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ORIGINAL ARTICLE ***Reproductive endocrinology***

Pre-mixing serum samples with assay buffer is a prerequisite for reproducible anti-Müllerian hormone measurement using the Beckman Coulter Gen II assay

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STUDY QUESTION: Does pre-mixing stored serum samples with assay buffer improve the reproducibility of the Beckman Gen II assay for anti-Müllerian hormone (AMH)?

SUMMARY ANSWER: Pre-mixing serum samples with assay buffer is a prerequisite for reproducible measurement of AMH in serum using the Beckman Coulter Gen II assay.

WHAT IS KNOWN ALREADY: Discrepancies in the results obtained from AMH assays have raised doubts concerning the clinical utility of measuring AMH. Sample storage conditions may be responsible for the lack of reproducibility of results obtained from the Gen II kit.

STUDY DESIGN, SIZE, DURATION: This was a prospective study in which serum samples were stored at three different temperatures and assayed for AMH at times 0, 4, 8, 12, 24, 48 h and 1 or 2 weeks after collection. Volunteers ($n = 28$) were healthy non-pregnant and early pregnant women aged 22–41 years. Anonymized long-term stored samples ($n = 42$, stored at -20° for 2 weeks) from fertility clinic attendees were also included. For determining the reference range, 179 samples from healthy pregnant women presenting for first trimester screening were used.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Thirty separate assays were performed by two operators using four different Gen II kit lots with both kit and in-house quality controls (QCs) included in each assay. In addition to the standard protocol, a modified protocol (pre-mixing samples with assay buffer) was used for selected sample groups.

MAIN RESULTS AND THE ROLE OF CHANCE: In non-pregnant women, AMH concentrations remained unchanged in serum stored for up to 8 h at room temperature, −20 and −80°C. At room temperature, levels started to rise by 24 h, increasing by up to 29% of the time 0 h value by 48 h and 26% after 1 week. Significant changes versus baseline (time 0 h) in measured AMH concentration were also observed after storage at −20 and −80°C (only at the 12 h time point). In the pregnant group, there was a 50% increase above baseline in samples stored for 48 h at room temperature. When samples were pre-mixed with assay buffer, AMH concentrations showed a consistent increase versus the standard assay in both non-pregnant (29%) and pregnant (280%) groups, regardless of storage conditions and duration, but concentrations remained constant during long-term storage (2 weeks). Stored fertility clinic patient samples also exhibited stability of AMH values after a consistent 2-fold increase following pre-mixing. Kit QCs were consistent over 30 weeks using either standard or modified protocols while the in-house pooled serum QC rose over time unless using the modified protocol. Overall, there was a 2-fold increase in medians in the pre-mixed reference range, with the biggest increase observed in the oldest age bracket (41–45 years, 3.4-fold).

LIMITATIONS, REASONS FOR CAUTION: The cause of the observed instability of AMH in stored serum samples requires further investigation, which is outside the scope of this publication. A larger and wider population study is necessary for a more reliable and clinically relevant reference range.

WIDER IMPLICATIONS OF THE FINDINGS: Our study has confirmed previous findings of lack of consistency in AMH concentrations when measured with the Gen II assay. Pre-mixing serum samples with assay buffer gave higher but also the most consistent results regardless of

storage conditions; therefore, we propose that all serum samples for AMH assay should be pre-mixed with assay buffer. Furthermore, clinical laboratories that offer AMH measurement as part of the assessment of endocrinopathies, such as polycystic ovary syndrome or premature ovarian failure, or for management of ovulation induction as part of assisted reproduction, must re-establish their own normal ranges using the modified method.

STUDY FUNDING/COMPETING INTEREST(S): No funding was obtained for this study. There are no conflicts of interest to declare.

Key words: anti-Mullerian hormone / Beckman Gen II assay / pre-mixing / ovarian reserve

Introduction

Measurement of anti-Müllerian hormone (AMH) is used widely in clinical practice as an indirect assessment of ovarian reserve and therefore reproductive capacity. Together with antral follicle count (AFC), AMH measurement allows practitioners to optimize stimulation protocols for women undergoing assisted reproduction technology with gonadotrophins (Nelson *et al.*, 2009; Yates *et al.*, 2011). This is particularly significant in identifying those women who are more likely to develop ovarian hyperstimulation syndrome, and those who may be likely to under-respond to stimulation. The most commonly used methodology for AMH measurement is the Beckman Coulter Gen II assay, which is a two-step 'sandwich' enzymatic microplate assay. This assay utilizes standards and antibodies derived from two previous AMH assays (IOT and DSL, respectively) (Kumar *et al.*, 2010). The two antibodies used in the assay are directed against epitopes in the mature and the pro-region of the molecule (Al-Qahtani *et al.*, 2005). The mature region of the molecule is considered to be less susceptible to proteolysis (Fleming *et al.*, 2013). A recent report (Rustamov *et al.*, 2012) has suggested that there may be considerable within-subject variability in AMH results, particularly concerning specific storage and assay conditions. Rustamov *et al.* (2012) reported a 58% increase in measured AMH concentration when serum was stored at room temperature and a 23% increase when serum was stored at −20°C. Pre-mixing of serum with assay buffer before assay also gave higher readings. Fleming *et al.* (2013) also demonstrated that storing whole blood at room temperature produced a significant increase in measured AMH (31%) but that when whole blood was stored at 4°C, the increase was modest (10%). Anectodal reports suggest that there is often a discrepancy between individual patients' AMH concentration and their AFC. Inconsistent results make it difficult to predict a woman's response to ovarian stimulation or to provide an estimation of ovarian reserve.

In routine laboratory practice, it is reasonable to expect some delay between the time when blood is collected and when it reaches the laboratory. Similarly, because the AMH assay is not run on an automated platform, it is routine practice to store samples and run assays in a batch. In this prospective study, we investigated in more detail the previously reported findings regarding the stability of AMH under varying storage and assay conditions. In particular, we performed an in-depth analysis of the significance of pre-analytical time delay under various storage conditions and whether the impact of this delay on the result can be minimized by manipulating assay conditions. Specifically, we investigated whether premixing samples with assay buffer prior to plating improves the stability and consistency of AMH results.

Materials and Methods

Participants and sample preparation

Five millilitres of whole blood was collected from each subject (in gold top Vacutainer tubes; BD ref 367954). Blood was allowed to clot at room temperature for at least 20 min and then centrifuged at 1000 *g* for 10 min at room temperature within 30 min of collection.

Where samples needed to be stored (at −20 or −80°C), serum was aliquoted into Eppendorf tubes in 150 μl aliquots. Primary tubes were kept at room temperature. No samples were re-frozen.

Subject groups were as follows:

Group I: 13 volunteers who were healthy non-pregnant women aged 22–41 years with no relevant co-morbidities or medications. One subject was taking the oral contraceptive pill.

Group II (patient group): 15 randomized subjects, both pregnant and non-pregnant attending clinics at the hospital.

Group III (fertility group): 42 patients attending a routine fertility clinic, whose AMH was requested as part of their clinical investigation.

Group IV (reference range group): 179 pregnant women attending the laboratory for first trimester screening.

AMH assay

The Beckman Coulter Gen II AMH assay was used throughout this study. Thirty (separate assays were performed by two operators using four different kit lot numbers prior to manufacturer's product recall in June 2013 (Beckman Coulter IPCA 20434-3). An internal pooled serum quality control (QC1) targeted at the lower end of the assay range (mean 2.0 pmol/l) was included in each assay, in addition to two kit controls. Following the manufacturer's product recall, a new in-house pooled serum quality control (QC2) (mean 3.0 pmol/l without pre-mixing) was run in 20 separate assays using the modified (pre-mixing) protocol. Samples and controls were run in duplicate and individual comparison tests were run in the same assay.

Sample stability studies

Samples were assayed at time 0 and 4, 8, 12, 24, 48, 168 h (1 week) thereafter. These samples were kept under three different conditions: room temperature, −20 and −80°C.

Pre-mixing studies

A modified protocol was used for pre-mixing studies in which each individual sample was pre-mixed with assay buffer in the same ratio as the standard protocol prior to assay. In brief, 60 μl sample plus 300 μl assay buffer was incubated in an Eppendorf tube for 10 min and 120 μl was then transferred to the AMH assay plate in duplicate. There were no other changes to the assay procedure.

Samples from Group II were assayed using both protocols, immediately after collection and after 48 h at room temperature or at −20°C.

Samples from Group III were assayed using both protocols on the day of collection as well as after 2-week storage at −20°C.

Reference range

Stored samples (−20°C) from the reference group were assayed using the modified protocol.

For all groups, when AMH level was undetectable (below the assay detection limit, usually <1.0 pmol/l) in the initial assay with fresh sample, subjects were excluded from further studies as numerical comparison of the results would be difficult.

Statistical analysis

AMH results for each individual were expressed as a percentage relative to the value assayed with fresh sample. A paired sample *t*-test was performed using the statistical function in Microsoft excel. A $P < 0.05$ was considered significant.

Results

Sample stability studies using standard method

When samples were stored at room temperature, AMH values remained relatively unchanged versus baseline for the first 8 h followed by a significant increase at all storage time points ($P < 0.05$) (Fig. 1).

For samples that were stored at −20°C, AMH was again relatively unchanged for the first 8 h but this was followed by inconsistent results for the remaining time points with both a significant increase observed at 12 h and a significant decrease at 24 h ($P < 0.05$) (Fig. 1).

Storage at −80°C produced similarly inconsistent results.

Pre-mixing studies

(i) In the non-pregnant group, pre-mixing of samples prior to assay tended to produce consistently higher AMH measurements compared with standard protocol, regardless of how samples were stored (Fig. 2A). When stored samples were assayed using the standard protocol, results were lower compared with fresh samples, for both room temperature and −20°C storage conditions.

(ii) In the pregnant group, again pre-mixed results were consistently higher (more than doubled) compared with those from standard protocol regardless of if samples were fresh or stored and this increment was consistent. When using the standard protocol, samples stored for 48 h at room temperature were 50% higher compared with fresh samples (Fig. 2B).

(iii) In the fertility group (Table I), pre-mixed samples had on average double the measured amount of AMH compared with standard protocol for both fresh and stored samples (mean B/A ratio 1.95; mean D/C ratio 2.00), but there was a wide distribution of ratios in this group and therefore a high % coefficient of variation (CV) (20 and 23%, respectively). Similarly, when results from stored versus fresh samples were compared in terms of AMH ratio, both standard and modified protocols had an average ratio of almost 1 (C/A ratio 0.96; D/B ratio 0.97) but only the modified method results were consistent for all subjects with a narrow distribution of these ratios and a CV of 8% compared with 31% for the standard protocol.

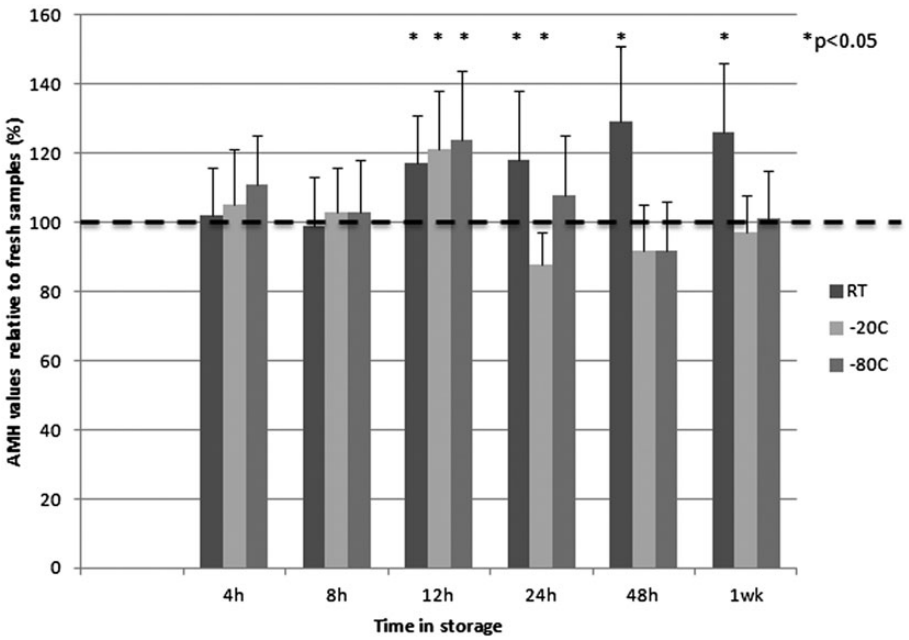

Figure 1 Stability of AMH in serum samples, measured using the standard method, with storage at room temperature (RT), −20 and −80°C. Results are expressed as a percentage ± SD relative to AMH measured in fresh samples (time 0 h). *$P < 0.05$ versus fresh sample. $n = 13$ (group I). Dotted line at 100% represents AMH levels at time 0 h.

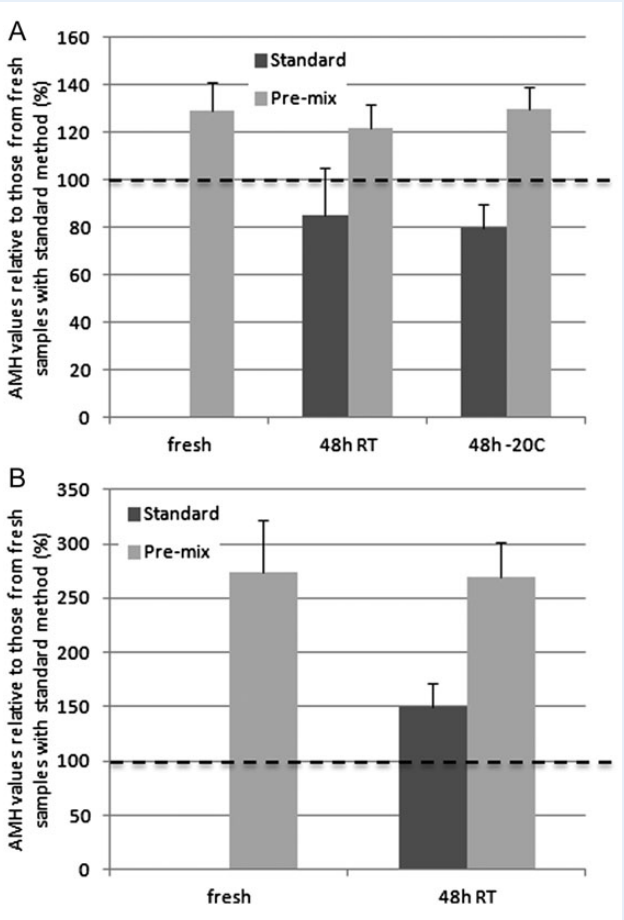

Figure 2 Serum AMH results for non-pregnant (**A**) ($n = 8$) and pregnant groups of women (**B**) ($n = 7$) using the standard and modified (i.e. pre-mixed with assay buffer) protocols for both fresh and stored samples. Results are expressed as a percentage ± SD relative to AMH measured in fresh samples using the standard method (dotted line).

(iv) AMH levels for both kit QCs did not change over a period of 30 weeks using the standard protocol (Fig. 3A) and the modified protocol (data not shown). AMH levels for the in-house pooled serum QC1 showed a steady increase over the same time period (Fig. 3B). However, when the modified protocol was introduced, the in-house pooled serum QC2 showed no change over a period of 20 weeks (Fig. 3C).

Reference range

As expected, results for the reference range using the modified method were higher than those for the standard method (Table II). A reference range was previously established in our laboratory from 483 healthy first trimester pregnant women using the standard method. Median values for all five age groups were higher when using the modified method but this increment was not consistent. The youngest age group (20–25 years) had only a slightly higher median (1.2-fold), while in the oldest age group (41–45 years), the new method median was 3.4-fold higher compared with that for the standard method (Table II).

Discussion

This is the largest entirely prospective study of the stability of AMH in serum when stored under various conditions and when measured using a modified 'pre-mixing' method. Our results support previous findings from a retrospective study (Rustamov *et al.* 2012), which indicated that AMH measurements tends to rise over time when samples are stored at room temperature. AMH also fluctuates significantly under storage at −20 and −80°C, although not at all time points. The changes in AMH were different for different subject groups even under the same storage conditions, with no obvious consistency within the different patient and volunteer groups. Our study included a series of samples from patients in early pregnancy. There have been several studies (La Marca *et al.*, 2005, Nelson *et al.*, 2010) which show no significant alteration in baseline AMH in early pregnancy when compared with the non-pregnant state. The consistency in AMH concentrations seen across the different patient groups that we studied supports inclusion of early pregnant samples in this stability study. Along with the findings of Rustamov *et al.* (2012) (and other stability studies that have been excluded from this paper for reasons of brevity), this study demonstrates the instability of the Beckman Gen II assay when performed according to the manufacturer's instructions, which was present until July 2013 (Fig. 1). Clinical laboratories that continue to use this methodology with stored samples may be reporting erroneous results.

However, pre-mixing samples with the assay buffer before assay produced a higher and stable measured AMH concentration which remained consistent over time, regardless of the method of storage.

One possible explanation for the variable AMH results observed for the same serum sample under different pre-analytical conditions is that sample storage may expose a second antibody-binding site (Rustamov *et al.*, 2012). The extent of such conformational changes occuring within the AMH molecule may differ between individuals. Regardless of what changes occur at a molecular level, pre-mixing of samples may allow such changes to become complete before assay, producing a homogenous AMH molecular structure and therefore more consistent and reliable results. Secondly, an unknown serum factor may be interfering with the assay. It is also possible that both these theories are applicable. The fact that data from this study and others showed the maximum increase in AMH values by pre-mixing is always 2-fold, supports the notion of a second antibody-binding site. However interference from another substance present in serum, may affect the degree of the exposure of this second antibody-binding site, so that a 2-fold increase in AMH values is not always observed unless the sample is pre-mixed with assay buffer prior to assay. We have also found that the length of for which whole blood is allowed to clot, time before serum is separated made a difference to AMH results and that pre-mixing of manufactured QC material (i.e. not serum) made no difference to the results (data not shown). Pre-mixing in-house pooled serum QC resulted in consistent and higher AMH values. All these findings lend support to the presence of an interfering factor in serum.

Recently, Beckman Coulter has introduced a modification of their Gen II assay procedure and it now includes a pre-mixing step. They claim that complement interferes with the assay which is eliminated by pre-mixing. It is therefore possible that our findings can be explained by interference from complement in the assay. Pre-mixing with the highly anionic buffer may inactivate the complement cascade and thereby prevent interference. Different subjects will exhibit different levels of complement activation which will explain our observation of inconsistencies between

Table I AMH results for women undergoing routine investigation at a fertility clinic, using both the standard and modified assay protocols.

Sample no.	AMH (pmol/l)				Ratio			
	A	B	C	D	B/A	D/C	C/A	D/B
1	34.8	64.9	34.7	63.2	1.86	1.82	1.00	0.97
2	18.6	28.5	14.9	27.3	1.53	1.83	0.80	0.96
3	25.1	36.1	20.2	32.6	1.44	1.61	0.80	0.90
4	18.6	26.0	16.8	26.5	1.40	1.58	0.90	1.02
5	25.2	34.0	22.6	33.0	1.35	1.46	0.90	0.97
6	14.8	26.2	13.9	24.1	1.77	1.73	0.94	0.92
7	12.1	21.0	12.8	20.1	1.74	1.57	1.06	0.96
8	18.1	34.2	17.3	31.5	1.89	1.82	0.96	0.92
9	2.5	4.7	1.6	4.0	1.88	2.50	0.64	0.85
10	20.8	35.1	21.9	34.5	1.69	1.58	1.05	0.98
11	11.4	17.3	7.3	16.6	1.52	2.27	0.64	0.96
12	13.3	19.1	8.5	16.4	1.44	1.93	0.64	0.86
13	4.2	6.8	3.5	7.3	1.62	2.09	0.83	1.07
14	37.8	61.5	27.9	59.4	1.63	2.13	0.74	0.97
15	3.5	6.8	2.6	6.3	1.94	2.42	0.74	0.93
16	24.5	46.6	32.9	52.3	1.90	1.59	1.34	1.12
17	1.3	3.4	2.1	3.7	2.62	1.76	1.62	1.09
18	1.2	3.3	1.9	3.7	2.75	1.95	1.58	1.12
19	8.4	15.1	7.6	14.2	1.80	1.87	0.90	0.94
20	42.7	96.4	48.3	92.3	2.26	1.91	1.13	0.96
21	25.5	40.6	25.1	39.3	1.59	1.57	0.98	0.97
22	5.6	12.2	4.2	10.6	2.18	2.52	0.75	0.87
23	4.4	8.5	4.1	7.4	1.93	1.80	0.93	0.87
24	2.4	5.0	2.2	4.7	2.08	2.14	0.92	0.94
25	5.3	9.9	4.2	10.2	1.87	2.43	0.79	1.03
26	15.5	25.0	14.1	22.1	1.61	1.57	0.91	0.88
27	9.3	19.9	7.3	17.4	2.14	2.38	0.78	0.87
28	7.5	16.6	6.1	15.0	2.21	2.46	0.81	0.90
29	1.5	3.5	1.0	3.3	2.33	3.30	0.67	0.94
30	9.7	16.0	7.9	15.5	1.65	1.96	0.81	0.97
31	12.7	22.8	11.1	20.7	1.80	1.86	0.87	0.91
32	17.6	31.7	14.2	31.2	1.80	2.20	0.81	0.98
33	11.2	21.4	8.9	24.1	1.91	2.71	0.79	1.13
34	3.4	7.5	3.0	7.2	2.21	2.40	0.88	0.96
35	21.2	46.0	22.7	52.4	2.17	2.31	1.07	1.14
36	14.2	32.6	10.7	32.7	2.30	3.06	0.75	1.00
37	2.4	5.4	2.4	5.8	2.25	2.42	1.00	1.07
38	8.7	17.8	7.7	16.9	2.05	2.19	0.89	0.95
39	4.5	12.1	5.1	10.9	2.69	2.14	1.13	0.90
40	7.5	17.0	10.9	17.3	2.27	1.59	1.45	1.02
41	3.6	11.0	7.8	10.0	3.06	1.28	2.17	0.91
42	32.1	53.4	28.5	46.1	1.66	1.62	0.89	0.86

Continued

Table I *Continued*

Sample no.	AMH (pmol/l)				Ratio			
	A	B	C	D	B/A	D/C	C/A	D/B
Mean					1.95	2.00	0.96	0.97
SD					0.39	0.44	0.30	0.08
% CV					20.00	23.00	31.00	8.00

A, fresh samples assayed with standard Gen II method; B, fresh samples assayed with modified (sample pre-mixed with assay buffer) Gen II method; C, samples stored at −20°C for 2 weeks and assayed with standard Gen II method; D, samples stored at −20°C for 2 weeks and assayed with modified Gen II method; CV, coefficient of variation.

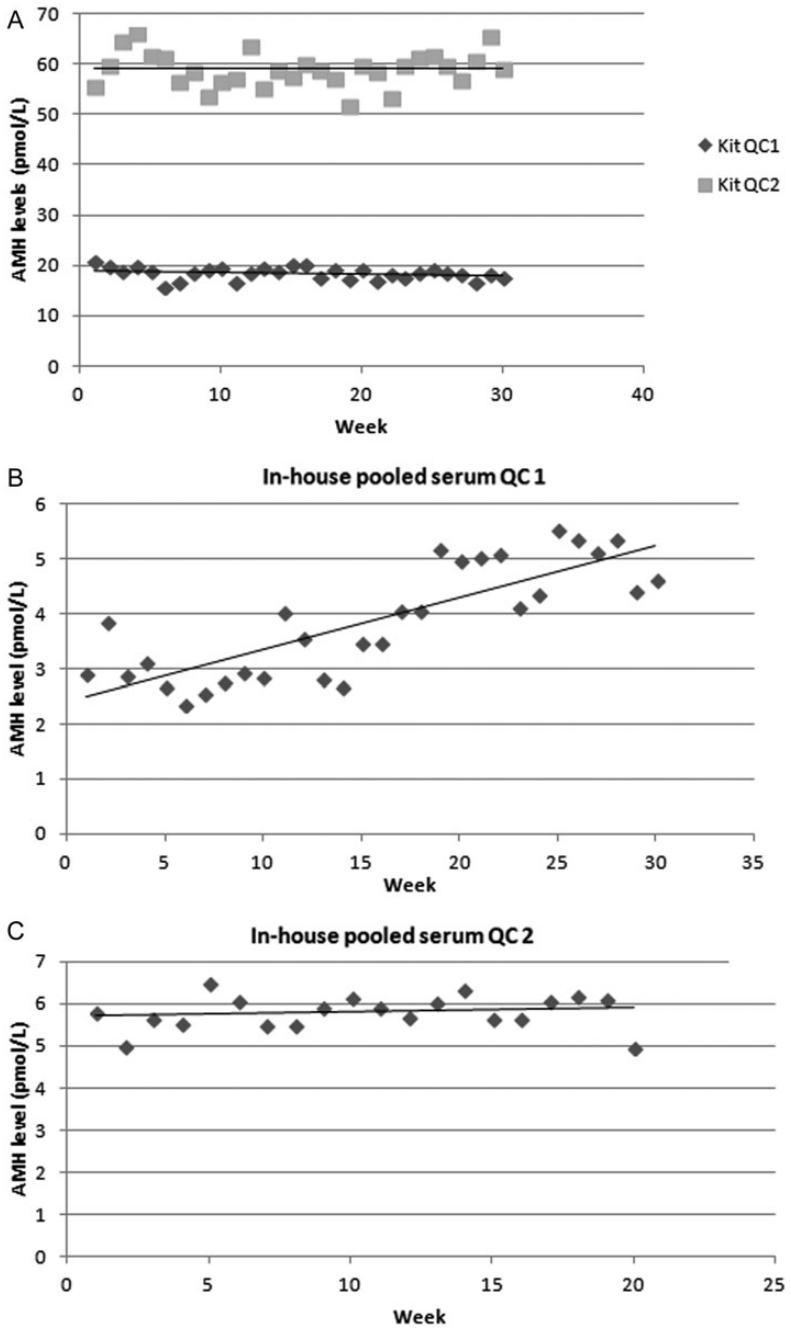

Figure 3 QC checks for the AMH assays. (**A**) AMH kit QCs over a period of 30 weeks using the standard protocol; (**B**) pooled serum QC1 over a period of 30 weeks using the standard protocol; (**C**) pooled serum QC2 over a period of 20 weeks using the modified (pre-mixing) protocol.

Table II Comparison of the reference range of serum AMH values (pmol/l) using standard and modified protocols.

Age (years)	Modified protocol				Standard protocol[a]			
	n	25th percentile	Median	75th percentile	*n*	25th percentile	Median	75th percentile
20–25	41	16.1	22.2	34.0	27	13.7	17.9	24.4
26–30	37	13.0	19.6	32.6	90	7.7	13.8	21.5
31–35	37	9.5	17.0	34.0	208	4.8	9.9	17.1
36–40	33	6.1	11.1	18.8	142	2.3	5.2	11.0
41–45	31	2.7	8.1	13.9	16	1.8	2.4	3.3

[a]These reference data were established previously in our laboratory from 483 healthy pregnant women in the first trimester.

different subjects in the magnitude of the effects of storage. We observed a particularly marked effect of storage on samples collected from healthy early pregnancies. Pregnancy is associated with increased activation of the complement system (Denny *et al.*, 2013), which may explain this finding. However, further work is necessary to determine the mechanism by which storage influences measurable AMH concentration by a mechanism which is nullified by pre-mixing with assay buffer.

Whilst pre-mixing should remove the problem of variation within samples during storage, it also requires a significant re-calculation of the normal ranges of serum AMH for women of different ages. This is essential since it appears that even in our relatively small subject group, the increment in median reference range values is not uniform for all ages. Scientific publications concerning AMH measurement should describe the assay methodology including whether pre-mixing has been used, and clinical laboratories that offer AMH measurement as part of the assessment of endocrinopathies, such as polycystic ovary syndrome or premature ovarian failure, or for management of ovulation induction as part of assisted reproduction, must re-establish their own reference ranges with a larger cohort of subjects.

We conclude that the inconsistency of AMH results seen with the Gen II assay may be attributed to an interplay of pre-analytical factors such as sample handling conditions and an unknown serum-based interfering factor such as complement. Exposure of a second antibody-binding site during storage may be further complicated by such interference and it is possible that this is subject to significant biological variation between individuals. Pre-mixing of serum with assay buffer prior to assay appears to eliminate these factors and therefore produces consistent AMH results, which is vital for clinical applications.

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None.

Authors' roles

X.H. was the laboratory lead responsible for study design, all laboratory-based experiments and for the routine analysis of clinical samples. X.H. was responsible for collation and analysis of all results including statistical analysis. X.H. was involved in preparation of the whole paper and submission of final manuscript. M.M. was involved with recruitment of volunteers, blood collection and sample processing. M.M. contributed to study design, data analysis, discussion, draft preparation and submission of final manuscript. R.S. was involved in most of the laboratory-based experiments. CW contributed to discussion and progression of drafts through to approval of final manuscript. W.L. oversaw the whole study, led on clinical aspects and contributed to data analysis, discussion and preparation of the whole paper.

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Conflict of interest

None declared.

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