

Development of a Sensitive Inhibin B ELISA Optimized to Eliminate False Positive Results

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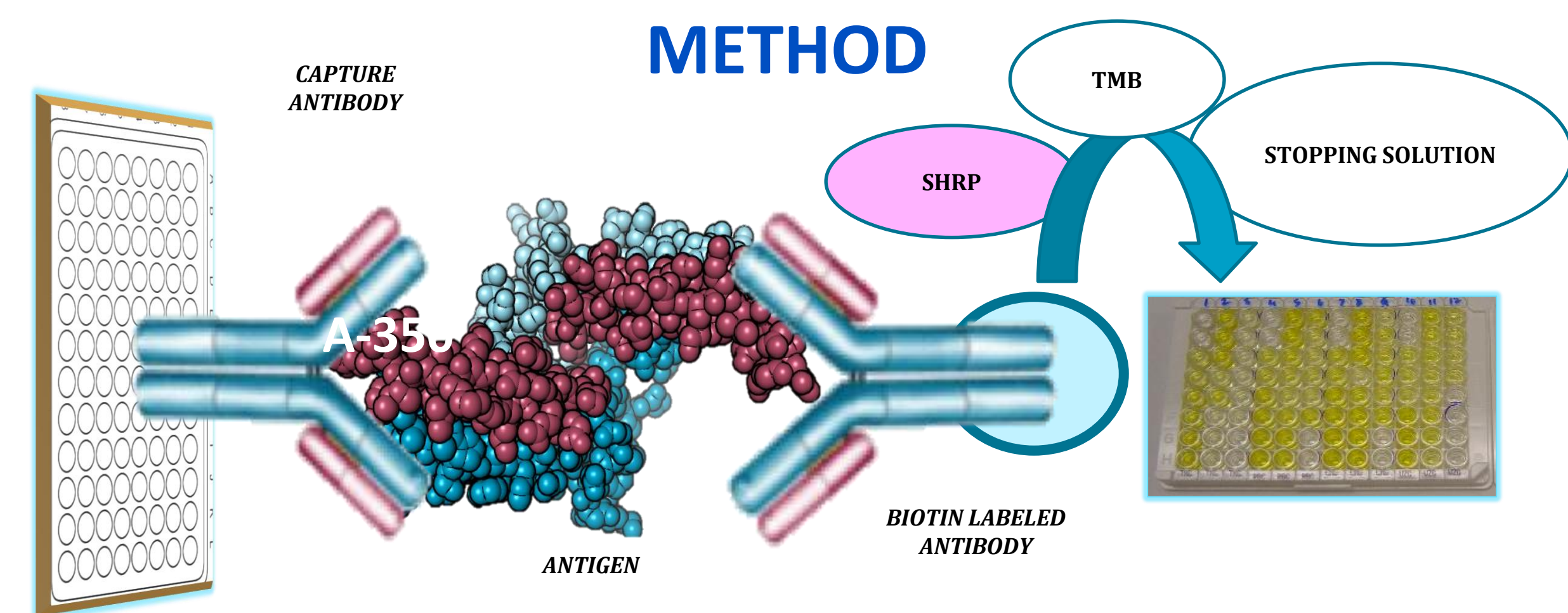
ABSTRACT

Relevance: Inhibins are heterodimeric protein hormones secreted by granulosa cells of the ovary in the female and Sertoli cells of the testis in the male. The role of inhibin B in male factor and female infertility has been extensively published. In males inhibin B is a potential marker for spermatogenesis and testicular function. In females inhibin B is a useful tool for assessment of ovarian reserve, oocyte quality, and granulosa cell tumors. Early commercial Inhibin B assays involved an overnight pre-treatment of samples with an oxidation reagent. This important pre-treatment step minimized the risk of false positive results by removing binding proteins, deactivating proteases and catalases and allow for full immunoreactivity with the inhibin B in the sample. However, these original assays requiring overnight incubation were time-consuming and procedurally cumbersome for laboratories. By incorporating the necessary oxidation step with the initial sample addition, we have been able to simplify the assay procedure.

Methodology: We have developed a three-step, sandwich-type enzymatic microplate assay to measure inhibin B levels. This convenient assay utilizes oxidation and binding protein-releasing reagents that eliminate potential false positive results. Results are generated in less than 3.5 hours with excellent precision. The assay measures inhibin B in 50 µL of serum or Li-Hep plasma samples. The assay uses human inhibin B calibrators (10-1200 pg/mL). The highly characterized dual monoclonal antibody pair is specific to inhibin B and does not detect inhibin A, activin A, activin B, activin AB, AMH, FSH, LH, follistatin 288 and 315 at two times their physiological concentrations.

Validation: The Ansh Labs Inhibin B ELISA assay was compared against two commercially available assays using 97 random male and female samples. The assay showed significant positive linear correlations to the Inhibin B Gen II assay and the AnshLite Inhibin B CLIA assay ($r=0.97$; $P<0.0001$; & $r=0.99$; $P<0.0001$, respectively). Method comparison to Inhibin B Gen II assay and AnshLite Inhibin B CLIA assays resulted in the following slope and intercept (Ansh Labs ELISA=0.93 Gen II +1.08 pg/mL & Ansh Labs ELISA = 1.01 AnshLite CLIA + 5.9 pg/mL), respectively. Matched serum and Li-Hep plasma samples ($n=40$) demonstrated a correlation coefficient of >0.99 and a slope of 1.04 with -0.23 intercept. Total imprecision calculated on three serum pools over fifteen runs, four replicates per run was 7.4% at 50.3 pg/mL, 5.48% at 109.5 pg/mL, 5.98% at 397.2 pg/mL. The LoD of the assay when calculated using six serum samples and twelve assays was 7.23 pg/mL. Dilution and spiking studies demonstrated average recoveries between 90-110%.

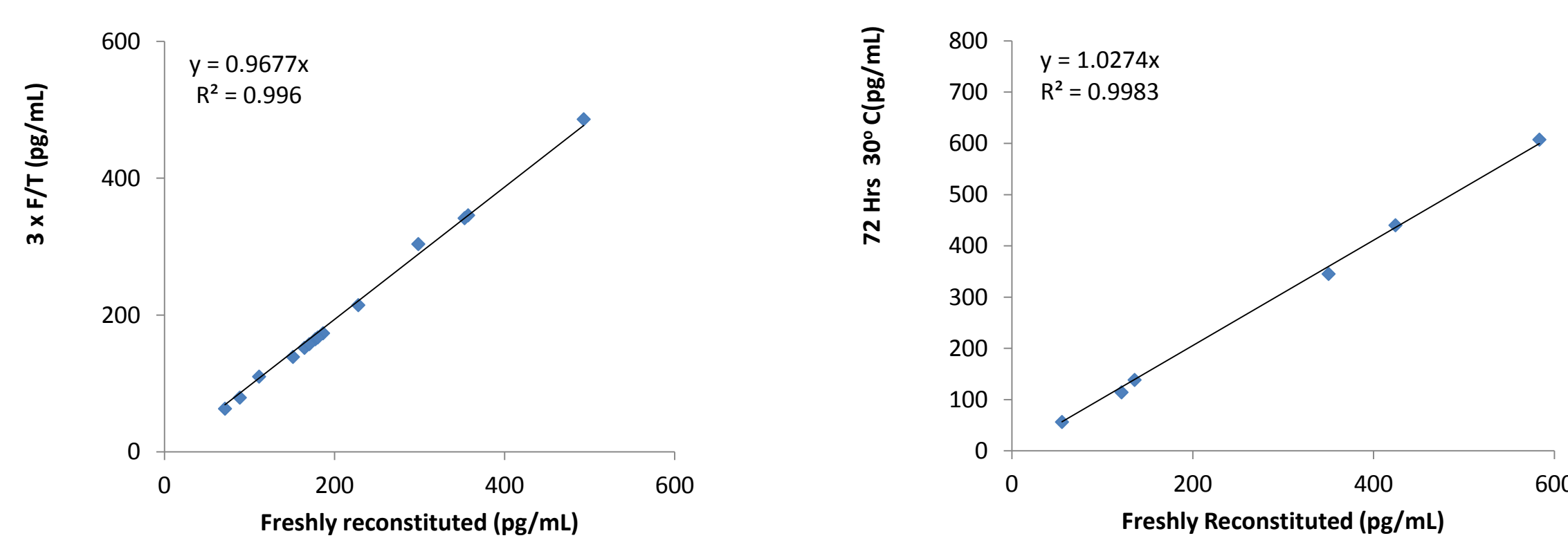
Conclusions: A highly specific, sensitive, reproducible and precise microplate Inhibin B assay has been developed to measure inhibin B in serum, plasma and follicular fluid. The assay has been optimized to eliminate false positive results that can result in incorrectly elevated values. The performance of the assay is ideal for investigation of the physiologic roles of inhibin B in men and women.



RESULTS

Assay Calibration:

The Inhibin B concentrations in calibrators are traceable to human Inhibin B WHO 96/784 preparation. The calibrators are stable upon reconstitution at 30°C for up to 72 hours and three freeze thaw cycles.



Limit of Detection:

The lowest amount of Inhibin B in a sample that can be detected with a 95% probability ($n=24$) is **7.23 pg/mL**. The value was determined by processing six serum samples in the range of 12.2 to 111.45 pg/mL following CLSI EP17-A guidelines. Twelve assay runs were performed over four days with all samples run in duplicate per run.

Imprecision:

Reproducibility of the Inhibin B assay was determined in a study using three serum pools and 2 kit controls. The study included a total of 15 assays, four replicates of each per assay ($n=60$). Representative data were calculated based on CLSI EP5-A2 guidelines.

Sample	Mean Conc. (pg/mL)	Within Run		Between Run		Total	
		SD	%CV	SD	%CV	SD	%CV
QC-1	121.13	6.327	5.22%	3.55	2.93%	7.25	5.99%
QC-2	359.88	13.46	3.71%	20.76	5.73%	24.74	6.83%
Pool-1	50.30	2.25	4.48%	3.02	6.00%	3.76	7.48%
Pool-2	109.49	3.20	2.92%	5.07	4.63%	5.99	5.48%
Pool-3	397.18	17.96	4.52%	15.54	3.91%	23.75	5.98%

Cross Reactivity and Interference

This monoclonal antibody pair used in the assay detects human, non-human primate, and rodent Inhibin B and does not cross react other related structure.

Cross-reactant	Concentration	% Cross-reactivity
Inhibin A	100 ng/mL	ND
Activin A	50 ng/mL	ND
Activin B	50 ng/mL	0.04%
Activin AB	50 ng/mL	ND

Interferents	Analyte Conc. (mg/mL)	Neat (pg/mL)	Spiked (pg/mL)	% Difference
Hemoglobin	1.35	133.99	124.83	-6.8
		31.83	30.01	-5.7
Triglycerides	5.0	133.99	141.57	5.7
		31.83	32.48	2.0

Spike Recovery:

Known amounts of Inhibin B were added to multiple serum samples containing different levels of endogenous Inhibin B. The concentration of Inhibin B was determined before and after the addition of exogenous Inhibin B and the percent recovery was calculated.

Sample	Endogenous (pg/mL)	Expected (pg/mL)	Observed (pg/mL)	% Recovery
1	50.86	112.44	106.53	95
		168.42	151.23	90
		219.530	209.90	96
2	66.97	127.78	127.75	100
		183.06	180.78	99
		233.54	239.06	102
3	144.88	201.98	190.5	94
		253.89	235.8	93
		301.29	301.8	100

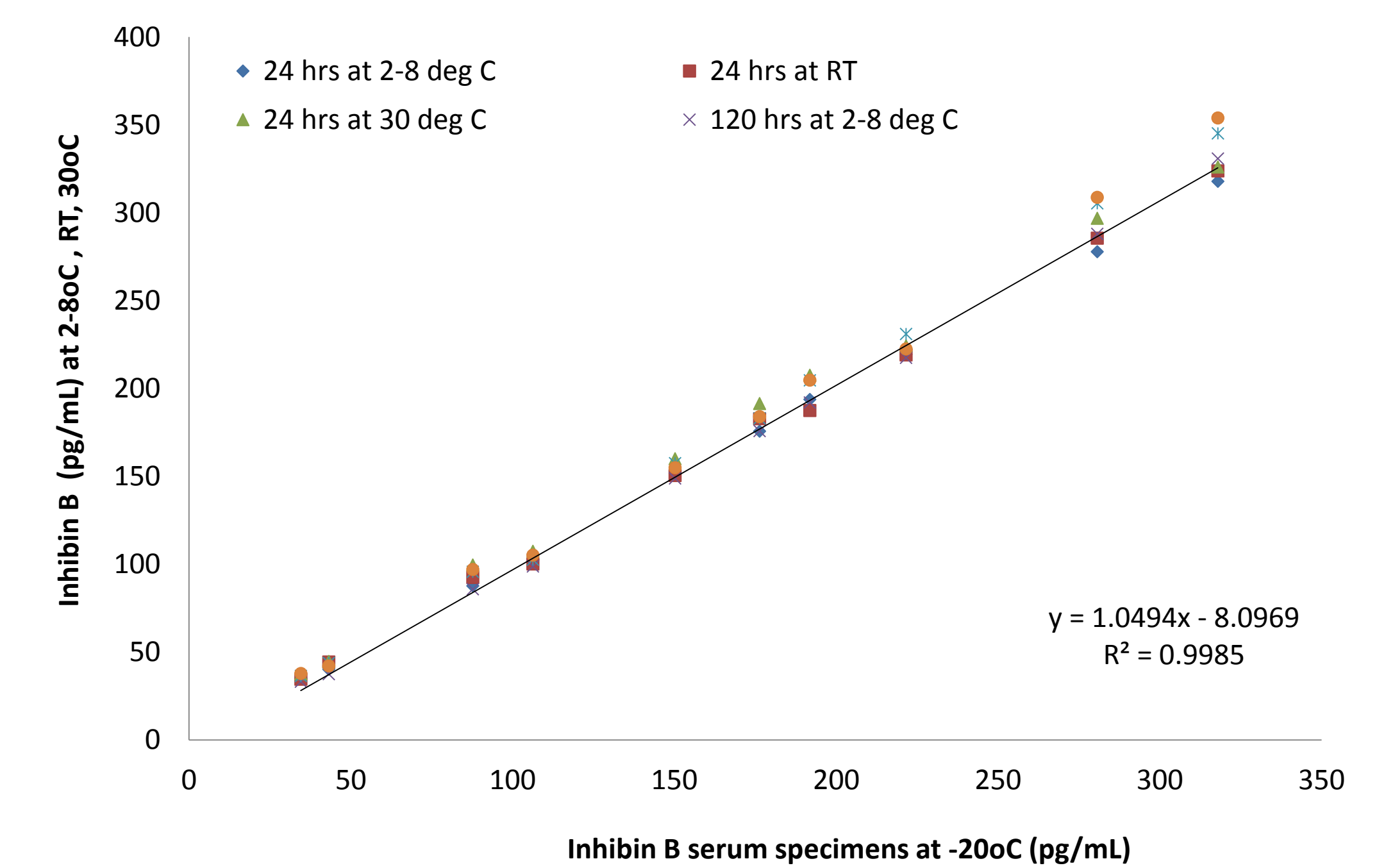
Linearity of Dilution:

Multiple dilutions of the four serum samples containing various Inhibin B levels were diluted with Calibrator A. The linearity of dilution is represented below.

Sample	Dilution Factor	Expected Conc. (pg/mL)	Observed Conc. (pg/mL)	% Recovery
1	Neat	1318.4	N/A	N/A
	1:2	659.2	674.6	102
	1:4	329.6	310.8	94
	1:8	164.8	173.3	105
	1:16	82.4	84.5	103
	1:32	41.2	47.1	114
2	Neat	319.8	N/A	N/A
	1:2	159.9	174.0	109
	1:4	79.9	91.9	115
	1:8	40.0	47.8	120
	1:16	20.0	19.3	97
	1:32	10.0	8.9	89
3	Neat	224.960	N/A	N/A
	1:2	112.480	122.580	109
	1:4	56.240	60.080	107
	1:8	28.120	30.970	110
	1:16	14.060	12.110	86

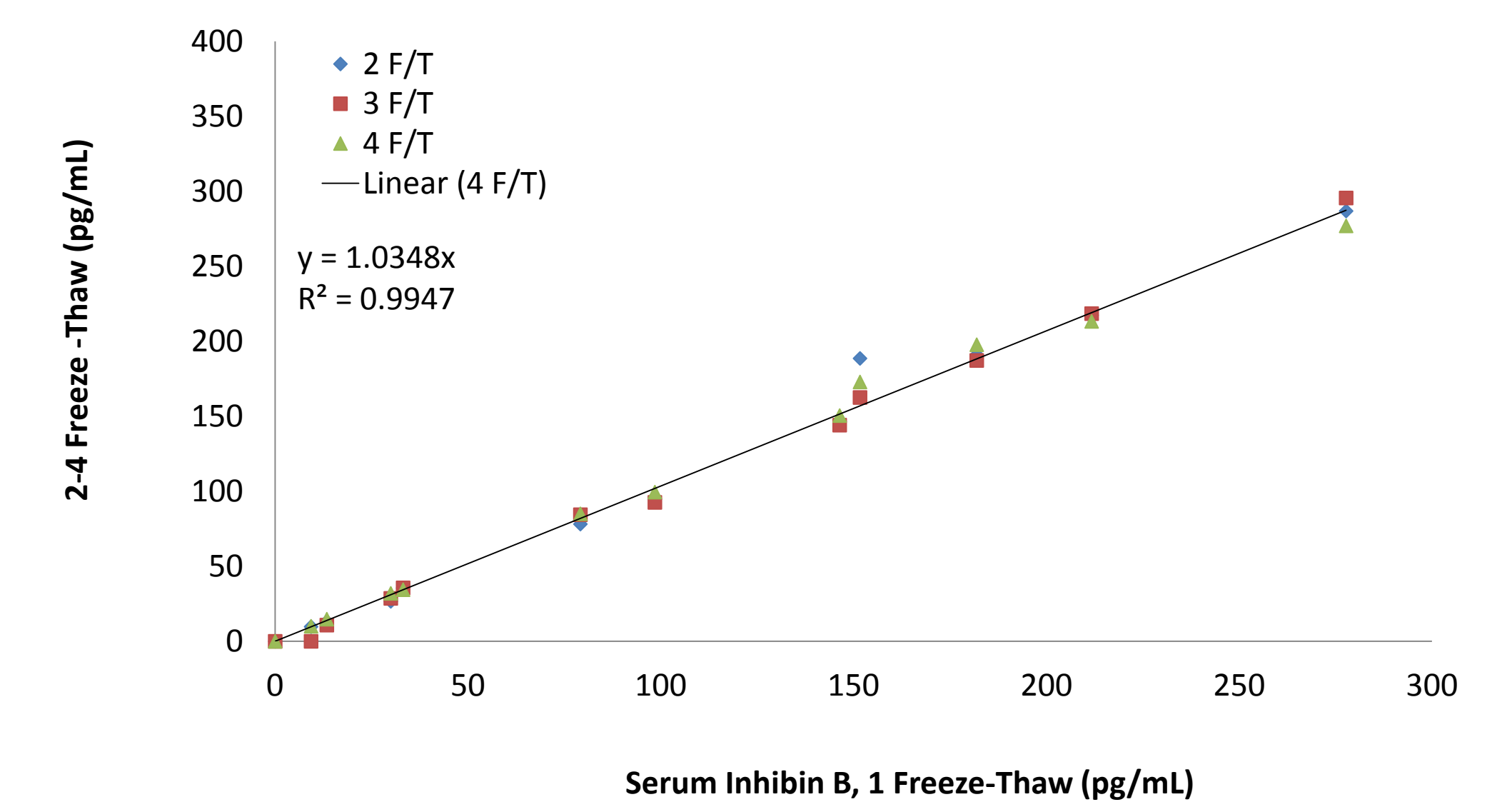
Sample Stability:

Fresh serum samples were aliquoted and tested over 5 days



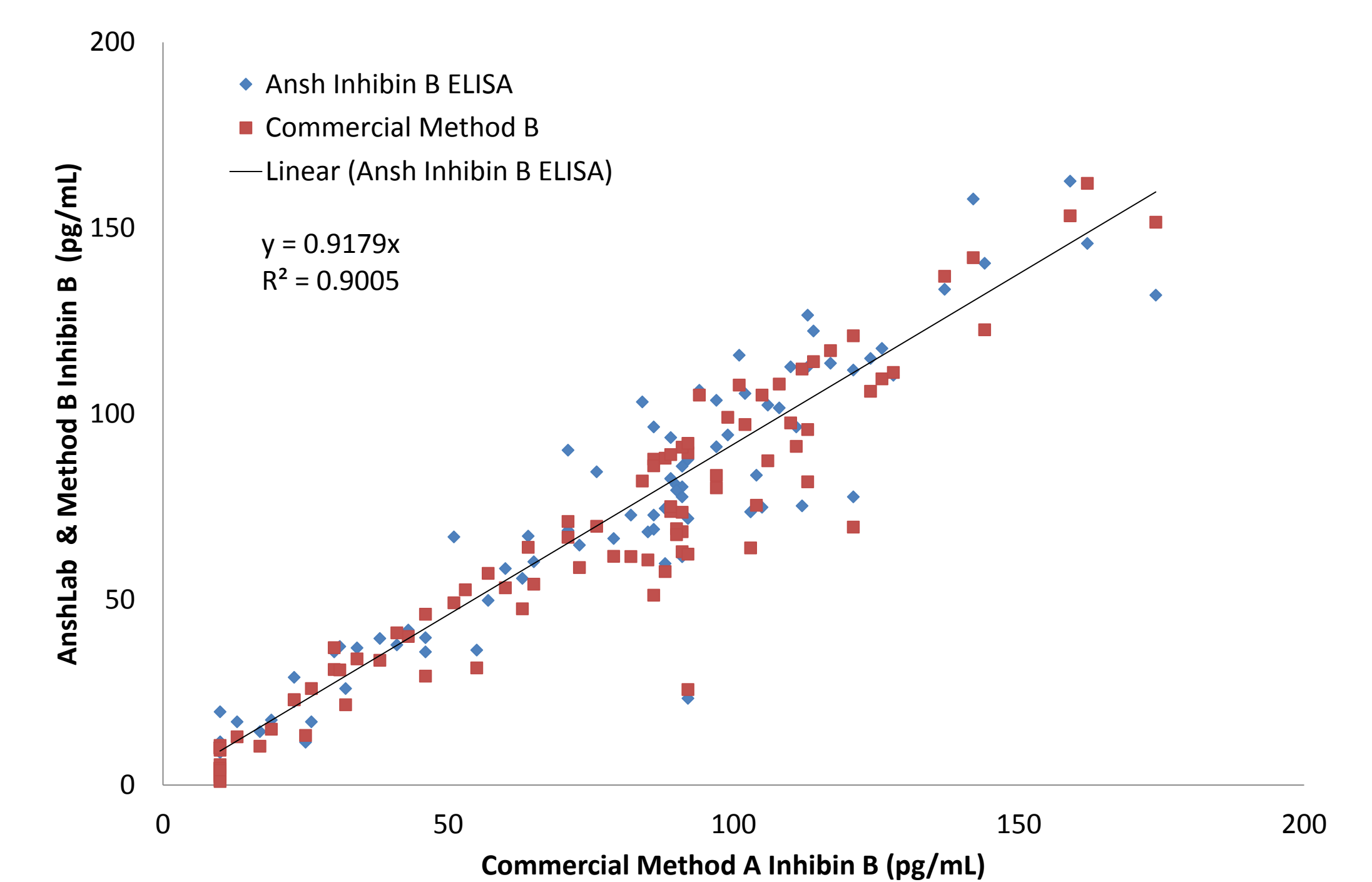
Sample Freeze Thaw Stability:

Fresh serum samples were aliquoted and tested over four freeze thaw cycles



Method Comparison:

The Inhibin B ELISA has been compared to commercial Inhibin B kits (Method A, Method B) using 97 random male and female serum samples in the range of 10-174 pg/mL. Discrepant samples in Method A showed heterophilic antibody interactions. Interference by heterophilic antibodies was not exhibited by the Ansh Labs Inhibin B assays in side-by-side comparisons.



CONCLUSIONS

- A sensitive, reliable and easy-to-run microplate Inhibin B assay has been developed to measure Inhibin B in serum and other biological fluids.
- The assay is optimized to minimize the risk of false positive results caused by interaction of Inhibin B with binding proteins, proteases and catalases.
- The approximate median Inhibin B levels found in healthy population can be measured within <5% CV using this assay. The assay is suitable for studies in the area of in-vitro fertilization, male infertility, granulosa cell tumors etc.

ACKNOWLEDGEMENTS

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