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WHO International Collaborative Study of the Proposed WHO IS for Mullerian Inhibiting Substance/Anti-Mullerian Hormone, human, recombinant

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NOTE:
This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by **27 September 2019** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technologies, Standards and Norms (TSN). Comments may also be submitted electronically to the Responsible Officer: **Dr Ivana Knezevic** at email: knezevici@who.int.

Summary

The World Health Organization (WHO) Expert Committee on Biological Standardization (ECBS) has recognized (2015)(1) the need for an International Standard (IS) for Mullerian Inhibiting Substance/Anti-Mullerian Hormone (AMH), used for the calibration of immunoassays to measure AMH in human serum and plasma. We report here, the evaluation of a candidate standard for AMH, human, recombinant, coded 16/190, by immunoassay in an international collaborative study carried out by seven laboratories in four countries.

The geometric mean of all laboratory estimates from valid assays for the AMH content of the candidate standard was 511 ng/amp (95% CI: 426 - 612, n=16, GCV 42%) with a robust geometric mean of 489 ng/amp. It is noted that these estimates are based on individual method calibrations. Measurement of a coded duplicate confirmed low intra-assay variability for each method, with the ratio of estimates for coded duplicates ranging from 0.91 to 1.08 in 30/32 valid assay runs. Measurement of a comparator sample (ampoule B) with a lower AMH content suggested that recognition of AMH was consistent within a method. Estimates of the AMH content of ampoule B relative to the candidate IS ranged from 0.41 to 0.65 in individual assays with an overall study geometric mean of 0.53 (95%CI: 0.50 – 0.56; GCV% 12%; n=16). Measurement of thermally accelerated degradation samples has demonstrated that the candidate is sufficiently stable to serve as an IS.

The study included an assessment of the impact of the new standard on the routine measurement of AMH in patient samples. All laboratories contributed data during the collaborative study through the concomitant measurement of the AMH immunoreactivity of seventeen human serum and five human plasma samples. The commutability with patient samples of the candidate IS at six nominal AMH concentrations, 0.5 – 16 ng/ml, was assessed by a difference in bias approach. Of the 16 valid methods contributed to the study, the candidate IS was considered commutable with patient samples in 6 methods, partially commutable in 3 methods and not commutable with patient samples in 7 methods. It is noted that 16/190 was commutable with patient samples in two methods which are considered to be in worldwide use. Non-commutability could result from differences in the approaches used to assign values to method calibrators which may be native, recombinant or bovine AMH or from differences in epitope recognition between native and recombinant AMH by some antibody combinations.

As the candidate standard is not commutable with patient samples in a number of the current methods, we do not propose for this material to be established as the WHO IS for human, recombinant AMH. However, to assist with further characterization of AMH immunoassays, we

propose a recommendation to the ECBS committee of WHO that the preparation in ampoules coded, **16/190**, is made available as the **WHO Reference Reagent for Mullerian Inhibiting Substance/Anti-Mullerian Hormone (AMH), human, recombinant** with a consensus geometric mean content based on current immunoassays of **489 ng per ampoule**. There may be concerns regarding the use of immunoassays rather than SI-traceable physicochemical reference methods to assign a mass value to a WHO-endorsed reference material. Should these concerns be insurmountable, our proposal would be to make 16/190 available as a NIBSC reagent. In both cases, the use of immunoassays to assign a value in terms of current method calibrations would be clearly stated in the Instructions for Use.

Introduction

Mullerian Inhibiting Substance, also known as Anti-Mullerian Hormone (AMH), is a homodimeric glycoprotein, expressed by the Sertoli cells of the testes and the granulosa cells of the ovary (2). Measurement of AMH in serum or plasma is considered useful for assessing ovarian reserve, the likely response to ovarian stimulation, menopausal status and in paediatric medicine, Sertoli cell function.

The AMH molecule is comprised of two identical, glycosylated subunits which are linked by disulfide bonds. Each monomer undergoes cleavage such that N-terminal and C-terminal disulfide-bonded dimers are formed which remain non-covalently associated. There are reports that both cleaved and uncleaved forms can be isolated from serum (3). To our knowledge, the antibodies used in current immunoassays that are used clinically, recognize the total AMH molecule.

Current immunoassays report in mass units (ng/ml) or in molar units (pmol) using the conversion factor 1ng/ml = 7.14pM based on the apparent molecular weight of the glycosylated dimer by SDS-PAGE (4,5). The traceability of the calibration of current assays is unclear and a SI-traceable reference method is not available to accurately assign a mass value to the protein. As such, the aims of this international collaborative study are:

1. To determine the AMH content of 16/190 by different immunoassay methods in terms of the calibration of each method
2. To assess the suitability of 16/190 to calibrate immunoassays of AMH
3. To assess the stability of 16/190 after accelerated thermal degradation (ATD)

Participants

Seven laboratories in four countries took part in the study and are listed alphabetically, by country, in Table 1. Throughout the study, each method provided by a participating laboratory is referred to by a code number. The code numbers were randomly assigned and do not reflect the order of listing.

Table 1: List of participants in order of country

CHINA	Dr Yu Ting National Institutes for Food and Drug Control, Institute for Medical Devices Control, No. 2 Tiantan Xili, Dongcheng District, Beijing, 100050.
FRANCE	Dr Nathalie Ripoll, bioMerieux, Chemin de L'Orme, 69280 Marcy L'Etoile.
GERMANY	Dr Verena Hofmann, Roche Diagnostics GmbH, Nonnenwald 2, D-82377 Penzberg.
USA	Dr Patrick Sluss Ansh Labs, 455 Medical Centre Blvd, Webster, TX 77598
USA	Ryan Masica Beckman Coulter Inc., 1000 Lake Hazeltine Drive, Chaska, MN 55318-1084
USA	Drs Hubert Vesper, Uliana Danilenko and Candice Ulmer Centre for Disease Control, Chamblee Campus Warehouse, 3719N Peachtree Road, Chamblee, GA 30341-2251.
USA	Dr Jelena Bogdanovic, Siemens Healthcare Diagnostics, 511 Benedict Avenue, Tarrytown, NY 10591

Bulk material and processing

Recombinant AMH was donated to NIBSC by Professor Patricia Donahoe (Director) and Dr David Pépin of the Pediatric Surgical Research Laboratories, Massachusetts General Hospital, USA. The donated recombinant AMH was purified from the culture media of a stable CHO cell line, LR-MIS, expressing the native human AMH sequence with the leader sequence from human serum albumin (HSA) and with a modification of the internal cleavage site at amino acids 423-428 from RAQR/S to RARR/S (Pépin, D *et al*., (2013)). The HSA leader sequence is cleaved during maturation. At NIBSC on August 12th, 2016, a 2100 ml volume of bulk formulation containing 0.24% (w/v) casein (Calbiochem), 0.5% (w/v) trehalose (Sigma) and nominally, 1 µg/ml AMH was distributed into 3 mL siliconized DIN ampoules as 0.5 ml aliquots. The ampoule contents were freeze-dried, secondarily desiccated and sealed under nitrogen according to WHO guidelines for the preparation of a reference material. The batch, coded 16/190, was stored at -20°C at NIBSC who will be custodians of the material.

Characterization of the freeze dried product

A total of 3814 ampoules, coded 16/190 were produced. Check-weights measured during filling demonstrated a mean fill weight of 0.4999 g (CV 0.58 %, n=12). The mean residual moisture content as determined by coulometric Karl Fischer titration was 1.810 % (CV 46.45 %, n=12), mean headspace oxygen was 0.21% (CV 52.37 %, n=12) and the mean dry weight was 0.0030 g (CV 5.78 %, n=5).

Collaborative study for the evaluation of 16/190

The collaborative study was organised by NIBSC. All participants were provided with ampoules of the candidate IS, 16/190 and the comparator sample, coded B. Thermally-accelerated degradation samples were available in limited numbers and were distributed to participants based on assay capacity and sample availability. A study protocol, shown in Appendix 2, and instructions for use were provided with the samples.

Participants were asked to measure the AMH content of the materials using the immunoassay(s) normally in use in their laboratory and, where possible, to perform at least two independent assays, using fresh ampoules, each assay to include all the preparations allocated, measured at no less than five of the specified dilutions in the linear part of their dose-response curve. In instances where there was not a fresh ampoule for subsequent assays, it was suggested that fresh dilutions be made from frozen stock solutions. Where dilutions of a stored stock solution were used, participants were asked to provide details of the freezing and thawing procedures used. Participants were asked to provide details of the assay method(s) used, the diluent and dilution steps, together with all raw assay data for central computation at NIBSC. Participants' own estimates of immunoreactivity as calculated by the method normally used in their laboratory were also requested**.** The ampoules provided for this study, which may be identified only by code letter, are listed in Table 2.

In addition, participants were provided with a panel of seventeen human serum samples coded AMHSerum1 to AMHSerum17 and five human plasma samples coded AMHPlasma18 to AMHPlasma22 with estimated AMH concentrations of <0.1 to >12 ng/mL. The assay methods contributed to the study are shown listed in alphabetical order in Table 3.

Table 2: Preparations provided to participants in the collaborative study.

Code	Preparation	Content
A, C	Candidate IS for AMH, 16/190	Nominally, 0.5 µg per ampoule
B	Comparator sample	Approximately half the content of the candidate IS
D, E, F, G, H	Accelerated thermal degradation (ATD) samples of 16/190 stored at +4°C, +45°C, +37°C and +20°C and -20°C (respectively) for 21m 13d	Content assumed to be identical to 16/190 stored at -20ºC

Table 3: AMH Assays contributed to the collaborative study in alphabetical order.

Access2 AMH immunoassay	Beckman-Coulter (performed by two laboratories)
Advia Centaur XP AMH immunoassay	Siemens
Architect i2000sr	Tellgen Corporation
Auto Lumo A2000 plus AMH immunoassay	Autobio Diagnostics Co Ltd
Caris200 AMH immunoassay	Guangzhou Darui Biotechnology Co. Ltd.
CI1000 AMH immunoassay	Beijing Leadman Biochemistry Co,.Ltd.

CIA200 AMH immunoassay	Taizhou Ze Cheng Biotechnology Co
CL2000i AMH immunoassay	Shenzhen Mindray BioMedical Electronics Co Ltd.
Cobas e411 AMH immunoassay	Roche (performed by two laboratories)
Cobas e801 AMH immunoassay	Roche
Dxl AMH immunoassay	Beckman Coulter
Gen II AMH ELISA	Beckman Coulter
iFlash3000	Shenzhen YHLO Biotech Co. Ltd.
Maglumi 4000 plus AMH immunoassay	SNIBE Co. Ltd.
MenoCheck picoAMH ELISA	Ansh Labs
Unicell-S AMH immunochromatography assay	Shenzhen YHLO Biotech Co. Ltd.
Union-CO718 AMH ELISA	Shenzhen YHLO Biotech Co. Ltd.
Vidas 30 AMH fluorescence immunoassay	bioMerieux
Vidas PC AMH fluorescence immunoassay	bioMerieux

Statistical Analysis

Assessment of the immunoreactivity of 16/190
Analysis was performed with AMH concentrations as reported by the participants, using results from the nominal concentration range of 0.125 - 16 ng/ml only. To determine if a method showed acceptable dilutional linearity, linear regression analysis was applied to each sample in each assay run to estimate the slope of $\log_{10}$ reported concentration against $\log_{10}$ nominal concentration. The r^2 value was confirmed to exceed 0.975 in all cases. Methods were considered invalid if the geometric mean slope for any of samples A, B or C was outside the range [0.91, 1.10]. In addition, an individual assay run was only concluded to be acceptable if the ratio of the coded duplicates [A:C] was between 0.91 and 1.10.

Results from all valid methods were corrected for dilution factor and combined to generate unweighted geometric mean (GM) estimates for each laboratory and these laboratory means were used to calculate overall unweighted geometric mean estimates. Variability between laboratories has been expressed using geometric coefficients of variation (GCV = $\{10^s-1\}\times100\%$ where s is the standard deviation of the $\log_{10}$ transformed estimates). Due to possible outliers and anomalous results, Huber's robust geometric mean was also calculated using the R package 'WRS2' (7).

Assessment of commutability
Data used for analysis. AMHPlasma18 and AMHPlasma22 were excluded from the analysis as the AMH content was below or above, respectively, the range of some assays (n=9 for AMHPlasma18, n=7 for AMHPlasma22). All reported results were $\log_{10}$ transformed for analysis in order to achieve approximately constant scatter over the range of concentrations used. A consensus value for each sample, shown in Appendix Table 6, was calculated as Huber's robust mean of laboratory means using the R package 'WRS2' (7). Bias values were then calculated for all reported results as the difference between the reported value and the study consensus value for that sample.

Determination of commutability criteria. The standard deviation of the bias values for patient samples only was calculated within each laboratory and a pooled value, s_P, was calculated across all laboratories. Commutability criteria representing the maximum acceptable difference in bias were then set as $\pm 2s_P$. Reference standards were to be concluded as commutable if the observed bias was within the commutability criteria. For this commutability assessment, the bias for patient samples has been assumed to be constant over the concentration range used.

Assessment of stability
The relative immunoreactivities of the accelerated thermal degradation samples were used to fit an Arrhenius equation relating degradation rate to absolute temperature assuming first-order decay (8), and hence predict the degradation rates when stored at a range of temperatures.

Results

Assay validity
In total, participants in the collaborative study performed 42 runs of the 19 method/platform combinations listed in Table 3. Two method/platform combinations were performed by two independent laboratories as indicated in Table 3, giving a total number of methods of 21. In addition, it is noted that (i) for method 2, all dilutions of ampoules A-C underwent a further 1/15 pre-analytical dilution as part of the validated assay protocol and (ii) for method 21, all dilutions of ampoules A-C underwent a single freeze-thaw cycle prior to measurement.

The following exclusions were made as the slopes of the fitted regression lines (Appendix Table 7) did not meet the validity criteria described above: both runs of method 8, 10, 11, 17 and 18. In addition, the following exclusions were made as the ratio of the coded duplicates, A:C (Appendix Table 8), was outside of the range [0.91, 1.10]; method 15, run 2 and 19, run 2.

Estimates of total AMH content of 16/190
From each of the 16 methods which met the validity criteria, a geometric mean laboratory estimate of the AMH content of 16/190 ampoule A and the coded duplicate, ampoule C, was determined (Table 4 and Figure 1). Estimates from individual runs are shown in Appendix Table 8. As summarized in Table 4, estimates ranged from 282 ng/amp to 1157 ng/amp with a geometric mean estimate of 511 ng/amp (95% CI: 426-612, n=16, GCV 42%) and a robust geometric mean of 489 ng/amp. The median estimate was 485 ng/amp with an interquartile range of 462 – 533 ng/amp.

Estimates of the AMH content of the comparator sample, coded B
Laboratory geometric mean estimates for the AMH content of the comparator sample, B, were calculated from the 16 laboratory methods which met the validity criteria (Table 4, Figure 2). Estimates from individual runs are shown in Appendix Table 9. The geometric mean relative potency for the comparator sample, B, relative to 16/190 was determined as 0.53 (95% CI: 0.50 - 0.56, GCV 12%, n=16) (Figure 3).

Assessment of the AMH content of patient samples and reference standard commutability
All participants measured the AMH immunoreactivity of 22 patient samples which comprised female (coded AMHSerum 6-16 and AMHPlasma 18-20), male (coded AMHSerum 4, 5, 17 and

AMHPlasma 21 and 22) and diluted male paediatric samples (coded AMHSerum 1-3). Twenty samples were included in the assessment of commutability. AMHPlasma18 and AMHPlasma22 were excluded as described above. Two laboratory methods, 1 and 13, required dilution of three of the patient samples, method 2 performed a 1/20 pre-analytical dilution as part of their validated protocol and method 6 omitted two serum samples (AMHSerum3 and AMHSerum4) due to assay capacity.

The commutability of 16/190 with patient samples was assessed in the 16 methods in which the regression slope validity criteria were met. Of these, for methods 2 and 3, participants measured different dilutions to those stated in the study protocol and laboratory method 12 used gravimetrically prepared dilutions. To enable these to be included in the analysis of commutability, interpolated values were used.

The limits for acceptable bias difference were determined as described above, giving ±0.051, or 0.892 to 1.121 on the untransformed scale, i.e. the bias for a reference standard must be demonstrated to be not less than 89.2% and not more than 112.1% of the bias observed for patient samples. The limits were applied to the mean patient sample bias of each method as shown in Figure 4. For each method, Figure 4 shows individual data points for the bias from the consensus values for each patient sample and each reference material dilution between 0.5 and 16 ng/ml. The bias values for each reference material dilution are also shown in Table 5a. Values in red indicate where a bias value is outside the limits for acceptable bias which for each method is shown in Table 5b. Table 5a also shows the slopes of $\log_{10}$ reported concentration against $\log_{10}$ consensus concentration which are expected to have a value of 1 (i.e. constant bias) for this difference in bias approach to be appropriate.

The candidate standard was commutable with patient samples in 6 of the 16 methods. These were laboratory methods 2, 4, 7, 14, 16 and 19. Of these, laboratory method 14 was the same assay method as laboratory method 7 but on a different platform. Laboratory method 2 reported data for (nominal) AMH concentrations from 3 – 12 ng/ml so values for the concentrations defined in the study protocol (0.5 – 8 ng/ml) were interpolated.

The candidate standard was not commutable with patient samples in 7 of the 16 methods. These were laboratory methods 1, 3, 5, 6, 9, 12 and 13. Of these, laboratory methods 1 and 13 were the same method on different platforms. Laboratory methods 5 and 4 (in which 16/190 was shown to be commutable with patient samples at all but the highest nominal dilution) were the same method on different platforms. Despite the bias for the reference material dilutions being similar in the two methods, in method 5, the average serum bias was more negative which resulted in the bias for the reference material dilutions being outside the upper limit of acceptable bias (Figure 4). As shown by the slope values in Table 5a, the bias values for laboratory method 13 are non-constant and it may be that this approach is not applicable for this method. An increasingly negative trend in bias was observed for the higher concentrations of 16/190 when measured by method 9.

For 3 of the 16 methods, 16/190 was not commutable with patient samples at two or three of the reference material dilutions. These were methods 15, 20 and 21. Laboratory method 15 is the same method as method 4 in which 16/190 was commutable with patient samples at nominal

AMH concentrations of 0.5 – 8 ng/ml. When measured by method 15, 16/190 was commutable at concentrations of 2 – 16 ng/ml. It is possible that 16/190 is also commutable with patient samples by method 15 and the observed results are due to experimental variation.

By method 20, the candidate standard was commutable with patient samples at nominal concentrations of 2 – 8 ng/ml but as shown in Table 5a and Figure 3, the bias for the patient samples may not be constant.

Method 21 is the same assay and platform as method 14 and the same method on a different platform as method 7. The candidate standard is commutable with patient samples when measured using methods 7 and 14. The candidate standard was not commutable with patient samples at nominal concentrations of 0.5 – 2 ng/ml when measured by method 21. However, it is noted that the dilutions were prepared, stored frozen and thawed before measuring which may have affected recovery at the lower concentrations causing the negative bias observed at these concentrations.

For all methods, it is important to note that the commutability criteria have been derived statistically, rather than based on clinical relevance, using only data obtained from the participating laboratories in this study. Thus, they may be too strict (or not strict enough) to fully define acceptable commutability.

Stability of 16/190

Estimates of the mean relative immunoreactivity of ampoules stored at elevated temperatures for a period of 21 months, 13 d and measured by Laboratories 1, 3, 4, 5, and 7 are summarized in Table 10. Analysis showed no significant change in relative immunoreactivity of 16/190 stored at elevated temperatures suggesting that 16/190 is likely to be highly stable when stored at -20˚C.

Discussion

Immunoassays to determine the AMH concentration in mass units (ng/ml or pmol) of patient samples have been available for clinical use since the early 2000s. Traceability of the mass unit of AMH is not clear but it is likely to have been first assigned by protein assay to the recombinant AMH calibrators of an ELISA developed by Immunotech-Coulter (IOT) (9) and transferred to more recent manual and automated assays by the process of serum value transfer. For this, the AMH concentration of a range of serum samples is determined using an established method. The values obtained are then assigned to the mean signal counts of the new method to assign values to the new method reference calibrators. Therefore, the assigned values will be dependent on the established method chosen and the relationship between the signal and the concentration of the calibrators for each method. A second, independent approach to assigning a mass value to AMH was reported by Savjani and Kumar (2014)(10) and Visser (2013)(11) who determined the concentration of a purified preparation of the AMH C-terminal dimer by optical density at 280nm and then used this preparation to value assign recombinant, human AMH calibrators. A physico-chemical reference method to assign SI-traceable units to AMH is not available although some progress has been made towards quantification by tryptic digest, isotope

dilution mass spectrometry (12). For these reasons, the aims of this collaborative study are limited to: (i) providing a consensus mean estimate of the AMH content as determined by current immunoassay methods in terms of their individual calibrations and (ii) to assess the suitability and stability of the candidate standard.

Our study to evaluate the candidate standard, 16/190, by 21 methods (19 different methods) showed that 16 methods exhibited an acceptable dose response for 16/190 and that, by these methods, estimates of the AMH content ranged from 282 - 1157 ng/amp with a geometric mean estimate of 511 ng/amp (95% CI: 426-612, n=16, GCV 42%) and a robust mean of 489 ng/amp. The variability in the recovery of the recombinant, human AMH in the candidate standard was demonstrated by the GCV of the mean of estimates (42%). For the valid assays, intra-method variability, as assessed by the ratio of the content estimates for coded duplicate samples A and C suggested that this is not contributing to the observed variability. Similarly, the all-method geometric mean of the relative AMH content of the comparator B to 16/190 varied with a much lower GCV of 12%, also suggesting that the proportionality of the response to the recombinant AMH is a not a major contributing factor. Thus, other possible explanations for the observed variability of the content estimates are differences in epitope recognition and binding rates of the recombinant AMH molecule by the antibody species at the concentrations and incubation times used as well as differences in the epitope recognition and the values assigned to the method calibrators, which may be of native, recombinant or bovine origin.

As may be expected from the use of serum value transfer to calibrate new methods, the bias observed for each patient sample from a consensus all-method value was for some methods, considerably different to the bias observed for each dilution of the 16/190. Thus, when assessed using the difference in bias approach, although 16/190 was commutable with patient samples in methods 2, 4, 7, 14, 16 and 19, it was outside the acceptable bias difference when measured by methods 1, 3, 5, 6, 9, 12, and 13. For three methods (methods 15, 20 21), 16/190 was not commutable at two or three of the nominal AMH concentrations. The methods in our study are anonymised, but it is likely that some of the methods use the same antibodies (antibodies F2B/7A and F2B/12H (13)) and it is interesting that 16/190 is commutable in some but not all of these suggesting the involvement of calibration rather than epitope recognition.

Despite 16/190 being demonstrated as being commutable in two methods that are in widespread global use, across all the methods submitted to the study, the commutability of 16/190 is unlikely to be acceptable for the establishment of 16/190 as a WHO IS. As there may be value in providing a recombinant, human AMH reference material to further investigate the calibration and performance of methods, our recommendation is that 16/190 is proposed to WHO for establishment as a WHO Reference Reagent with a consensus AMH content estimate of 489 ng/amp. Of the seven laboratories who participated in the study, six were in agreement with the proposal. One laboratory suggested that 16/190 is also not suitable for a WHO Reference Reagent without basing the assigned units on a SI traceable physicochemical method. We acknowledge that WHO guidelines recommend this approach. However, until a physicochemical reference method is available, we propose that the consensus immunoassay estimate should be used with clear notification in the Instructions for Use. Should this approach not be acceptable to WHO, 16/190 can be made available as a NIBSC reagent.

Proposal

It is recommended that the preparation in ampoules coded 16/190 is provided as a **WHO Reference Reagent for Mullerian Inhibiting Substance/Anti-Mullerian Hormone (AMH), human, recombinant** with an estimated, consensus geometric mean content by immunoassay of **489 ng per ampoule**.

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Table 4. Laboratory estimates of the AMH content in ng/amp of ampoules A, B and C, the geometric mean of A,C and the ratio of B to [A,C] as measured by the 16 methods that met the validity criteria.

Lab	Sample A	Sample C	GM(A,C)	Sample B	Ratio of B to GM(A,C)
1	337	335	336	150	0.45
2	623	624	624	283	0.45
3	1094	1074	1084	548	0.51
4	523	542	533	293	0.55
5	520	537	528	284	0.54
6	1182	1133	1157	554	0.48
7	484	456	470	252	0.54
8					
9	546	553	549	298	0.54
10					
11					
12	499	506	502	324	0.65
13	277	288	282	141	0.50
14	481	470	475	253	0.53
15	453	471	462	268	0.58
16	461	467	464	203	0.44
17					
18					
19	379	381	380	210	0.55

20	472	498	485	312	0.64
21	410	432	421	228	0.54
Geometric Mean	508	513	511	269	0.53
95% Confidence Limits	417 – 619	430 – 612	426 – 612	221 – 328	0.50 – 0.56
GCV	45%	41%	42%	45%	12%
Robust Geometric Mean	487	494	489	265	0.53

Table 5: (a) Mean bias values obtained for 16/190. Values in red indicate where the bias for the dilution of 16/190 is outside the acceptable bias difference from the mean value observed for patient samples. (b) The mean serum bias for patient samples for each method and the upper and lower limts defining acceptable bias difference.

(a)

Nominal [AMH] (ng/ml)	Laboratory															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**9**	**12**	**13**	**14**	**15**	**16**	**19**	**20**	**21**
0.5	-0.151	0.156	0.332	0.035	0.039	0.343	0.003	0.071	0.015	-0.256	-0.018	-0.029	-0.009	-0.114	-0.007	-0.100
1	-0.157	0.146	0.338	0.029	0.032	0.370	-0.026	0.114	0.012	-0.246	-0.032	-0.032	-0.002	-0.109	-0.011	-0.105
2	-0.154	0.143	0.350	0.029	0.037	0.378	-0.038	0.074	0.014	-0.223	-0.029	-0.017	0.004	-0.113	-0.006	-0.067
4	-0.151	0.136	0.355	0.029	0.025	0.398	-0.028	0.059	0.010	-0.209	-0.029	-0.014	0.000	-0.109	0.003	-0.054
8	-0.130	0.130	0.360	0.032	0.024	0.441	-0.038	0.042	0.005	-0.184	-0.022	-0.004	-0.001	-0.102	-0.007	-0.030
16			0.356	0.044	0.040		-0.048	0.030	-0.009		-0.019	0.005	-0.004	-0.116	-0.016	-0.023
Mean	-0.149	0.143	0.348	0.033	0.033	0.386	-0.029	0.065	0.008	-0.223	-0.025	-0.015	-0.002	-0.111	-0.008	-0.063
16/190 Slope	1.02	0.98	1.02	1.01	1.00	1.08	0.97	0.96	0.99	1.06	1.00	1.02	1.00	1.00	1.00	1.06
Patient Samples Slope	1.04	1.08	0.98	1.04	1.06	1.07	0.98	0.95	0.97	1.06	0.97	1.04	0.93	1.03	0.93	0.97

(b)

Laboratory	Mean Patient Sample Bias	Lower Limit	Upper Limit
1	-0.029	-0.078	0.021
2	0.137	0.088	0.187
3	0.045	-0.005	0.094
4	-0.014	-0.063	0.036
5	-0.044	-0.094	0.005
6	0.109	0.060	0.159
7	-0.025	-0.074	0.025
9	0.121	0.072	0.171
12	-0.064	-0.114	-0.015
13	-0.041	-0.090	0.009
14	-0.009	-0.059	0.040
15	0.023	-0.027	0.072
16	0.007	-0.042	0.057
19	-0.078	-0.127	-0.028
20	0.042	-0.008	0.091
21	-0.017	-0.066	0.033

Figure 1. Laboratory geometric mean estimates of the AMH content of 16/190 ampoules A (candidate) and C (coded duplicate)

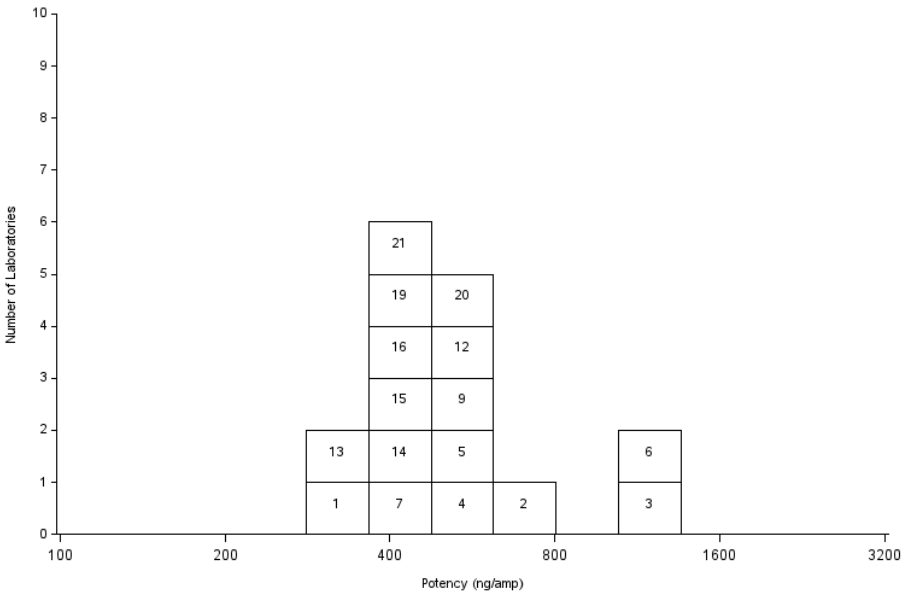

Figure 2. Laboratory geometric mean estimates of the AMH content of 16/190 ampoule B (comparator)

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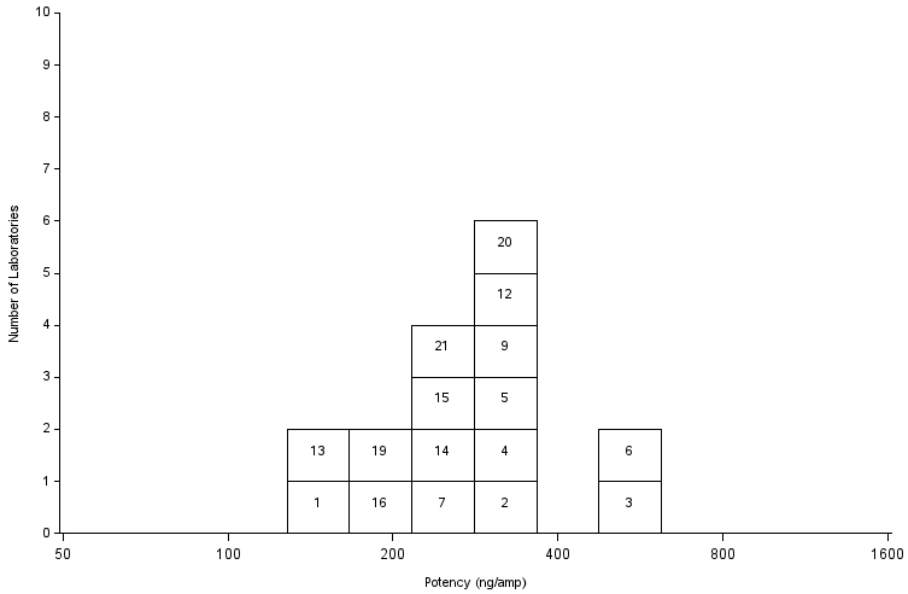
Number of Laboratories
Potency (ng/amp)
0
1
2
3
4
5
6
7
8
9
10
50
100
200
400
800
1600
1
16
7
2
3
13
19
14
4
6
21
9
15
5
20
12

Figure 3. Laboratory geometric mean estimates of the AMH content of 16/190 ampoule B (comparator) relative to geometric mean of samples A and C

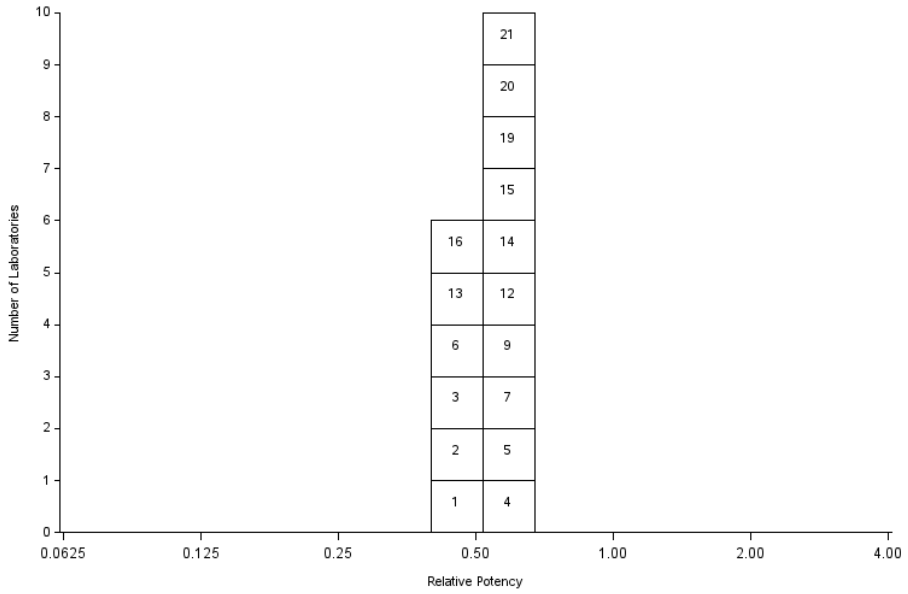

Figure 4. For each valid method, comparison of the laboratory average bias (·····) for the patient samples (●) with the bias observed for each dilution of 16/190 ampoule A (●) (candidate) and ampoule C (●) (coded duplicate) at dilutions of 0.5 – 16 ng/ml. The upper and lower limits of commutability (·····) are +/- $2s_P$ where s_P is the pooled standard deviation of the laboratory bias for the patient samples for each method.

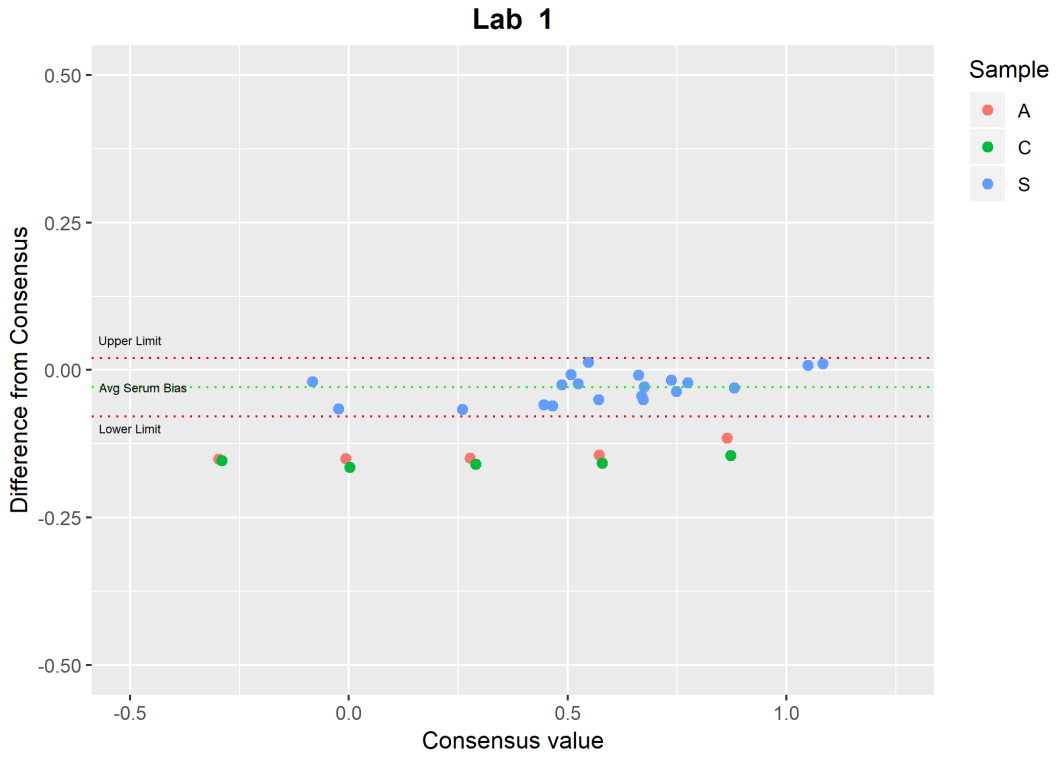

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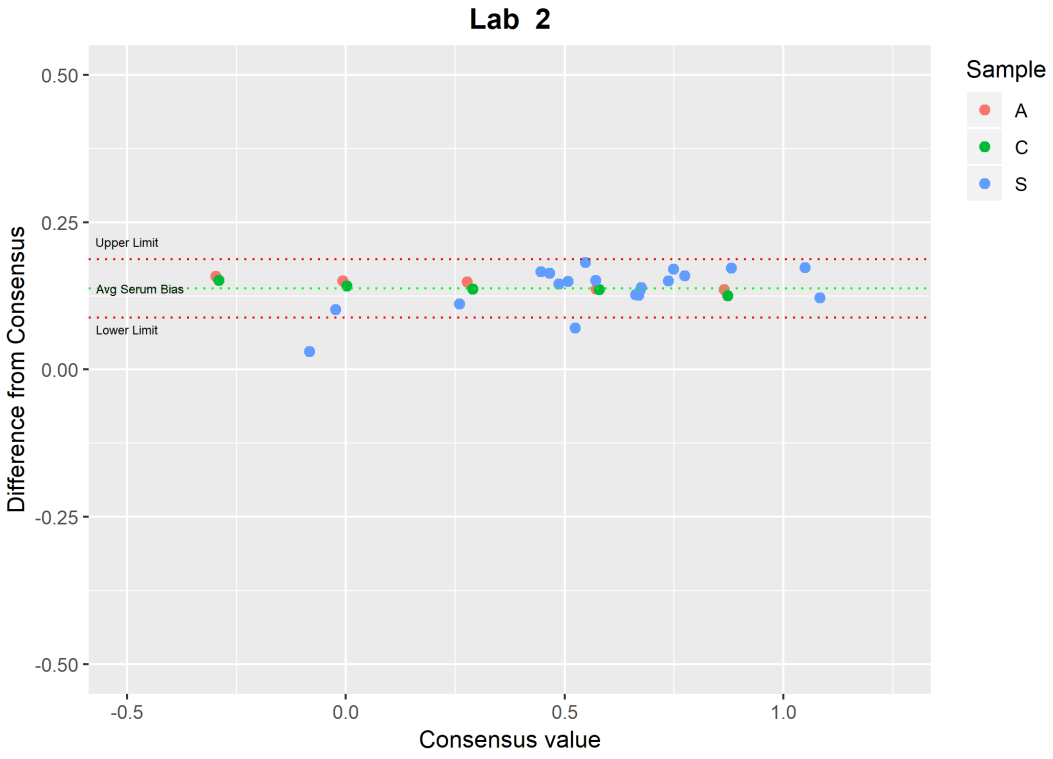
Figure 4 continued.



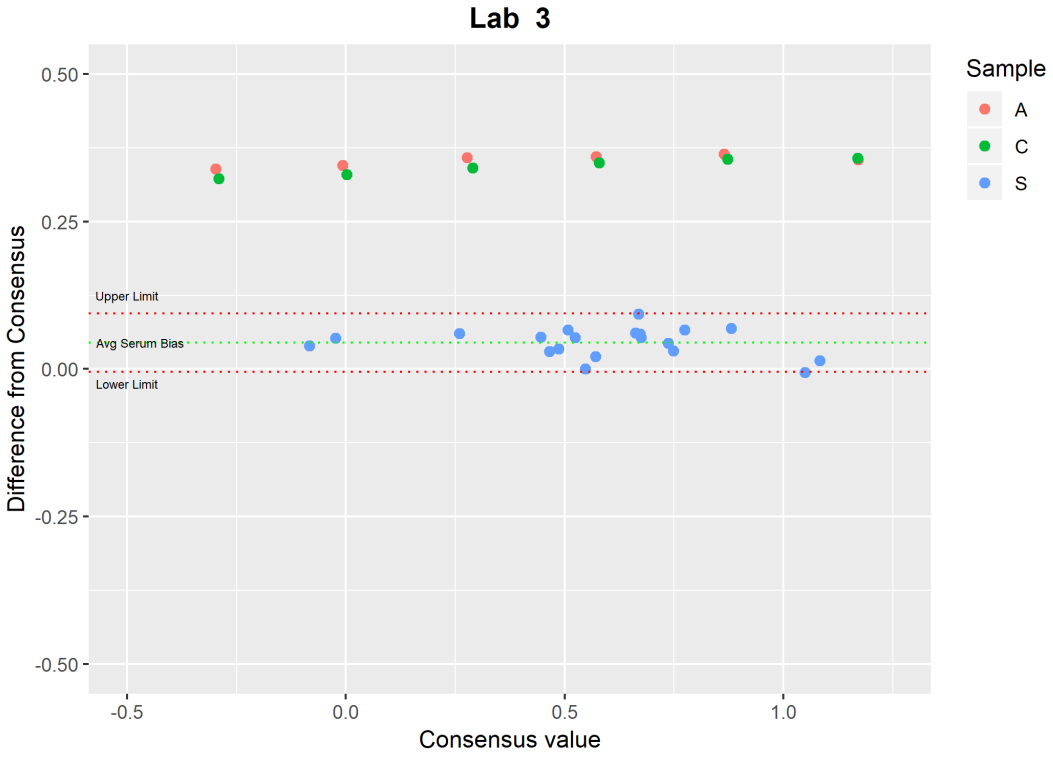


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Figure 4 continued.

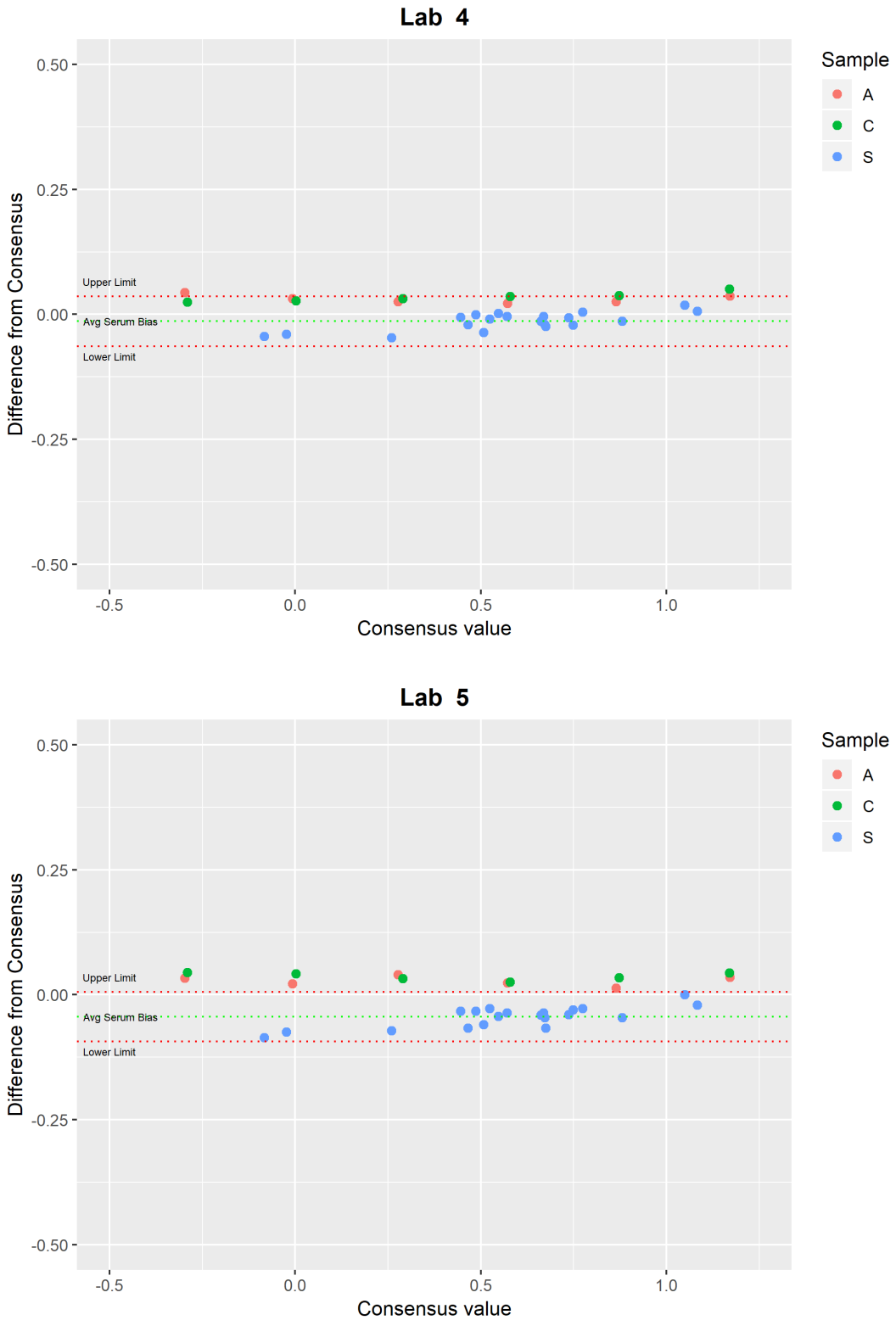

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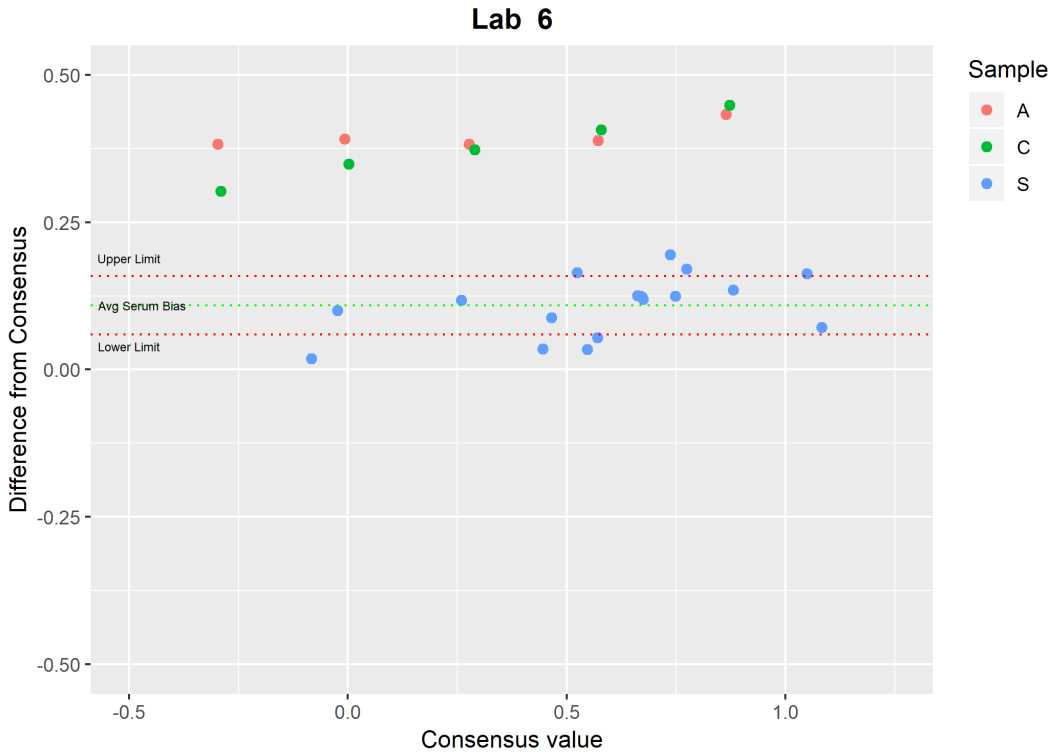
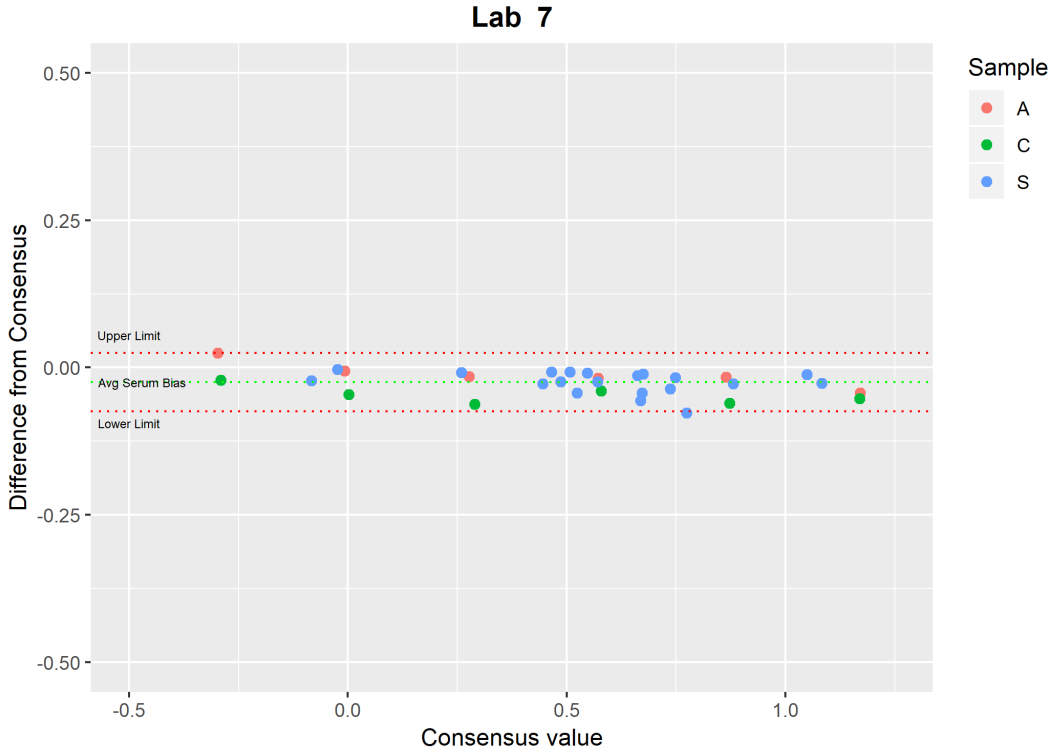
Figure 4 continued.





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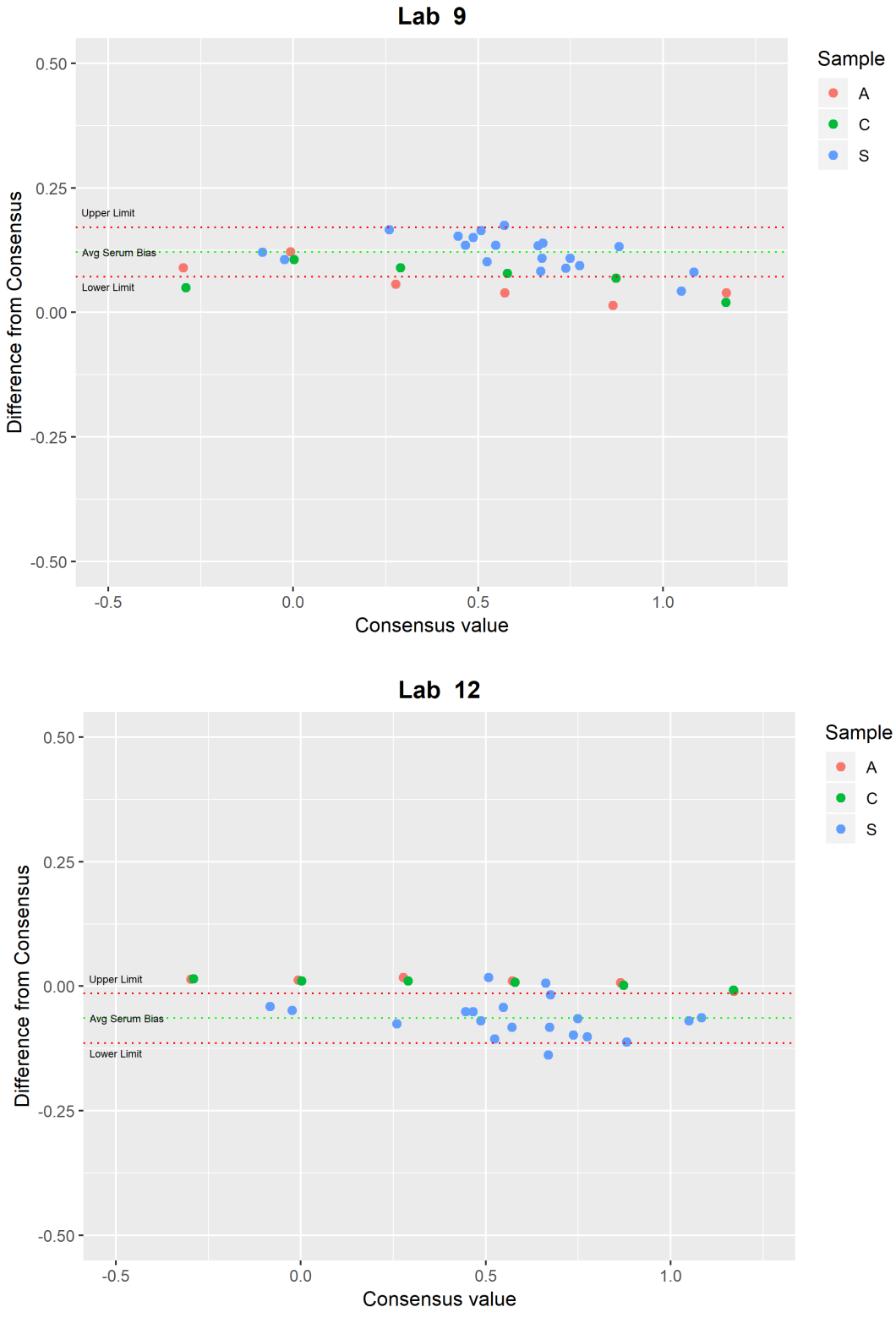

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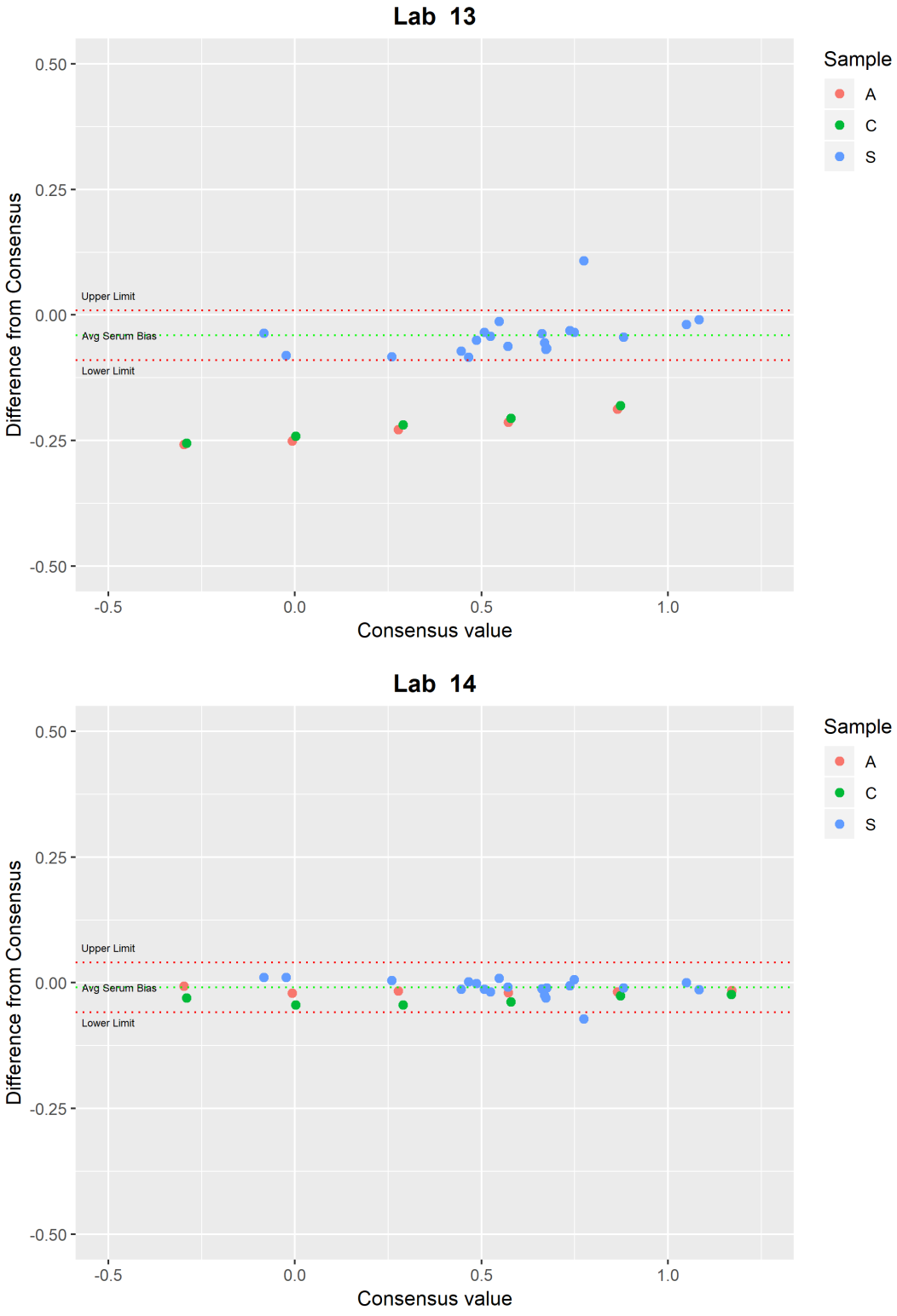

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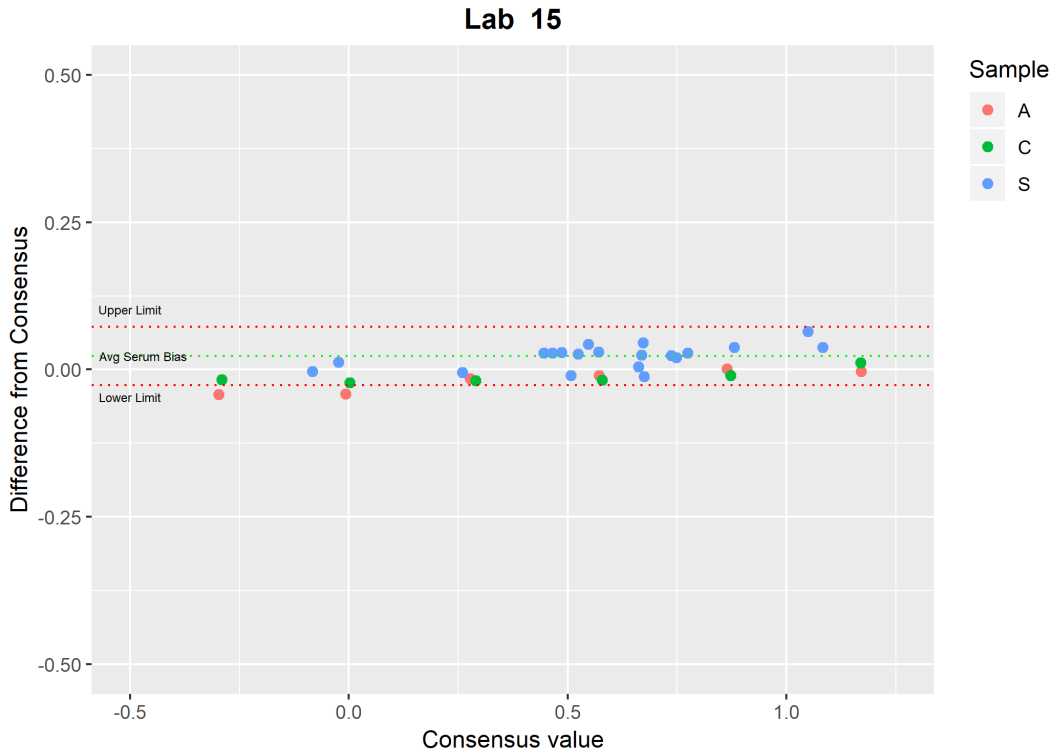
Figure 4 continued.



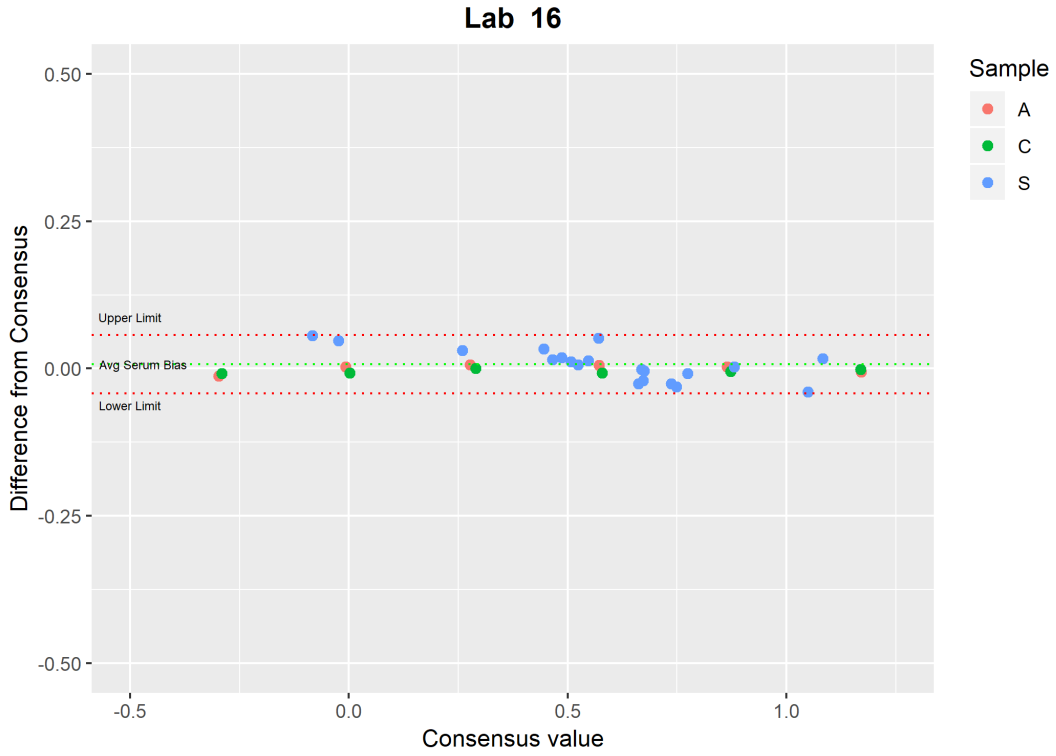


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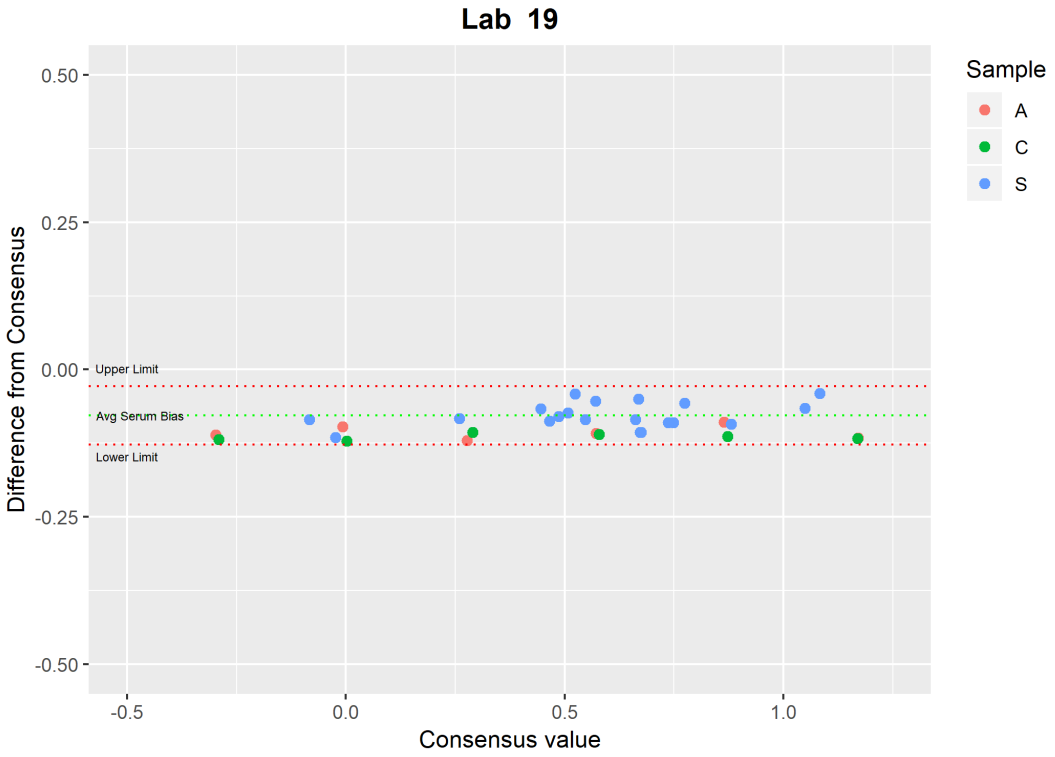
Figure 4 continued.



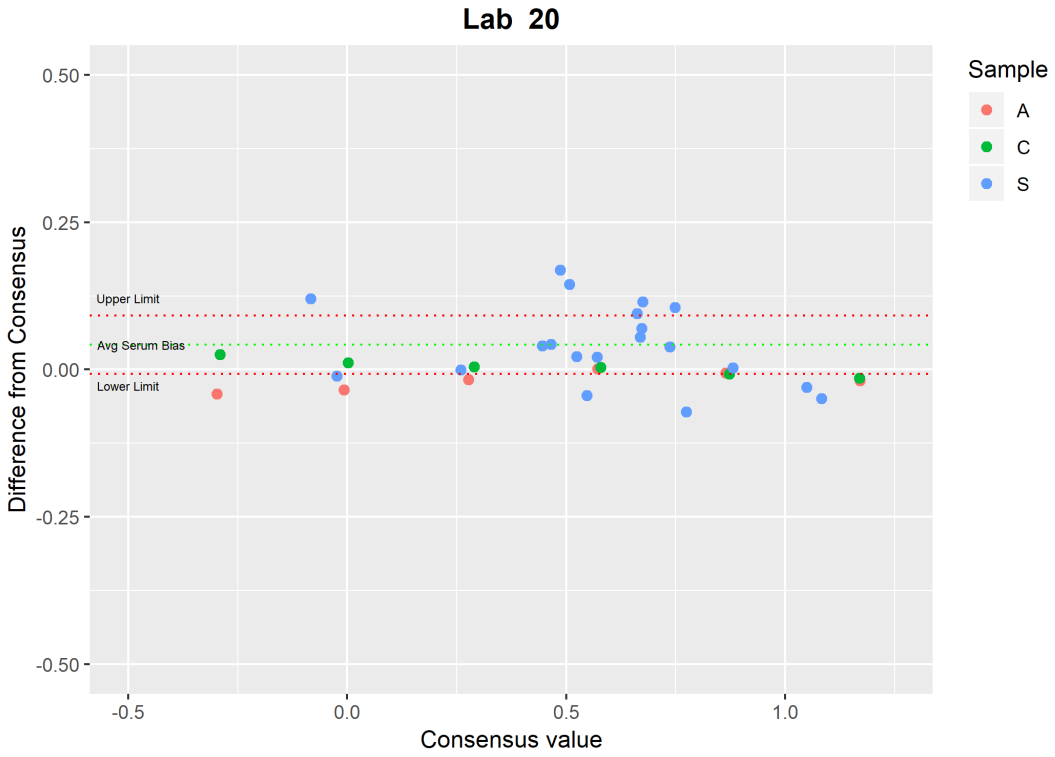


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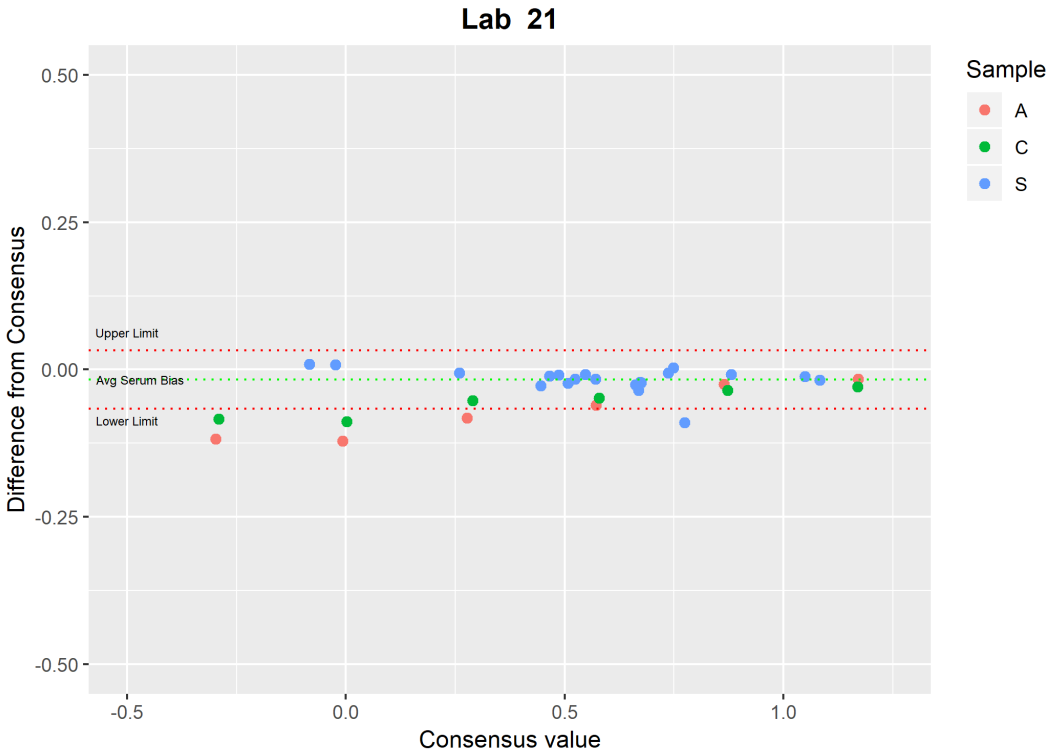
Figure 4 continued.



Appendix 1

Table 6. Log consensus values obtained for all samples as used for the assessment of the commutability of 16/190 by difference in bias. AMHPlasma18 and AMHPlasma22 were excluded from the analysis.

Patient Sample	**Robust mean** (log_{10} ng/ml)	**Robust geometric mean** (ng/ml)	**16/190 Nominal dilution** (ng/ml)	**Robust mean** (log_{10} ng/ml)	**Robust geometric mean** (ng/ml)
AMHSerum1	0.66	4.59	0.5	-0.29	0.51
AMHSerum2	0.67	4.73	1	0.00	0.99
AMHSerum3	0.51	3.22	2	0.28	1.92
AMHSerum4	0.49	3.06	4	0.58	3.76
AMHSerum5	0.77	5.94	8	0.87	7.39
AMHSerum6	0.55	3.53	16	1.17	14.80
AMHSerum7	0.45	2.79			
AMHSerum8	0.57	3.72			
AMHSerum9	1.05	11.19			
AMHSerum10	0.47	2.92			
AMHSerum11	0.88	7.60			
AMHSerum12	0.26	1.82			
AMHSerum13	0.74	5.45			
AMHSerum14	-0.02	0.95			
AMHSerum15	-0.08	0.83			
AMHSerum16	1.08	12.10			
AMHSerum17	0.75	5.60			
AMHPlasma19	0.67	4.66			
AMHPlasma20	0.52	3.34			
AMHPlasma21	0.67	4.70			

Table 7. Fitted linear regression slopes for $\log_{10}$ reported concentration against $\log_{10}$ nominal concentration for 16/190 ampoule A (candidate IS), C (coded duplicate) and B (comparator) for each run of each method. Methods were considered invalid if geometric mean slope for any of the samples was outside the range [0.91, 1.10]

Lab	Run	Slope A	Slope C	Slope B
1	1	1.02	1.02	1.06
1	2	1.02	1.00	1.04
2	1	0.92		0.98
2	2	0.93	0.96	.
2	3	0.96		0.97
2	4	0.95	0.92	.
3	1	0.99	0.99	0.99
3	3	0.99	1.00	0.98
4	1	0.97	0.97	0.97
4	2	0.96	0.95	0.96
5	1	0.96	0.98	0.97
5	2	0.97	0.96	0.94
6	1	1.01	1.05	1.07
6	2	1.01	1.07	1.09
7	1	0.93	0.93	0.92
7	2	0.93	0.94	0.91
8	**1**	**0.87***	**0.87***	**0.84***
8	**2**	**0.87***	**0.87***	**0.84***
9	1	0.97	1.05	1.02
9	2	0.93	1.02	1.00
10	**1**	**0.83***	**0.99***	**0.94***
10	**2**	**0.87***	**0.96***	**0.88***
11	**1**	**0.82***	**0.83***	**0.75***
11	**2**	**0.82***	**0.81***	**0.74***
12	1	0.96	0.96	0.95
12	2	0.95	0.95	0.93
13	1	1.02	1.02	1.07
13	2	1.04	1.04	1.16
14	1	0.94	0.95	0.91
14	2	0.95	0.95	0.92
15	1	1.01	0.99	1.00
15	2	1.01	1.00	1.01
16	1	1.03	1.02	1.16
16	2	1.01	1.00	1.05
17	**1**	**0.93***	**0.92***	**0.91***
17	**2**	**0.90***	**0.89***	**0.90***
18	**1**	**0.89***	**0.90***	**0.83***
18	**2**	**0.91***	**0.95***	**0.84***
19	1	0.97	0.97	0.96
19	2	0.91	0.96	0.95
20	1	0.97	0.97	1.00
20	2	0.97	0.96	0.98
21	1	1.01	1.00	0.96
21	2	1.01	0.98	0.98

* Laboratory excluded from further analyses due to mean slope values being outside of chosen range of validity

Table 8: Individual estimates of the AMH content of 16/190 (ng/amp) ampoule A and ampoule C, the geometric mean of these and the ratio of A:C.

Lab	Run	Sample A	Sample C	GM of A & C	Ratio of A:C
1	1	357	340	348	1.05
1	2	318	330	324	0.96
2	1	630		630	
2	2	629	632	631	0.99
2	3	613		613	
2	4	621	615	618	1.01
3	1	1104	1100	1102	1.00
3	3	1084	1049	1066	1.03
4	1	523	520	522	1.01
4	2	524	566	544	0.93
5	1	528	521	524	1.01
5	2	512	553	533	0.93
6	1	1142	1116	1129	1.02
6	2	1224	1149	1186	1.07
7	1	482	456	469	1.06
7	2	486	456	471	1.07
8	1	723*	733*	728*	0.99*
8	2	713*	725*	719*	0.98*
9	1	546	598	571	0.91
9	2	546	512	529	1.07
10	1	512*	571*	541*	0.90*
10	2	493*	482*	488*	1.02*
11	1	510*	526*	518*	0.97*
11	2	503*	503*	503*	1.00*
12	1	498	495	497	1.01
12	2	500	516	508	0.97
13	1	286	281	283	1.02
13	2	269	295	282	0.91
14	1	514	478	496	1.08
14	2	450	462	456	0.98
15	1	453	471	462	0.96
15	**2**	**495**↓	**437**↓	**465**↓	**1.13**↓
16	1	467	468	467	1.00
16	2	454	466	460	0.97
17	1	841*	902*	871*	0.93*
17	2	822*	859*	840*	0.96*
18	1	864*	820*	841*	1.05*
18	2	870*	808*	838*	1.08*
19	1	379	381	380	0.99
19	**2**	**494**↓	**438**↓	**465**↓	**1.13**↓
20	1	470	494	481	0.95
20	2	474	503	488	0.94
21	1	387	414	400	0.93
21	2	434	451	442	0.96

* Laboratory excluded from further analyses due to mean slope values being outside of chosen range of validity.↓ Assay excluded from further analyses due to inconsistent estimates between coded duplicates

Table 9: Individual estimates of the AMH content of the comparator sample B (ng/amp) and the ratio of B to the geometric mean of A and C for each run.

Lab	Run	Sample B	Ratio of B to GM(A,C)
1	1	156	0.45
1	2	144	0.45
2	1	287	0.46
2	2		
2	3	279	0.46
2	4		
3	1	554	0.5
3	3	541	0.51
4	1	293	0.56
4	2	292	0.54
5	1	284	0.54
5	2	285	0.54
6	1	550	0.49
6	2	559	0.47
7	1	251	0.53
7	2	253	0.54
8	**1**	**424***	**0.58***
8	**2**	**440***	**0.61***
9	1	305	0.53
9	2	292	0.55
10	**1**	**265***	**0.49***
10	**2**	**318***	**0.65***
11	**1**	**303***	**0.59***
11	**2**	**299***	**0.59***
12	1	324	0.65
12	2	324	0.64
13	1	150	0.53
13	2	133	0.47
14	1	265	0.54
14	2	242	0.53
15	1	268	0.58
15	**2**	**250**[ǂ]	**0.54**[ǂ]
16	1	194	0.41
16	2	213	0.46
17	**1**	**537***	**0.62***
17	**2**	**523***	**0.62***
18	**1**	**516***	**0.61***
18	**2**	**538***	**0.64***
19	1	210	0.55
19	**2**	**242**[ǂ]	**0.52**[ǂ]
20	1	305	0.63
20	2	318	0.65
21	1	220	0.55
21	2	236	0.53

[*] Laboratory excluded from further analyses due to mean slope values being outside of chosen range of validity. [ǂ] Assay excluded from further analyses due to inconsistent estimates between coded duplicates

Table 10: Laboratory estimates of the relative immunoreactivity (n=5) of accelerated thermal degradation samples relative to Sample H, stored at -20˚C.

Lab	Temp	Relative Immunoreactivity	95% Confidence Limits
1	4	1.00	(0.92 - 1.09)
1	20	1.00	(0.90 - 1.11)
1	37	1.03	(0.93 - 1.15)
1	45	0.96	(0.87 - 1.04)
3	4	0.98	(0.97 - 1.00)
3	20	1.01	(1.00 - 1.02)
3	37	0.99	(0.98 - 1.00)
3	45	0.99	(0.95 - 1.02)
4	4	0.99	(0.89 - 1.09)
4	20	1.04	(1.00 - 1.09)
4	37	0.99	(0.84 - 1.17)
4	45	0.93	(0.82 - 1.05)
5	4	0.97	(0.91 - 1.04)
5	20	1.01	(0.97 - 1.06)
5	37	1.03	(0.94 - 1.12)
5	45	0.94	(0.87 - 1.00)
7	4	0.94	*
7	20	0.94	*
7	37	0.94	*
7	45	0.92	*

*****Laboratory 7 performed a single run.

Appendix 2

Study Protocol
Collaborative Study to evaluate a candidate preparation for the 1st WHO International Standard for Mullerian Inhibiting Substance/Anti-Mullerian Hormone

Introduction

Measurements of serum concentrations of Mullerian Inhibiting Substance, also called Anti-Mullerian Hormone (AMH), are increasingly used in reproductive medicine as a biomarker of ovarian response and ovarian reserve and there is a need for a reference material for AMH to calibrate and to support the harmonization of immunoassay methods. Current AMH immunoassays report in mass units (ng/ml or pmol).

A project to develop a WHO International Standard for AMH at NIBSC was endorsed by the WHO Expert Committee for Biological Standardization in 2014 and a candidate standard, coded 16/190, has been prepared using human, recombinant AMH donated to NIBSC for this purpose. We now intend to organize a collaborative study with expert laboratories to evaluate the candidate standard.

The aims of the study are:

1. To determine the AMH content of 16/190 by different immunoassay methods in terms of their method calibration
2. To assess the suitability of 16/190 to calibrate immunoassays of AMH
3. To assess the stability of 16/190 after accelerated thermal degradation (ATD)

According to WHO guidelines (WHO, 2006), assignment of the AMH content of 16/190 in mass units (ng/ml) requires the use of a reference method such that the assignment is traceable to the *System Internationale* (SI) unit of mass. Immunoassay technologies rely on a binding equilibrium of the antibody and analyte and therefore, are not considered reference methods. Thus, the data from this study will be used to evaluate the suitability of the candidate standard and will give an indication of a consensus value of content as determined by immunoassay methods. This data will be evaluated alongside estimates of AMH polypeptide content by isotope dilution mass spectrometry. We will invite input from all participants and other interested parties as we evaluate the anonymised data with careful consideration of the impact on patients.

Materials

Participants will receive the materials listed in Table 1. Each participant will be allocated a set of preparations based on assay capacity and sample availability. The minimum allocation will be **three ampoules coded A, B** and **C** and **17 test samples of human serum** and **5 test samples of human plasma (Li-Hep)** per assay. Where assay capacity and sample availability allow, participants will an additional set of **five coded ampoules (D-H)** to evaluate the stability of the candidate standard.

Preparation	Content
Candidate standard – three samples coded A, B, C.	Nominally, 500 ng AMH per ampoule
Accelerated thermal degradation samples of the candidate standard – five ampoules coded D-H	Nominally, 500 ng AMH per ampoule
Human serum samples (n=17)	Volumes of 0.6 -0.8 ml are provided according to assays requirements. The estimated content of the serum samples is 0.9 - 17.0 ng/ml AMH by ELISA.
Human plasma samples (Li-Hep) (n=5)	Volumes of 0.6 - 0.8 ml are provided according to assays requirements. The estimated content of the plasma samples is <0.1-12.0 ng/ml AMH by ELISA.

Table 1: Materials provided to participants

Candidate standard
The candidate standard contains the residue after free-drying of a 0.5 ml solution which contained 1.2 mg bovine casein, 2.5 mg trehalose and nominally, 500 ng recombinant, human AMH. The recombinant, human AMH was donated by Professor Patricia Donahoe (Director) and Dr David Pépin of the Pediatric Surgical Research Laboratories, Massachusetts General Hospital. Preparation of the recombinant, human AMH is described in Pépin et al., (2013) in which it is referred to as LR-MIS. The country of origin of the milk used to prepare the bovine casein (EMD Millipore Cat: 218682, Lot 2626982) was Australia.

Accelerated thermal degradation samples
Ampoules of the candidate standard which have been incubated at -20˚C, +4°C, +20°C, +37°C and +45°C for 20 months will be included in the study to assess the stability of the candidate standard.

Human Samples
Samples coded AMHSerum1 to AMHSerum17 contain human serum with an AMH content of between 0.9 and 17.0 ng/ml. Samples coded AMHPlasma18 to AMHPlasma22 contain human plasma (Li-Hep) with an AMH content of between <0.1 and 12 ng/ml. Samples were obtained from either First Link UK (Wolverhampton, UK) or TCS Biosciences (Buckingham, UK) or CERBA Specimen Services (Saint-Ouen l'Aumône, France). Some specimens were diluted in post-menopausal human serum (TCS Biosciences). Human serum samples from First Link UK and TCS Biosciences were certified by the supplier to have been tested and found non-reactive for antibodies to HIV 1 and 2, HIV p24, HBsAg, Anti-HCV and Syphilis TP. Clinical remnant samples from Cerba Specimen Services, diluted in post-menopausal serum, were tested at NIBSC and found to be non-reactive for HBsAg, HIV antibody and HCV RNA by PCR.

This material is to be used only for this study and in accordance with the Human Tissue Act or equivalent national legislation and is to be destroyed at the end of the collaborative study.

All material of human origin should be considered as potentially hazardous and handled with appropriate care. It should be used and discarded according to your own laboratory's safety procedures.

Handling of the Preparations

Ampoules:

- Store at -20°C or below until use. Allow contents to reach room temperature before opening.
- To reconstitute, add a 1 ml volume of distilled water to the ampoule and leave at room temperature for 20 minutes.
- Prepare test dilutions of the concentrations defined below in your assay diluent. The diluent should include protein to prevent surface adsorption (typically 0.1 % (w/v) bovine serum albumin or 0.1 % (w/v) human serum albumin).

Please provide details of the reconstitution of the ampoules, all pre-dilutions and the dilutions used to prepare the test samples.

Storage of reconstituted ampoule contents:

In cases where reconstituted ampoules require storage for use in subsequent runs (**Sample B** for some laboratories) the conditions below have been shown to retain AMH immunoreactivity by ELISA.

- Reconstitute ampoule contents as described above.
- Prepare aliquots of 300 µl in low binding 0.5 ml microfuge tubes (e.g. Protein Lo-Bind Tube 0.5 ml, Eppendorf (Hamburg, Germany))
- Freeze on dry ice and store at -20˚C or below.
- When required, thaw at room temperature and mix gently by pipetting.

Test samples of human serum:

Store at -20°C or below until use. Allow contents to thaw and reach room temperature.
Mix contents gently before measuring. Please do not re-freeze. Use a fresh aliquot for each run.

Test samples of human plasma (Li-Hep):

Store at -20°C or below until use. Thaw by placing in a waterbath at 37˚C for 6 mins.
Centrifuge at 1500 rpm (250 *xg*) for 10 mins and remove supernatant for testing.
Do not re-freeze. Use a fresh aliquot for each run.

Tests Requested

Participants are asked to perform **two independent runs** of the assay method(s) in use in their laboratory and which are used clinically for the measurement of patient samples.

An independent run consists of the measurement of one set of dilutions prepared from each of ampoules A, B and C and one set of serum and plasma samples (n=22) which have been thawed specifically for that run. An independent run will use a single calibrated kit, integral or 96-well plate as required for your method.

An independent run of the accelerated thermal degradation samples will consist of the measurement of one set of dilutions prepared from each of ampoules coded D-H.

Participants are asked to prepare dilutions of the ampouled preparations and to measure, **in triplicate**, the AMH content of these and the content of the serum and plasma samples. The test concentrations are described in **Common test sample concentrations** overleaf.

Common test samples concentrations
In order to compare different assay methods, participants are asked to measure a minimum of eight dilutions of 16/190 which are common to all participants. These are **16, 8, 4, 2, 1, 0.5, 0.25** and **0.125** ng/ml AMH with additional dilutions to ensure that a minimum of **six points in the linear part of the dose response curve are measured**.

We request that participants to provide details of the reconstitution of the ampoules, all pre-dilutions and the volumes used to prepare the test samples.

Submission of data
Participants are asked to submit data as an Excel file.
For each run, participants are required to provide details of:
- the diluent used prepare the test samples
- the volumes used to prepare the dilution series of each vial/ampoule
- the signal (e.g. absorbance, RLU, counts) for each replicate of the kit or in-house calibrators
- the signal (e.g. absorbance, RLU, counts) for each replicate of the test samples
- the signal (e.g. absorbance, RLU, counts) for each replicate of the serum samples
- the reported concentration (ng/mL) for each replicate of the test samples
- the reported concentration (ng/mL) for each replicate of the serum samples

Suggested reporting tables are shown in **Appendix 1**.

Participants' estimates of the total AMH content of the ampouled preparations are requested, as calculated by the method normally used in their laboratory. However, it must be noted that data from all participating laboratories will be analysed at NIBSC and this may result in small differences between participants' estimates and the values reported after central computation.

Use of Samples
The use of the ampoules of the candidate standard, 16/190 and the ampoules coded A to H and the serum and plasma samples, AMHSerum1 to AMHSerum17 and AMHPlasma18 to AMHPlasma22 is restricted to this study.

Publication
The publication of data arising from the use or analysis of the candidate standard or serum samples is not permitted.

Report
A preliminary report will be prepared and circulated to all participants for comment.
Participating laboratories will be identified by a laboratory number only and any requests to treat

information in confidence will be respected. To proceed to establishment of the standard, the report will be submitted to the Expert Committee on Biological Standardization of the WHO.

Further information

For further information, please contact Dr Jackie Ferguson (e-mail: jackie.ferguson@nibsc.org)
National Institute for Biological Standards and Control (http://www.nibsc.org)
Tel: 44 (0) 1707 641135

References

WHO Expert Committee on Standardization; 55th report. WHO 2006; 932, Annex 2, 75-130.
Pépin, D et al., (2013) Technology 2013; 1, 63-71.

Appendix 1: Example data reporting table for recording the AMH content of the test samples

		Platform:			**Method:**		
Run No.:		**RLU/Absorbance Units/Counts**			**Reported AMH concentration (ng/mL)**		
***Sample**	**Nominal AMH (ng/mL)**	**1**	**2**	**3**	**1**	**2**	**3**
Baselines	0						
Kit standard 1							
Kit standard 2							
Kit standard 3							
Kit standard 4							
Expand table for additional kit standards							
Ampoule [Code] dil^n 1	16						
Ampoule [Code] dil^n 2	8						
Ampoule [Code] dil^n 3	4						
Ampoule [Code] dil^n 4	2						
Amopule [Code] dil^n 5	1						
Ampoule [Code] dil^n 6	0.5						
Ampoule [Code] dil^n 7	0.25						
Ampoule [Code] dil^n 8	0.125						
Expand table for additional ampoules							
AMHSerum1							
AMHSerum2							
AMHSerum3							
AMHSerum4							
AMHSerum5							
AMHSerum6							
AMHSerum7							
AMHSerum8							
AMHSerum9							
AMHSerum10							
AMHSerum11							
AMHSerum12							
AMHSerum13							
AMHSerum14							
AMHSerum15							
AMHSerum16							
AMHSerum17							
AMHPlasma18							
AMHPlasma19							
AMHPlasma20							
AMHPlasma21							
AMHPlasma22							

Appendix 3

WHO Reference Reagent
Mullerian Inhibiting Substance (Anti-Mullerian Hormone), human, recombinant
NIBSC Code: 16/190
DRAFT instructions for use
(Version1, Dated XX/11/2019)

1. INTENDED USE

This consists of a batch of lyophilized ampoules containing purified, recombinant human Mullerian Inhibiting Substance, also known as Anti-Mullerian Hormone, AMH. The preparation was established as the first WHO Reference Reagent at the 2019 meeting of the Expert Committee on Biological Standardization. The intended use is for the characterization of AMH assays.

2. CAUTION

This preparation is not for administration to humans or animals in the human food chain.
The preparation contains material of bovine origin. As with all materials of biological origin, this preparation should be regards as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials to avoid cuts.

3. UNITAGE

By collaborative study, immunoassay estimates provided a robust geometric mean content estimate of 489 ng/amp.

4. CONTENTS

Country of origin of biological material: USA
Each ampoule contains:
Recombinant, human AMH
2.5 mg trehalose
1.2 mg bovine casein

5. STORAGE

Unopened ampoules should be stored at -20°C.
Please note:because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

DIN ampoules have an "easy-open" coloured stress point, where the narrow ampoule stem joins the wider ampoule body. Tap the ampoule gently to collect the material at the bottom (labelled) end. Ensure that the disposable ampoule safety breaker provided is pushed down on the stem of the ampoule and against the shoulder of the ampoule body. Hold the body of the ampoule in one

hand and the disposable ampoule breaker covering the ampoule stem between the thumb and first finger of the other hand. Apply a bending force to open the ampoule at the coloured stress point, primarily using the hand holding the plastic collar. Care should be taken to avoid cuts and projectile glass fragments that might enter the eyes, for example, by the use of suitable gloves and an eye shield. Take care that no material is lost from the ampoule and no glass falls into the ampoule. Within the ampoule is dry nitrogen gas at slightly less than atmospheric pressure. A new disposable ampoule breaker is provided with each DIN ampoule.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution. For all practical purposes each ampoule contains the same quantity of the substances listed above. Depending on the intended use, dissolve the total contents of the ampoule in a known volume of a suitable diluent. Users should make their own investigations into the type of diluent suitable for their use. If extensive dilutions are prepared, a carrier protein should be added. The ampoules do not contain bacteriostat and solutions of the material should not be assumed to be sterile.

8. PREPARATION OF AMPOULES

Recombinant AMH was purified from the culture media of a stable CHO cell line, LR-MIS, expressing the native human AMH sequence with the leader sequence from human serum albumin (HSA) and with a modification of the internal cleavage site at amino acids 423-428 from RAQR/S to RARR/S (Pépin, D *et al*., (2013)). The HSA leader sequence is cleaved during maturation. A 2100 ml volume of bulk formulation containing 0.24% (w/v) casein (Calbiochem), 0.5% (w/v) trehalose (Sigma) and nominally, 1 µg/ml AMH was distributed into 3 mL siliconized DIN ampoules as 0.5 ml aliquots. The ampoule contents were freeze-dried, secondarily desiccated and sealed under nitrogen.

The batch of ampoules, coded 16/190, was evaluated in a collaborative study by immunoassays performed by seven laboratories in four countries providing 21 data sets, 16 of which met the assay validity criteria of slope [0.91, 1.10] and ratio of coded duplicates [0.91, 1.10]. Reported estimates of content in terms of method calibrations gave a geometric mean estimate of 511 ng/amp (95% CI: 426-612 ng/amp, n=16, GCV 42.0%) and a robust geometric mean of 489 ng/amp. Commutability was assessed by the concomitant measurement of 22 patient samples. By the difference in bias approach, dilutions of 16/190 were shown to be fully within the statistically-defined limits of commutability for 6 of the 16 methods. Of the remaining methods, some dilutions of 16/190 were within the limits for 3/16 methods whereas no dilutions were within the defined limits for 7/16 methods (Ferguson *et al.*, 2019). Manufacturers are recommended to perform an assessment of the commutability of 16/190 with patient samples when measured by their method.

9. STABILITY

Analysis of accelerated thermal degradation samples stored for 21 months and measured by participants in the collaborative study showed no significant loss of AMH immunoreactivity suggesting that 16/190 is likely to be highly stable when stored at -20ºC. NIBSC follows the policy of WHO with respect to its reference materials. It is the policy of WHO not to assign an expiry date to their international reference materials. They remain valid with the assigned

potency and status until withdrawn or amended. Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference materials should be stored on receipt as indicated on the label. In addition, once reconstituted, diluted or aliquoted, users should determine the stability of the material according to their own method of preparation, storage and use. Users who have data supporting any deterioration in the characteristics of any reference preparation are encouraged to contact NIBSC.

10. REFERENCES

Pépin, D *et al*., (2013) An albumin leader sequence coupled with a cleavage site modification enhances the yield of recombinant C-terminal Mullerian Inhibiting Substance. Technology 2013; 1, 63-71

Ferguson *et al*., (2019) WHO International Collaborative Study of the Proposed WHO IS for Mullerian Inhibiting Substance/Anti-Mullerian Hormone, human, recombinant. https://www.who.int/Biologicals/BS.2019.XXXX

11. ACKNOWLEDGEMENTS

We gratefully acknowledge Professor Patricia Donahoe (Director) and Dr David Pépin of the Pediatric Surgical Research Laboratories, Massachusetts General Hospital for the donation of the bulk material, the important contributions of all the participants in the collaborative study, the Standardization Science Group at NIBSC for preparation of trial materials, the Standards Processing Division at NIBSC for the preparation and dispatch of the ampouled materials and the Biostatistics Group at NIBSC for analysis of the collaborative study data.

12. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.org
WHO Biological Standards: http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.org/products/biological_reference_materials/frequently_asked_questions/how_are_international_units.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering_information/frequently_asked_ questions.aspx NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx

13. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.org

14. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

15. MATERIAL SAFETY SHEET

Physical and Chemical properties (at room temperature)	
Physical appearance : Freeze dried powder	Corrosive: No
Stable: Yes	Oxidising: No
Hygroscopic: Yes	Irritant: No
Flammable: No	Handling: See caution, Section 2
Other (specify)	
Toxicological properties	
Effects of inhalation: Not established, avoid inhalation	
Effects of ingestion: Not established, avoid ingestion	
Effects of skin absorption: Not established, avoid contact with skin	
Suggested First Aid	
Inhalation: Seek medical advice	
Ingestion: Seek medical advice	
Contact with eyes: Wash with copious amounts of water. Seek medical advice.	
Contact with skin: Wash thoroughly with water.	
Action on Spillage and Method of Disposal	
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biologically hazardous waste.	

16. LIABILITY AND LOSS

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.
Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11of the Conditions.

17. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 0.003 g
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable. Attached: No

18. CERTIFICATE OF ANALYSIS

NIBSC does not provide a Certificate of Analysis for WHO Biological Reference Materials because they are internationally recognised primary reference materials fully described in the instructions for use. The reference materials are established according to the WHO

Recommendations for the preparation, characterization and establishment of international and other biological reference standards http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_biolefstandardsrev2004.pdf (revised 2004). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.