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Pre-analytical assessment of AMH stability in human serum using a well-characterized midpro-mature immunoassay

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INTRODUCTION

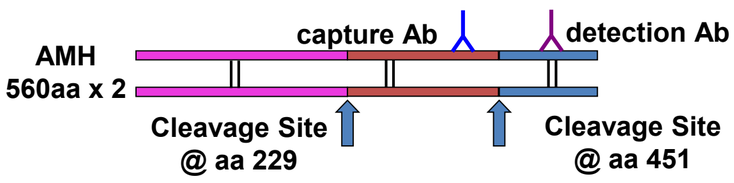
Study Question: How can the AMH stability be addressed in routine clinical practice?

Summary answer: AMH in serum is mostly pro-mature associated form. The kinetics of association of pro and mature is rapid. Assay design that includes stable epitope antibodies and is not impacted by molecule association will measure reproducible results.

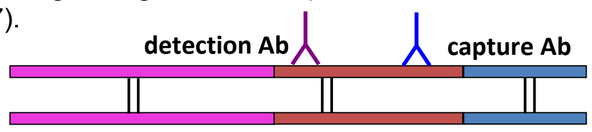
What is already known: AMH is a homodimeric glycoprotein composed of two 55kDa N-terminal and two 12.5kDa C-terminal homodimers, mostly linked non-covalently in circulation. Recently, there have been multiple field safety notices for AMH Gen II ELISA related to sample stability and antibody complement binding interferences. This has generated numerous debates and publications related to reproducibility of AMH measurements. To date no publication has clearly stated if the AMH variability is related to sample processing (pre-analytical) or the assay.

METHOD

- ❑ Ansh Labs US AMH sandwich ELISAs.
- ❑ Standardized to recombinant human AMH Calibrators.
- ❑ Human specific, linear epitope capture and detection Abs in the stable midpro-region and mature region of AMH, respectively (combination mAbs identified as 24-32).

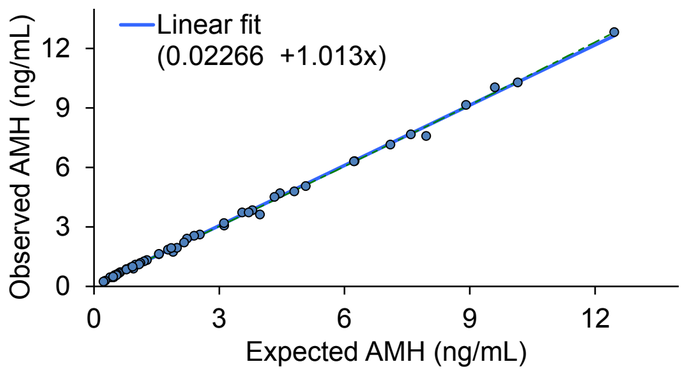


- ❑ Linear epitopes for capture and detection Abs in the stable midpro-region region of AMH (combination mAbs identified as 24-37).



Antibody clone / Isotype	AMH Epitope	Affinity (Region)
AMH Capture (24) IgG2b	358- 369	+++ (Midpro)
AMH Detection (32) IgG1	491-502	+++ (Mature)
AMH Detection (37) IgG2b	260-271	+++ (Midpro)

- ❑ Measures clinically relevant associated pro mature AMH form.
- ❑ Parallelism between native and recombinant hAMH.



Sample Collection and Preparation: Samples were collected from volunteers (with informed consent) at Ansh Labs, Webster, TX or were residual serum from a lab at Women and Infants Hospital, Providence, RI, USA.

- ❑ 10 mL of blood was collected from each subject using BD vacutainer SST tubes. The tubes were inverted six times and left at room temperature for 30-40 minutes for clotting. The tubes were then centrifuged at 3500 rpm for 12 minutes at 20°C and were kept at room (23-25°C) for a minimum of 30 minutes. The separated serum were then poured off into plastic micro centrifuge tubes and the processing of all samples were completed within 3 hours (labelled as 3 hrs).
- ❑ A total of 8 assays were performed on the same kit lot. Controls were included in every run.

Study Design-1: 16 Serum samples freshly poured off the tube (3 hrs) and the same sample treated with assay buffer for 30 minutes were assayed against the same calibration curve. AMH concentration was measured by Ansh Labs US AMH ELISAs (24-32 and 24-37).

Study Design-2: Multiple aliquots of 16 Serum samples (8 adult males and 8 adult females) were stressed at different storage conditions (Table-1) and assayed using Ansh Labs US AMH ELISAs (24-32 and 24-37).

Table-1:

Time from Draw	23-25° C	2-8° C	-20° C
3 Hrs	X		
7 Hrs	X	X	X
10 Hrs	X	X	X
24 Hrs	X	X	X
48 Hrs	X	X	X
168 Hrs (Day 7)	X	X	X
336 Hrs (Day14)	X	X	X

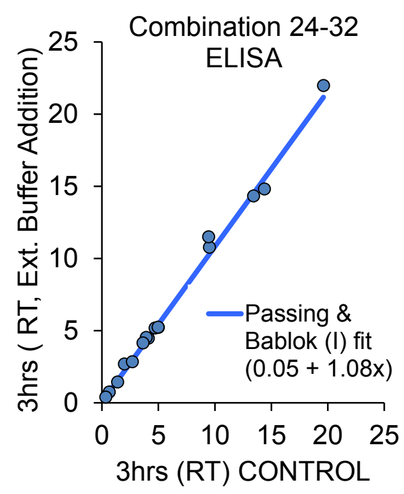
Study Design-3: Four aliquots of 4 serum samples (site-1) and 5 independent serum samples (site-2) were frozen at day zero and thawed as per table-2 and were measured by Ansh Labs US AMH ELISA (24-32).

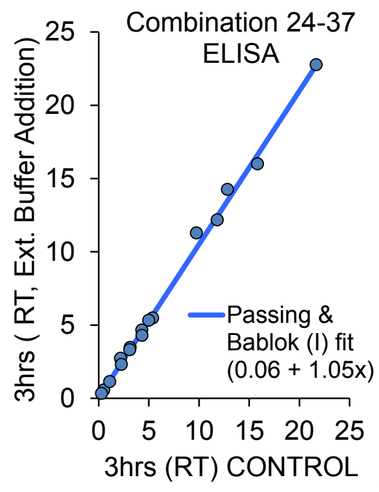
Table-2:

Day 0	4 vials frozen per sample	Control set
Day 1	3 vials thawed and re-frozen	1 F/T
Day 2	2 vials re-thawed and frozen	2 F/T
Day 7	1 vial re-thawed and frozen	3 F/T
Day 10	Run all on same plate	1-4F/T

RESULTS

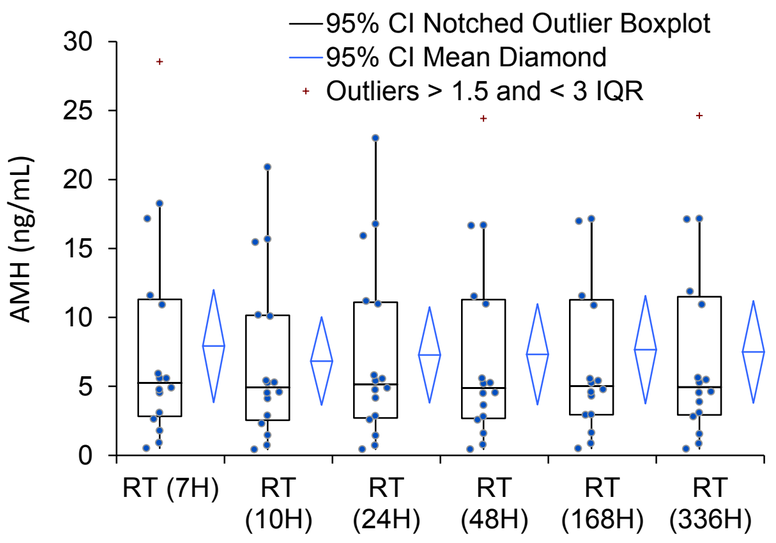
Study Design-1 Results: AMH values were the same regardless of being added to the plate with or without addition of assay buffer to the sample. External buffer treatment is not required for AMH measurement in these assays.



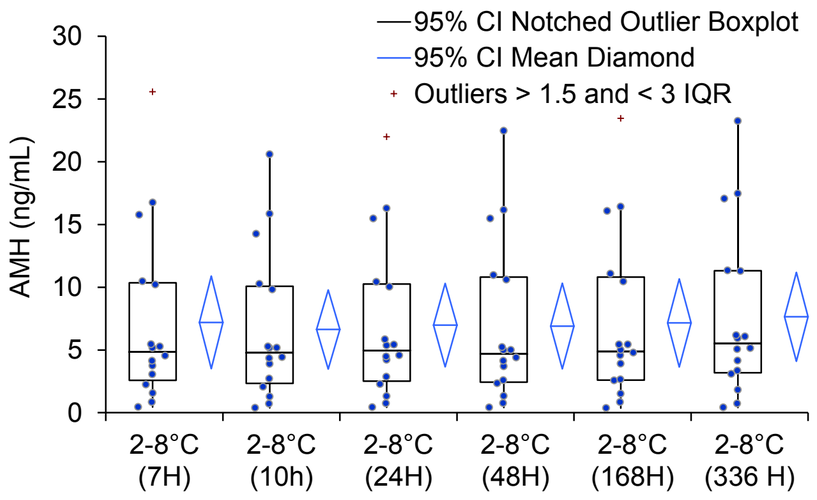


Study Design-2 Results: AMH measurement is stable between 7hrs and 14 days from -20° C to 25° C.

Sample Stability (Combination 24-32) at 23-25° C.

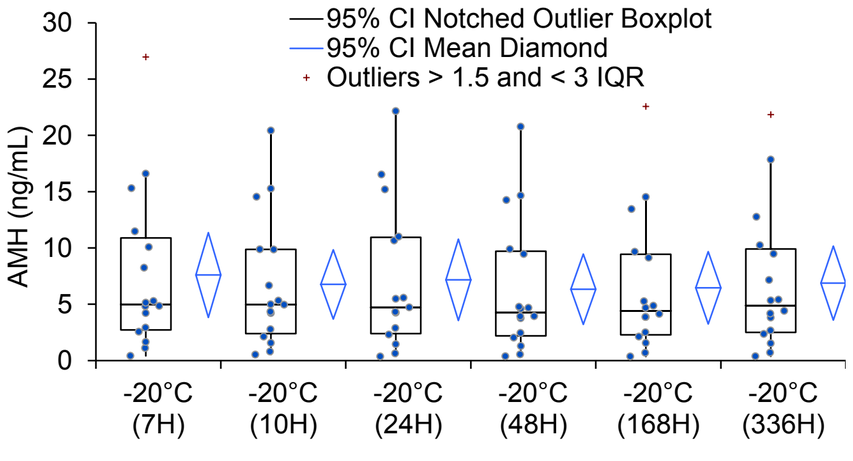


Sample Stability (Combination 24-32) at 2-8° C

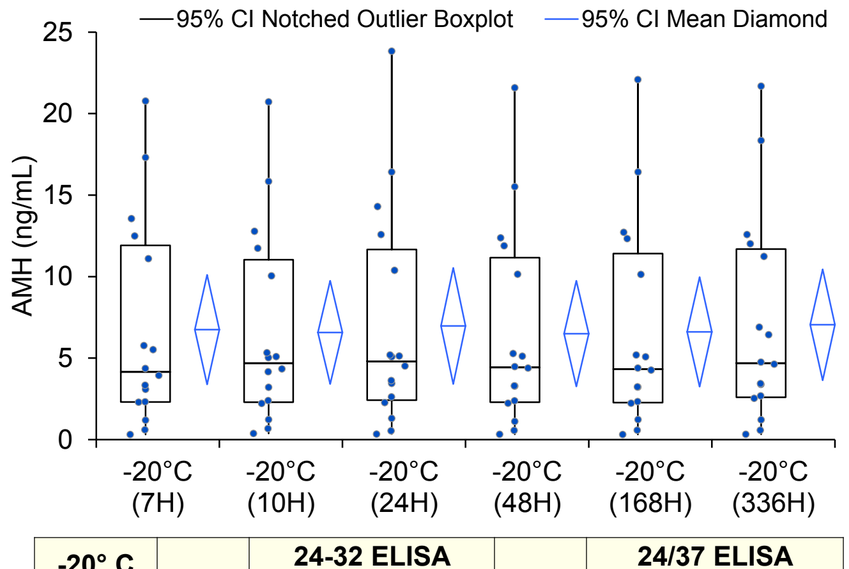


Time from Draw	n	AMH (ng/mL) @ RT		AMH (ng/mL) at 2-8° C	
		Mean	Median	Mean	Median
7 Hrs	16	7.9	5.2	7.2	4.8
10 Hrs	16	6.8	4.9	6.6	4.8
24 Hrs	16	7.3	5.1	7.0	5.0
48 Hrs	16	7.3	4.9	6.9	4.7
168 Hrs	16	7.7	5.0	7.1	4.9
336 Hrs	16	7.5	4.9	7.6	5.5

Sample Stability at -20° C (Combination 24-32)

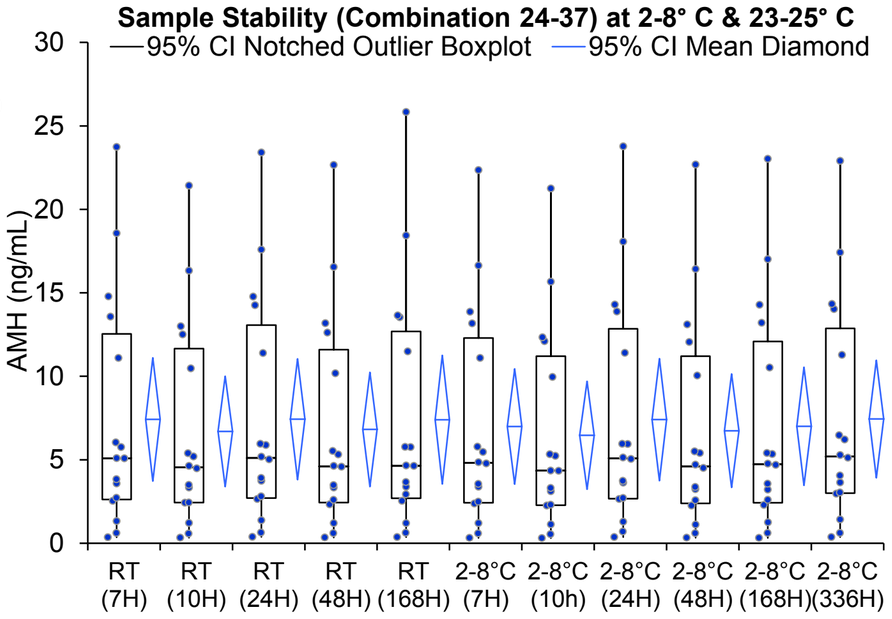


Sample Stability (Combination 24-37) at -20° C



-20° C stored for	(n)	24-32 ELISA AMH (ng/mL)		(n)	24/37 ELISA AMH (ng/mL)	
		Mean	Median		Mean	Median
7 Hrs	16	7.6	5.0	16	6.7	4.2
10 Hrs	16	6.8	5.0	16	6.6	4.7
24 Hrs	16	7.2	4.7	16	7.0	4.8
336 Hrs	16	6.9	4.9	16	7.0	4.7

Sample Stability (Combination 24-37) at 2-8° C & 23-25° C



Time from Draw (24-37)	n	AMH (ng/mL) at RT		AMH (ng/mL) at 2-8° C	
		Mean	Median	Mean	Median
7 Hrs	16	7.4	5.1	7.0	4.8
10 Hrs	16	6.7	4.6	6.5	4.3
24 Hrs	16	7.4	5.1	7.4	5.1
48 Hrs	16	6.8	4.6	6.7	4.6
168 Hrs	16	7.4	4.6	7.0	4.7
336 Hrs	16	7.2	4.7	7.4	5.2

Study Design-3 Results : AMH is stable after 1-4 freeze-thaw cycles.

Freeze Thaw	Site-1 (n)	AMH (ng/mL)		Site-2 (n)	AMH (ng/mL)	
		Mean	Median		Mean	Median
1 F/T	5	5.1	4.5	6	2.0	1.2
2 F/T	5	5.3	4.7	6	2.4	1.2
3 F/T	5	5.4	4.8	6	1.6	1.3
4 F/T	5	5.4	4.8	6	2.0	1.3

Imprecision: Reproducibility of the assay runs were determined using two kit controls. The study included a total of 8 assays; two replicates of each per assay (n=16).

SUMMARY:		Within run		Between run		Total	
Sample	Mean	SD	CV	SD	CV	SD	CV
Control I	1.50	0.05	3.6%	0.04	2.8%	0.07	4.6%
Control II	4.35	0.12	2.8%	0.10	2.4%	0.16	3.6%

Strength: This is a prospective study and uses two well-characterized antibody based AMH assays (with epitopes to midpro and midpro-mature regions of AMH).

Wider implications of the Finding: AMH is stable in serum stored under different conditions. Well-characterized assays with good pre-analytical methods will produce reproducible clinically relevant results.

CONCLUSIONS

- ❑ The US AMH ELISA method is a well-characterized, human-specific, easy-to-perform and reliable immunoassay.
- ❑ The assay is not impacted by interfering factors (i.e., serum component, or non-human AMH or other proteins).
- ❑ External buffer treatment is not required for AMH measurement.
- ❑ The AMH form in circulation for normal subjects is predominantly associated pro-mature AMH. Assay to measure specific isoforms of AMH in disease state is important towards understanding the biology and its utility (PCT/US2013/069172).