

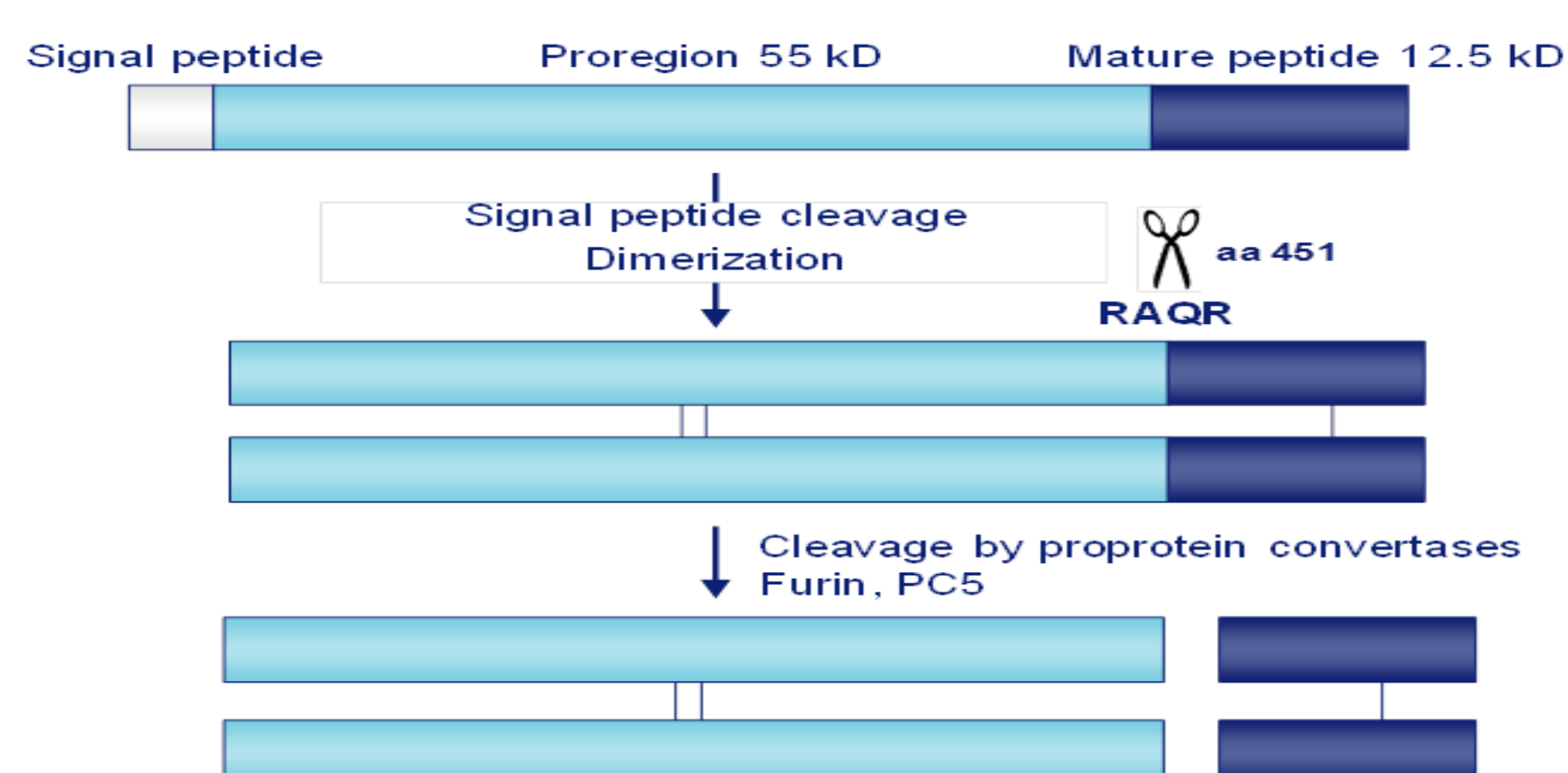
Development of a Well Characterized Ultra-Sensitive Human Anti-Müllerian Hormone (AMH) ELISA

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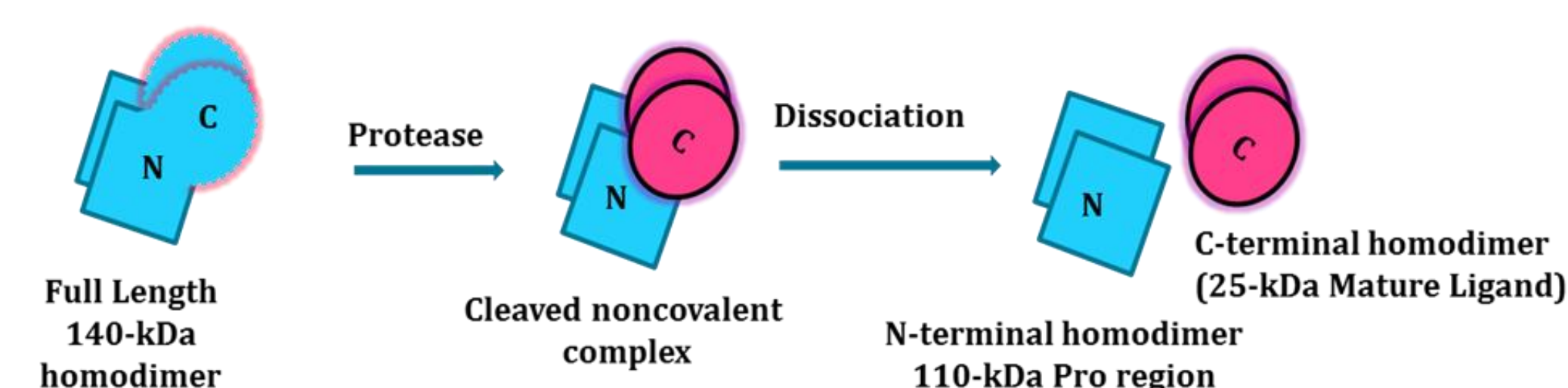
ABSTRACT

Relevance: AMH is a member of the transforming growth factor- β family (TGF- β) responsible for the regression of Müllerian ducts in the male embryo. In female embryos, the Müllerian ducts give rise to the uterus, Fallopian tubes, and upper part of the vagina. AMH is produced in small amounts by ovarian granulosa cells after birth until menopause, and then becomes undetectable. In the adults, AMH also plays a role in Leydig cell differentiation and function and follicular development.

Like other TGF- β superfamily members, AMH is produced as a large homodimeric precursor 140-kDa linked by disulfide bridges. Cleavage at the monobasic site generates 110-kDa N-terminal and 25-kDa C-terminal homodimers prior to cytoplasmic transit. In circulation, the N-terminal and C-terminal homodimers associate in a noncovalent complex.



Recent studies by Di Clemente et al. have shown that the AMH C-terminal homodimer is much less active than the noncovalent complex, but almost full activity can be restored by adding back the N-terminal pro-region, which reforms a complex with the mature C-terminal dimer. The finding raises the possibility that the AMH noncovalent, associated complex is the active form of the protein.

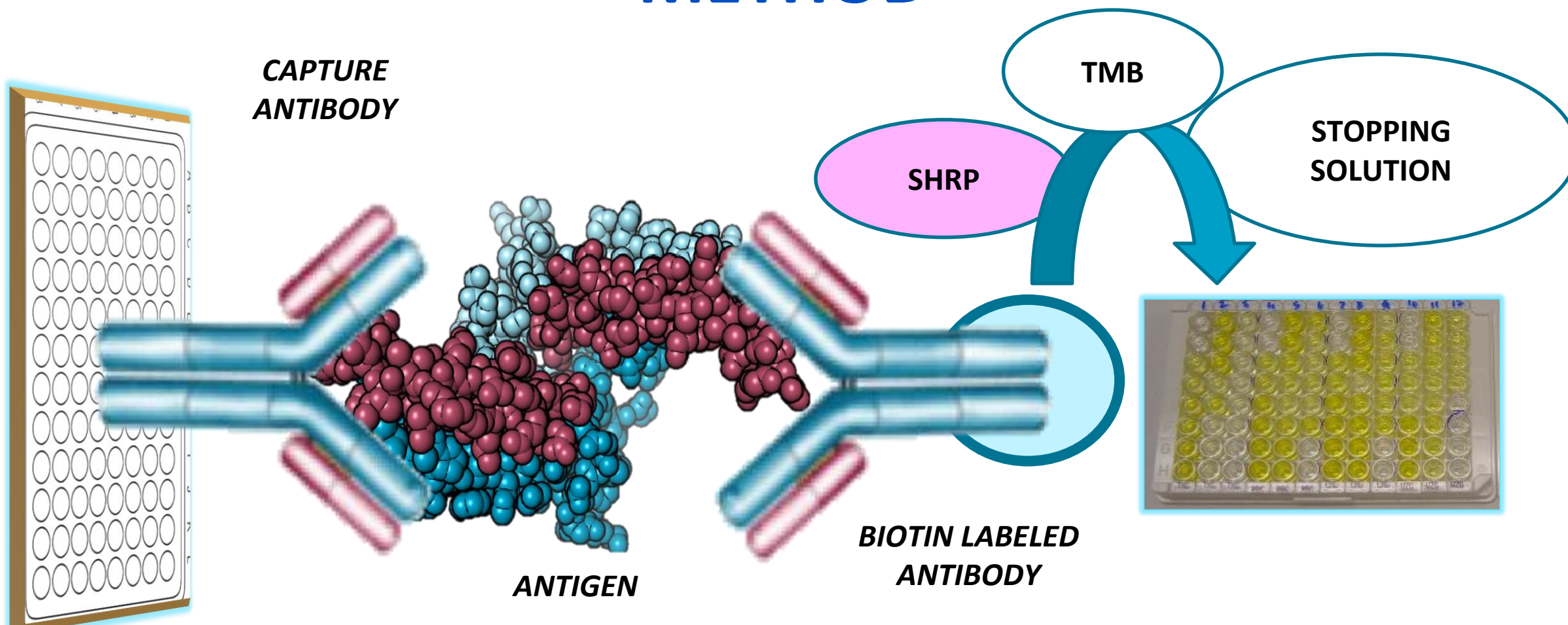


Methodology: A three-step, sandwich-type enzymatic microplate assay has been developed to measure human AMH levels in 25 μ L of sample in less than 3.5 hours. The assay uses stabilized recombinant human AMH as calibrators (0.06-14 ng/mL). The assay measures the noncovalent complex of human AMH and does not detect inhibin A, inhibin B, activin A, activin B, activin AB, FSH, LH, TSH, α 2M, progesterone, estradiol, prolactin, myostatin at two times their physiological concentrations.

Validation: The Ultra-Sensitive AMH ELISA, when compared to AMH Gen II using 90 serum samples in the range of 0.1-13 ng/mL yielded a correlation coefficient of >0.98 and a slope of 1.1 with an intercept of 0.06 ng/mL. Forty matched Lithium heparin plasma and serum specimens in the range of 0.13-13.01 ng/mL yielded a correlation coefficient of >0.99 and a slope of 1.06 with an intercept of -0.1 ng/mL. Total imprecision, calculated on 3 serum samples and 2 kit controls over 40 runs, 2 replicates per run, was 5.4% at 0.51 ng/mL, 5.7% at 0.71 ng/mL, 5.6% at 1.05 ng/mL and 5.5% at 1.1 ng/mL, 5.9% at 2.7 ng/mL, respectively. The functional sensitivity calculated at 20% CV was 0.023 ng/mL. Dilution and spiking studies showed an average recovery of 90-110%. When potential interferents (hemoglobin, triglycerides and bilirubin) were added at twice their physiological concentrations, AMH concentrations were within \pm 10% of the control.

Conclusions: A highly sensitive, specific and reproducible microplate AMH assay has been developed that measures the non-covalent complex of human AMH. The performance of the AMH assay is ideal for research involving neonatal gender determination, ovarian reserve assessment, premature ovarian failure (POF), primary ovarian insufficiency (POI), polycystic ovary syndrome (PCOS), peri-menopausal transition, testicular function, and monitoring of granulosa cell tumor therapy.

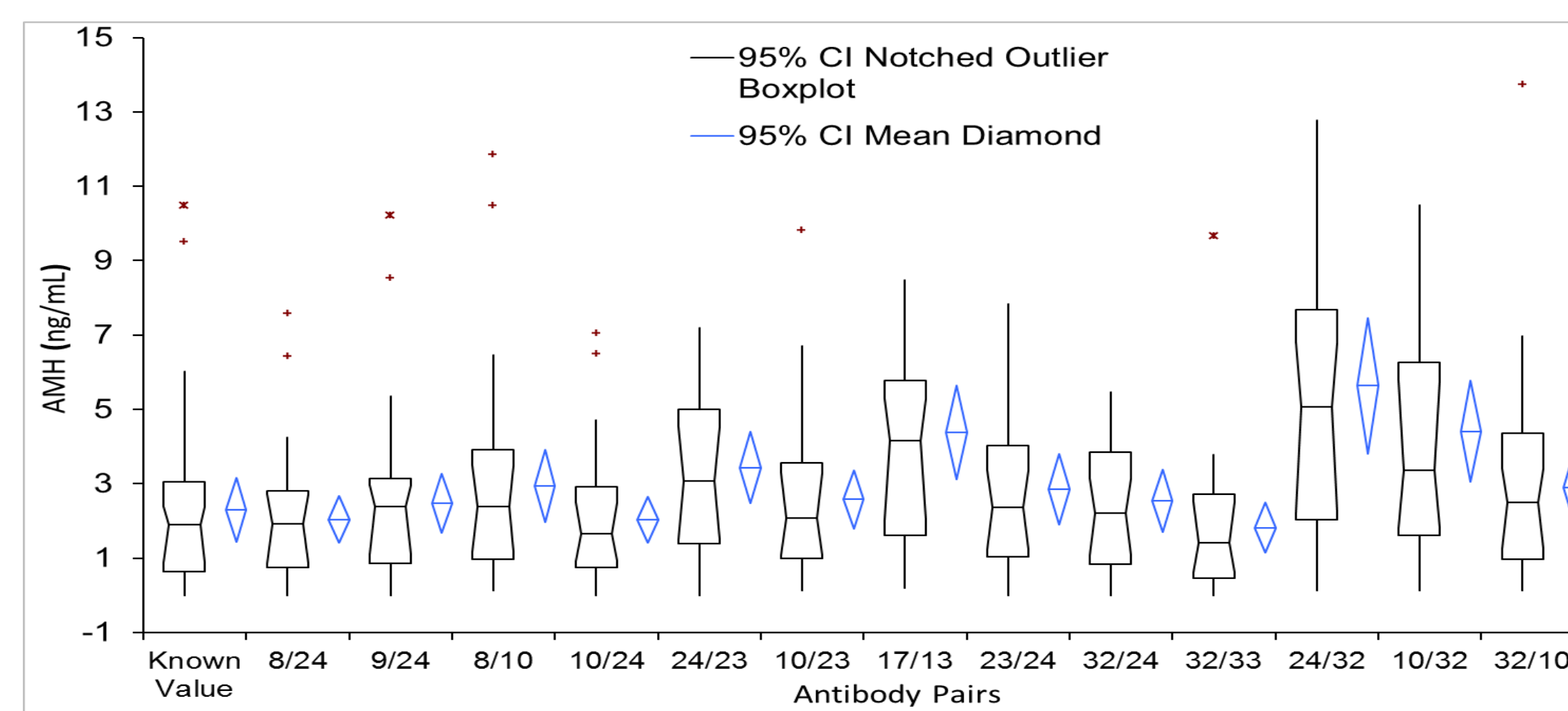
METHOD



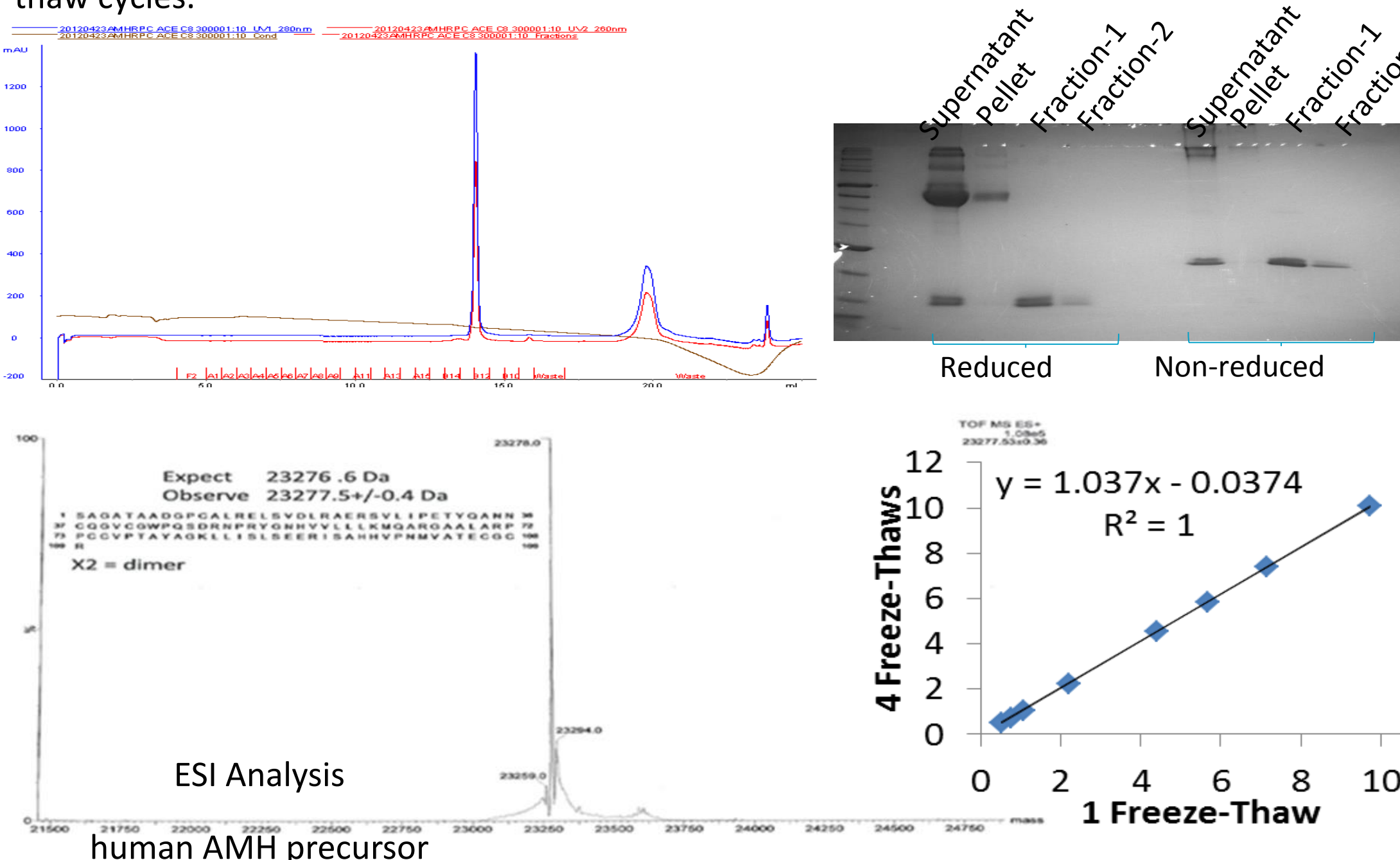
RESULTS

Antibody Selection: 33 Female serum samples were tested on multiple optimized antibody pairs and compared to a commercial AMH assay. The antibody pair was selected based on:

- Linear epitopes in stable pro-region and mature region.
- High affinity and specificity to human AMH



Assay Calibration: The recombinant AMH concentrations in calibrators are standardized to purified mature AMH preparation that is characterized by mass spectroscopy and optical density at 280nm. The calibrators are stable upon reconstitution at -20°C or below and up to four freeze thaw cycles.



Limit of Quantitation: The estimated minimum dose achieved at 20% total imprecision is 0.037 ng/mL. The value was determined by processing thirteen samples in the range of 0.078 - 7.18 ng/mL over 40 runs, 2 replicates per run over 20 days (n=80).

Imprecision: Reproducibility of the US AMH/MIS ELISA assay was determined on two kit controls and three serum pools over 40 runs, 2 replicates per run over 20 days (n=80).

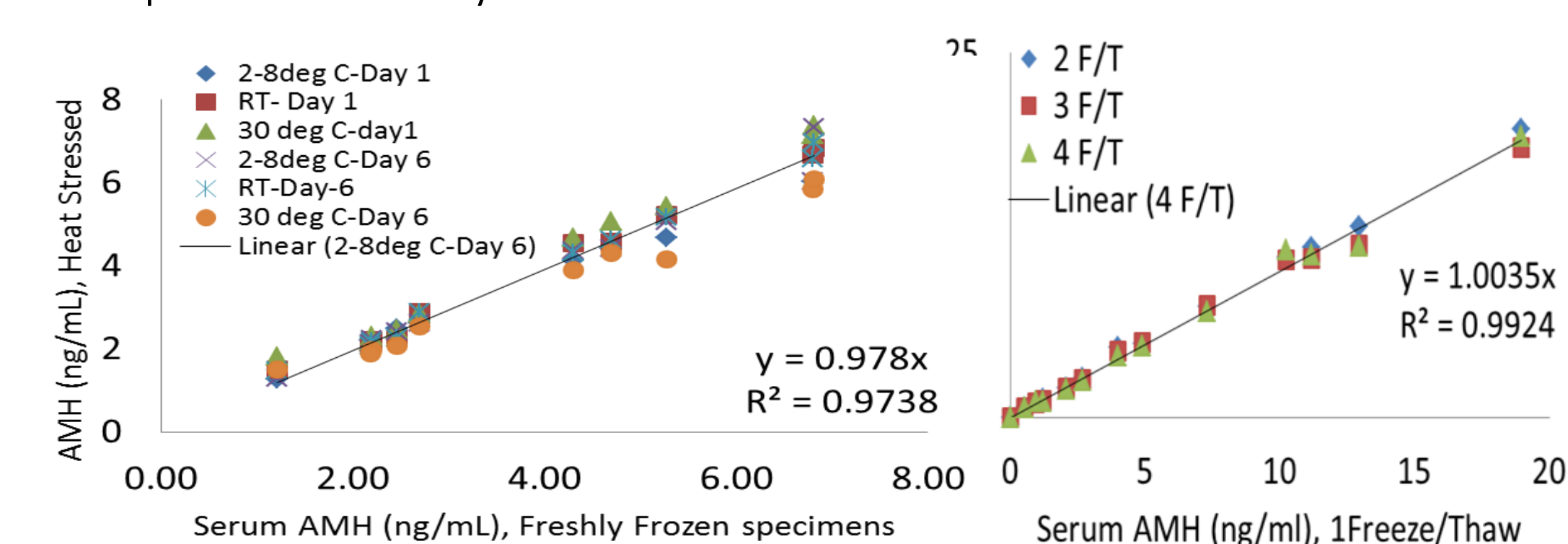
Sample	Mean Conc. (ng/mL)	Within Run		Between Run		Total	
		SD	%CV	SD	%CV	SD	%CV
QC-1	1.110	0.052	4.72	0.032	2.85	0.061	5.51
QC-2	2.661	0.129	4.84	0.088	3.29	0.156	5.86
Pool-1	0.507	0.018	3.50	0.021	4.12	0.027	5.41
Pool-2	0.708	0.019	2.73	0.034	4.84	0.039	5.56
Pool-3	1.048	0.035	3.32	0.047	5.55	0.058	5.55

Linearity of Dilution: Multiple dilutions of the four serum samples containing various AMH/MIS levels were diluted with Calibrator A/sample diluent. The linearity of dilution is represented below.

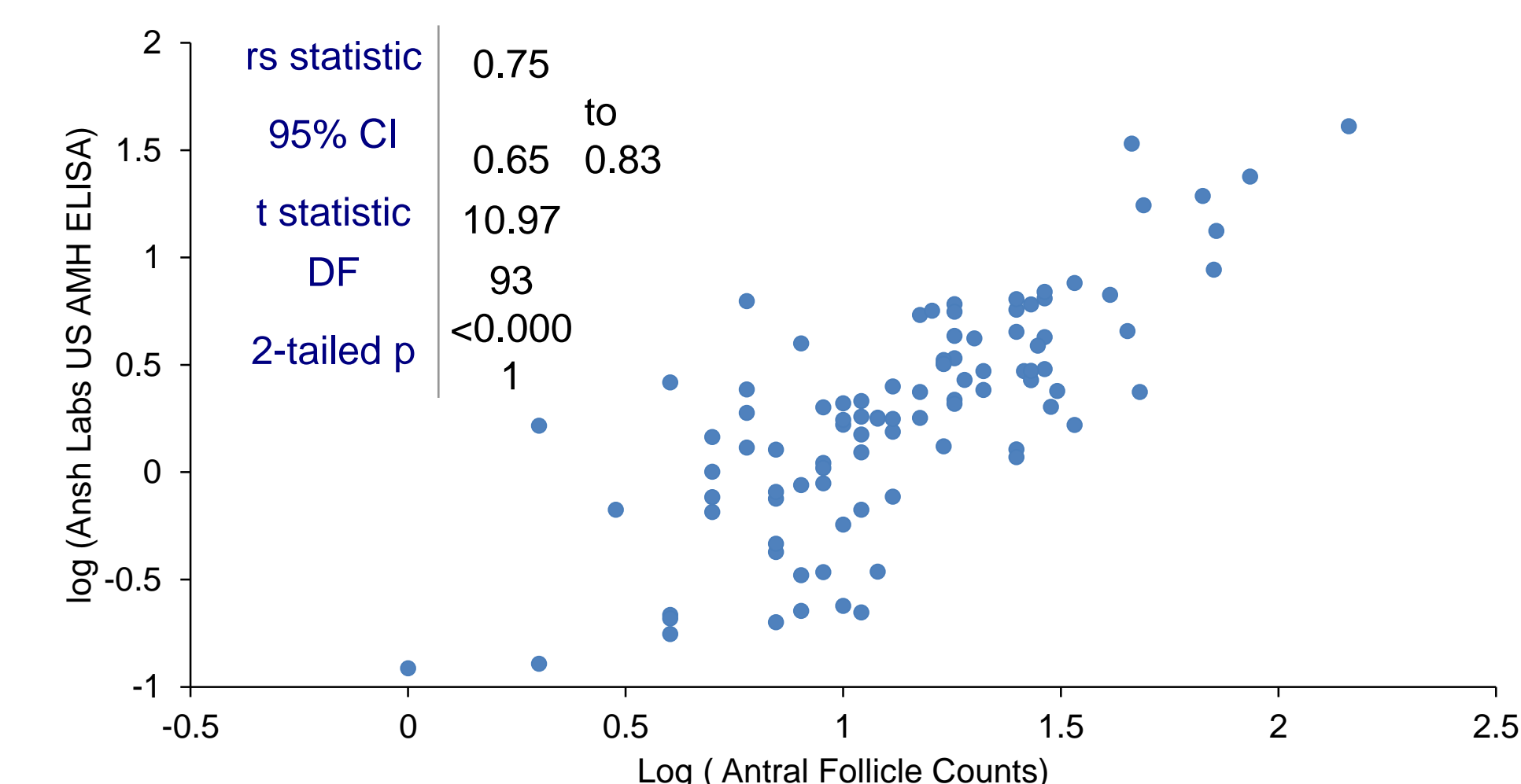
Sample	Dilution Factor	Expected Conc. (ng/mL)	Observed Conc. (ng/mL)	% Recovery
Calibrator F (Antigen)	NEAT VALUE	14.200	NEAT	NA
	1:2	7.100	7.154	101%
	1:4	3.550	3.735	105%
	1:8	1.775	1.850	104%
	1:16	0.888	0.964	109%
Sample (Female)	NEAT VALUE	8.650	NEAT	NA
	1:2	4.325	4.519	104%
	1:4	2.163	2.217	103%
	1:8	1.081	1.114	103%
	1:16	0.541	0.561	104%
Sample (Pediatric Male)	NEAT VALUE	20.295	NEAT	NA
	1:16	10.148	10.287	101%
	1:32	5.074	5.061	100%
	1:64	2.537	2.617	103%
	1:128	1.268	1.325	104%
1:256	0.634	0.722	114%	

Cross Reactivity and Interference: The antibody pair used in the assay is specific to human AMH and does not cross react to mouse, rat, bovine, ovine, canine and other species or other structurally related proteins. Hemoglobin, triglycerides and bilirubin when added at twice their physiological concentrations, AMH concentrations were within \pm 5% of the control.

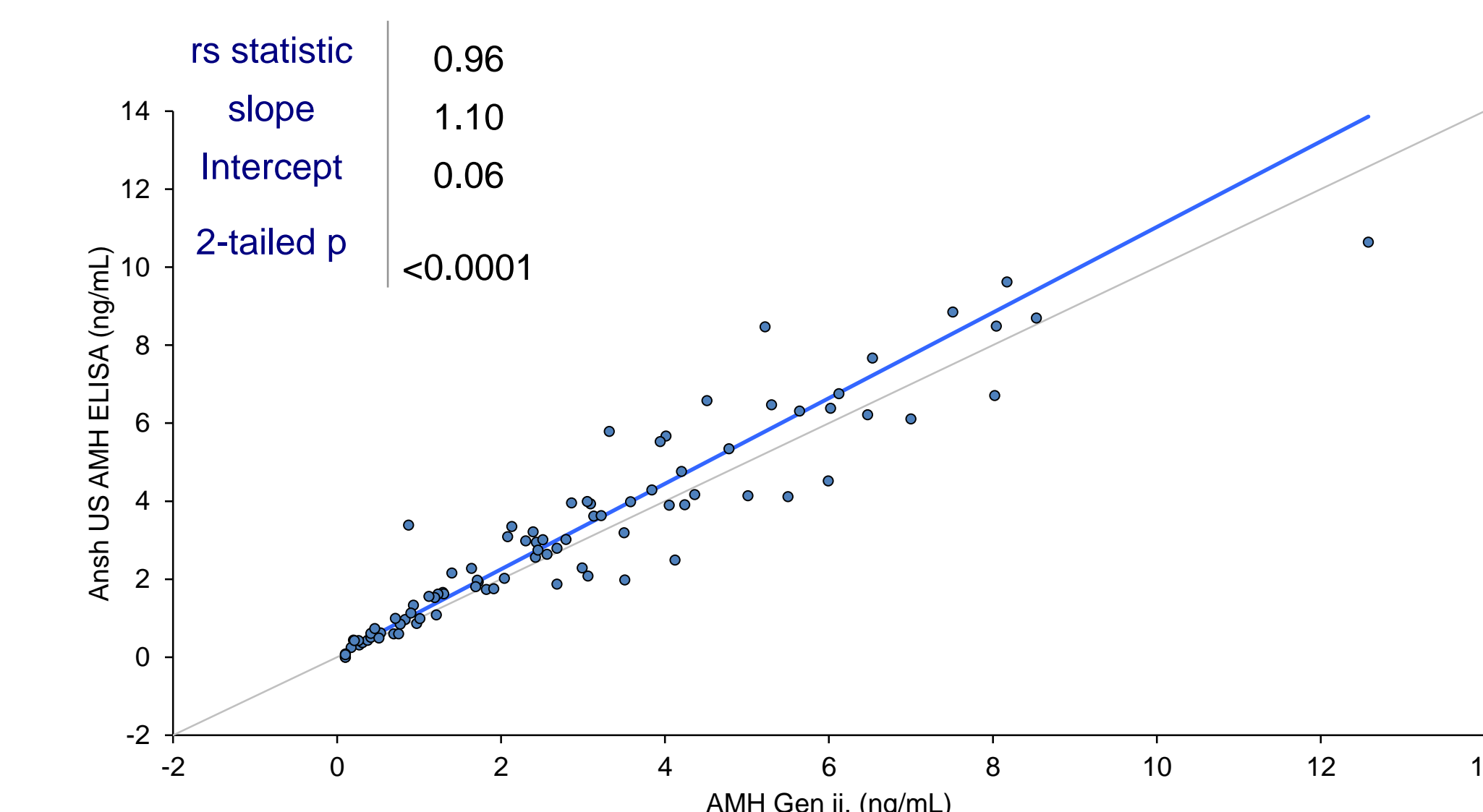
Sample stability: Fresh serum specimens were stressed at 2-8°C, room temperature, 30°C and up to 4 freeze thaw cycles.



Method Comparison: Ansh Labs USAMH ELISA, was compared against total antral follicle counts (96 specimens).



Method Comparison: The UltraSensitive AMH/MIS ELISA has been compared to AMH Gen II assay using 90 serum samples in the range of 0.1-13 ng/mL. Passing & Bablok analysis of the results yielded the following Regression:



CONCLUSIONS

- A sensitive, reliable and easy-to-run microplate AMH assay has been developed to measure AMH in serum and other biological fluids.
- The approximate median AMH levels found in healthy population can be measured within <5% CV using this assay.
- The assay exhibits excellent analytical performance and is suitable for studies in the area of in-vitro fertilization, polycystic ovary syndrome, primary ovarian insufficiency, granulosa cell tumors, menopause, etc.

ACKNOWLEDGEMENTS

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