Development of a Broad-range, Sensitive, and Specific Immunoassay for Anti-Müllerian Hormone (AMH) with No Dilution Issues

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Abstract

Objective: To develop a highly accurate chemiluminescent immunoassay for AMH quantitation that dilutes linearly even at high AMH concentrations.

Background: AMH is a 140 kDa glycoprotein belonging to the transforming growth factor-β (TGF-β) superfamily. In males, AMH is produced in the testis from embryogenesis to puberty. In females, small amounts of AMH are produced by the ovaries from birth to menopause. AMH promotes involution of the Müllerian ducts and inhibits female gonadogenesis. It also controls primordial follicle recruitment by limiting the responsiveness of the growing follicles to follicle stimulating hormone (FSH). A commonly used AMH assay, AMH Gen II, is a sandwich immunoassay using two monoclonal antibodies. Although the assay is reproducible, it does not show parallel dilution with standards at high AMH concentrations, thereby producing falsely elevated results. To address these dilution problems, we have developed an assay using two monoclonal antibodies, one specific for the pro region of AMH and the other specific for the mature region.

Methodology: An immunochemiluminometric assay was developed using a capture antibody specific for the pro region of AMH (Clone 39/6C) and a detection antibody specific for the mature region of AMH (Clone 39/30A). The capture antibody was coated on microtiter plates; serum samples, calibrators, and controls were added to the coated plates and incubated for 90 minutes. AMH was detected using a biotinylated detection antibody, streptavidin-labeled horseradish peroxidase, and a luminol chemiluminescent substrate. Emitted light was measured in relative light units using a luminometer. Sample results were calculated using a standard curve based on human recombinant AMH. Samples with >12 ng/mL AMH were diluted.

Results: This new assay has a sensitivity of 0.027 ng/mL and a reportable range of 0.03 to 440 ng/mL. Serum samples with high AMH concentrations diluted in parallel with the standard curve. This assay was highly reproducible, with an interassay CV of 6.4%. The assay was also highly specific for human AMH, has no cross-reactivity with other members of the TGF-β superfamily and no interference with lipemic, icteric, and hemolysed samples. Correlation with the AMH Gen II immunoassay was good at concentrations <10 ng/mL ($y = 1.00x + 0.03$; $R^2 = 0.85$). However, correlation deviated from the line of unity for samples >10 ng/mL ($y = 0.43x + 5.33$; $R^2 = 0.71$). This poor correlation is likely due to the Gen II dilution problem* and its lack of parallelism for human samples.

Conclusion: We have developed a highly sensitive and reproducible immunoassay to quantify all levels of AMH, including high concentrations. The assay is specific for human AMH and uses human recombinant AMH for quantitation. Because of its wide range, this assay could be used in various pathophysiological conditions.

* See Beckman Coulter product clarification PCA-20434.

Introduction

Anti-Müllerian hormone (AMH) belongs to the transforming growth factor-β (TGF-β) family of proteins involved in regulating cell growth. Prior to secretion, mature AMH undergoes glycosylation and dimerization to produce a 140-kDa dimer of identical disulphide-linked 70-kDa monomer subunits; each contains an N-terminal ("pro") and a C-terminal ("mature") domain.

AMH plays an important role in sexual differentiation and its measurement provides a useful serum marker for intersex disorders in children, delayed or precocious puberty, cryptorchidism, and anorchidism. It is also used to monitor patients with granulosa cell tumors, for predicting the success of in vitro fertilization, and for providing diagnostic information in cases of suspected polycystic ovary syndrome when ultrasonographic examination is not possible.

We developed a sensitive chemiluminescence immunoassay using a pair of monoclonal antibodies, one directed to the pro region and the other to the mature region of the AMH molecule. This laboratory developed test (LDT) was compared with the AMH Gen II ELISA kit (Beckman Coulter, Brea, CA), which uses a pair of monoclonal antibodies directed to epitopes in the mature region only.

Method

- A capture antibody specific for the pro region of AMH (Clone 39/6C) and a detection antibody specific for the mature region of AMH (Clone 39/30A) (Ansh Laboratories, Inc, Webster, TX) were used.
- AMH was measured in relative light units (RLU) using a luminometer. Originally de-identified samples were used in this study. Sample results were calculated using a standard curve based on human recombinant AMH.
- AMH LDT method imprecision was validated using 3 different levels of controls (n=25). Accuracy and linearity were evaluated using various concentrations of standard material.
- The performance of our AMH chemiluminescence LDT was compared with that of the AMH Gen II ELISA kit. Samples with RLU values higher than the highest standard were appropriately diluted using sample diluent, and retested with the LDT.
- Reference ranges were established based on sex and age.

Results

- Table 1 summarizes the performance characteristics of the chemiluminescent AMH assay. The assay exhibited good inter- and intra-assay precision and a broad analytical measurable range, with high accuracy (recovery 100±10%) and CV <10% **(Table 2)**.
- Comparison of our AMH chemiluminescence immunoassay with the AMH Gen II assay demonstrated good correlation and slope ($y=0.9983x + 0.0258$; $R^2 = 0.8466$) when limiting the comparison to samples with AMH concentrations <10ng/mL **(Figure 1A)**. Samples with high concentrations (>10ng/mL) exhibited poor correlation and slope ($y = 0.4275x + 5.3276$; $R^2 = 0.7117$) **(Figure 1B)**. Differences in antigen targets may partially explain the latter observation.
- AMH analytical specificity studies showed no cross-reactivity with the substances tested **(Table 3)**.
- Reference intervals are summarized in **Table 4**.

Table 1. Performance Characteristics AMH LDT

Intraassay variation (n=15)	All 3 controls: < 3.7%
Total imprecision coefficient of variation (CV) (n= 25)	Low control (0.11 ng/mL): 7.6% Medium control (1.01 ng/mL): 6.4% High control (2.33 ng/mL): 5.7%
Limit of detection	0.027 ng/mL
Limit of quantitation	0.030 ng/mL
Analytical measurement range	0.03 to 11.0 ng/mL
Reportable range	0.03 to 440 ng/mL
Stability	Refrigerated (2-8 °C): 15 days Frozen (-20 °C): 30 days

SI conversion factor for AMH: 1 ng/mL = 7.14 pmol/L

Table 2. AMH LDT Linearity: Accuracy and Precision Over Linear Range

Target Value, ng/mL	Mean, ng/mL	CV, %	Accuracy, %
0.030	0.030	0.0	100.0
0.045	0.046	3.1	102.2
0.210	0.200	7.8	95.2
1.100	1.045	0.5	95.0
2.290	2.296	2.0	100.3
3.500	3.297	1.1	94.2
4.580	4.453	0.1	97.2
6.870	6.997	0.8	101.8
9.130	9.126	1.6	100.0
11.000	10.605	1.0	96.4
$y = 0.9837x + 0.003$; $R^2 = 0.9989$			

Results (cont)

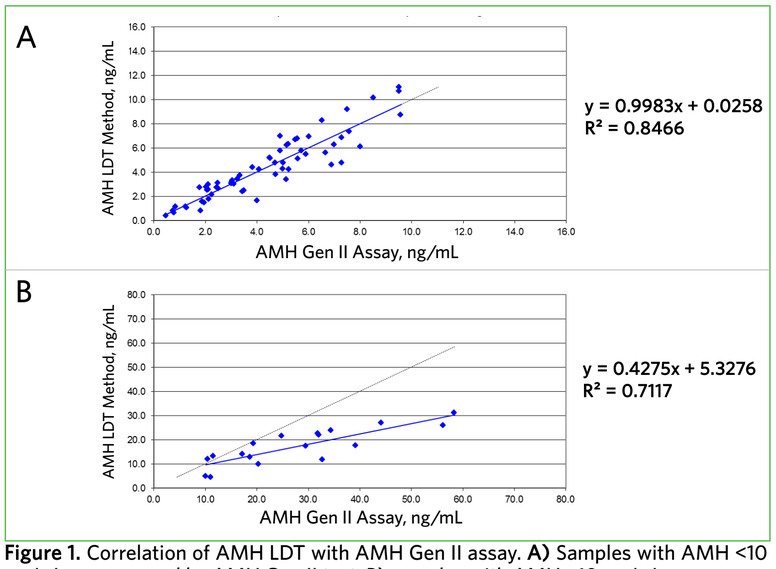

Figure 1. Correlation of AMH LDT with AMH Gen II assay. **A)** Samples with AMH <10 ng/mL as measured by AMH Gen II test; B) samples with AMH >10 ng/mL.

Table 3. Specificity of the AMH LDT

Analytes	Concentration Used, ng/mL	Cross-reactivity, %
Human AMH	44	100
Complexed form of human pro and mature AMH subunits	11.3	98
Human ProAMH (116 kDa)	40	3.8
Mature AMH (24 kDa)	40	1.96

This assay exhibited no cross-reactivity with the following analytes (various concentrations): inhibin A, inhibin B, activin A, activin B, activin AB, follistatin 288, follistatin 315, myostatin, and alpha-2 macroglobulin.

Table 4. AMH Normal Reference Ranges Determined Using the AMH LDT

Age	Mean AMH Concentration, ng/mL	AMH Reference Range, ng/mL
Male		
< 1 y (n=17)	150.40	37.20 - 345.67
1- 6 y (n=26)	158.28	59.54 - 320.65
7 - 11 y (n=22)	113.50	40.99 - 203.67
12 - 17 y (n=21)	18.08	< 128.29
> 17 y (n=23)	6.29	1.15 - 15.23
Female		
< 14 y (n=22)	1.70	0.49 - 3.15
14 - 19 y (n=53)	5.94	1.28 - 16.37
20 - 29 y (n=130)	5.15	0.76 - 11.34
30 - 39 y (n=132)	3.26	< 9.24
40 - 49 y (n=115)	0.85	< 4.50
≥ 50 y (n=44)	0.03	< 0.45

Conclusions

- We have developed and validated a highly sensitive and reproducible chemiluminescence immunoassay for the quantitative detection of AMH levels, including high concentrations. This LDT is highly specific for human AMH, with assay LOD = 0.030 ng/mL or 0.21 pmol/L. Because of its wide reportable range, this assay could be used in various pathophysiological conditions.

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