POSTER A-229

Development of a sensitive Dried Blood Spot Anti-Müllerian Hormone (AMH) ELISA

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ABSTRACT

Objective: The aim of this study was to develop a highly sensitive and simple dried blood spot human AMH ELISA to assess ovarian reserve.

Relevance: AMH has been reported to be strongly associated with age, antral follicle counts (AFC), FSH, and has emerged as a clinically useful biomarker of ovarian reserve. Recently, there have been concerns related to AMH stability in serum/plasma and complement interferences affecting the end result. This has generated numerous debates and publications related to reproducibility of AMH measurements and impact of pre-analytical sample handling. Dried blood spot specimens stability makes it a practicable alternative to venous blood. It opens new possibilities in AMH testing, such as comparison of historical to current patient results; simplified blood sampling for patients in remote locations or for those who are homebound. Instead of traveling to a clinic to get blood drawn, a blood spot sample can be taken at a convenient site and mailed to a laboratory. This technology will be especially useful for monitoring ovarian function of physically challenged cancer patients undergoing chemotherapy.

Methodology: A three-step, sandwich-type enzymatic microplate assay has been developed to measure AMH levels in two 7.9 mm dried blood spot disc in less than 6 hours. The assay measures human AMH and uses stabilized recombinant human AMH as calibrators (7-1000 pg/mL). This method uses a drop of whole blood collected on filter paper from a simple finger stick. The sample is eluted from the dried blood spot in an extraction solution and is added directly to the well. The assay measures the bio-essential AMH and does not exhibit interference by hematocrit in the extracted spot.

Validation: Ansh Labs DBS AMH ELISA (AL-129), when compared to Ansh Labs US AMH ELISA (AL-105) using 56 matched serum and dried blood spot samples in the range of 62-18443 pg/mL yielded a correlation coefficient of 0.98 ($p < 0.0001$) and a slope of 0.96 with an intercept of -0.01 pg/mL. DBS AMH ELISA (AL-129) when compared to Ansh Labs picoAMH ELISA (AL-124) using 65 matched serum and dried blood spot samples in the range of 5-5240 pg/mL yielded a correlation coefficient of 0.99 ($p<0.0001$) and a slope of 1.02 with an intercept of -4.7 pg/mL. Serial dilution of seven extracted dried blood specimens (5000-11000 pg/mL) in the sample diluent showed an average recovery of 87-105%. Total imprecision, calculated on 3 controls over 40 runs, 2 replicates per run, was 5.84% at 22.58 pg/mL, 3.15% at 86.51 pg/mL and 4.34% at 373.18 pg/mL. The functional sensitivity of the assay calculated at 20% CV was 3.9 pg/mL..

Conclusions: A highly simplified, sensitive, specific and reproducible dried blood spot AMH assay has been developed to assess ovarian reserve in females of reproductive age. The DBS results are comparable to serum-based assays. The specimen stability, ease and low cost of collection and transportation makes it a very attractive sample type for epidemiologic and other research studies.

INTRODUCTION

Measurement of AMH in community-based studies, or studies requiring multiple blood draws at present requires venous blood. Venipuncture blood draws are costly, invasive and must be performed by a trained phlebotomist in close proximity to a facility where blood samples can be centrifuged, separated and frozen.

Dried blood spots (DBS)—drops of whole blood collected on filter paper following a simple finger stick—represent a minimally invasive alternative .The participant's finger is cleaned, pricked with a sterile, disposable lancet of the type commonly used to monitor blood glucose, and up to five drops of whole blood are applied to the paper. Samples are allowed to dry, and then stacked and stored in plastic bags prior to shipment to the laboratory.

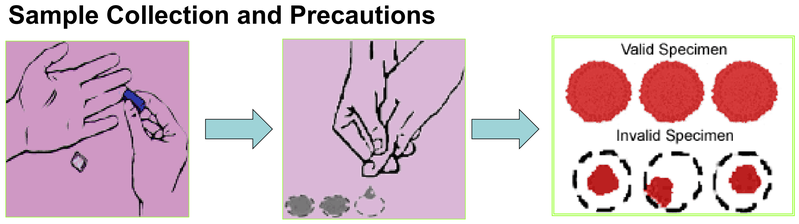
A major advantage of DBS sampling is that it is relatively painless and noninvasive, low cost, and can be implemented by non-medically trained personnel in the participant's home or other non-clinical setting. The test will help researchers monitor the ovarian function of patients with polycystic ovary syndrome, granulosa cell tumor subjects undergoing chemotherapy, etc.

METHOD

- ❑ Ansh Labs Dried Blood Spot (DBS) AMH sandwich ELISA (AL-129)
- ❑ Standardized to recombinant human AMH Calibrators.
- ❑ Can be measured reproducibly on Ahlstrom 226 and Whatman 903 filter paper
- ❑ Human specific, linear epitope capture and detection Abs in the stable midpro-region and mature region of AMH.

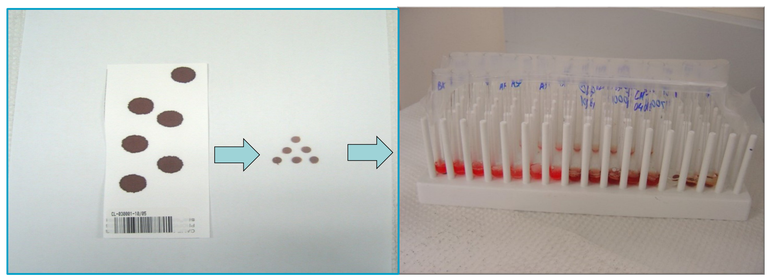
Antibody clone / Isotype	AMH Epitope	Affinity (Region)
AMH Capture (24) IgG2b	358- 369	+++ (Midpro)
AMH Detection (32) IgG1	491-502	+++ (Mature)

Sample Collection and Precautions



- ❑ All blood spots should be inspected for quality. Do not use spots if the circle is not completely filled and impregnated with blood.
- ❑ Do not use irregular shaped spots, spots that are not impregnated throughout, or spots with multiple spotting.
- ❑ Do not use spots that have not been properly dried.

Sample Preparation



- ❑ Label two 12 X 75 culture tubes for each unknown dried blood sample.
- ❑ Punch out two filter paper disc (7.9 mm), impregnated with the unknown dried blood specimen, onto a clean surface and transfer the disc using clean tweezers into the corresponding tube.
- ❑ Add 450 µL of the DBS AMH Extraction Buffer to each tube, vortex well.
- ❑ Place the tubes in a tight fitting tube rack and incubate the tubes, shaking at a slow speed (400-450 rpm) at room temperature for 60 minutes.
- ❑ Transfer the liquid from one tube into the corresponding second labeled tube leaving the blood spot in the initial tube. The extracted sample is ready to be analyzed in AL-129 AMH ELISA.

AL-129 DBS AMH ELISA Procedure

The DBS AMH ELISA is a quantitative three-step sandwich type immunoassay.

- ❑ Add 150 µL of calibrators and extracted dried blood spot samples to AMH antibody coated microtiter wells, incubated for 3 hrs and wash.
- ❑ Add 100 µL of botinylated AMH antibody solution, incubate for 1hr and wash.
- ❑ Add 100 µL of SHRP conjugate solution, incubate for 30 mins and wash.
- ❑ Add 100 µL of TMB solution, incubate for 10 mins and add 100 µL of acidic stopping solution.
- ❑ Measure the optical density and calculate the AMH concentrations against the calibration curve.

RESULTS

Limit of Detection: The lowest amount of AMH in a serum that can be detected with a 95% probability (45 runs in duplicate, n=90) is 1.2 pg/mL and the corresponding Dried Blood Spot equivalent is 35 pg/mL.

Cross-Reactivity: Recombinant and native AMH antigens were run as unknown in the assay and the % cross-reactivity was calculated.

Cross-Reactant	Concentration	% Cross-reactivity
Rodent/Bovine/Equine/Canine AMH	130 ng/mL	0.00
Full-Length AMH dimer	1000 pg/mL	100
Human mature AMH (>99.9%)	600 pg/mL	0.00
pro-mature AMH dimer (>90%)	610 pg/mL	91.3

Linearity: Multiple dilutions of the three dried blood samples and calibrator F containing various AMH/MIS levels were diluted with extraction buffer/sample diluent. The % recovery on individual samples is represented in

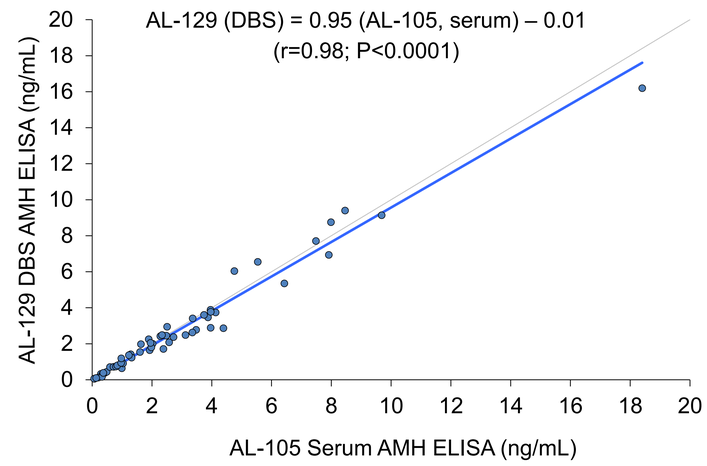
Sample	Dilution Factor	Expected Conc. (ng/mL)	Observed Conc. (ng/mL)	% Recovery
1	NEAT VALUE	11.967	NEAT	NA
	1:2	5.984	5.701	95%
	1:4	2.992	3.084	103%
	1:8	1.496	1.566	105%
	1:16	0.748	0.746	100%
2	NEAT VALUE	7.066	NEAT	NA
	1:2	3.533	3.384	96%
	1:4	1.767	1.768	100%
	1:8	0.883	0.813	92%
	1:16	0.442	0.419	95%
3	NEAT VALUE	5.101	NEAT	NA
	1:2	2.551	2.321	91%
	1:4	1.275	1.170	92%
	1:8	0.638	0.586	92%
	1:16	0.319	0.318	100%
Calibrator F	NEAT VALUE	14.200	NEAT	NA
	1:2	7.100	7.154	101%
	1:4	3.550	3.735	105%
	1:8	1.775	1.850	104%
	1:16	0.888	0.964	109%

Imprecision: Reproducibility of the DBS AMH ELISA assay was determined in a study using two kit controls and three serum pools. The study included a total of 40 assays, two replicates of each per assay (n=80). Representative data were calculated based on NCCLS EP5-A guidelines and are presented in the following table.

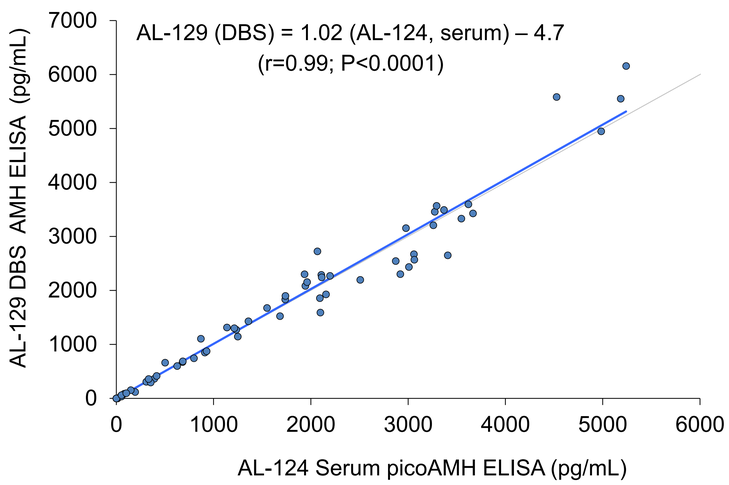
Sample	Conc. (pg/mL)	Within run		Between run		Total	
		SD	CV	SD	CV	SD	CV
Control I	64.1	1.8	2.9%	2.0	3.2%	2.7	4.3%
Control II	186.4	6.5	3.5%	5.8	3.1%	8.7	4.7%
QC1	22.6	0.8	3.7%	1.0	4.5%	1.3	5.8%
QC2	86.5	1.9	2.2%	1.9	2.2%	2.7	3.1%
QC3	373.2	7.7	2.1%	14.2	3.8%	16.2	4.3%

Method Comparison

The DBS AMH ELISA has been compared to Ultra-Sensitive AMH/MIS ELISA using 56 matched serum and dried blood spot samples in the range of 0.6 - 18.5 ng/mL. Passing-Bablok analysis of the results yielded the following Regression:



The DBS AMH ELISA (AL-129) has been compared to picoAMH/MIS ELISA (AL-124) using 55 matched serum and dried blood spot samples in the range of 600-5500 pg/mL. Passing-Bablok analysis of the results yielded the following



CONCLUSIONS

- ❑ The DBS AMH ELISA method is a well-characterized, sensitive human-specific, easy-to-perform, and reliable immunoassay.
- ❑ The dried blood spot sample results are comparable to serum-based assays.
- ❑ DBS sampling is noninvasive and can be implemented by non-medically trained personnel in the participant's home or other non-clinical setting.
- ❑ The specimen stability, ease, and low cost of collection and transportation makes it a very attractive sample type for epidemiologic and other research studies.