) for porcine, 0.06 for ovine, 0.12 for bovine, and 0.09 for human). The SPICA also detects dimeric inhibin-A in serum samples of mouse, rat, and monkey, as well as mouse ovarian, testes, kidney, and placental tissue extracts, and rat ovarian tissue extracts (data not shown).

**Molecular variants of bovine inhibin.** Molecular mass variants of purified bovine inhibin were provided by and prepared as described by Good et al. [7]. In brief, bovine follicular fluid was incubated with Affi-Gel 10 linked to affinity-purified bovine anti-bovine \( \alpha_c \) Gly-Tyr antibodies. The gel was washed, and the proteins were eluted and subjected to 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under nonreducing conditions. Protein bands were cut from the gel and electroeluted to provide the variants. Dilutions of the resulting molecular variants (32 to \( >160 \) kDa) were all immunoreactive in the assay (Fig. 8), although the preparations were less potent (10\(^{-5}\)- to 10\(^{-6}\)-fold) than rc-hINH on a molar basis. Dimeric bovine 32-kDa inhibin showed the greatest reaction. The 29-kDa form (consisting primarily of \( \alpha \)-inhibin) and electroeluted BSA did not cross-react in this assay. Other forms exhibited various degrees of activity as shown in the Figure. All other molecular mass forms except the 32-kDa form were parallel to rc-hINH (\( P = 0.66 \) for 38 kDa, 0.31 for 49 kDa, 0.25 for 53 kDa, 0.12 for 77 kDa, 0.77 for 88 kDa, 0.76 for 110 kDa, and 0.05 for \( >160 \) kDa).
Fig. 8. Ability of SPICA to detect various molecular-mass forms of bovine inhibins.

Shown are the calibrion curve for rc-hINH-A (1) and cross-reactivity of various isolated molecular-mass forms of bovine inhibin (29 to >160 kDa) as determined by the SPICA method. Electrophoretically purified BSA was used as a negative control. Note that the 29-kDa α subunit is not detected.

**Discussion**

The difficulties in designing a workable immunoassay for inhibin stem from its physiochemistry: (a) The α and β subunits are both evolutionarily conserved such that even exposed sites rarely induce production of specific, high-affinity antibodies [24]; (b) inhibin is highly hydrophobic with potential epitopes buried, and thereby poorly antigenic [25]; (c) detecting solely the dimer is difficult because inhibins and activins share a common β subunit, and inhibin exists as both the dimer and free subunit in vivo [1-5]; (d) inhibin exists in several different biological forms [6, 7]; in human follicular fluid this includes a 100-kDa pre-pro- form, a 60-kDa first cleavage form, and the 32-kDa bioactive protein [26]. This diversity has made it difficult to choose, let alone purify, a bioactive inhibin form for generation of antibodies.

cDNA clones encoding the α- and β-chains of inhibin were isolated in 1985 [27] and 1986 [28]. Antibodies against porcine, bovine, murine, and human inhibin were soon produced and the first RIAs for inhibin developed [12, 13]. These assays included 31- and 58-kDa native bovine inhibin as antigens and 125I-labeled 31-kDa bovine inhibin as a tracer. Additional anti-inhibin antibodies have been developed against synthetic peptide fragments. The majority of developed antibodies, however, have been to antigenic peptides on the N-terminus of the α-portion of the dimer [1-5, 12, 13].

Initial studies aimed at quantifying inhibin in human follicular fluid [12], serum, and plasma quickly led to theories about the role of inhibin in menstrual cycle physiology [1-5, 29], during pregnancy [30-32], and even as an ovarian tumor marker [33]. However, in 1988, Robertson et al. compared the bioactivity of inhibin fractions in an in vitro bioassay with its immunoreactivity in the available RIAs [13] and demonstrated differences between the bio- and immunoactivities. Subsequently, studies of Robertson et al. [34] and Schneyer et al. [35] documented that the widely used Monash assay was not specific for the dimer but detected inhibin α subunit equally well. This study, coupled with demonstrations of immunoreactive but not bioactive fragments of inhibin in normal circulation, indicated that the α-specific assay could not be used for specific detection of dimeric inhibin, and established the need for a two-site inhibin assay.

Shortly thereafter, Knight et al. reported a two-site IRMA involving antibodies raised against synthetic peptides from the 1-32 region of the α subunit and the 82-114 region of the βA-subunit [36]. Although this assay showed low cross-reactivity with free inhibin α subunits, a large discrepancy was observed between bioassay and RIA estimates. Sandwich-method ELISA better accommodated the α-β two-site detection necessary for accurate dimer quantification [37, 38]. However, immunosorbent assays designed with anti-α-INH antibodies bound to the plate showed similar cross-reactivity problems, and the reverse architecture with anti-β-INH antibodies as capture antibodies resulted in an insensitive assay. By using an amplified detection system and hydrazide plates, Groome overcame these obstacles [14] and reported an assay specific for the dimeric inhibin-A molecule with detection of 2 ng/L of the 32-kDa inhibin-A dimer [37].

Building upon similar logic and using the identical pair of monoclonal antibodies but different methodology, this study introduces a SPICA protocol that provides results very similar to Groome’s amplified ELISA [14]. By using chemiluminescent detection, this protocol avoids problems inherent with both radioimmunometric and enzyme-linked methods; instead of radioactive tracers with short shelf-lives and safety hazards, this chemiluminescent procedure involves a stable, innocuous acridinium ester tracer that yields highly sensitive results. The chemiluminescent assay involves fewer steps than the ELISA and eliminates the need for amplification procedures, both of which have the potential of contributing to higher precision, accuracy, and reproducibility. Since microparticle PMP can be dispensed precisely, variability normally observed in attachment to microplate surfaces is reduced. By keeping particles in suspension, this approach also overcomes some of the solid-phase steric restrictions posed by the ELISA static solid phase, a factor that may improve the valence of second antibody reactions [39]. This protocol, like that of Groome’s ELISA, uses an anti-β antibody for capture. Free β-inhibin does not appear to be present in biological fluids in high concentrations, as is free α-inhibin; therefore, anti-β capture extends the useful range of the assay. The SPICA has been validated for the measurement of dimeric inhibin-A in serum and shows little cross-reactivity with other members of the TGF-β family. In contrast to a previous report [40], which indicated that introduction of α2-M at 200 ng/L led to a small underestimation of inhibin, we have verified that in this format, α2-M at physiological (1 g/L) concentrations does not interfere or cross-react in the assay.

In a two-site assay it is imperative that one not only measures the extent to which a related protein is detected, but also the degree to which it interferes with the binding of the intended analyte to either of the antibodies. Such interference will lead to an unrecognized underestimation of the analyte. Earlier assays
Table 1. Most commonly used inhibin immunoassays, with attributes, specificity, cross-species applicability, and ability of the respective assays to detect various molecular-mass variants.

<table>
<thead>
<tr>
<th>Assay ref</th>
<th>Assay format</th>
<th>Antibody ref.</th>
<th>Dimer-specific assay</th>
<th>Cross-reactivity</th>
<th>Interference</th>
<th>Molecular mass variants</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>RIA</td>
<td>12</td>
<td>No</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>RIA</td>
<td>13</td>
<td>No</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>RIA</td>
<td>43</td>
<td>No</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>RIA</td>
<td>44</td>
<td>No</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>RIA</td>
<td>45</td>
<td>No</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>RIA</td>
<td>46</td>
<td>No</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>RIA</td>
<td>47</td>
<td>No</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>ELISA</td>
<td>37</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>IRMA</td>
<td>23</td>
<td>Yes</td>
<td>No</td>
<td>1.00%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50*</td>
<td>ELISA</td>
<td>50</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>ELISA</td>
<td>24</td>
<td>Yes</td>
<td>No</td>
<td>0.01-0.06%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>This SPICA report</td>
<td>37</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

—. Not tested.
* Same assay format as [37].
* Oxidized format.
* Assessed subsequently [34,35].
* Bovine 294Da α.
* ACT-A and ACT-B.

[14, 38] have characterized cross-reactivity aspects in depth, but have failed to address fully this interference issue. Recent studies tested this possibility with α2-M [40], but not at physiological concentrations. Our preincubation studies demonstrate that two of the known binding proteins for inhibin, α2-M, and follistatin [41, 42] do not interfere with binding of antibodies. The absence of such interference indicates that the SPICA measures both free and bound dimeric inhibin. This indicates that the immuno-reactive sites do not include those that are involved in binding follistatin or α2-M.

In addition to the assays discussed above, several other assays for inhibin have been validated. Table 1 lists the most widely used assays, summarizes their features, compares and contrasts observed cross-reactions and interferences, and identifies their ability to measure dimeric variants of inhibin in different species. Although dimer-specific assays have been validated, none has addressed interference issues, applicability to a range of other species, and ability to detect the range of dimeric molecular variants reported here.

With this specific assay, serum samples from men, women in the follicular phase, and postmenopausal women contained very little circulating inhibin-A (at or below detection limit), as was previously reported by the ELISA [48]. The seeming 10-fold differences in concentrations of inhibin measured by the SPICA and ELISA in men and postmenopausal women are a function of the respective assay sensitivities. Inhibin concentrations were higher in the luteal phase than the follicular phase, confirming earlier observations of Groome et al. [37], Lambert-Messerlian et al. [48], and Muttkrishna et al. [40]. With the SPICA, dimeric inhibin has been reported to fluctuate episodically during the luteal phase of normally cycling women [49]. As with reports with α-specific assays [31, 32], samples from pregnant women had the highest concentration of dimeric inhibin (~200-fold higher than in normally cycling women during their follicular phase). During IVF treatment, concentrations of dimeric inhibin were ~20-fold higher than in normally cycling women, similar to reports with α-specific assays [50]. A 20-fold increase in dimeric inhibin after 6 days of treatment with human menopausal gonadotropin has also been reported with Groome's ELISA format [40]. Since changes in dimeric inhibin after IVF therapy closely parallel changes in estradiol [51], inhibin may be another useful marker for monitoring IVF therapy as previously suggested by McLaughlin et al. [52] using an α-directed assay.

In addition to providing a useful means for detecting dimeric inhibin-A in human samples, the SPICA is capable of measuring inhibin-A in ovine, bovine, and porcine samples, and dose-response curves with follicular fluids from these species were parallel with the rc-hINH-A calibration curve. With the ELISA, the antibodies have been previously shown to recognize human [37, 40, 48] and bovine [7, 53] inhibin forms. The SPICA also detects dimeric inhibin in serum from female rats (N.B. Schwartz, Northwestern Univ., Evanston, IL, unpublished observations) and monkeys [54].

The lower reactivity of bovine inhibin forms, including the 32-kDa form, presumably results from the few known differ-
ences in amino acid sequence between the human and bovine α subunit [4]. Five amino acids within the region of the potential antibody recognition site differ between human and bovine species. Since the sequence and hence the epitopic site of the β, subunit are identical in both species, the difference in affinity must be due to differences in the amino acid sequence in the α chain [4]. Because the assay detects a variety of molecular mass forms of bovine inhibin, it may also be capable of revealing diverse forms of human inhibin, including pre- and proproteins in the circulation. Studies of Muttukrishna et al. [40] involving the ELISA format have indeed demonstrated that the ELISA, which utilizes the same two antibodies as the SPICA, is capable of detecting human inhibin-A variants in the molecular range of 30–200 kDa.

In this study we used overnight incubation at room temperature. In other experiments, increasing the temperature to 37 °C allowed a significant reduction in incubation time with no loss of sensitivity (data not shown). Automation of this modification might permit reporting serum inhibin concentrations with turnaround times as short as 15 min. With the same PMP and chemiluminescent label, this is done routinely with the Ciba Corning Diagnostics ACS:180 immunoassay analyzer for a wide range of analytes. Such a development could provide a major impact on monitoring the course of IVF therapy. In IVF programs, the major cost is the surgical procedure, follicular aspiration. If monitoring of inhibin-A concentrations could occur rapidly in the clinic, suboptimal cycles could be canceled for the aspiration procedure, decreasing the overall cost.

In conclusion, we have developed a two-site chemiluminescent immunoassay specific for dimeric inhibin-A. This approach was selected to avoid the use of radioactive inhibin-A. This approach was selected to avoid the use of radioactive inhibin-A. This approach was selected to avoid the use of radioactive inhibin-A. This approach was selected to avoid the use of radioactive inhibin-A. This approach was selected to avoid the use of radioactive inhibin-A. This approach was selected to avoid the use of radioactive inhibin-A. This approach was selected to avoid the use of radioactive inhibin-A. This approach was selected to avoid the use of radioactive inhibin-A.

Supported by the National Cooperative Program for Infertility Research at Michigan (NIH U54 HD-29184) and the Center for the Study of Reproduction (NIH P30 HD-18258). We acknowledge Genentech, especially Jennie P. Mather, who generously provided rc-hINH and rc-hACT for use in this assay. We are indebted to Patricia Donohue and David MacLaughlin for providing rc-hMIS. We also thank Katherine Reese for her expert technical assistance and David Mauger, Biostatistics Core of the Center for Study of Reproduction, for assistance with statistical analysis.

References


