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Proposed 6th WHO International Standard for human chorionic gonadotrophin

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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 **5 October 2020** 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

Stocks of the 5th International Standard for human chorionic gonadotrophin (hCG), 07/364, are exhausted, and the World Health Organisation (WHO) Expert Committee on Biological Standardization (ECBS) has recognised (2019) the urgent need for a replacement IS to be prepared for the calibration of assays to control the quality and potency of hCG used in the treatment of infertility, and for the calibration of assays used in the diagnosis of pregnancy and a range of other clinical conditions.

We report here the evaluation of a candidate preparation of purified intact hCG, filled into ampoules coded 18/244, in an international collaborative study carried out by 16 laboratories in 10 countries. The evaluation of the candidate preparation included assessment of the candidate preparation by both bioassay and immunoassay in comparison with the existing International Standard, 07/364, calibrated in IU and molar units, and the WHO Reference Reagent for intact CG, coded 99/688, calibrated in molar units.

Participants in the bioassay part of the study (6 laboratories) were sent duplicate ampoules of the candidate standard, 18/244, alongside the current International Standard, 07/364. All laboratories were in good agreement, with laboratory geometric means ranging from 149.1 to 171.1 IU/amp giving an overall geometric mean for the candidate standard of 158.5 IU/amp (95% confidence interval 153.5 – 163.8 IU/amp, inter-laboratory GCV 5.6%). These data support a value assignment of 159 IU/amp for bioassays of hCG and indicate the candidate standard is suitable to serve as a reference preparation for the calibration of bioassays of hCG.

For evaluation of the candidate standard by immunoassay, participants were sent duplicate ampoules of the candidate standard, 18/244, the current International Standard, 07/364, the WHO Reference Reagent for intact hCG, 99/688, and a panel of 10 human serum samples. In total, 12 laboratories participated in the immunoassay part of the study, with one laboratory unable to return data due to the Covid19 pandemic. From the data provided by the 11 remaining laboratories, a total of 17 different immunoassay methods were analysed. The two standards behaved in a similar manner in the immunoassays included in this study, with the relative potencies of the 5th IS and candidate standard 18/244, as reported by laboratories, in reasonable agreement with an overall geometric mean of 191.5 IU/amp (inter-laboratory GCV 13.6%) for the 5th IS, 07/364, and 199.8 IU/amp (inter-laboratory GCV 14%) for the candidate standard 18/244. When calculated relative to the 5th IS, the relative potencies of 18/244 were in very good agreement, with an overall geometric mean of 185.8 IU/amp (95% confidence intervals 183.3 – 188.4 IU/amp) and inter-laboratory GCV 3.1%. The data demonstrated that the candidate IS, 18/244, with an assigned potency of 186 IU/amp, to be immunoreactive and behave in a similar manner to the 5th IS, 07/364, in the immunoassays included in this study, indicating that

continuity of hCG measurements would be achieved with the introduction of 18/244 as the replacement standard.

The candidate standard was also assigned a potency relative to the WHO Reference Reagent for intact hCG, 99/688, to give an assigned content in molar units. The geometric mean of laboratory estimates for the candidate standard calculated relative to the WHO RR 99/688, was 0.413 nmol/amp (95 % confidence interval 0.399-0.428, GCV 7.4%). Laboratories were in good agreement whether the candidate standard was expressed relative to 07/364 or 99/688, giving a geometric mean of 0.41 nmol/amp in both cases (GCV 3.1% and 7.4% respectively). This is in very close agreement with the expected content of 0.39 nmol/amp for 18/244. In addition, analysis of the stability of the candidate standard was performed by immunoassay of accelerated thermal degradation (ATD) study samples. No significant loss of activity was demonstrated in samples stored at elevated temperatures for 7 months, therefore prediction of long-term stability during storage at -20ºC was not possible. However, the lack of observed degradation at elevated temperatures does indicate that the candidate IS, 18/244, is highly stable and suitable for use as an International Standard.

The immunoassay part of the study included an assessment of the impact of the new standard on the routine measurement of hCG in human serum samples. These serum samples were measured in parallel with the candidate standard 18/244, the 5th International Standard 07/364, and the WHO Reference Reagent, 99/688, by all laboratories contributing immunoassays. The assessment of commutability was performed using a difference in bias approach. This assessment found the candidate standard 18/244 and 5th IS behaved similarly in the immunoassays included in this study, with both standard preparations commutable with patient samples in 11 out of 17 assay methods. Of the remaining 6 laboratories, in 1 laboratory (Lab 7) 18/244 was commutable, but 07/364 was not. Importantly for the 5 laboratory methods in which 18/244 was non-commutable with serum samples, the 5th IS 07/364 was also found to be non-commutable.

Taken together, these results indicate that the candidate standard 18/244 is suitable to serve as an International Standard for the measurement of hCG by both bioassay and immunoassay, and that it is suitable to serve as a replacement for the 5th International Standard, 07/364, for the continued calibration of bioassays and immunoassays for hCG. The assignment of molar units will continue to enable the transition of these methods to the use of substance concentration for reporting of hCG values by immunoassay in the future.

Therefore, it is proposed that the candidate preparation in ampoules coded 18/244 is established as the 6th International Standard for hCG with an assigned content of **159 IU/amp for bioassay and 186 IU/amp; 0.41 nmol/amp for immunoassay.**

Introduction

Human chorionic gonadotrophin (hCG) is a heterodimeric glycoprotein hormone. Intact hCG molecules are composed of an α and β subunit with a molecular weight of approximately 36 kDa. hCG shares an almost identical α subunit with members of the glycoprotein hormone family, LH, FSH and TSH, with the unique β subunit in the respective hormones accounting for their biological specificity [1]. hCG is produced by the developing embryo in pregnancy, synthesised by syncytiotrophoblasts. Its early role is to support the corpus luteum and thereby maintain the levels of progesterone that are required for pregnancy [1].

Human urinary derived hCG is an important biotherapeutic, used to promote the final maturation of ovarian follicles and ovulation for the treatment of infertility and in assisted reproductive technology (ART). Detection of hCG is also an important diagnostic tool: in addition to measurements of serum hCG by immunoassay for the diagnosis of pregnancy and ectopic pregnancy, hCG measurement is also used in pre-natal screening for Down's syndrome and as a marker for other clinical conditions such as gestational trophoblastic diseases and some germ cell tumours [2].

The current 5th WHO International Standard for hCG in ampoules coded 07/364 was established in 2009 [3] and has been widely used for the calibration of therapeutic preparations of hCG by bioassay, in addition to its use as a calibrant for immunoassays of hCG. The current standard, 07/364, was assigned a dual unitage of 162 IU/amp for bioassay and 179 IU/amp for immunoassay. To enable the diagnostic community to calibrate assays for hCG in substance concentrations in the future, the current standard 07/364 was also assigned a value of 0.39 nmol/amp by calibration with the WHO Reference Reagent for intact hCG, 99/688. The WHO Reference Reagent was prepared in 2001 as part of a collaboration with the International Federation of Clinical Chemists (IFCC) Working Group for the standardisation of immunoassays of hCG. This collaboration involved the production of a panel of purified isoforms of hCG, including intact hCG (99/688), for the intended purpose of investigating and characterizing the specificity of existing hCG immunoassays, and were assigned a molar content by amino acid analysis of stock solutions that were then corrected for loss on freeze drying, as measured by immunoassay [4-6].

Stocks of the 5th International Standard are now almost exhausted and there is an urgent requirement to replace the standard. To prepare a candidate replacement International Standard for hCG, a batch of purified human urinary hCG was filled into ampoules (NIBSC code 18/244), following procedures recommended by WHO [7]. This batch of ampoules was evaluated in an

international collaborative study with expert laboratories by both bioassay for the calibration of therapeutic preparations of hCG, and by immunoassay to determine the immunoreactivity of the candidate standard and assess its suitability to serve as a calibrant for immunoassays of hCG content. Human serum samples containing a range of hCG concentrations were included in the immunoassay phase of the study in order to assess commutability of the candidate standard with native samples. To maintain continuity, it was intended to calibrate the candidate standard 18/244 by both bioassay and immunoassay, in terms of the 5th International Standard 07/364, and in molar units in terms of the WHO Reference Reagent, 99/688.

The aims of the study were therefore:

1) To calibrate the candidate standard 18/244:

    a. Relative to the 5th International Standard for hCG, 07/364, by *in vivo* bioassay for hCG.

    b. Relative to the 5th International Standard for hCG, 07/364, and the WHO Reference Reagent 99/688, by immunoassay

2) To assess the suitability of the candidate preparation 18/244 to serve as the 6th International Standard for the calibration of therapeutic preparations of hCG by bioassay.

3) To assess the suitability of the candidate preparation 18/244 to serve as the 6th International Standard for the calibration of immunoassays for hCG.

    To assess the stability of the preparation of 18/244 by comparison with ampoules stored at elevated temperatures as part of an accelerated degradation stability study in both bioassay and immunoassay systems.

Participants

In total, 17 laboratories in 10 countries agreed to participate in either the bioassay or immunoassay phases of the study. These laboratories are listed alphabetically by country in Table 1. Of these 17 laboratories, one was unable to participate or return data due to extraneous circumstances with COVID-19 (Dr Nathalie Ripoll, BioMerieux, France).

Throughout the study, each of the final sixteen participating laboratories 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

ARGENTINA	Dr Claudio Wolfenson Instituto Massone Arias 4431, Buenos Aires, 1430
BELGIUM	Dr Stefaan Marivoet Tosoh Europe N.V., Transportstraat 4, 3940 Tessenderlo
BELGIUM	Dr Michel Hars, DiaSource Immunoassays Rue du bosquet 2, 1348 Louvain-la Neuve
CHINA	Dr Yuan Zhang NIFDC No. 31 Huatuo Road, Daxing District, Beijing, 102629
FRANCE	Dr Nathalie Ripoll bioMerieux, Chemin de L'Orme, 69280 Marcy L' Etoile
GERMANY	Dr Sven Michael-Cords and Dr Simone Hoeger Bioassay GmbH IM Neuenheimer Feld 515, Heidelberg, 69120
GERMANY	Dr Matthias Herkert DRG Instruments Frauenbergstrasse 18, 35039 Marburg
GERMANY	Dr Stefan Hutzler Roche Diagnostics GmbH Nonnenwald 2, 82377 Penzberg
IRELAND	Dr Paul Dowling and Dr Rosa Alshammari Abbott Diagnostics Division Co. Longford
ITALY	Dr Cristina Pierini and Dr Silvia Pompili Merck Merck Ivrea – RBM S.p.A, Via Ribes n.1, Colleretto Giacosa (TO) 1001
SWITZERLAND	Dr Walter De Matteo IBSA Institut Biochimique SA Via Al Ponte 13, Massagno, 6900
UK	Haf Saxby,

	Siemens Siemens Healthcare Diagnostics Products Ltd, Glyn Rhonwy, Llanberis, LL55 4EL
UK	Dr Melanie Moore and Katherine Partridge NIBSC Blanche Lane, South Mimms, Potters Bar, EN6 3QG
USA	Ryan Masica Beckman Coulter Inc, 322 Lake Hazeltine Dr, Chaska, MN 55318
USA	Dr Qian Ding and Bethany Novick Ortho Clinical Diagnostics 100 Indigo Creek Drive, Rochester, NY 14623
USA	Fred Masulli and Mary Beth Stankevich Siemens Healthineers, PO Box 6101, Building 700, Newark, DE 19702
USA	Tanja Dubravcic, Alicia Plumer and Kelsey Murray Siemens Healthcare Diagnostics Inc., 333 Coney Street, Walpole, MA 02032

Materials and Methods

Bulk materials and processing

A batch of intact hCG, highly purified to remove other forms of hCG, was kindly donated with permission from the International Federation of Clinical Chemists (IFCC). This batch is the same batch of hCG used to prepare both the 5th International Standard, 07/364, and the WHO Reference Reagent for intact hCG, 99/688. A 300 mg portion of hCG material was dissolved in 7500 ml of a buffer containing 2 mg/ml human plasma albumin, 50 mM sodium phosphate buffer, pH7.4, and 10 mg/ml trehalose. Aliquots of 0.5 ml (nominally 20 µg/ampoule) were then dispensed into glass ampoules, lyophilised and sealed according to procedures recommended by WHO [7] and stored at -20 ºC in the dark at NIBSC.

The bulk hCG preparation and human plasma albumin were tested and found negative for anti-HIV 1 and 2, HBsAg and HCV by NAT assay.

Product characterisation

A total of 14109 ampoules, coded 18/244, were produced and are stored at -20 ºC under temperature-controlled conditions at NIBSC (Blanche Lane, Potters Bar, EN6 3QG). Check weights measured during filling demonstrated a mean fill weight of 0.5190 g (CV 0.18%, n=468). The mean dry weight was 0.00922 g (CV 1.87%, n=6), mean residual moisture content

was 1.0% (CV 23.06%, n=12) and mean oxygen headspace was 0.18% (CV 73.90%, n=12). There was no detectable microbial contamination in the pre or post-filled material.

Collaborative study design for the value assignment of 18/244 by bioassay and immunoassay

Materials

The collaborative study was organised by NIBSC. All participants performing bioassays of hCG in the study were provided with a set of samples consisting of the 5th International Standard 07/364 and duplicates of the candidate standard 18/244 as described in Table 2. A study protocol detailing the handling of all materials and the tests requested was also sent to participants and a copy is provided in Appendix 1.

Table 2. Preparations supplied for bioassays

Ampoule code	hCG preparation	Ampoule content
Not coded	5th IS for hCG, 07/364	162 IU per ampoule for bioassay
F, G Duplicates	Candidate 6th IS, 18/244, stored at -20ºC	Nominally 20 µg hCG per ampoule (assumed to be approximately 162 IU per ampoule)

Abbreviations: International Standard (IS), Accelerated thermal degradation (ATD)

All participants performing immunoassays for the measurement of hCG in the study were provided with a set of samples consisting of the 5th IS 07/364, the WHO RR 99/688, duplicates of the candidate standard 18/244, and a panel of 10 human serum samples. The set of materials are listed in Table 3. A study protocol detailing the handling of all the materials and the tests requested was also sent to participants. The study protocol also provided an approximate hCG content for the human serum samples, as analysed in-house, to enable approximate dilutions to be performed as required by each assay. A copy of the study protocol is provided in Appendix 2.

Table 3. Preparations supplied for immunoassays

Ampoule code	hCG preparation	Ampoule content
Not coded	5th IS for hCG, 07/364	179 IU per ampoule for immunoassay 0.39 nmol per ampoule for immunoassay

Not coded	WHO RR for intact hCG, 99/688	1.88 nmol per ampoule
F, G Duplicates	Candidate 6th IS, 18/244, stored at -20ºC	Nominally 20 µg hCG per ampoule (assumed to be approximately 179 IU per ampoule hCG)
hCGSerum 1 through hCGSerum10	Ten human serum samples	Approximate hCG content was provided to participants in the study protocol (Appendix 3)
H, I, J and K	ATD samples of 18/244 stored at +4ºC, +20ºC, +37ºC and +45ºC, coded	Content assumed identical to 18/244 stored at -20ºC

Abbreviations: International Standard (IS), Reference Reagent (RR)

Methods contributed

Methods contributed are summarised in Table 4 below. Participants were requested to perform the *in vivo* bioassay or immunoassay normally used in house for the measurement of hCG. Bioassay participants were requested to perform at least two independent assays: each independent assay to include all preparations allocated (Table 2) plus any controls if possible/appropriate (assay buffer only/ in house control), with each preparation assayed at three dose levels in the linear part of the dose response curve. Of the 6 bioassay methods contributed, 5 were based on seminal vesicle weight gain in rats and one was based on uteri weight gain in mice.

In order to assess commutability, immunoassay participants were requested to test a set of common dilutions of each of the ampouled preparations provided (Table 3) in parallel with each set of the human serum samples (hCGSerum1 to hCGSerum10). The core dilutions were 1000, 500, 250, 125, 62.5, 31.25, 15.63 and 7.81 mIU/ml, and a guide for their preparation was provided in the assay protocol (Appendix 2). Participants were requested to measure the hCG content of these and the set of human serum samples plus in-house standard/controls, ensuring that a minimum of five points in the linear part of the dose response curve were included. These measurements formed one independent assay, and where possible, participants were requested to perform at least three of these independent assays. Ten laboratories contributed immunoassay data to the study, using a total of 15 different immunoassay methods, and are summarised in Table 4 below.

All participants were requested to provide details of their assay methods, including details of reconstitution and dilution steps made.

Table 4. Assay methods contributed.

Method type	Labs contributing	Assay	HCG immunoassay specificity*
Bioassay	Lab 1, 2, 3, 5, 6	Seminal vesicle weight gain in rats	
Bioassay	Lab 4	Uterine weight gain in mice	
Immunoassay	Labs 2, 7-16	Abbott ARCHITECT, Total β hCG	Total hCG
		Abbott Alinity I, Total β hCG	Total hCG
		Beckman Access2, Total β hCG	Total hCG
		Beckman Dxl, Total β hCG	Total hCG
		Demeditec, Intact hCG	Intact hCG (α and β hCG)
		Diasorin Liaison XL, hCG and β hCG	Total hCG
		DiaSource, Intact hCG	Intact hCG (α and β hCG)
		DRG, β hCG	Total hCG
		Ortho Clinical Diagnostics, Total β hCG	Total hCG
		Roche Elecsys, hCG and β hCG	Total hCG
		Siemens Immulite/Immulite 2000	Total hCG
		Siemens Dimension Exl, HCG	Intact hCG (α and β hCG)
		Siemens Dimension Vista, β hCG	Total hCG
		Siemens ADVIA Centaur, Total (intact and free β) hCG	Total hCG
		Siemens Atellica, Total (intact and free β) hCG	Total hCG
		TOSOH ST AIA-PACK, β-HCG	Total hCG

		TOSOH ST AIA-PACK, HCG	Intact hCG (α hCG)

*Where: Intact HCG detects the presence of α hCG or both α hCG and β hCG in the intact molecule as specified; Total hCG detects the intact hCG molecule and free β chain

Stability assessment

A thermally accelerated degradation (ATD) study was performed in house by immunoassay. The samples used in the ATD study are listed in Table 3 above. Samples were stored at elevated temperatures of +4ºC, +20ºC, +37ºC and +45ºC for 7 months.

Data and Statistical Analysis

Participants performing both bioassays and immunoassays were asked to return all raw assay data in electronic form for central computation at NIBSC, plus participants' own estimates of activity as calculated by the method normally used in their laboratory.

Potency assignment of 18/244 and 07/364 by hCG bioassays

Organ weight data from bioassays were analysed (untransformed) using a parallel line model in order to determine the potency of 18/244 (samples coded F or G) relative to IS 07/364. Calculations were performed using CombiStats v6.1 (CombiStats v6.1, EDQM – Council of Europe, www.combistats.eu) and tests of invalidity (significance of non-linearity and non-parallelism) were performed at the 1% level ($p < 0.01$). Estimates from valid assays were combined to generate semi-weighted geometric mean (GM) estimates (EP Chapter 5.3). Variability between laboratories has been expressed using geometric coefficients of variation (GCV = $\{10^s\text{-}1\}$ x 100%), where s is the standard deviation of the $\log_{10}$ transformed estimates).

Potency assignment of 18/244 and 07/364 by hCG immunoassays

Estimates from immunoassays as reported by the participants were used directly for analysis after correction for dilution factor. Final GM estimates from each laboratory were combined to obtain overall semi-weighted GM estimates (based on intra- and inter-laboratory variation) for each sample. Variability between laboratories has been expressed using geometric coefficients of variation (GCV = $\{10^s\text{-}1\}$ x 100%), where s is the standard deviation of the $\log_{10}$ transformed estimates).

Assessment of commutability

Commutability of the candidate IS, 18/244, and the current IS, 07/364, was assessed using a difference in bias approach. Geometric mean estimates for serum and plasma samples were calculated from reported estimates and estimates relative to both 18/244 and 07/364. Median values, calculated from $\log_{10}$ transformed estimates for analysis in order to achieve approximately constant scatter over the range of concentrations used, were used as the study consensus values for each sample in the analysis. Bias values were calculated as the laboratory GM estimate as % of the study median value for the sample. In order to derive an acceptable bias range (for analysis of this study only), the standard deviation of the log transformed bias values was calculated within each laboratory, and a pooled (median) value, s_P, was calculated across all laboratories. Criteria representing the maximum acceptable bias were then set as $\pm 3s_P$. Reference standards were to be concluded as commutable if the observed difference in bias was within the commutability criteria. For this commutability assessment, the bias for plasma and serum samples has been assumed to be constant over the concentration range used.

Assessment of stability

Samples stored at elevated temperatures (+4, +20, +37, +45ºC) and a reference temperature (-20ºC), were analysed via immunoassay, with the intention of fitting an Arrhenius equation relating degradation rate to absolute temperature assuming first-order decay [8], and thus predict the degradation rates when stored at a range of temperatures.

Results

Data returned for analysis

Bioassays

A total of 38 estimates of potency for the coded duplicates of 18/244-F and 18/244-G were returned for analysis from the 6 laboratories performing bioassays. Valid estimates of potency (18/244 relative to the 5th IS, 07/364) were obtained from all bioassays with the following exceptions which was omitted from further analysis:

- Lab 3, Assay 1, Sample G, showed significant non-parallelism

Immunoassays

A total of 52 immunoassays were returned for central analysis from 11 laboratories that performed 18 different immunoassay methods. All assays included kit controls/standards and met the associated acceptance criteria.

Estimated potency of the candidate IS, 18/244, by bioassay

Potency estimates (IU/ampoule) and laboratory geometric means from the bioassays contributed are summarised in Table 5. Laboratory geometric means for both coded duplicates 18/244-F and 18/244-G ranged from 149.1 IU/amp to 171.1 IU/amp across the 6 laboratories, with an overall geometric mean of 158.5 IU/amp (95% CI 153.5 – 163.8 IU/amp). As the candidate standard was filled to the same specifications as the 5th IS, 07/364, the estimated potency of the candidate standard was expected to be approximately that of 07/364, at 162 IU/amp, and the overall potency estimate of 158.5 IU/amp is in very good agreement with this expected potency. Laboratory means were also in very good agreement with each other, with an inter-lab GCV of 5.6%.

Table 5. Potency estimates (IU/ampoule) for 18/244 from bioassays calculated relative to 07/364

Lab	Assay 1		Assay 2		Assay 3		Lab GM (95% CI)
	F	G	F	G	F	G	
1	151.6	142.1	145.8	152.7	146.0	156.6	149.1 (142.7-155.7)
2	144.8	158.7	167.9	142.5			151.8 (126.0-183.0)
3	161.5	NP	144.3	119.8	154.2	160.5	148.2 (133.0-165.1)
4	147.2	158.5	163.1	167.4			159.2 (146.4-173.1)
5	153.1	170.0	169.6	157.5	154.2	160.0	159.9 (152.2-168.1)
6	187.0 162.9	177.2 185.9	181.3 160.4	165.9 140.0	173.1 165.0	176.4 177.4	171.1 (163.7-178.7)
Overall GM (95% CI) **Inter-lab GCV**							158.5 (153.5 - 163.8) 5.6%

GM: Semi-weighted Geometric Mean
CI: Confidence Interval
GCV: Inter-lab Geometric Coefficient of Variation (%)
NP: Non-parallel

Estimated potency of the candidate IS, 18/244, by immunoassay

The analysis of immunoassay data was based on the results from nominal concentrations 7.8 to 1000 mIU/ml (07/364 and 18/244) or 16.4 to 2100 pmol/ml (99/688) that the participants were requested to run. Dilutional linearity (parallelism with kit standards) was assessed for each standard in each laboratory by calculating the slope of the fitted regression line for log estimated concentration against log nominal concentration. The results are shown in Table A3.1 (Appendix 3) and demonstrate, with the exception of laboratory 15b, broadly acceptable parallelism with all slope ratios in the range 0.92-1.08 (majority in range 0.95-1.05 as highlighted). A greater degree of parallelism was observed between 18/244, 07/364 and 99/688 with all slope ratios in the range 0.97-1.04 and an inter-lab GCV of only 1.2-1.3%. Due to the lack of parallelism evident relative to kit standards, laboratory 15b were excluded from further analysis of the immunoassay results.

Potency estimates (IU/ampoule) for 07/364 (calculated relative to kit standards) and 18/244 (calculated relative to kit standards or 07/364) are summarised in Table 6 and Figures 1 and 2. Laboratory 7 was excluded from overall calculations due to discrepant results when compared to all other laboratories. Relative to kit standards, laboratory geometric means for the 5th IS 07/364 ranged from 161.7 IU/amp - 231.4 IU/amp, with an overall geometric mean of 191.5 IU/amp (95% CI 179.7 – 204.1 IU/amp) (Table 6, Figure 2). The candidate standard (coded duplicates F and G) showed a similar range of reported estimates, with geometric means ranging from 165.4 IU/amp to 244.8 IU/amp and an overall geometric mean of 199.8 IU/amp (95% CI 187.2 – 213.3 IU/amp) (Table 6, Figure 1). Although for both standards, the estimated potency calculated in terms of the kit standards were slightly higher than the expected potency of 179 IU/amp, laboratories were in reasonable agreement, with an inter-laboratory GCV of 13.6% for 07/364 and 14.0% for 18/244, and showed a similar range of potencies for both standards across the laboratory methods. The potency of the candidate standard 18/244 calculated relative to 07/364 (Table 6, Figure 1), however, is in very good agreement with the expected potency, with an overall geometric mean of 185.8 IU/amp (95% CI 183.3 – 188.4 IU/amp) and very good agreement between laboratories, with an inter-laboratory GCV of 3.1%. The similar improvement in agreement between laboratories is illustrated in Figure 2 for the 5th IS, 07/364, when calculated relative to the candidate standard 18/244. The improvement in laboratory agreement when calculating relative to either the 5th or candidate 6th IS is likely a reflection of the range of current calibration status for the immunoassays in this study to either the 3rd, 4th or 5th IS. The data indicates that the candidate standard, 18/244, behaves in a similar manner to the 5th IS 07/364, and that its introduction as the 6th IS will enable continued calibration of immunoassays for hCG.

Table 6. Potency estimates (IU/ampoule) for 07/364 and 18/244 from immunoassays

Lab	Calculated relative to kit standards											Calculated relative to 07/364						
	07/364				18/244							18/244						
					Assay 1		Assay 2		Assay 3			Assay 1		Assay 2		Assay 3		
	Assay 1	Assay 2	Assay 3	Lab GM	F	G	F	G	F	G	Lab GM	F	G	F	G	F	G	Lab GM
2a	178.0	155.5	175.3	169.3	183.7	183.1	163.9	185.7	170.5	178.8	177.4	184.8	184.1	188.7	213.7	174.1	182.6	187.6
2b	214.2	169.1	197.1	192.6	225.1	201.0	179.9	217.3	196.1	192.6	201.5	188.1	168.0	190.5	230.0	178.1	174.9	187.3
7	179.3	147.6	228.7	182.2	145.8	156.8	137.0	132.4	148.2	144.0	143.9	145.6	156.6	166.2	160.6	116.0	112.7	141.3
8	208.1	180.0	202.0	196.3	214.7	226.0	202.6	219.6	239.0		220.1	184.7	194.4	201.5	218.4	211.8		201.8
9	224.9	233.7	227.7	228.8	238.6	237.2	243.3	242.4	234.6	235.1	238.5	189.9	188.7	186.3	185.6	184.4	184.8	186.6
10a	178.3	160.9		169.4	179.7	180.0	161.8				173.6	180.4	180.7	180.0				180.4
10b	170.0	181.3		175.5	171.8	176.6	185.1	192.6			181.3	181.0	186.0	182.8	190.1			184.9
11a	167.0	160.0	158.2	161.7	188.1	181.1	161.3	169.5	168.0	166.7	172.2	201.6	194.1	180.5	189.7	190.2	188.7	190.7
11b	166.7	161.2	159.7	162.5	188.9	181.7	162.9	170.5	168.1	165.6	172.7	202.8	195.2	180.8	189.3	188.4	185.7	190.2
12a	216.1	215.9	208.6	213.5	219.0	227.4	204.7	209.9	223.3	210.8	215.7	181.4	188.3	169.8	174.0	191.7	180.9	180.9
12b	215.5	206.2	201.7	207.7	208.2	214.7	196.0	201.6	215.8	207.0	207.1	172.9	178.4	170.2	175.0	191.5	183.7	178.5
13a	226.4	240.7	227.4	231.4	246.9	240.4	246.3	244.2	248.5	242.9	244.8	195.2	190.1	183.1	181.6	195.6	191.2	189.4
13b	195.5	190.0	191.5	192.3	209.2	207.1	202.0	199.2	195.0	199.5	201.9	191.5	189.6	190.3	187.7	182.3	186.5	188.0
14	152.4	180.2	203.9	177.6	147.2	181.2	193.5	188.7	206.1	206.3	186.0	172.8	212.8	192.2	187.4	181.0	181.2	187.5
15a	165.5	166.3	165.9	165.9	164.8	162.7	164.2	161.0	172.8	166.8	165.4	178.2	175.9	176.7	173.3	186.5	180.0	178.4
16a	235.4	217.7	217.3	223.3	233.5	244.1	222.5	214.5	230.3	244.1	231.2	177.6	185.6	182.9	176.4	189.7	201.1	185.4
16b	232.0	210.2	206.3	215.9	243.0	241.8	215.8	209.4	224.8	238.4	228.5	187.5	186.5	183.7	178.3	195.0	206.9	189.4

Summary statistics (excludes lab 7)	Calculated relative to kit standards		Calculated relative to 07/364
	07/364	18/244	18/244
Overall GM (95% CL)	191.5 (179.7 – 204.1)	199.8 (187.2 – 213.3)	185.8 (183.3 – 188.4)
Inter-lab GCV	13.6%	14.0%	3.1%

GM: Geometric Mean

CI: Confidence Interval

GCV: Inter-lab Geometric Coefficient of Variation (%)

Figure 1. Potency estimates (IU/ampoule) for 18/244 from immunoassays

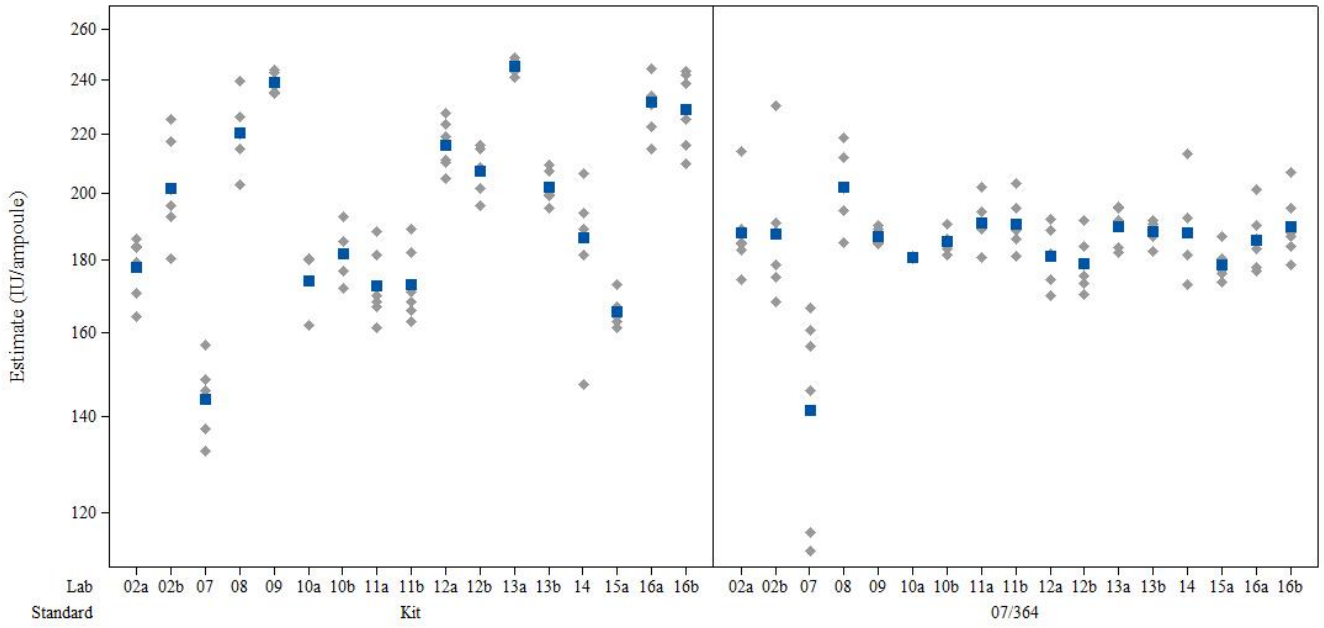
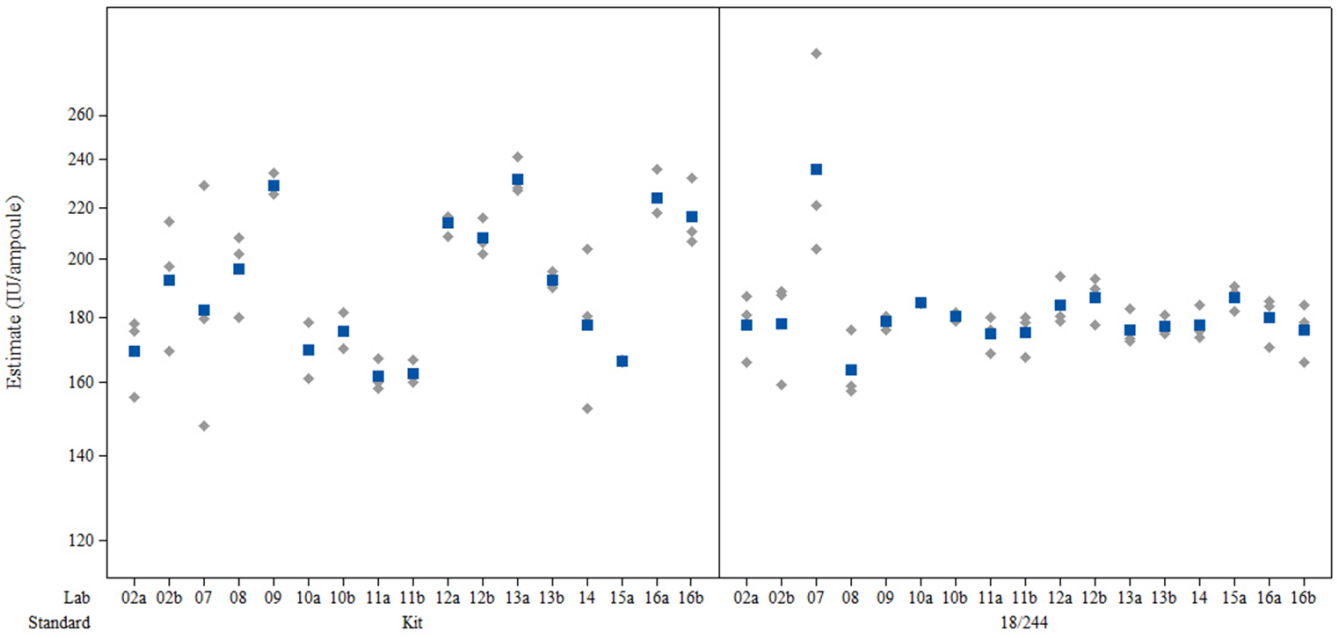


Figure 2. Potency estimates (IU/ampoule) for 07/364 from immunoassays; 18/244 as standard assumed to contain 186 IU/ampoule



Estimated content of the candidate IS, 18/244, by immunoassay

The inclusion of the WHO Reference Reagent for intact hCG, 99/688, also enabled a content to be assigned to the candidate standard in molar units. Content estimates (nmol/ampoule) for 18/244 (calculated relative to 07/364 or 99/688) are summarised in Table 7 and Figure 3.

The geometric mean of laboratory estimates for the candidate standard duplicates F and G, calculated relative to the 5th IS, 07/364, ranged from 0.389 nmol/amp to 0.440 nmol/amp, with an overall geometric mean of 0.405 nmol/amp (95% CI 0.399 – 0.411 nmol/amp). The geometric mean of laboratory estimates for the candidate standard duplicates F and G calculated relative to the WHO RR 99/688, ranged from 0.351 nmol/amp to 0.460 nmol/amp, with an overall geometric mean of 0.413 nmol/amp (95% CI 0.399-0.428 nmol/amp). As shown in Figure 2, laboratories were in good agreement when expressed relative to 07/364 or the WHO RR 99/688, with inter-laboratory GCVs of 3.1% and 7.4% respectively. Indeed, an overall geometric mean for the candidate standard of 0.41 nmol/amp was observed whether the candidate standard content was calculated relative to either 07/364 or 99/688 and is in very close agreement with the expected content of 0.39 nmol/amp.

Figure 3. Content estimates (nmol/ampoule) for 18/244 from immunoassays

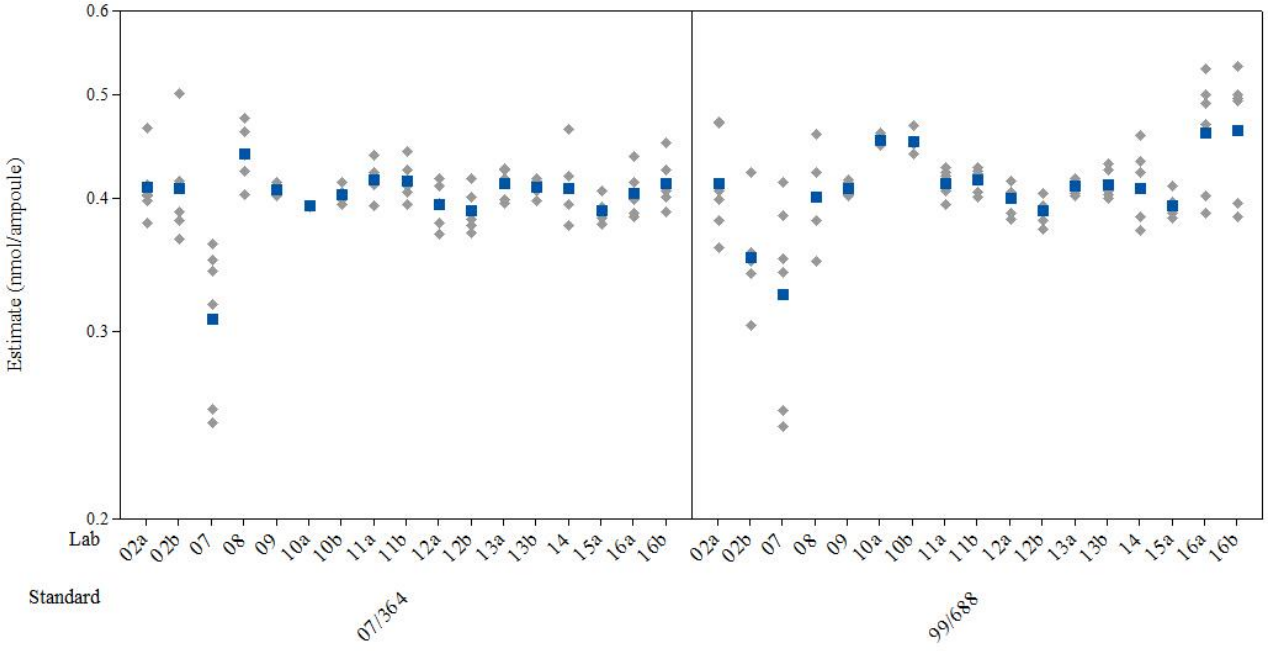


Table 7. Content estimates (nmol/ampoule) for 18/244 from immunoassays

Lab	18/244 calculated relative to 07/364							18/244 calculated relative to 99/688						
	Assay 1		Assay 2		Assay 3		Lab GM	Assay 1		Assay 2		Assay 3		Lab GM
	F	G	F	G	F	G		F	G	F	G	F	G	
2a	0.403	0.401	0.411	0.466	0.379	0.398	0.409	0.472	0.470	0.359	0.406	0.380	0.399	0.412
2b	0.410	0.366	0.415	0.501	0.388	0.381	0.408	0.339	0.303	0.350	0.423	0.355	0.349	0.351
7	0.317	0.341	0.362	0.350	0.253	0.246	0.308	0.385	0.414	0.252	0.244	0.350	0.341	0.325
8	0.403	0.424	0.439	0.476	0.461		0.440	0.381	0.401	0.423	0.459	0.348		0.401
9	0.414	0.411	0.406	0.404	0.402	0.403	0.407	0.416	0.414	0.403	0.402	0.408	0.409	0.409
10a	0.393	0.394	0.392				0.393	0.449	0.450	0.460				0.453
10b	0.394	0.405	0.398	0.414			0.403	0.439	0.452	0.450	0.468			0.452
11a	0.439	0.423	0.393	0.413	0.414	0.411	0.416	0.409	0.394	0.406	0.427	0.423	0.419	0.413
11b	0.442	0.425	0.394	0.412	0.411	0.405	0.415	0.417	0.401	0.405	0.424	0.427	0.421	0.416
12a	0.395	0.410	0.370	0.379	0.418	0.394	0.394	0.387	0.402	0.404	0.414	0.405	0.382	0.399
12b	0.377	0.389	0.371	0.381	0.417	0.400	0.389	0.381	0.393	0.393	0.404	0.389	0.374	0.389
13a	0.425	0.414	0.399	0.396	0.426	0.417	0.413	0.415	0.404	0.417	0.414	0.411	0.401	0.410
13b	0.417	0.413	0.415	0.409	0.397	0.406	0.410	0.406	0.402	0.431	0.425	0.400	0.409	0.412
14	0.377	0.464	0.419	0.408	0.394	0.395	0.409	0.372	0.458	0.433	0.422	0.383	0.384	0.408
15a	0.388	0.383	0.385	0.378	0.406	0.392	0.389	0.392	0.387	0.391	0.383	0.410	0.396	0.393
16a	0.387	0.404	0.398	0.384	0.413	0.438	0.404	0.469	0.490	0.401	0.387	0.500	0.530	0.460
16b	0.409	0.406	0.400	0.389	0.425	0.451	0.413	0.496	0.493	0.395	0.384	0.501	0.531	0.463

Summary statistics (excludes lab 7)	Calculated relative to 07/364 18/244	Calculated relative to 99/688 18/244
Overall GM (95% CL)	0.405 (0.399 – 0.411)	0.413 (0.399 – 0.428)
Inter-lab GCV	3.1%	7.4%

Commutability of the candidate IS, 18/244, and the current IS, 07/364

The commutability of the candidate IS, 18/244, and the current IS, 07/364, with human serum samples was assessed for all methods included in the study, with the exception of Laboratory 15b, omitted due to non-parallelism.

Data used for the assessment of commutability are shown in Appendix 3, Tables A3.2, A3.3 and A3.4. Table A3.2 shows the geometric mean reported estimates for the serum samples in each laboratory. Geometric mean estimates relative to the candidate IS, 18/244, and the current IS, 07/364, are shown in Tables A3.3 and A3.4 respectively. Median values, calculated using log transformed estimates, are shown for each sample and have been used as the study consensus values for each sample in the analysis. Bias values were calculated as the laboratory GM estimate as a % of the study median value for the sample and are shown in Tables 8-9 and Figures 4-5.

The limits for acceptable bias of $\pm 3S_P$ were calculated as described in the Statistical Analysis section, giving ±0.097, or 0.800 to 1.249 on the untransformed scale, i.e. the bias should be demonstrated to be not less than 80.0% and not more than 124.9% to be considered acceptable. Log transformed bias values for estimates relative to a standard are equivalent to the difference in bias between the test sample and the standard, so values within the acceptance criteria can be taken to indicate commutability of the standard with serum and plasma samples for that laboratory.

Table 8. Bias estimates for serum samples relative to 07/364; laboratory GM estimate as % of study median value for sample. Shaded cells indicate that the bias for the estimates of these samples is outside the limits of acceptable bias of 80.0% to 124.9%.

Sample	Lab																
	2a	2b	7	8	9	10a	10b	11a	11b	12a	12b	13a	13b	14	15a	16a	16b
Serum1	100.0%	68.8%	66.7%	54.5%	73.7%	112.0%	110.5%	115.1%	113.3%	107.6%	112.3%	71.0%	86.6%	102.8%	126.3%	77.1%	73.9%
Serum2	76.2%	86.2%	74.7%	124.5%	85.4%	100.0%	103.4%	123.7%	122.7%	102.6%	107.8%	76.1%	79.8%	109.4%	121.0%	76.0%	82.3%
Serum3	72.6%	144.3%	72.5%	111.4%	85.4%	98.0%	100.0%	126.7%	122.8%	101.0%	107.1%	73.2%	70.3%	102.0%	123.7%	72.8%	79.8%
Serum5	119.8%	101.0%	93.2%	109.7%	80.3%	99.3%	108.8%	116.9%	115.8%	99.5%	100.2%	89.3%	89.5%	100.0%	121.3%	72.2%	76.9%
Serum6	100.0%	59.0%	61.8%	91.9%	76.8%	101.8%	104.6%	113.6%	110.7%	102.5%	100.2%	75.6%	85.1%	102.4%	126.4%	76.4%	79.4%
Serum7	100.0%	63.1%	66.2%	45.9%	70.9%	104.0%	103.0%	115.6%	114.0%	108.8%	103.7%	71.8%	82.1%	100.8%	121.5%	82.6%	73.8%
Serum8	87.2%	63.9%	77.8%	100.0%	86.9%	105.2%	107.2%	133.2%	129.4%	111.8%	116.0%	73.5%	74.4%	113.3%	140.8%	89.6%	89.6%
Serum9	101.8%	70.9%	68.1%	94.2%	88.1%	105.3%	112.1%	132.6%	123.2%	111.6%	109.1%	87.2%	85.6%	100.0%	128.6%	82.1%	81.1%
Serum10	77.8%	54.3%	69.9%	92.5%	98.1%	136.6%	144.8%	135.9%	125.5%	102.3%	100.0%	81.8%	78.8%	121.3%	142.7%	98.4%	109.3%

Table 9. Bias estimates for serum samples relative to 18/244; laboratory GM estimate as % of study median value for sample. Shaded cells indicate that the bias for the estimates of these samples is outside the limits of acceptable bias of 80.0% to 124.9%.

Sample	Lab																
	2a	2b	7	8	9	10a	10b	11a	11b	12a	12b	13a	13b	14	15a	16a	16b
Serum1	100.0%	68.9%	88.6%	51.0%	74.1%	114.6%	112.1%	113.3%	111.7%	111.7%	118.0%	70.4%	86.5%	102.8%	132.8%	78.1%	73.1%
Serum2	74.5%	84.5%	96.9%	113.8%	84.0%	100.0%	102.6%	119.0%	118.3%	104.1%	110.8%	73.7%	77.9%	107.1%	124.5%	75.3%	79.7%
Serum3	71.5%	142.5%	94.9%	102.7%	84.7%	98.8%	100.0%	122.8%	119.4%	103.3%	110.9%	71.5%	69.2%	100.6%	128.2%	72.6%	77.9%
Serum5	116.8%	98.7%	120.7%	100.0%	78.7%	99.1%	107.6%	112.1%	111.4%	100.7%	102.7%	86.3%	87.1%	97.6%	124.4%	71.2%	74.3%
Serum6	100.0%	59.1%	82.1%	85.9%	77.2%	104.1%	106.1%	111.8%	109.2%	106.3%	105.3%	74.9%	84.9%	102.4%	132.9%	77.3%	78.7%
Serum7	100.0%	63.2%	87.8%	42.9%	71.3%	106.3%	104.5%	113.7%	112.5%	112.8%	109.0%	71.2%	81.9%	100.9%	127.7%	83.6%	73.1%
Serum8	84.4%	61.9%	100.0%	90.5%	84.5%	104.1%	105.2%	126.7%	123.4%	112.2%	118.0%	70.5%	71.9%	109.7%	143.3%	87.7%	85.9%
Serum9	101.8%	71.0%	90.3%	88.1%	88.5%	107.6%	113.7%	130.3%	121.4%	115.7%	114.7%	86.3%	85.4%	100.0%	135.1%	83.0%	80.2%
Serum10	74.1%	51.8%	88.3%	82.3%	93.8%	132.9%	139.7%	127.2%	117.7%	100.9%	100.0%	77.1%	74.8%	115.4%	142.7%	94.8%	103.0%

Figure 4. Bias in estimates for serum and plasma samples relative to 07/364 (lab GM estimate as % of study median value for sample)

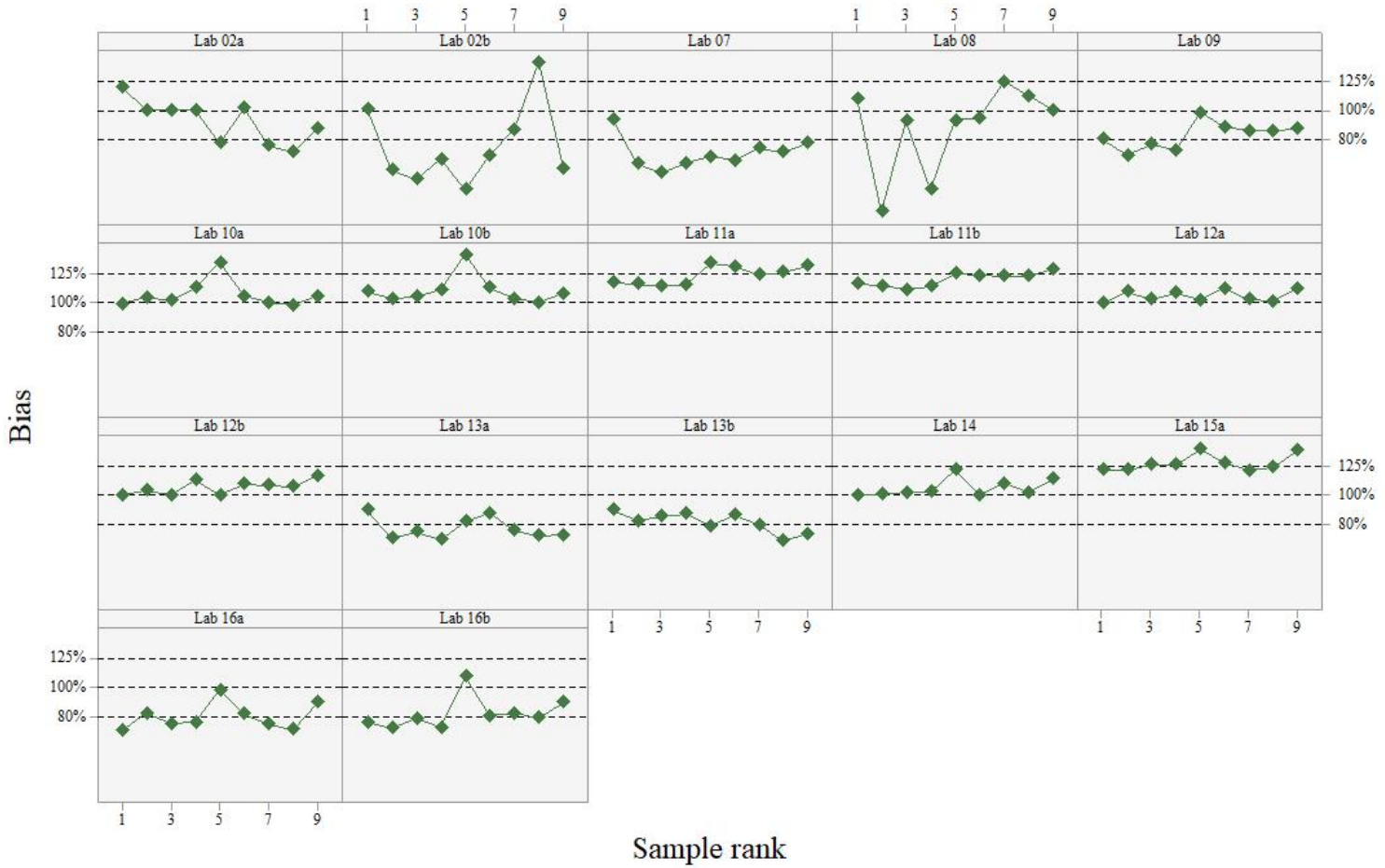
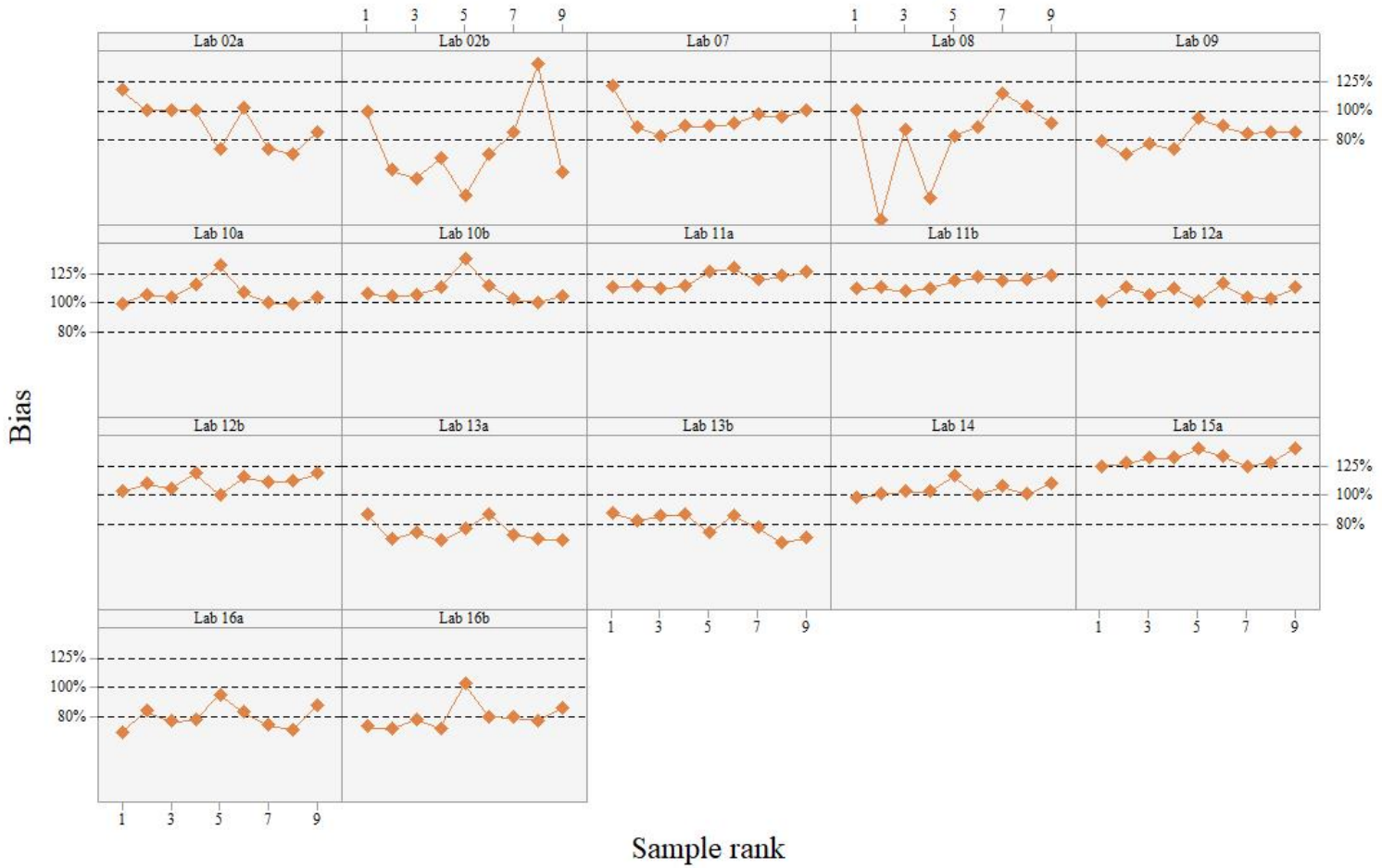


Figure 5. Bias in estimates for serum and plasma samples relative to 18/244 (lab GM estimate as % of study median value for sample)



Bias values for estimates relative to the current IS, 07/364, and candidate IS, 18/244 (Tables 8 and 9 respectively) are equivalent to the difference in bias between the test sample and the standard, so values within the acceptance criteria can be taken to indicate commutability of the standard with serum samples for that laboratory. This data is also represented graphically in Figures 4 and 5. The shaded cells show where the bias for a particular sample relative to 18/244 or 07/364 is outside the acceptable commutability criteria. It is notable that in the majority of labs, the serum samples expressed relative to either the candidate IS or 5th IS preparations behaved in a similar manner. For both standards, 8 out of 17 laboratory methods show bias values for the majority of serum samples are within the acceptance criteria, suggesting that in these 8 methods (Labs 2a, 8, 10a, 10b, 11b, 12a, 12b and 14) both 18/244 and 07/364 are commutable. In a further 3 laboratories (Labs 9, 11a and 13b), both standards showed a more borderline commutability, with just over half the serum samples (5 or 6 from 9 samples) within the acceptance criteria. Of those samples that were outside of the acceptance criteria in Labs 9 and 13b, these were just outside the lower limit of commutability, and in Lab 11a were just above the upper limit of commutability. In one laboratory method, Lab 7, the bias in serum sample estimates for this laboratory are within the acceptable limits when expressed relative to 18/244, but show the majority have negative bias when expressed relative to 07/364. In the remaining 5 laboratories (Laboratories 2b, 13a, 15a, 16a and 16b) the bias in serum sample estimates expressed relative to either 18/244 or 07/364 are outside the limits of commutability for over half the samples, suggesting that both standards are non-commutable with these methods.

Further details of these laboratories are outlined below:

- Lab 2b is the same method as that performed by Lab 8, in which the samples were commutable
- Lab 7 showed negative bias for samples expressed relative to 07/364, but not 18/244. It is notable that this laboratory also showed a lower estimate for 07/364. This laboratory has commented that this may be due to the use of a PBS/BSA diluent used to prepare the standard dilutions compared with a serum-based diluent used for samples.
- Lab 13a showed a negative bias below the lower limit of commutability for the majority of serum samples when expressed relative to the either IS.
- Conversely, for Lab 15a, the majority of serum samples showed a positive bias above the upper limits of commutability when expressed relative to the either IS.
- For laboratories 16a and 16b, there was a tendency to show negative bias, with just over half the samples falling just below the lower limit of commutability when expressed relative to either IS.

It is interesting to note that the direction of bias (positive or negative) of the serum samples is opposite to that of the standards in some laboratory methods, leading to a conclusion the standard is non commutable for that laboratory. For example, Laboratory 15a gave geometric means of 165.9 IU/amp for 18/244 and 165.3 IU/amp for 07/364 (Table 6), which are lower

than the median values of 192.3 IU/amp and 201.5 IU/amp for these standards respectively, whereas serum samples showed a positive bias from study median values (Tables 8 and 9). Conversely, in Laboratories 16a and 16b, a higher geometric mean for the standards was observed (223 IU/amp and 231 IU/amp for 18/244 and 07/364 in Lab 16a, and 215 IU/amp and 228 IU/amp for 18/244 and 07/364 in Lab 16b, Table 6), whereas a negative bias was observed in the serum samples (Tables 8 and 9). Similarly, for Lab 13a, a higher geometric mean of 231 IU/amp and 245 IU/amp was observed for 18/244 and 07/364 respectively (Table 6), whereas the majority of samples gave a negative bias (Tables 8 and 9).

Two further laboratories show this trend (Lab 9 with a higher geometric mean for the standards, and Lab 11a with a lower geometric mean for the standards), but in these cases the majority of serum samples (albeit borderline at 5/9 samples) lay within the acceptable limits of bias. For those laboratories in which the standard was commutable, the standards and samples showed a similar trend and remained within the acceptability limits.

In summary, both standards were commutable with 11/17 methods and non-commutable with 5/17 methods. In 1 method (lab 7), 18/244 demonstrated commutability but 07/364 did not. In those methods in which the standards were non commutable, the standard and serum samples may be recognized differently by these assays, as demonstrated by the difference in bias observed. Importantly, the two reference materials behaved in a similar manner with regard to commutability with the immunoassays included in this study. Taking this together with the results of the immunoassay estimates, the data indicates that the candidate 6th IS, 18/244, is suitable as a replacement for 07/364, for the continued calibration of immunoassays for hCG.

Stability of 18/244

Due to the Covid-19 pandemic, it was not possible to analyse accelerated degradation study samples by bioassay, or in a larger number of immunoassays. Ampoules of 18/244 stored at elevated temperatures of +4ºC, +20ºC, +37ºC and +45ºC for 7 months, were therefore analysed by a single immunoassay method. Potencies of hCG content at each elevated temperature were calculated relative to that of ampoules stored at -20ºC, and the data is summarized in Table 10. No significant loss of activity was observed at elevated temperatures (Table 10) therefore it was not possible to predict the rate of degradation during long-term storage at -20ºC [8]. However, the lack of observed degradation at elevated temperatures does indicate that the candidate IS, 18/244, is highly stable, and therefore suitable for use as an International Standard.

Table 10. Potencies (HCG content by immunoassay) of ATD samples relative to the reference sample (stored at -20ºC).

ATD sample	Relative potency	95% confidence interval
+4 ºC	0.941	0.883 – 1.002
+20 ºC	0.957	0.898 – 1.019
+37 ºC	0.960	0.901 – 1.023
+45 ºC	0.932	0.875 – 0.993

Discussion

The current 5th IS for hCG, 07/364, has been widely used for the calibration of bioassays of hCG for the potency assignment of therapeutic preparations of hCG, as well as for the calibration of immunoassays for hCG. Stocks of this standard are now exhausted, and a replacement standard is urgently required. This study describes the preparation and evaluation of a candidate 6th IS, coded 18/244. The study was designed to enable continuity of value assignment and unitage that had been assigned to the current IS. The candidate standard was therefore assigned an individual value for both bioassays and immunoassays, and the dual unitage of IU and nmol for immunoassay to meet both the current requirement for an International Standard calibrated in IU and to continue to provide an International Standard to enable future calibration of hCG immunoassays in substance concentration[5-6]. The intact hCG Reference Reagent, 99/688, was value assigned by amino acid analysis, a reference method, and can therefore be used to assign a content in molar units to the candidate standard.

There were 6 laboratories who provided organ weight data from 16 valid hCG bioassays. This data was analysed using a parallel line model to determine the potency of 18/244 in terms of the 5th IS, 07/364, giving an overall geometric mean of 158.5 IU/amp (95% CI 153.5 – 163.8 IU/amp) for 18/244 in bioassays for hCG. This potency estimate is in very good agreement with the expected potency of 162 IU/amp, and the 6 laboratories were in very good agreement with each other with an inter-laboratory GCV of 5.6%, giving confidence that the introduction of the candidate standard as the 6th IS for hCG will maintain continuity of calibration for therapeutic preparations of hCG by bioassay.

Immunoassay data from a total of 11 laboratories (17 laboratory methods) were also analysed to determine the potency of 18/244 in terms of the 5th IS, 07/364. Although higher than the expected potency of 179 IU/amp, estimates of the standard preparations 07/364 and 18/244, when calculated relative to kit standards, were in reasonable agreement with overall geometric means of 191.5 IU/amp (95% CI 179.7 – 204.1 IU/amp) and 199.8 IU/amp (95% CI 187.2 – 213.3 IU/amp) and inter-laboratory GCVs of ~14%. When calculated relative to the 5th IS, the overall potency estimate for 18/244 was 185.8 IU/amp (95% CI 183.3 – 188.4) with a very low inter-laboratory GCV of 3.1%, indicating improved agreement in potency estimates of the standard when expressed relative to the 5th IS. A similar improved agreement between laboratories was observed when the 5th IS was expressed relative to the candidate standard. The immunoassay methods performed for this study are currently calibrated using a range of previous international standards: the 3rd IS 75/537, 4th IS 75/589 and 5th IS 07/364. Although all prepared from very similar materials, the 3rd IS, 75/357 and 4th IS, 75/589, were prepared from the same batch of urinary purified hCG material that is known to have contained other hCG isoform impurities, namely nicked hCG and the free β subunit [3-5], whereas the 5th IS 07/364, and now candidate 6th IS, are a preparation of intact hCG that has been highly purified to remove these impurities [3-5]. It is anticipated that the improvement in

agreement between laboratories seen here will be reflected over time as immunoassays are recalibrated to the candidate 6th IS in the future.

The immunoassay data was also analysed to determine the content of 18/244 in nmol/amp, in terms of the WHO Reference Reagent, 99/688. The overall geometric mean potency estimate for 18/244 in terms of either 07/364 or 99/688 was 0.41 nmol/amp (with 95% CI 0.399 – 0.411 nmol/amp relative to 07/364 and 0.399 – 0.428 nmol/amp relative to 18/244). This is in very good agreement with the expected concentration of 0.39 nmol/amp, and laboratories were also in good agreement with GCVs of 3.1% and 7.4% when relative to 07/364 and 18/244 respectively.

Both the candidate standard 18/244 and the 5th IS 07/364 were analysed in comparison with patient samples by immunoassay to determine the commutability of the standards with patient samples in these assays using a difference in bias approach. Of the 17 immunoassay methods contributed, both 18/244 and 07/364 demonstrated commutability in 11 laboratory methods. In one method (Lab 7) 18/244 was commutable with patient samples, but 07/364 was not, although this may be the result of the diluent used to prepare the standards. Of the laboratory methods in which the standards were commutable, these methods were calibrated to either the 3rd, 4th or 5th IS preparations.

In the remaining 5 laboratory methods, the bias in the majority of serum sample estimates reported in terms of both 18/244 and 07/364 were outside the limits of commutability, and therefore both standards were deemed non-commutable in these methods. In 4 of these laboratory methods (13a, 15a, 16a and 16b) there was a difference between the potency estimates of the standard and the estimates of the serum sample concentration: when the standard potency was over or underestimated from the median, the bias of the serum samples expressed relative to the standards was negative or positive respectively. This difference in recognition of the standard and samples may reflect a difference in how these laboratory methods recognise intact hCG in the standards compared with a mixture of hCG isoforms that may be present in the samples [6], which have all been obtained from pregnant individuals in different trimesters. Although these 4 laboratory methods with which 07/364 and 18/244 were non commutable were calibrated to the 3rd and 4th IS, overall the commutability of the 18/244 and 07/364 standards does not appear to depend specifically on the calibration status of the immunoassay (to 3rd, 4th or 5th IS) or on assay specificity (intact or intact + free β hCG), indicating the purified intact hCG used to produce the 5th and candidate 6th IS does not, in itself, have an adverse impact on calibration or commutability. Another potential reason for the standard and serum samples to behave differently in these 4 assays may be the use of other internal calibration methods (e.g. serum panels) that has resulted in a drift away from calibration in terms of the IS.

It is important to note that the commutability criteria for the difference in bias approach have been derived statistically, rather than based on clinical relevance, and are therefore directly related to the bias seen in patient samples in each assay. There is a potential for intra-assay

variability between methods to have had an impact on the statistically derived definitions of commutability. This variability may be influenced, for example, by experimental variation caused by a number of external factors such as assay procedures and dilution buffers for example. Dilution may be of particular relevance for the hCG immunoassay, as due to the very large range of concentrations of hCG during pregnancy, serum sample dilution into the range of the assay is often required. However, it is not possible within the scope of a collaborative study to perform the intra- and inter-method comparisons that would be required to further examine these potential causes.

In summary, the candidate standard 18/244 was shown to exhibit the expected bioactivity and demonstrated similar behaviour to the 5th IS 07/364 in the bioassays for hCG. In immunoassays, the candidate standard also exhibited the expected immunoreactivity and importantly, demonstrated similar behaviour to the 5th IS, 07/364 in the immunoassays contributed. In addition, although prediction of long-term stability during storage at -20ºC was not possible due to no significant loss of activity observed in ATD samples stored at elevated temperature, the lack of observed degradation does indicate that the candidate IS, 18/244, is highly stable and suitable for use as an International Standard.

Taken together, the laboratory estimates from both bioassays and immunoassays contributed indicate that the candidate standard acts in a similar manner to the current IS, 07/364, and is suitable for the continued calibration of hCG bioassays and immunoassays for hCG.

Proposal

The product coded 18/244 is recommended as the 6th International Standard for hCG with an assigned value of 159 IU/amp for bioassay and 186 IU/amp; 0.41 nmol/amp for immunoassay.

Comments from participants

Comment 1 - to provide a more uniform description of immunoassay specificity. Table 4 was updated to reflect this.
Comment 2 – to add a note that bioassay data were not transformed for analysis.
Remaining comments were to minor errors in text and have been incorporated.

Acknowledgements

We gratefully acknowledge the IFCC, who kindly donated the material, and the important contribution of all invited participants, and in particular we would like to thank them for their continued support during the Covid-19 pandemic. We would also like to acknowledge the important contributions of our NIBSC colleagues: the Standardisation Science Group for preparation of trial fill materials, the Biological Services Division for performing hCG bioassays and the Standards Processing Division for the preparation and dispatch of ampouled materials.

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2. Berger and Sturgeon (2014) Pregnancy testing with hCG – future prospects. *Trends Endocrinology and Metabolism, 25 (12): 637 – 648.*

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APPENDIX 1 – Bioassay study protocol

PROPOSED INTERNATIONAL COLLABORATIVE STUDY TO ESTABLISH THE 6TH WHO INTERNATIONAL STANDARD FOR HUMAN CHORIONIC GONDATROPHIN BIOASSAY PHASE STUDY PROTOCOL

INTRODUCTION

Human chorionic gonadotrophin (hCG) is a heterodimeric glycoprotein hormone. Intact hCG molecules are composed of an α and β subunit with a molecular weight of approximately 36 kDa protein. hCG shares an almost identical α subunit with members of the glycoprotein hormone family, LH, FSH and TSH, with the unique β subunit in the respective hormones accounting for their biological specificity [1]. hCG is produced by the developing embryo in pregnancy, synthesised by syncytiotrophoblasts. Its early role is to support the corpus luteum and thereby maintain the levels of progesterone that are required for pregnancy [1].

Human urinary derived hCG is an important biotherapeutic, used to promote the final maturation of ovarian follicles and ovulation for the treatment of infertility and in assisted reproductive technology (ART). Detection of hCG is also an important diagnostic tool: in addition to measurements of serum hCG by immunoassay for the diagnosis of pregnancy and ectopic pregnancy, hCG measurement is also used in pre-natal screening for Down's syndrome and as a marker for other clinical conditions such as gestational trophoblastic diseases and some germ cell tumours[2].

The 5th WHO International Standard (IS) for hCG in ampoules coded 07/364 was established in 2009 [3] and has been widely used for the calibration of therapeutic preparations of hCG by bioassay, in addition to its use as a calibrant for immunoassays of hCG. Stocks of the 5th IS are now almost exhausted and there is an urgent requirement to replace the standard.

A new preparation of hCG has been filled into ampoules (NIBSC code 18/244), following procedures recommended by WHO [4]. It is now intended to initiate an international collaborative study with expert laboratories to aid in the value assignment of the proposed 6th International Standard. The current 5th IS, 07/364, was assigned a value by both bioassay, in units of IU/ampoule by comparison with the 4th IS, 75/589, and by immunoassay in units of both IU/ampoule in comparison with the 4th IS, 75/589, and in nmol/ampoule by comparison with the WHO Reference Reagent for intact hCG, 99/688. We propose to value assign the candidate 6th IS, 18/244, in a similar manner by both bioassay and immunoassay. Human serum samples containing a range of hCG concentrations will be included in the immunoassay phase of the study in order to assess commutability of the candidate standard with native samples.

The aims of the study are therefore:

4) To calibrate the candidate standard 18/244:
    a. Relative to the 5th IS for hCG, 07/364, by *in vivo* bioassay for hCG.

b. Relative to the 5th IS for hCG, 07/364, and the WHO Reference reagent 99/688, by immunoassay

5) To assess the suitability of the candidate preparation 18/244 to serve as the 6th IS for the calibration of therapeutic preparations of hCG by bioassay.

6) To assess the suitability of the candidate preparation 18/244 to serve as the 6th IS for the calibration of immunoassays for hCG.

7) To assess the stability of the preparation of 18/244 by comparison with ampoules stored at elevated temperatures as part of an accelerated degradation stability study in both bioassay and immunoassay systems.

MATERIALS

Preparations supplied to participants in the bioassay phase of the collaborative study

The materials for the bioassay phase of the study, which may be identified only by code letter, are listed in Table 1. Where appropriate, each participant will be allocated a set of core preparations: the 5th IS, 07/364, and two coded preparations of the candidate standard. These coded preparations of 18/244 will be labelled CS655(1) sample F and CS655(1) sample G. A further selection of accelerated thermal degradation samples will be allocated based on assay capacity and sample availability.

Table 1 – preparations for bioassay phase

hCG preparation	Ampoule content
5th IS for hCG, 07/364	162 IU per ampoule for bioassay
Coded preparations of the candidate 6th IS, 18/244, stored at -20°C	Nominally 20 µg hCG per ampoule (assumed to be approximately 162 IU per ampoule)
Accelerated thermal degradation (ATD) samples of 18/244 stored at +4°C, +20°C, +37°C and +45°C, coded	Content assumed identical to 18/244 stored at -20°C

5th WHO International Standard, 07/364

A bulk preparation of intact human chorionic gonadotrophin, highly purified to remove other forms of hCG, was generously donated to the WHO by the IFCC. A portion of the bulk preparation was formulated in a buffer containing 2mg/ml human plasma albumin, 50mM sodium phosphate buffer, pH 7.4 and 10mg/ml trehalose. Aliquots of 1.0 ml (nominally 20µg/ampoule hCG) were then dispensed into glass ampoules, lyophilised and sealed according to procedures recommended by WHO [4] and stored at -20°C in the dark at NIBSC. The bulk hCG preparation and human plasma albumin were tested and found negative for anti-HIV 1 and 2, HBsAg and HCV by NAT assay.

Candidate standard, 18/244

The bulk material used to prepare the candidate standard (with permission from the IFCC) is the same batch as that used to prepare the 5th IS for hCG, 07/364.

A portion of this bulk hCG material was formulated in a buffer containing 2mg/ml human plasma albumin, 50mM sodium phosphate buffer, pH7.4, and 10mg/ml trehalose. Aliquots of 0.5ml (nominally 20 µg/ampoule) were then dispensed into glass ampoules, lyophilised and sealed according to procedures recommended by WHO [4] and stored at -20^{o}C in the dark at NIBSC.
The bulk hCG preparation and human plasma albumin were tested and found negative for anti-HIV 1 and 2, HBsAg and HCV by NAT assay.

Accelerated thermal degradation samples
Ampoules of the candidate standard which have been incubated at -20^{o}C, +4^{o}C, +20^{o}C, +37^{o}C and +45^{o}C for 7 months will be included in the study to assess the stability of the candidate standard.

Handling of the preparations
Upon receipt, ampoules should be stored at -20^{o}C or below until use. Allow contents to reach room temperature before opening. Reconstitute with 1ml volume of appropriate assay diluent (e.g. your own assay buffer, PBS or saline, preferably with 0.05 – 0.1% added to protein such as bovine serum albumin or human serum albumin to reduce adsorption). Leave at room temperature for 10 minutes to fully dissolve.
Appropriate dilutions should be made from this stock using the assay diluent according to the assay protocol used.

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.

TESTS REQUESTED - Bioassay
Participants are requested to carry out the *in vivo* bioassay method for hCG normally in use in their laboratory, and where possible, to perform at least two independent assays. Each independent assay should use fresh ampoules (not a stored aliquot) and should include all the preparations allocated (e.g. 5th IS and coded duplicates of the candidate standard) plus a blank control (assay buffer only) at preferably **no less than three dose levels in the linear part of the dose response curve** in order to provide information on parallelism.
Participants are asked to provide details of the assay methods used, including details of reconstitution and dilution steps made and **all raw assay data** in electronic excel spreadsheet format for central computation at NIBSC. Participants' own estimates of activity as calculated by the method normally used in their laboratory are also requested.

REPORT
A preliminary report will be prepared and circulated to all participants for comment before submission to the Expert Committee on Biological Standardisation of WHO. In the report, participating laboratories will be identified by a laboratory number only and any requests to treat information in confidence will be respected.

REFERENCES

1. Cole, L.A. (2010) Biological functions of hCG and hCG-related molecules. *Reproductive Biology and Endocrinology, 8: 102-115*.
2. Berger and Sturgeon (2014) Pregnancy testing with hCG – future prospects. *Trends Endocrinology and Metabolism, 25 (12): 637 – 648*.
3. Burns, C.B., Moore, M., Sturgeon, C., Hockley J. and Rigsby, P. (2009) WHO International Collaborative study of the proposed 5th International Standard for Chorionic Gonadotrophin. *WHO/BS/09.2107*. *EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION, Geneva, 19 – 23 October 2009.*
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APPENDIX 2 – Immunoassay study protocol

INTERNATIONAL COLLABORATIVE STUDY TO ESTABLISH THE 6TH WHO INTERNATIONAL STANDARD FOR HUMAN CHORIONIC GONDATROPHIN
IMMUNOASSAY PHASE STUDY PROTOCOL

INTRODUCTION

Human chorionic gonadotrophin (hCG) is a heterodimeric glycoprotein hormone. Intact hCG molecules are composed of an α and β subunit with a molecular weight of approximately 36 kDa protein. hCG shares an almost identical α subunit with members of the glycoprotein hormone family, LH, FSH and TSH, with the unique β subunit in the respective hormones accounting for their biological specificity [1]. hCG is produced by the developing embryo in pregnancy, synthesised by syncytiotrophoblasts. Its early role is to support the corpus luteum and thereby maintain the levels of progesterone that are required for pregnancy [1].

Human urinary derived hCG is an important biotherapeutic, used to promote the final maturation of ovarian follicles and ovulation for the treatment of infertility and in assisted reproductive technology (ART). Detection of hCG is also an important diagnostic tool: in addition to measurements of serum hCG by immunoassay for the diagnosis of pregnancy and ectopic pregnancy, hCG measurement is also used in pre-natal screening for Down's syndrome and as a marker for other clinical conditions such as gestational trophoblastic diseases and some germ cell tumours[2].

The 5th WHO International Standard (IS) for hCG in ampoules coded 07/364 was established in 2009 [3] and has been widely used for the calibration of therapeutic preparations of hCG by bioassay, in addition to its use as a calibrant for immunoassays of hCG. Stocks of the 5th IS are now almost exhausted and there is an urgent requirement to replace the standard.

A new preparation of hCG has been filled into ampoules (NIBSC code 18/244), following procedures recommended by WHO [4]. It is now intended to initiate an international collaborative study with expert laboratories to aid in the value assignment of the proposed 6th International Standard. The current 5th IS, 07/364, was assigned a value by both bioassay, in units of IU/ampoule by comparison with the 4th IS, 75/589, and by immunoassay in units of both IU/ampoule in comparison with the 4th IS, 75/589, and in nmol/ampoule by comparison with the WHO Reference Reagent for intact hCG, 99/688. We propose to value assign the candidate 6th IS, 18/244, in a similar manner by both bioassay and immunoassay. Human serum samples containing a range of hCG concentrations will be included in the immunoassay phase of the study in order to assess commutability of the candidate standard with native samples.

The aims of the study are therefore:

1. To calibrate the candidate standard 18/244:
    a. Relative to the 5th IS for hCG, 07/364, by *in vivo* bioassay for hCG.
    b. Relative to the 5th IS for hCG, 07/364, and the WHO Reference reagent 99/688, by immunoassay

2. To assess the suitability of the candidate preparation 18/244 to serve as the 6th IS for the calibration of therapeutic preparations of hCG by bioassay.

3. To assess the suitability of the candidate preparation 18/244 to serve as the 6th IS for the calibration of immunoassays for hCG.

4. To assess the stability of the preparation of 18/244 by comparison with ampoules stored at elevated temperatures as part of an accelerated degradation stability study in both bioassay and immunoassay systems.

MATERIALS

Preparations supplied to participants in the immunoassay phase of the collaborative study

The materials for the immunoassay phase of the study are listed in Table 1. Each participant will be allocated a minimum set of samples consisting of the 5th IS, 07/364, the WHO Reference Reagent 99/688, duplicates of 18/244 and a panel of ten human serum samples. Where assay capacity and sample availability allow, participants will receive an additional set of five coded ampoules to determine the stability of the candidate standard.

Table 1 – preparations for immunoassay phase

hCG preparation	Ampoule content
5th IS for hCG, 07/364	179 IU per ampoule for immunoassay 0.39 nmol per ampoule for immunoassay
WHO Reference Reagent for intact hCG, 99/688	1.88 nmol per ampoule
Coded preparations of the candidate 6th IS, 18/244, stored at -20°C	Nominally 20 µg hCG per ampoule (assumed to be approximately 179 IU per ampoule hCG)
Accelerated thermal degradation (ATD) samples of 18/244 stored at +4°C, +20°C, +37°C and +45°C, coded	Content assumed identical to 18/244 stored at -20°C
Ten human serum samples labelled hCGSerum 1 to hCGSerum 10	0.2ml or 0.3ml aliquots human serum

WHO Reference Reagent, 99/688

A bulk preparation of intact human chorionic gonadotrophin, highly purified to remove other forms of hCG, was generously donated to the WHO by the IFCC. A portion of the bulk preparation was formulated in a buffer containing 2mg/ml human plasma albumin and 50mM sodium phosphate buffer, pH 7.4. Aliquots of 1.0 ml (nominally 2nmol/ampoule hCG) were then dispensed into glass ampoules, lyophilised and sealed according to procedures recommended by WHO [4] and stored at -20°C in the dark at NIBSC.

The bulk hCG preparation and human plasma albumin were tested and found negative for anti-HIV 1 and 2, HBsAg and HCV by NAT assay.

5th WHO International Standard, 07/364

The bulk material used to prepare the 5th IS 07/364 (with permission from the IFCC) is the same batch as that used to prepare 99/688. A portion of the bulk preparation was formulated in a buffer containing 2mg/ml human plasma albumin, 50mM sodium phosphate buffer, pH 7.4 and 10mg/ml trehalose. Aliquots of 1.0 ml (nominally 20µg/ampoule hCG) were then dispensed into glass ampoules, lyophilised and sealed according to procedures recommended by WHO [4] and stored at -20°C in the dark at NIBSC. The bulk hCG preparation and human plasma albumin were tested and found negative for anti-HIV 1 and 2, HBsAg and HCV by NAT assay.

Candidate standard, 18/244

The bulk material used to prepare the candidate standard (with permission from the IFCC) is the same batch as that used to prepare both the previous 5th IS for hCG, 07/364, and the WHO Reference Reagent for intact hCG, 99/688.

A portion of this bulk hCG material was formulated in a buffer containing 2mg/ml human plasma albumin, 50mM sodium phosphate buffer, pH7.4, and 10mg/ml trehalose. Aliquots of 0.5ml (nominally 20 µg/ampoule) were then dispensed into glass ampoules, lyophilised and sealed according to procedures recommended by WHO [4] and stored at -20°C in the dark at NIBSC.

The bulk hCG preparation and human plasma albumin were tested and found negative for anti-HIV 1 and 2, HBsAg and HCV by NAT assay.

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 7 months will be included in the study to assess the stability of the candidate standard.

Human samples

Samples were purchased from TCS Biosciences (Buckingham, UK), BioIVT (West Sussex, UK) and FirstLink (Wolverhampton, UK). Samples are coded hCGSerum 1 to hCGSerum 10. Please note samples **have not been tested** for blood borne pathogens.

Samples labelled hCGSerum1-3 and hCGSerum7-10 are from pregnant donors and contain > 1000mIU/ml hCG. hCGSerum4 is from a non-pregnant donor and contains <1 mIU/ml hCG. hCGSerum5 and 6 are pregnant donor serum diluted in non-pregnant donor serum to provide samples at approximately 130 and 1200mIU/ml respectively. Participants are requested to pre-dilute samples based on the approximate concentrations as necessary and in accordance with their in-house protocols. Please provide all details of pre-dilution steps where necessary. A table of approximate hCG content of each sample is provided in Appendix 1.

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.

Handling of materials

Upon receipt, ampoules should be stored at -20°C or below until use. Allow contents to reach room temperature before opening. Reconstitute with 1ml volume of appropriate

assay diluent (e.g. your own assay buffer, PBS or saline, preferably with 0.05 – 0.1% added protein such as bovine serum albumin or human serum albumin to reduce adsorption). Leave at room temperature for 10 minutes to fully dissolve. Dilutions should be prepared from this stock using your own assay diluent or PBS with protein cover as defined in common test sample concentrations below. A detailed protocol for reconstitution and dilution of the standards is provided in Appendix 1.

Please provide details of the reconstitution of the ampoules and the dilutions used to prepare the test samples.

Upon receipt, test samples of human serum should be stored at -20^{o}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.

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.

TESTS REQUESTED – Immunoassay

Participants are asked to perform **three independent runs** of the assay method (s) in use in their laboratory. An independent run should consist of the measurement of one set of dilutions prepared from each of the ampoules provided (07/364, coded preparations 18/244-F and 18/244-G and 99/688), one set of serum samples (n=10) which have been thawed specifically for that run and assay kit calibrators and controls where applicable. 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 the ampoules coded 18/244 A-E.

Participants are asked to prepare dilutions of the ampouled preparations and measure, in duplicate, the hCG content of these and the content of the serum samples. The test concentrations are described in common test sample concentrations below, and in further detail in Appendix 1. A suggested ELISA plate layout is also provided in Appendix 1.

Common test samples concentrations

In order to assess commutability across different assay methods, participants are asked to measure a minimum number of dilutions of the coded 18/244 preparations, 07/364 and 99/688 that are common to all participants. **These core dilutions are 1000, 500, 250, 125, 62.5, 31.3, 15.6 and 7.8 mIU/ml**. Additional dilutions should be included in order to ensure that a minimum **of five points in the linear part of the dose response curve are measured**.

Data submission

Participants are requested to provide **all raw assay data** in an electronic spreadsheet format for central computation at NIBSC, along with participants' own estimates of activity as calculated by the method normally used in their laboratory, and all details of the

reconstitution and dilution volumes used to prepare the test samples. A suggested reporting table is shown in Appendix 1, Table A4.

REPORT

A preliminary report will be prepared and circulated to all participants for comment before submission to the Expert Committee on Biological Standardisation of WHO. In the report, participating laboratories will be identified by a laboratory number only and any requests to treat information in confidence will be respected.

REFERENCES

1. Cole, L.A. (2010) Biological functions of hCG and hCG-related molecules. *Reproductive Biology and Endocrinology, 8: 102-115*.
2. Berger and Sturgeon (2014) Pregnancy testing with hCG – future prospects. *Trends Endocrinology and Metabolism, 25 (12): 637 – 648*.
3. Burns, C.B., Moore, M., Sturgeon, C., Hockley J. and Rigsby, P. (2009) WHO International Collaborative study of the proposed 5th International Standard for Chorionic Gonadotrophin. *WHO/BS/09.2107. EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION, Geneva, 19 – 23 October 2009*.
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Appendix 1

Assay buffer

For dilution steps below, please use your appropriate assay buffer, ensuring protein cover is provided to prevent adsorption. PBS plus 0.1% bovine serum albumin (BSA) or 0.1% human serum albumin (HSA) may be used as an alternative.

Standard and sample processing

The following provides details on the reconstitution of the standards, along with example dilution steps to generate the working stock solution of the standards for use in the assay. These example dilution steps, or your own in-house dilution methods may be used, but please provide all details of reconstitution and dilution steps taken in your report.

A fresh ampoule of 07/364, 99/688 or coded duplicate of 18/244 (coded CS655(2) 18/244-F or CS655(2) 18/244-G) should be used for each independent run.

A. Reconstitution and dilution of ampoules of 5th IS 07/364 and candidate standard 18/244

1. Before opening, ampoules should be brought to room temperature to minimize moisture uptake.
2. Reconstitute each ampoule in 1 ml diluent. Dilute with a further 9 ml diluent to provide a stock solution that of 17.9 IU/ml.
3. Dilute the stock solution from step 2 to provide a 2000 mIU/ml working stock solution. You may use the method normally in use in your laboratory. (For example, 200 µl of the 17.9 IU/ml stock solution may be diluted 1.59 ml assay buffer).
4. The working stock solution at 2000 mIU/ml will form dilution 1 and the solution from which serial dilutions should be made.
    - Prepare serial dilutions (1:2) of this working stock solution to provide dilutions 2 to 10. Table A1 below provides the full details of the dilutions and their expected concentrations.
    - To enable comparison across different immunoassays at the same dilution point, participants are asked to include the **8 core concentrations (dil 2-9) highlighted in bold** which should be included in **all assays**. If assay space permits, additional concentrations should be included.

Table A1 07/364 and 18/244 dilution table

07/364 dilution	07/364 and 18/244 concentration (mIU/ml)
1	2000
2	**1000**
3	**500**
4	**250**
5	**125**
6	**62.5**
7	**31.3**
8	**15.6**
9	**7.8**
10	3.9

B. Reconstitution and dilution of ampoules of WHO Reference Reagent 99/688

1. Before opening, ampoules should be brought to room temperature to minimize moisture uptake.
2. Reconstitute each ampoule in 1 ml diluent. Dilute with a further 9 ml diluent to provide a stock solution of 0.188 nmol/ml for 99/688.
3. Dilute the solution from step 2 1:5 to provide a stock solution of 0.0376 nmol/ml (e.g. 1ml of the solution from step 2. to 4 ml diluent).
4. Dilute the solution in step 3 to provide a 0.0042 nmol/ml working stock solution. You may use the method normally in use in your laboratory. For example, 200 µl of the 0.0376 nmol/ml stock solution may be diluted to 1.59 ml assay buffer.
5. The working stock solution at 0.0042 nmol/ml, or 4200pM, will form dilution 1 and the solution from which serial dilutions should be made.

i. Prepare 1:2 serial dilutions of this working stock solution to provide dilutions 2 to 10. Table A2 below provides the full details of the dilutions and their expected concentrations.
ii. To enable comparison across different immunoassays at the same dilution point, participants are asked to include the **8 core concentrations highlighted in bold** which should be included in **all assays**. If assay space permits, additional concentrations should be included.

Table A2 99/688 standard dilution table

99/688 dilution	99/688 concentration (pM)
1	4200
2	**2100**
3	**1050**
4	**525**
5	**263**
6	**131**
7	**65.6**
8	**32.8**
9	**16.4**
10	8.2

C. Preparation of serum samples hCGSerum1 to hCGSerum 10

Upon arrival, please store all serum samples at -20^{o}C or below until use. Serum samples should be thawed at room temperature and mixed well prior to assay. Serum samples should be diluted in your own assay buffer according to your standard assay protocol for measurement of hCG. To aid with these dilutions Table A3 below provides approximate hCG content of each sample.

Table A3 Approximate serum sample hCG content

Sample	Approx. hCG content (mIU/ml)
hCGSerum1	1600
hCGSerum2	>10000 (~12000)
hCGSerum3	>10000 (~13000)
hCGSerum4	<1 (negative)
hCGSerum5	130
hCGSerum6	1200
hCGSerum7	1100
hCGSerum8	>10000 (~18000)
hCGSerum9	5000

hCGSerum10	2800

D. Assay design and plate layout

Alongside local standards and controls, each assay/each plate should ideally include **core dilutions of 07/364, 99/688 and coded preparations of the candidate standard 18/244-F and 18/244-G, plus 1 set of serum samples, hCGSerum1-10.** All samples should be tested in duplicate according to the in-house method. In instances where assay capacity is limited and it is not possible to include all preparations alongside the hCGSerum sample set then **only core dilutions of sample 18/244-F** should be included in the set of preparations alongside the core dilutions of 07/364, 99/688 and hCGSerum1-10. For an example, see 96 well ELISA plate layout, Figure A1.

To enable us to gather data regarding inter and intra-assay variability within each laboratory, participants are requested to perform three independent assays with the samples provided. Due to limited stocks, only 0.2 or 0.3 ml of each human serum sample can be provided, and should be diluted in the appropriate assay buffer as per your usual assay protocol.

Figure A1 Suggested plate map for ELISA plate format*

07/364 dil 2	07/364 dil 2	99/688 dil 2	99/688 dil 2	18/244-F dil 2	18/244-F dil 2	Kit cal	Kit cal	hCG2	hCG2	hCG10	hCG10
07/364 dil 3	07/364 dil 3	99/688 dil 3	99/688 dil 3	18/244-F dil 3	18/244-F dil 3	Kit cal	Kit cal	hCG3	hCG3		
07/364 dil 4	07/364 dil 4	99/688 dil 4	99/688 dil 4	18/244-F dil 4	18/244-F dil 4	Kit cal	Kit cal	hCG4	hCG4		
07/364 dil 5	07/364 dil 5	99/688 dil 5	99/688 dil 5	18/244-F dil 5	18/244-F dil 5	Kit cal	Kit cal	hCG5	hCG5		
07/364 dil 6	07/364 dil 6	99/688 dil 6	99/688 dil 6	18/244-F dil 6	18/244-F dil 6	Kit cal	Kit cal	hCG6	hCG6		
07/364 dil 7	07/364 dil 7	99/688 dil 7	99/688 dil 7	18/244-F dil 7	18/244-F dil 7	Kit cntrl	Kit cntrl	hCG7	hCG7		
07/364 dil 8	07/364 dil 8	99/688 dil 8	99/688 dil 8	18/244-F dil 8	18/244-F dil 8	Kit cntrl	Kit cntrl	hCG8	hCG8		
07/364 dil 9	07/364 dil 9	99/688 dil 9	99/688 dil 9	18/244-F dil 9	18/244-F dil 9	hCG1	hCG1	hCG9	hCG9		

*Please note, if assay capacity permits, dilutions of 18/244-F and 18/244-G should **both** be included in each independent run.

E. Data reporting

Estimates of the hCG content of the candidate standard 18/244-F and 18/24-G, the 5th IS 07/364 and the WHO reference Reagent 99/688 should be calculated in comparison with the in-house assay kit standard. Participants are requested to provide details of the assay method used, including dilution steps, together with all the raw data e.g. counts for each sample, in electronic for (excel file) if possible. Please also clarify if/where serum sample dilutions have been taken into account when calculating the results. Participant's own

calculated estimates of insulin concentration are also requested. A sample reporting table is provided below in Table A4.

Table A4 Data reporting table for recording hCG content of test samples

Assay Run No.	***Platform:***				***Method:***			
	RLU/Absorbance Units/Counts				***Reported hCG concentration (mIU/ml)***			
****Sample***	***1***	***2***		***Avg***	***1***	***2***		***Avg***
Baselines								
Kit standard 1								
Kit standard 2								
Kit standard 3								
Kit standard 4								
Kit standard 5*								
99/688 dil 1 etc*								
07/364 dil 1 etc*								
Candidate F dil 1 etc*								
Candidate G dil 1 etc*								
hCGSerum1								
hCGSerum2								
hCGSerum3								
hCGSerum4 etc*								

* Final reporting table should be expanded according to finalised core dilutions, serum sample numbers and assay specific kit standards and controls.

APPENDIX 3

Table A3.1. Fitted slope-ratios from immunoassays for parallelism assessment of different standards; shaded cells are outside range 0.95-1.05

Lab	07/364 vs kit std	18/244 vs kit std	99/688 vs kit std	18/244 vs 07/364	18/244 vs 99/688
2a	0.945	0.952	0.951	1.008	1.001
2b	0.985	0.974	0.935	0.989	1.042
7	1.018	1.024	1.043	1.007	0.982
8	0.966	0.937	0.937	0.970	1.000
9	0.998	0.993	0.996	0.995	0.997
10a	1.040	1.044	1.040	1.003	1.004
10b	1.048	1.052	1.051	1.004	1.001
11a	0.997	0.988	0.987	0.991	1.001
11b	0.990	0.980	0.983	0.990	0.997
12a	0.958	0.966	0.958	1.009	1.008
12b	0.967	0.978	0.969	1.012	1.010
13a	0.964	0.967	0.974	1.002	0.993
13b	0.972	0.964	0.955	0.991	1.010
14	1.078	1.072	1.075	0.995	0.997
15a	1.002	1.021	1.023	1.019	0.999
15b	0.956	0.853	0.866	0.893	0.985
16a	0.931	0.937	0.948	1.005	0.988
16b	0.930	0.924	0.935	0.994	0.988
GCV	4.2%	4.4%	4.6%	1.2%	1.3%

GCV: Inter-lab Geometric Coefficient of Variation (%), excluding lab 15b

Table A3.2 Geometric mean reported estimates (mIU/ml) for serum samples

Sample	Lab																	Median	Sample rank
	2a	2b	7	8	9	10a	10b	11a	11b	12a	12b	13a	13b	14	15a	16a	16b		
Serum1	1577	1234	1132	997	1570	1768	1808	1734	1715	2141	2173	1531	1552	1701	1952	1604	1486	1604	4
Serum2	11779	15171	12428	22325	17846	15473	16582	18269	18216	20006	20463	16085	14015	17748	18344	15513	16234	16582	7
Serum3	11728	26524	12615	20880	18657	15849	16755	19544	19050	20591	21231	16177	12911	17280	19593	15512	16445	17280	8
Serum5	159	153	133	169	144	132	150	148	148	167	163	162	135	139	158	127	130	148	1
Serum6	1448	971	964	1543	1504	1475	1571	1572	1539	1872	1781	1497	1399	1555	1794	1459	1467	1504	3
Serum7	1151	826	820	612	1102	1197	1230	1271	1260	1579	1464	1130	1073	1217	1370	1253	1083	1197	2
Serum8	18143	15118	17429	24119	24424	21896	23125	26450	25831	29334	29605	20900	17584	24728	28704	24582	23777	24119	9
Serum9	5455	4318	3924	5853	6377	5644	6229	6781	6335	7535	7174	6385	5211	5618	6749	5798	5538	5853	6
Serum10	2981	2366	2880	4108	5076	5232	5748	4969	4613	4940	4698	4280	3426	4871	5353	4971	5336	4871	5

Table A3.3 Geometric mean estimates for serum samples relative to 18/244

Sample	Lab																	Median
	2a	2b	7	8	9	10a	10b	11a	11b	12a	12b	13a	13b	14	15a	16a	16b	
Serum1	9.32	6.41	6.21	5.08	6.86	10.44	10.30	10.73	10.56	10.03	10.46	6.62	8.07	9.58	11.77	7.18	6.88	9.32
Serum2	69.58	78.78	68.20	113.73	78.01	91.35	94.46	113.01	112.10	93.70	98.50	69.51	72.88	99.95	110.56	69.47	75.20	91.35
Serum3	69.27	137.74	69.23	106.37	81.55	93.57	95.45	120.89	117.23	96.45	102.20	69.90	67.14	97.32	118.09	69.46	76.17	95.45
Serum5	0.94	0.79	0.73	0.86	0.63	0.78	0.85	0.92	0.91	0.78	0.79	0.70	0.70	0.79	0.95	0.57	0.60	0.79
Serum6	8.55	5.04	5.29	7.86	6.57	8.71	8.95	9.72	9.47	8.77	8.57	6.47	7.28	8.76	10.81	6.53	6.80	8.55
Serum7	6.80	4.29	4.50	3.12	4.82	7.07	7.00	7.86	7.75	7.40	7.05	4.88	5.58	6.85	8.26	5.61	5.02	6.80
Serum8	107.17	78.51	95.64	122.87	106.76	129.28	131.73	163.61	158.96	137.40	142.51	90.31	91.44	139.26	173.00	110.08	110.13	122.87
Serum9	32.22	22.42	21.54	29.82	27.87	33.32	35.48	41.94	38.98	35.30	34.53	27.59	27.10	31.64	40.67	25.96	25.65	31.64
Serum10	17.61	12.29	15.80	20.93	22.19	30.89	32.75	30.73	28.39	23.14	22.62	18.49	17.81	27.43	32.26	22.26	24.72	22.62

Table A3.4 Geometric mean estimates for serum samples relative to 07/364

Sample	Lab																	Median
	2a	2b	7	8	9	10a	10b	11a	11b	12a	12b	13a	13b	14	15a	16a	16b	
Serum1	8.89	6.13	7.87	4.53	6.58	10.18	9.97	10.07	9.93	9.93	10.49	6.25	7.69	9.14	11.81	6.94	6.50	8.89
Serum2	66.38	75.31	86.40	101.45	74.83	89.13	91.44	106.07	105.47	92.74	98.79	65.69	69.40	95.42	110.94	67.09	71.05	89.13
Serum3	66.09	131.66	87.69	94.89	78.23	91.29	92.39	113.47	110.30	95.46	102.50	66.07	63.93	92.90	118.49	67.08	71.97	92.39
Serum5	0.90	0.76	0.93	0.77	0.60	0.76	0.83	0.86	0.86	0.77	0.79	0.66	0.67	0.75	0.96	0.55	0.57	0.77
Serum6	8.16	4.82	6.70	7.01	6.30	8.50	8.66	9.12	8.91	8.68	8.60	6.11	6.93	8.36	10.85	6.31	6.42	8.16
Serum7	6.49	4.10	5.70	2.78	4.62	6.90	6.78	7.38	7.29	7.32	7.07	4.62	5.31	6.54	8.29	5.42	4.74	6.49
Serum8	102.25	75.04	121.16	109.61	102.41	126.13	127.52	153.56	149.56	135.99	142.93	85.36	87.07	132.94	173.59	106.30	104.06	121.16
Serum9	30.74	21.44	27.28	26.60	26.74	32.51	34.35	39.37	36.68	34.93	34.63	26.08	25.81	30.20	40.81	25.07	24.24	30.20
Serum10	16.80	11.75	20.02	18.67	21.28	30.14	31.70	28.85	26.71	22.90	22.68	17.48	16.96	26.19	32.37	21.50	23.36	22.68

APPENDIX 4 - Draft IFU

6th WHO International Standard for human chorionic gonadotrophin
18/244
(version 1, dated XX/XX/XXXX)

1. INTENDED USE

The 6th International Standard for human chorionic gonadotrophin (hCG), coded 18/244, is intended for use in the calibration of bioassays and immunoassays for hCG. It replaces the 5th IS, coded 18/244, stocks of which are now exhausted. [The 6th IS was established by the Expert Committee on Biological Standardisation of the World Health Organisation at its 71st meeting in October 2020].

2. CAUTION

THIS PREPARATION IS NOT FOR ADMINISTRATION TO HUMANS OR ANIMALS IN THE HUMAN FOOD CHAIN:

The preparation contains material of human origin, which has been tested and found negative for HBsAg, HIV antibody, HCV antibody and HCV RNA by PCR.

As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures probably will 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

Each ampoule of the International Standard contains:
159 IU/ampoule for bioassay
186 IU/ampoule for immunoassay
0.41 nmol/ampoule for immunoassay

4. CONTENTS

Country of original of biological material: USA (purified hCG), Italy (human plasma albumin)

Each ampoule contains the residue after freeze-drying of 0.5 mL of a solution that contained:

hCG	approximately 20 µg
Trehalose	10 mg/ml
Sodium phosphate pH 7.4	50mM
Human plasma albumin	2 mg/ml

5. STORAGE

Unopened ampoules should be stored at -20ºC.
Please note: because if 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 AND COLLABORATIVE STUDY

A 300 mg portion of hCG material was dissolved in 7500 ml of a buffer containing 2 mg/ml human plasma albumin, 50 mM sodium phosphate buffer, pH7.4, and 10 mg/ml trehalose. Aliquots of 0.5 ml (nominally 20 µg/ampoule) were then dispensed into glass ampoules, lyophilised and sealed according to procedures recommended by WHO [1] and stored at -20 ºC in the dark at NIBSC

This batch of ampoules, coded 18/244, was evaluated in a collaborative study [2] to 1) assign a potency to the standard in terms of the 5th IS in IU/amp for bioassay, 2) assign a potency to the standard in terms of the 5th IS in IU/amp for immunoassays, 3) assign a content in terms of the WHO Reference Reagent 99/688 in nmol/amp for immunoassay, 4) to assess its immunoreactivity and suitability to serve as an International Standard by immunoassay in comparison with the 1st IS, 07/364, and a panel of human serum and plasma samples and 5) assess its stability by an accelerated thermal degradation study.

The results of the bioassays performed in the study gave an overall potency estimate of 158.5 IU/amp (95% CI 153.5 – 163.8 IU/amp), with all laboratories in very good agreement (GCV 5.6%). Both 18/244 and 07/364 were found to behave in a similar manner in the immunoassays used. When calculated relative to the 5th IS, the overall potency estimate for 18/244 was 185.8 IU/amp (95% CI 183.3 – 188.4) with a very good inter-laboratory GCV of 3.1%. In terms of the WHO Reference Reagent, 99/688, the overall potency estimate for 18/244 was 0.41 nmol/amp (95% CI 0.399 – 0.428 nmol/amp). The commutability of 18/244 with patient samples in the immunoassay methods used was also assessed using a difference in bias approach. In the 17 different laboratory methods performed, 18/244 was commutable with 12 methods. Of the 5 methods with which 18/244 was non commutable, it is notable that the 5th IS was also found non commutable with the same methods. It is important to note that the commutability criteria for the difference in bias approach have been derived statistically, rather than based on clinical relevance. It is not possible, within the confines of a collaborative study, to fully assess commutability of the candidate IS, 18/244, in all

immunoassay methods. It is therefore recommended that manufacturers make their own assessment of the commutability of 19/166 with their assay method.

A thermally accelerated degradation study was also performed. Data from immunoassay analysis of accelerated thermal degradation samples of 18/244 found ?, indicating that the candidate is sufficiently stable when stored at -20ºC to serve as an International Standard.

In conclusion, the candidate IS, 18/244, was shown to behave in a very similar manner to the 5th IS, 07/364, in bioassays and immunoassays of hCG, the latter in terms of both immunoreactivity and commutability. The candidate standard 18/244 was therefore considered suitable as a replacement for 07/364 for the continued calibration of bioassays and immunoassays for hCG.

9. CITATION

In any circumstance where the Recipient publishes a reference to NIBSC materials, it is important that the title of the preparation and any NIBSC code number, and the name and address of NIBSC are cited correctly.

10. LIABILITY AND LOSS

9.1 Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (http://www.nibsc.org/terms_and_conditions.aspx) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference.

9.2 Unless the context otherwise requires, the definitions in the Conditions shall apply.

9.3 Nothing in this document or the Conditions shall limit or exclude NIBSC's liability for fraud or fraudulent misrepresentation, death or personal injury caused by its negligence, or the negligence of its employees. Subject to clause 9.1:

9.3.1 NIBSC shall under no circumstances whatsoever be liable to the Recipient, whether in contract, tort (including negligence), breach of statutory duty, or otherwise, for any loss of data, loss of profit, loss of business or goodwill, or any indirect or consequential loss or damage suffered or incurred by the Recipient arising in relation to the supply of the Materials or the use, keeping, production or disposal of the Materials or any waste products arising from the use thereof by the Recipient or by any other person; and

9.3.2 NIBSC's total liability to the Recipient in respect of all other losses arising under or in connection with the Contract, whether in contract, tort (including negligence), breach of statutory duty, or otherwise, shall in no circumstances exceed 100% of the fees paid to NIBSC for the Materials.

9.4 The Recipient shall defend, indemnify and hold NIBSC, its officers, employees and agents harmless against any loss, claim, damage or liability including reasonable legal costs and fees (of whatsoever kind or nature) made

against NIBSC which may arise as a result of the wilful act, omission or negligence of the Recipient or its employees, the breach of any of the terms of the Contract, or the use, keeping, production or disposal of the Materials or any waste products arising from the use thereof by the Recipient or on its behalf.

11. REFERENCES

[1] WHO recommendation for ref preparation
[2] WHO ECBS report to be referenced

12. MATERIAL SAFETY SHEET

Physical properties (at room temperature)			
Physical appearance	White powder		
Fire hazard	None		
Chemical properties			
Stable	Yes	Corrosive:	No
Hygroscopic	No	Oxidising:	No
Flammable	No	Irritant:	No
Other (specify)	Contains material of human orign		
Handling:	See caution, section 2		
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 a virucidal agent. Rinse area with a virucidal agent followed by water.

Absorbent materials used to treat spillage should be treated as biologically hazardous waste.