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WHO International Collaborative Study of the Proposed 4th International Standard for Prolactin, Human

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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 **16 September 2016** 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 C M Nübling** at email: nueblingc@who.int.

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Summary
The World Health Organization (WHO) Expert Committee on Biological Standardization (ECBS) has recognized (2015) the need for a replacement International Standard (IS) for prolactin for the calibration of immunoassays to measure prolactin in human serum and plasma. We report here, the calibration of a candidate standard for prolactin by immunoassay in terms of the current, 3rd IS for Prolactin, human, 84/500, by an international collaborative study carried out by ten laboratories in seven countries. The unweighted geometric mean of the laboratory estimates of the prolactin immunoreactivity of the candidate standard, coded 83/573, is 67.2 mIU per ampoule (GCV = 8.1%, n=18) in terms of the current standard 84/500. The results of this study also indicate that the candidate standard appears sufficiently stable, on the basis of a thermally accelerated degradation study, to serve as an IS. The study included an assessment of the impact of the new standard on the routine measurement of prolactin in human serum samples. Nine of the ten laboratories contributed data during the collaborative study through the concomitant measurement of the prolactin content of sixteen human serum samples. The results suggest that the candidate standard is suitable for the continued calibration of immunoassay methods for the measurement of prolactin.

Therefore, it is proposed that the candidate preparation in ampoules coded, **83/573** is established as the **4th IS for prolactin, human, for immunoassay** with an assigned content of **67 mIU** per ampoule.

WHO are unlikely to receive another donation of human pituitary-derived prolactin for the eventual replacement of the 4th IS for prolactin once it has been established. Therefore, to provide information for future prolactin standardisation strategies, the WHO Reference Reagent for human, recombinant prolactin, 97/714, was included in the study. The estimated content of 97/714 in terms of the current IS, 84/500, was determined as 1315.0 mIU/amp (GCV 20.7%, n=18) which is comparable both with the assigned value of 1400 mU/amp and to the value obtained by immunoassays only in the original collaborative study of 2001 of 1265.9 mU/amp (GCV 27%, n=16). The variability in the mean prolactin concentration of human serum samples measured in terms of the recombinant standard was comparable to the variability currently observed between methods suggesting that the introduction of a recombinant IS may not have a significant impact on the clinical measurement of serum prolactin. However, the future introduction of the recombinant preparation may require a more detailed assessment of its commutability with human serum samples, since apparently a degree of non-commutability was demonstrated with these samples in two of the methods included in the current study.

Introduction
Secreted primarily by the anterior pituitary gland, prolactin was first identified by its action of stimulating lactation and its involvement in other reproductive processes and behaviours. Subsequently, homeostatic roles have been proposed such as osmoregulation, immune regulation and angiogenesis, and additional secretory sites have also been identified including the brain, mammary gland, lymphocytes and the placenta and decidua in pregnant females [1]. Clinically, prolactin is measured by immunoassay for the purposes of evaluating pituitary gland function, diagnosing and monitoring prolactinomas and investigating galactorrhoea, visual disturbance and headache.

The 3rd IS for prolactin, human, coded 84/500, was established by the WHO Expert Committee on Biological Standardization (ECBS) in 1988 and has been widely used for the calibration of immunoassays to measure prolactin in serum and plasma. This preparation contained prolactin purified from human pituitary glands. It was calibrated as part of the collaborative study to establish the 2nd IS for prolactin, coded 83/562, and was established upon exhaustion of stocks of the 2nd IS [2,3]. Stocks of the 3rd IS are low and there is a requirement to replace the standard.

In the absence of a donation of human prolactin with which to manufacture a new WHO IS, a batch of ampoules, coded 83/573, has been evaluated in this collaborative study to determine its immunoreactivity using current methods and to assess its suitability to serve as an IS. In addition, to inform and promote discussion of future options for the calibration of prolactin assays, the WHO Reference Reagent for Prolactin, human, recombinant, coded 97/714 [4], was included in the collaborative study.

Finally, participants were provided with human serum samples containing a range of normal and elevated prolactin concentrations.

The aims of this study were therefore:

1. To calibrate the candidate standard, 83/573, in terms of the 3rd IS, 84/500, by immunoassay.

2. To assess the suitability of the candidate standard, 83/573, to serve as the 4th IS for the calibration of immunoassays of prolactin.

3. To determine the stability of the candidate standard, 83/573, by comparison with ampoules stored at elevated temperatures as part of an accelerated degradation stability study.

4. To assess the immunoreactivity of a recombinant source of prolactin, 97/714, in current prolactin immunoassays, to inform future standardization strategies.

Participants

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

Table 1: List of participants in order of country

BELGIUM	Michel Hars Diasource S.A., Rue du Bosquet 2,1348 Louvain-la-Neuve.
BELGIUM	Dr Stefaan Marivoet Tosoh Europe N.V., Transportstraat 4, 3980 Tessenderlo.
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USA	Dr Craig Hixson Siemens Healthcare Division, 700 GBC Drive, Newark, DE 19702.
USA	Ryan Masica Beckman Coulter, Mail Stop R-530-B, 1000 Lake Hazeltine Drive, Chaska, MN 55318-1084.

Bulk materials and processing

A bulk preparation of highly purified pituitary extract containing 1.06 mg/ml prolactin was generously donated to the WHO by KabiVitrum through the good offices of Dr L. Fryklund. As described in [2], 12 ml of the bulk preparation was dissolved in 4 L of a solution containing 0.1% (w/v) human serum albumin, 0.5% (w/v) lactose, 0.01M ammonium formate to give a solution containing approximately 3.2 µg/ml human prolactin (based on the stated protein concentration of the donated bulk). The solution was distributed into ampoules as 1.0 ml aliquots. The ampoule contents were freeze-dried, secondarily desiccated and sealed under nitrogen. Ampoules were stored at -20°C.

Characterization of the freeze dried product

A total of 3840 ampoules of human prolactin, coded 83/573 were produced, of which there are 1975 remaining. Checkweights measured during filling demonstrated a mean fill weight of 1.002 g with a range of ± 0.003g. Estimates of moisture ranged from 2.6 – 12.8 µg/amp (mean 6.5 µg/amp; n=8) [2]. Using currently available tests, ampoules of the candidate standard, 83/573, were tested at NIBSC and found negative for the presence of HIV1 and HIV2 by serological assay, hepatitis B surface antigen (HBsAg) by serological assay and Hepatitis C (HCV) by molecular assay.

Collaborative study for the calibration of 83/573

The collaborative study was organised by NIBSC. All participants were provided with ampoules of the current and candidate IS, 84/500 and 83/573, respectively, and ampoules of the WHO Reference Reagent for Prolactin, human, recombinant, coded 97/714. Thermally-accelerated degradation samples were available in limited numbers and were distributed to participants based on assay capacity and sample availability. A study protocol, shown in Appendix 2, and instructions for use were provided with the samples.

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

Table 2: Ampouled preparations provided to participants in collaborative study.

Code	Preparation	Content
Not coded	3rd IS for prolactin, human (84/500)	53 mIU per ampoule
C	Candidate 4th IS for prolactin, human (83/573) stored at -20°C	Nominally, 63 mIU per ampoule
A, B, D, E	Accelerated thermal degradation (ATD) samples of 83/573 stored at +4°C, +45°C, +20°C and +37°C (respectively) for 10 months	Content assumed to be identical to 83/573 stored at -20ºC
Not coded	WHO Reference Reagent for Prolactin, human, recombinant (97/714)	1400 mU per ampoule

In addition to the ampouled preparations, participants were provided with a panel of sixteen human serum samples coded S1 to S16 which, when measured in-house prior to the study, contained 200 – 5000 mIU/L prolactin. Serum samples were obtained from either: (i) First Link UK (Wolverhampton, UK) and were certified non-reactive for HIV 1/2, HIV p24, HBsAg, anti-HCV and Syphilis TP by the supplier or, (ii) Equitech Enterprises Inc. (Kerrville, USA) and were certified non-reactive at the donor level for RPR (Syphilis), HBsAg, HBcAB, HCV, HIV I/II Ab, HBV, HCV and HIV PCR (NAT) and HTLV I/II. In the absence of serum from prolactinoma patients, sera from women in the third trimester of pregnancy were used to provide samples with a high prolactin concentration.

The assays provided by each laboratory are shown in Table 3.

Table 3: Immunoassay methods contributed

Lab	Method	Details of calibration of kit/method	Total Number of assays	Number of assays with ATDs	Number of assays with serum samples	Number of serum samples provided
1	Automated chemiluminescent immunoassay	Calibrated to 84/500	2	0	2	16
2a	In house, ^{125}I-radioimmunoassay	Prepared from recombinant human prolactin, calibrated against 84/500	2	0	2	16
2b	Automated chemiluminescent immunoassay	Calibrated to 84/500	2	0	2	16
3	Automated chemiluminescent immunoassay	Calibrated to 84/500	4	4	4	16
4	^{125}I-Immunoradiometric assay	Calibrated to 84/500	2	0	2	16
5	Automated one-step immunoassay	Calibrated to WHO 2nd IS for Prolactin, 83/562. Reported in terms of 84/500	4	2	4	16
6a	Automated chemiluminescent immunoassay	Calibrated to 84/500	2	2	2	16
6b	Automated chemiluminescent immunoassay	Calibrated to 84/500	2	2	2	16
7a	Automated magnetic particle chemiluminescent immunoassay	Calibrated to 84/500	2	0	0	0[1]
7b	Automated chemiluminescent immunoassay	Calibrated to 84/500	2	0	0	0
7c	Automated chemiluminescent immunoassay	Calibrated to 84/500	2	0	0	0
7d	Automated chemiluminescent immunoassay	Calibrated to 84/500	2	0	0	0
7e	Automated electrochemiluminescent immunoassay	Calibrated to 84/500	2	0	0	0
7f	Automated chemiluminescent immunoassay	Calibrated to 84/500	2	0	0	0
7g	Enzyme linked immunosorbent assay	Traceable to a national standard for human Prolactin (which is calibrated in terms of 84/500)	4	0	0	0
7h	^{125}I-radioimmunoassay	Traceable to a national standard for human Prolactin (which is calibrated in terms of 84/500)	2	0	0	0
7i	Time-resolved fluoroimmunometric assay	traceable to a national standard for human Prolactin (which is calibrated in terms of 84/500)	2	0	0	0
8a	Automated chemiluminescent immunoassay	Calibrated to 84/500	2	2	2	16
8b	Automated chemiluminescent immunoassay	Calibrated to 84/500	2	2	2	16
9	Automated enzyme-linked fluorescence immunoassay	Calibrated to 84/500	2	2	2	16
10	Automated chemiluminescent immunoassay	Calibrated to 84/500	3	1	2	16

[1] Participant 7 was unable to receive serum samples due to import restrictions.

Assessment of homogeneity of content of 83/573
An assessment of the homogeneity of content of 83/573 was assessed by Laboratory 10 by the reconstitution, dilution and measurement of twelve ampoules of 83/573 at six dilutions in duplicate using an automated chemiluminescent immunoassay method. A fresh ampoule of 84/500 was reconstituted and measured at six dilutions in duplicate at the beginning and end of the run. Content was calculated in terms of the current IS, 84/500, by estimation of the geometric mean and GCV% as described below.

Statistical analysis

Assessment of immunoreactivity of 83/573, 97/714 and current WHO IS, 84/500, in terms of kit standards
Estimates in mIU at each sample dilution as reported by participants were used directly in the analysis to calculate the immunoreactivity of the candidate IS (83/573), the current IS (84/500) and the WHO Reference Reagent for recombinant, human prolactin (97/714) relative to kit standards. A geometric mean of results corrected for dilution was calculated for each sample in each assay run, excluding any dilutions not on a linear section where necessary. Dilutional linearity (parallelism with kit standard) was concluded if a linear relationship with a fitted slope between 0.91 and 1.10 was observed for log estimated concentration against log dilution. Where this was not observed, no estimate relative to kit standard has been reported.

Assessment of immunoreactivity of 83/573 and 97/714 in terms of the current WHO IS, and 97/714 in terms of 83/573.
Final immunoreactivity estimates relative to kit standards from each assay run were used directly to determine relative activities of the study samples, following verification of acceptable parallelism. Where the ratio of fitted slopes (log estimated concentration against log dilution) for the samples under consideration was not in the range 0.91 to 1.10, non-parallelism was concluded and no relative estimate has been reported. Where one or both samples were non-parallel against the kit standard, a parallel-line model [5] was used to estimate relative immunoreactivity. This method was also used in the homogeneity assessment of 83/573 described above.

Combination of results
Results from all valid assay runs were combined to generate unweighted geometric means for each laboratory and these laboratory means were used to calculate overall unweighted geometric mean immunoreactivity estimates. Variability between laboratories has been expressed using geometric coefficients of variation (GCV = $\{10^{s}-1\}\times100\%$ where s is the standard deviation of the $\log_{10}$ transformed estimates).

Assessment of stability
The relative immunoreactivities of the accelerated thermal degradation samples were used to fit an Arrhenius equation relating degradation rate to absolute temperature assuming first-order decay [6], and hence predict the degradation rates when stored at a range of temperatures.

Assessment of commutability
Two approaches to assessing the commutability of the standards 84/500, 83/573 and 97/714 have been applied. Firstly, the level of inter-laboratory variability in the reported estimates relative to kit standards for each human serum sample was compared to the level observed when serum sample results were expressed relative to a common standard. Secondly, a

comparison of the bias observed for serum samples to that observed for the standards was made using elements of the approach reported by Korzun et al., 2015 [7]. For the purpose of this approach, all reported results were log transformed for analysis and consensus mean values for each serum sample were taken as the mean of laboratory means. Bias values for each serum sample within each laboratory were calculated as the difference between the laboratory mean result and the study consensus value for that sample. The mean (b_P) and standard deviation (s_P) of the serum sample bias values were calculated for each laboratory and a pooled value of the standard deviation (s) used to define commutability criteria as $\pm 2s$ for the maximum allowable difference in bias for a commutable reference standard. Consensus values for different dilutions of the standards were calculated in the same way and the bias (b_R) determined for each dilution in each assay run. A measure of commutability of the standard for each laboratory was calculated as the difference in bias $d = b_P - b_R$.

Results

Data returned for analysis

Data were contributed by 10 laboratories who performed in total 49 individual assays from 21 methods (16 different methods) which provided 18 sets of results for the calibration of the candidate IS, 83/573, Sample C. Geometric mean immunoreactivity estimates for the current IS 84/500, the candidate standard, 83/573 and the WHO Reference Reagent for recombinant human prolactin 97/714 calculated relative to the kit standards, the current IS 84/500 or the candidate IS 83/573 are summarized in Table 4 and Figures 1 and 2. Results from individual assays are given in Appendix Table A1.1 and A1.3.

Assay validity

The majority of assays met linearity and parallelism criteria, thus allowing valid estimates of relative immunoreactivity to be calculated. Tables showing individual assay results, slopes and slope ratios are included in Appendix Tables A1.1 – A1.4 for information. Instances of non-linearity or non-parallelism, accounting for less than 10% of cases are indicated in Tables A1.1 and A1.3. These include Method 2a where 83/573 was not parallel with 84/500 and Method 7h where both non-linearity and non-parallelism were observed. Data from these methods were therefore excluded from the analysis of immunoreactivity where appropriate, although data from Method 2a were included in the assessment of reference standard commutability in this method.

Data from Laboratory 6 showed low recovery from all ampouled preparations (approximately 50% of the expected content) although the measured prolactin content of the serum samples by both methods performed by this participant were comparable to other laboratories. Method 6b was also performed by Laboratory 7 (method 7e) where the expected content of the current standard, 84/500, was recovered (Appendix Table A1.1). Without an obvious explanation of the reason for the low recovery seen by Laboratory 6, these data sets were not included in the analysis.

Immunoreactivity of 83/573 and 97/714 calculated relative to 84/500

As shown in Table 4 and Figure 1, analysis of the candidate IS, 83/573, in terms of the current IS, 84/500, gave a geometric mean immunoreactivity estimate of 67.2 mIU/amp (n=18; GCV 8.1%). The geometric mean immunoreactivity estimate of the WHO Reference Reagent for human, recombinant Prolactin, 97/714 in terms of the current IS, 84/500 was determined as 1315.0 mIU/amp (n=18; GCV 20.7%) as shown in Table 4 and Figure 2.

Stability of 83/573
Estimates of the immunoreactivity of ampoules stored at elevated temperatures for a period of 10 months are summarized in Table 5. Analysis showed a predicted loss of immuno-reactivity per year of 0.007% when stored at -20°C.

Homogeneity assessment of 83/573
Using one automated chemiluminescent immunoassay method, dilutions prepared from twelve ampoules of the candidate IS, 83/573, and one ampoule of the current IS, 84/500, were measured in a single assay. This provided an estimate of the between ampoule variability GCV of 5.4% for the content, reconstitution, dilution and measurement of the candidate IS. The method-specific, geometric mean estimate of immunoreactivity of 83/573 in terms of 84/500 was 72.7 mIU/amp which is comparable with other estimates obtained by Participant 10 as shown in Table A1.3. Individual assay estimates from the twelve ampoules are shown in Appendix A1.6

Assessment of the prolactin concentration of human serum samples and reference standard commutability
Sixteen human serum samples with a range of prolactin concentrations measured in-house as 200 - 5000 mIU/L were supplied to nine laboratories. Eight laboratories contributed data from ten methods to the assessment of commutability and geometric mean estimates in mIU/L determined relative to kit standards are shown in Table A.1.5. A summary of inter-laboratory GCV values for each serum sample calculated using kit standards or a common standard (84/500, 83/573 or 97/714) is shown in Table 6.

For the difference in bias approach, dilutions of the reference standard to obtain target concentrations of 125, 250, 500, 1000 and 2000 mIU were used in the analysis. Lab 2a was excluded as an outlier from the calculation of mean consensus values for both serum samples and standards, but included in the assessment of bias difference. Commutability criteria were calculated as described above giving ±0.12 as the maximum allowable difference in bias between standards and serum samples. Estimated differences in bias for each reference standard dilution in each assay run are shown in Figures 3i – iii and the proportion concluded to be commutable is summarised in Table 7.

Summary

Candidate Standard, Prolactin, human pituitary, 83/573
International reference materials for the calibration of prolactin by immunoassay have been available since the late 1970s when the first IRP for Prolactin for Immunoassay, coded 75/504 was established [8]. The candidate IS, 83/573, was first assessed in a collaborative study in 1986 alongside 83/562 (2nd IS) and 84/500 (3rd IS) [2]. In this study, a value of 53 mIU was assigned to the 2nd IS, 83/562 in terms of the IRP using data from five laboratories who held stocks of this exhausted preparation. Seventeen laboratories performed radioimmunoassays comparing the three preparations (83/562, 84/500 and 83/573) to each other which determined that the content of 83/573 was 1.2 times the prolactin-like activity of 83/562 and it was therefore assigned a value of 63 mIU/amp. Coded duplicates of each preparation were included in the study and the report concluded that estimates of the coded duplicate of 83/573, 'Y', in terms of 83/573 did not suggest any consistent differences between the two estimates.

The candidate IS, 83/573, was also evaluated in 2001 in the collaborative study to establish the WHO Reference Reagent for human, recombinant prolactin, 97/714 [4]. Eleven laboratories performed immunoassays and the variety of assays provided (ELISA, IFMA, ICLMA, IRMA, RIA) reflects the development of multiple methods between the collaborative studies reported in 1986 and 2001. Five laboratories also performed cell-based proliferation bioassays. Although the aim of the 2001 study was not to alter the assignment of 83/573 which had no official status, the value determined by immunoassay in terms of 84/500 was 58.7 mIU/amp (GVC 16%, n=16) and by bioassays 77.1 mIU/amp (GCV 14%, n=6). In this study, the minimal level of variability present in the assay systems was assessed by including coded duplicates of the current IS, 84/500. Potency estimates of the coded duplicates relative to one another ranged from 0.60 – 1.58 with a geometric mean of 0.97 (GCV 10%). Pooled within-laboratory (between assay) variability was determined as 8% (GCV).

The aim of the collaborative study reported here was to assign a value to 83/573 using current immunoassay methods and to assess the potential impact of introducing the new standard by the concomitant measurement of sixteen human serum samples. Estimates for the content of 83/573 were in good agreement (Table 4, Figures 1 and 2) and the content in terms of the current IS, 84/500, was determined as 67.2 mIU per ampoule (GCV, 8.1%; n=18). The low value for between-laboratory variation strongly suggests that, despite being a legacy preparation that was filled according to the quality standards of the time, 83/573 exhibits a homogeneity of recovered content that is acceptable for its use as an IS. A further evaluation of recovered content in twelve ampoules of 83/573 tested in a single run by an automated chemiluminescent immunoassay demonstrate a between-ampoule GCV% of 5.4% which is lower than the intra-assay variability of 10% found for coded duplicates of the current IS, 84/500, in the collaborative study reported in 2001 [4].

Diagnostic standards require an assessment of commutability to evaluate if there is equivalence in the relationship between the assay response to dilutions of the reference material and the response to representative clinical samples. To assess this, all participants were provided with a set of sixteen human serum samples for each of the assays offered to NIBSC. Data from eight laboratories using ten methods were included in the analysis of commutability.

First, calculation of the concentration of the serum samples in terms of the kit standards, current IS or candidate IS allowed a broad view of the impact on the laboratory mean variability of prolactin measurements upon introduction of the candidate IS. As can be seen in Table 6, expressing the concentration of the serum samples in terms of the current IS, 84/500, or the candidate IS, 83/573, improved the GCV% both for each serum sample and for the overall pooled GCV from 22% (kit standards) to 19% and 16% respectively for 84/500 and 83/573, suggesting that the introduction of the candidate standard would not increase the variability of serum prolactin measurements and may harmonize prolactin measurements reported in International Units.

Secondly, to assess if the candidate IS, 83/573, exhibits a degree of commutability with human serum samples that is comparable with the current IS, 84/500, a difference in bias approach was used [7]. By this method, the bias from mean consensus values for each dilution of the reference material is compared to that observed for the serum samples for each laboratory/method. Figures 3i-iii illustrate that the majority of reference preparation dilutions for each method are within limits of ±0.12 defined as the maximum allowable difference in bias, suggesting that the two pituitary preparations exhibit a similar degree of commutability.

Finally, the stability of the candidate IS was assessed by the measurement of accelerated thermal degradation samples by five laboratories using six methods (Table 5). The candidate IS, 83/573, was predicted to exhibit a loss of immunoreactivity per year of 0.007% when stored at -20°C indicating that it is sufficiently stable to be a WHO IS. The stability of the material is also supported by the close agreement in IU of the content determined in this study to that determined in the original study of 1986 [2].

WHO Reference Reagent for recombinant, human prolactin, 97/714

The first WHO Reference Reagent for human, recombinant prolactin was established in 2001 with the aim of providing a reference preparation to support a change from pituitary-derived to recombinant human prolactin calibrators in assays kits. It was assessed alongside preparations containing glycosylated prolactin and non-glycosylated prolactin, coded 98/580 and 98/582, respectively, to further characterise systems which recognise these components differently. However, as this was not established as the IS, it is understandable that assays have continued to be calibrated to the pituitary preparation and as such, this has prevented the confusion and potential increase in assay variability which would result from having two IS preparations available.

In the 2001 collaborative study, the content of 97/714 was assigned as 1400 mU/amp by cell proliferation bioassays (Mean 1407.9 mU/amp; GCV 20%, n=5) [4]. The preparation was also assessed using a variety of immunoassay methods, by which the content in terms of 84/500 was determined as 1265.9 mU/amp (GCV 27%, n=16). The value determined by immunoassays in the current study of 1315 mU/amp (GCV 20.7%, n=18) was in close agreement with this value, the decrease in variability perhaps reflecting the consolidation of assay methods to predominantly automated chemiluminescence technologies. However, as may be expected given the different nature of the materials, the GCV remains higher than that determined for the pituitary preparation, 83/573 (GCV 8.1%). Despite this, there is good agreement between the laboratory mean estimates for the content of the serum samples calculated in terms of kit standards with those in terms of 97/714. This supports the potential use of a recombinant preparation as a future IS for assays reporting in International Units. However, the individual commutability assessment of the ten methods contributed for this analysis and shown in Figure 3iii, suggests that a recombinant reference preparation may exhibit non-commutability with the methods contributed by Participants 4 and 5.

WHO is unlikely to receive a donation of human, pituitary-derived prolactin for the eventual replacement of the candidate standard, 83/573. Commercial sources are available but the high cost of such preparations may require financial support from manufacturers. This approach would allow adherence to the guidelines of WHO which recommend that an IS matches, as closely as possible, the measured analyte. Alternatively, further discussion and evaluation by manufacturers is suggested to support the introduction of a recombinant prolactin IS with minimal impact on the measurement of patient samples.

Proposal

It is recommended that the preparation in ampoules coded 83/573 is established as the 4th IS for human, pituitary prolactin with an assigned immunoreactivity of 67 mIU/amp.

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Table 4: Laboratory geometric mean estimates of prolactin immunoreactivity in mIU/amp for the current IS 84/500, the candidate standard, 83/573 and the WHO Reference Reagent for recombinant human prolactin 97/714 calculated relative to the kit standards, the current IS 84/500 or the candidate IS 83/573*.

*assuming 63 mIU/amp

Lab	Relative to Kit			Relative Potency		
				vs 84/500		vs 83/573.C
	84/500	83/573.C	97/714	83/573.C	97/714	97/714
01	50.9	66.2	1418.5	69.0	1478.4	1436.0
02a		42.3			1062.0	1402.2
02b	63.7	93.1	1587.8	77.4	1320.2	1143.0
03	66.8	90.8	1515.5	72.0	1203.2	1118.9
04	53.5	64.4	1642.0	63.8	1642.6	1699.6
05	60.7	70.7	1126.3	61.7	998.8	1070.3
07a	52.0	63.8	2101.6	65.0	2141.1	2205.6
07b	52.9	64.8	1284.9	65.0	1287.8	1328.0
07c	49.4	58.8	1149.5	63.7	1233.9	1300.6
07d	55.5	70.7	1285.3	67.4	1226.8	1218.7
07e	55.8	73.8	1372.1	70.1	1302.5	1245.5
07f	53.4	61.1	1056.0	60.5	1047.9	1145.6
07g	51.1	64.1	1166.5	66.6	1210.9	1218.7
07h				64.2		
07i	56.0	64.2	1560.1	60.8	1477.5	1627.3
08a	49.7	69.1	1150.9	73.6	1226.2	1115.5
08b	53.5	65.1	1218.3	64.6	1208.0	1253.0
09	52.1	77.9	1702.2	79.3	1731.0	1463.2
10	67.1	81.1	1638.1	68.2	1293.3	1271.2
GM	55.3	68.1	1386.8	67.2	1315.0	1246.6
GCV	9.8%	19.0%	20.5%	8.1%	20.7%	19.8%
N	17	18	17	18	18	18

Table 5: Prolactin immunoreactivity in mIU/amp of accelerated degradation study samples of 83/573 (10 months incubation), calculated relative to 84/500

Lab	83/573 **-20**°C	A **+4**°C	D **+20**°C	E **+37**°C	B **+45**°C
3	90.8	91.9	79.2	59.2	
5	71.2	72.4	67.1	53.7	40.9
8a	69.1	64.6	64.4	50.3	37.4
8b	65.3	66.7	67.0	51.4	37.5
9	77.9	79.8	73.0	67.3	
10	71.5	102.0	105.4	89.1	97.8

Table 6: Geometric mean and between-laboratory variability of the laboratory estimates for the prolactin content of each of the human serum samples, S1 to S16, expressed in terms of the kit standards, the current standard, 84/500, the candidate standard, 83/573* and the WHO Reference Reagent for recombinant, human prolactin, 97/714**

* assuming 63 mIU/amp **assuming 1400 mIU/amp

Serum Sample	Prolactin relative to in-house ref/kit standards (mIU/L)		Prolactin relative to 84/500 (mIU/L)		Prolactin relative to 83/573 (mIU/L)		Prolactin relative to 97/714 (mIU/L)	
	GM	**GCV**	**GM**	**GCV**	**GM**	**GCV**	**GM**	**GCV**
S1	391.9	21%	364.7	19%	327.0	16%	385.2	19%
S2	845.5	21%	786.8	17%	705.5	13%	831.0	18%
S3	275.3	28%	256.1	24%	229.7	21%	270.5	22%
S4	213.5	19%	198.6	15%	178.1	15%	209.8	22%
S5	1623.6	21%	1510.7	17%	1354.8	15%	1595.6	19%
S6	3415.5	19%	3178.1	15%	2850.0	13%	3356.6	17%
S7	479.3	29%	445.9	27%	399.9	22%	471.0	24%
S8	336.1	18%	312.7	15%	280.4	13%	330.3	22%
S9	117.4	25%	109.2	23%	97.9	20%	115.4	23%
S10	266.7	21%	248.1	18%	222.5	16%	262.1	22%
S11	153.4	22%	142.7	18%	128.0	17%	150.7	20%
S12	2523.7	21%	2348.3	16%	2105.8	14%	2480.2	19%
S13	1939.5	23%	1804.6	18%	1618.3	16%	1906.0	20%
S14	374.8	21%	348.7	18%	312.7	16%	368.3	22%
S15	384.9	22%	358.1	20%	321.2	17%	378.3	21%
S16	4321.4	20%	3969.9	11%	3621.5	14%	4340.8	20%
Pooled		**22%**		**19%**		**16%**		**21%**

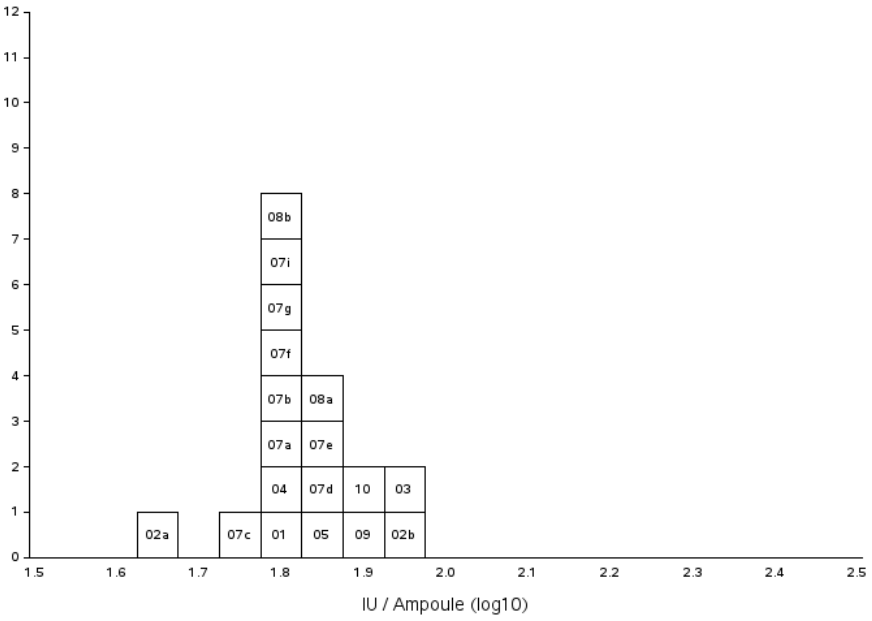
Table 7: Proportion (top) or percentage (bottom) of reference standard dilutions concluded to be commutable using a difference in bias approach

Lab	01	02a	02b	03	04	05	08a	08b	09	10
84/500	10/10	5/8	10/10	10/10	10/10	10/10	10/10	10/10	1/9	10/10
97/714	10/10	7/10	10/10	10/10	3/10	2/10	10/10	10/10	9/9	10/10
83/573	10/10	9/10	10/10	10/10	10/10	10/10	5/10	10/10	9/9	10/10

Lab	01	02a	02b	03	04	05	08a	08b	09	10
84/500	100%	63%	100%	100%	100%	100%	100%	100%	11%	100%
97/714	100%	70%	100%	100%	30%	20%	100%	100%	100%	100%
83/573	100%	90%	100%	100%	100%	100%	50%	100%	100%	100%

Figure 1: Estimates of the prolactin immunoreactivity in mIU/amp for candidate standard, 83/573 in terms of (i) kit standards and (ii) the current standard, 84/500

i.



ii.

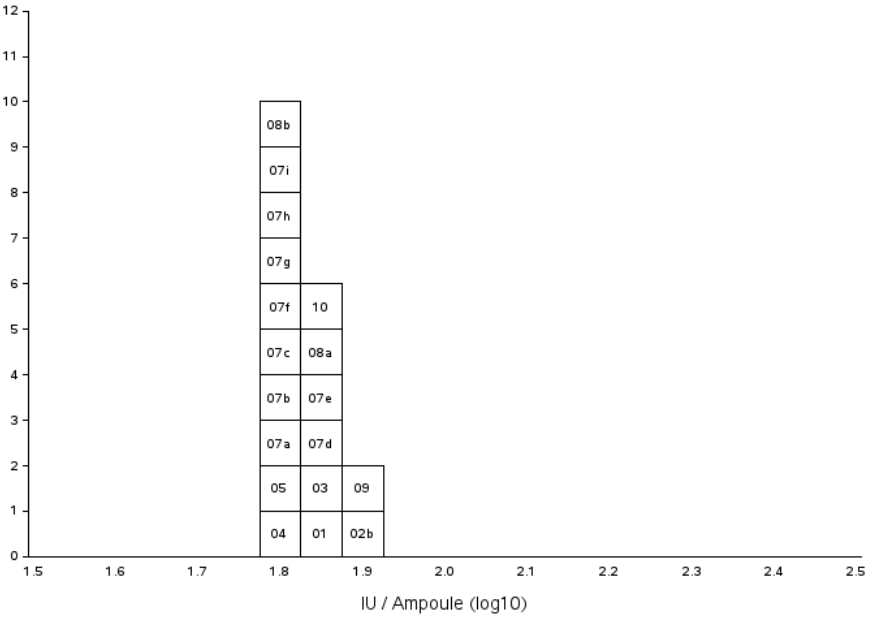
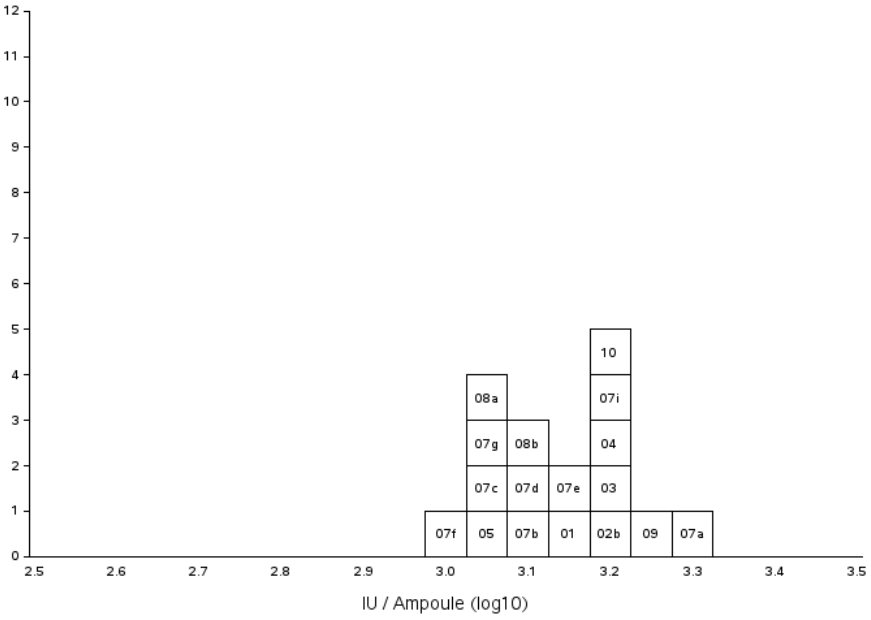


Figure 2: Estimates of the prolactin immunoreactivity in mIU/amp for the WHO Reference Reagent for recombinant, human prolactin, 97/714 in terms of (i) kit standards and (ii) the current standard, 84/500

i.



ii

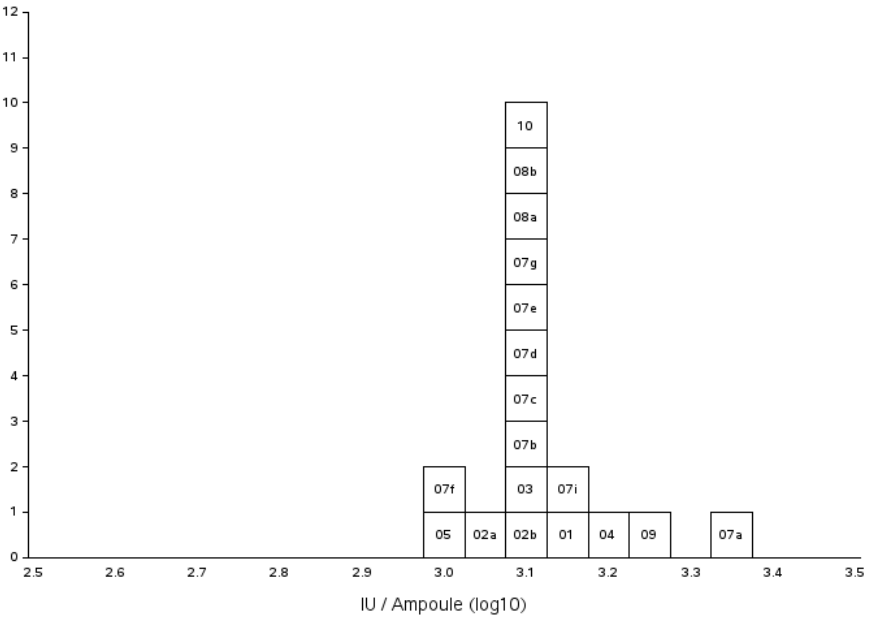


Figure 3i: Estimated differences in bias between serum samples and the current standard, 84/500. Blue shading in the key represents the different concentrations of the reference material.

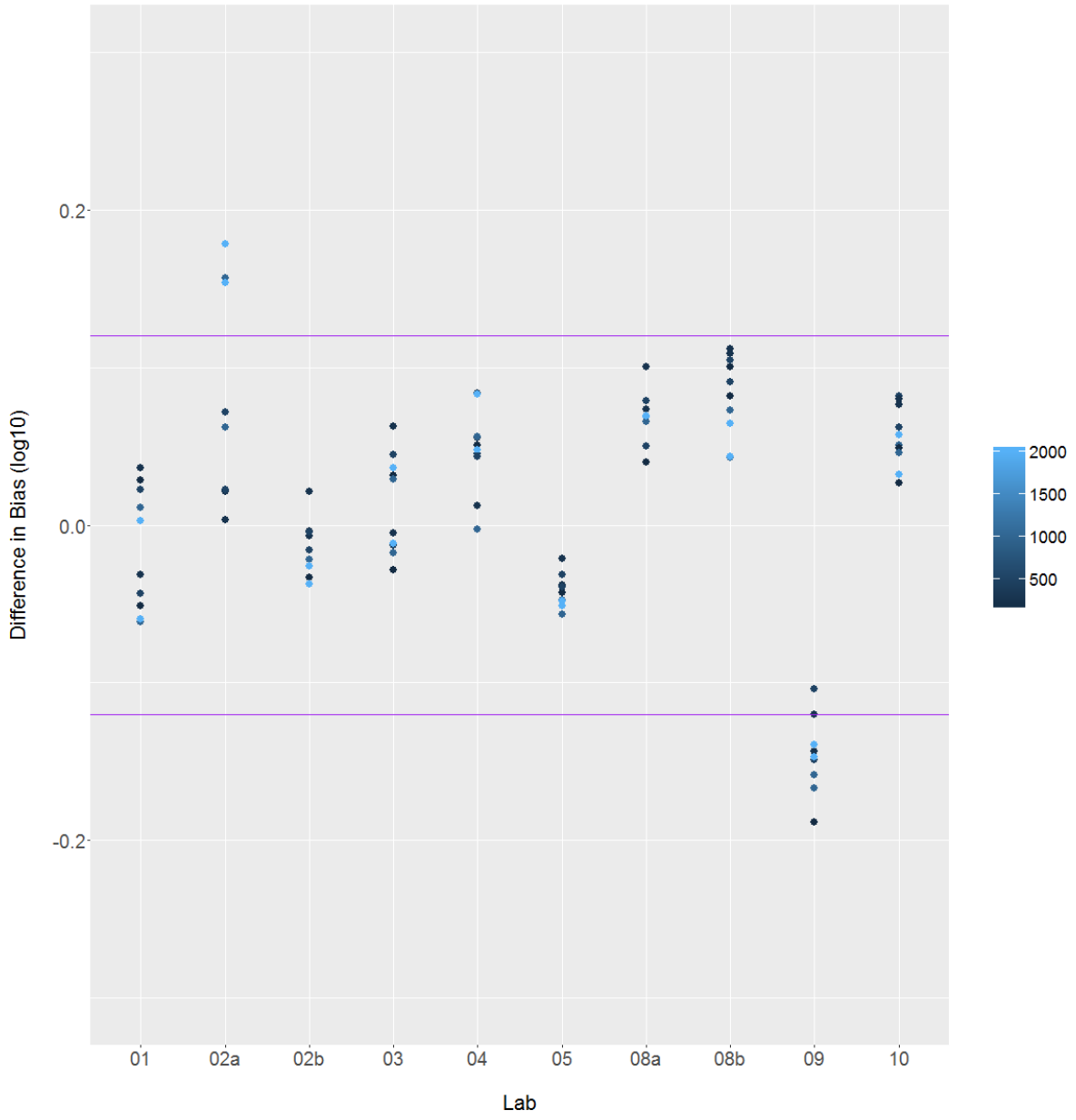


Figure 3ii: Estimated differences in bias between serum samples and the candidate standard, 83/573. Blue shading in the key represents the different concentrations of the reference material.

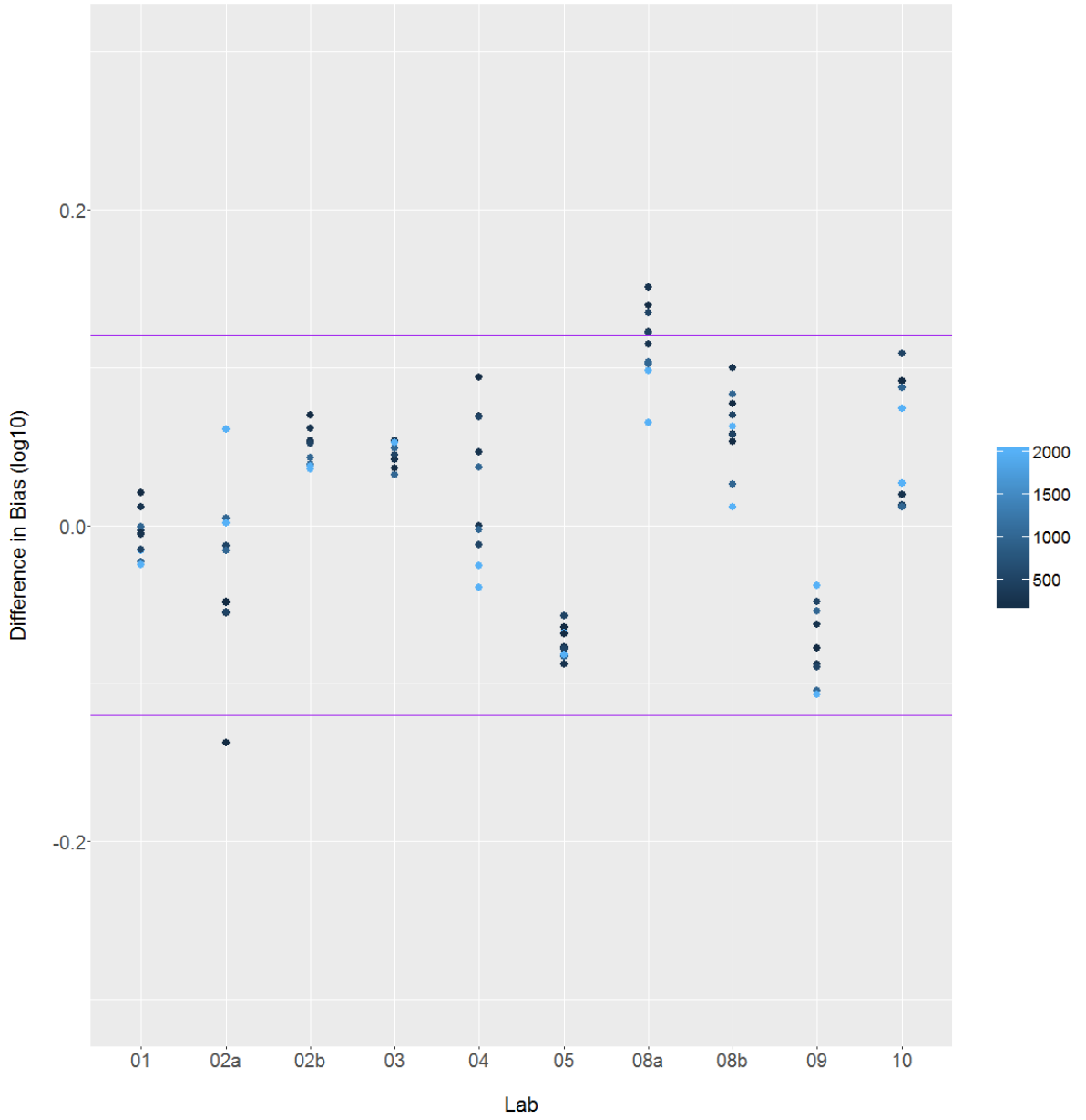
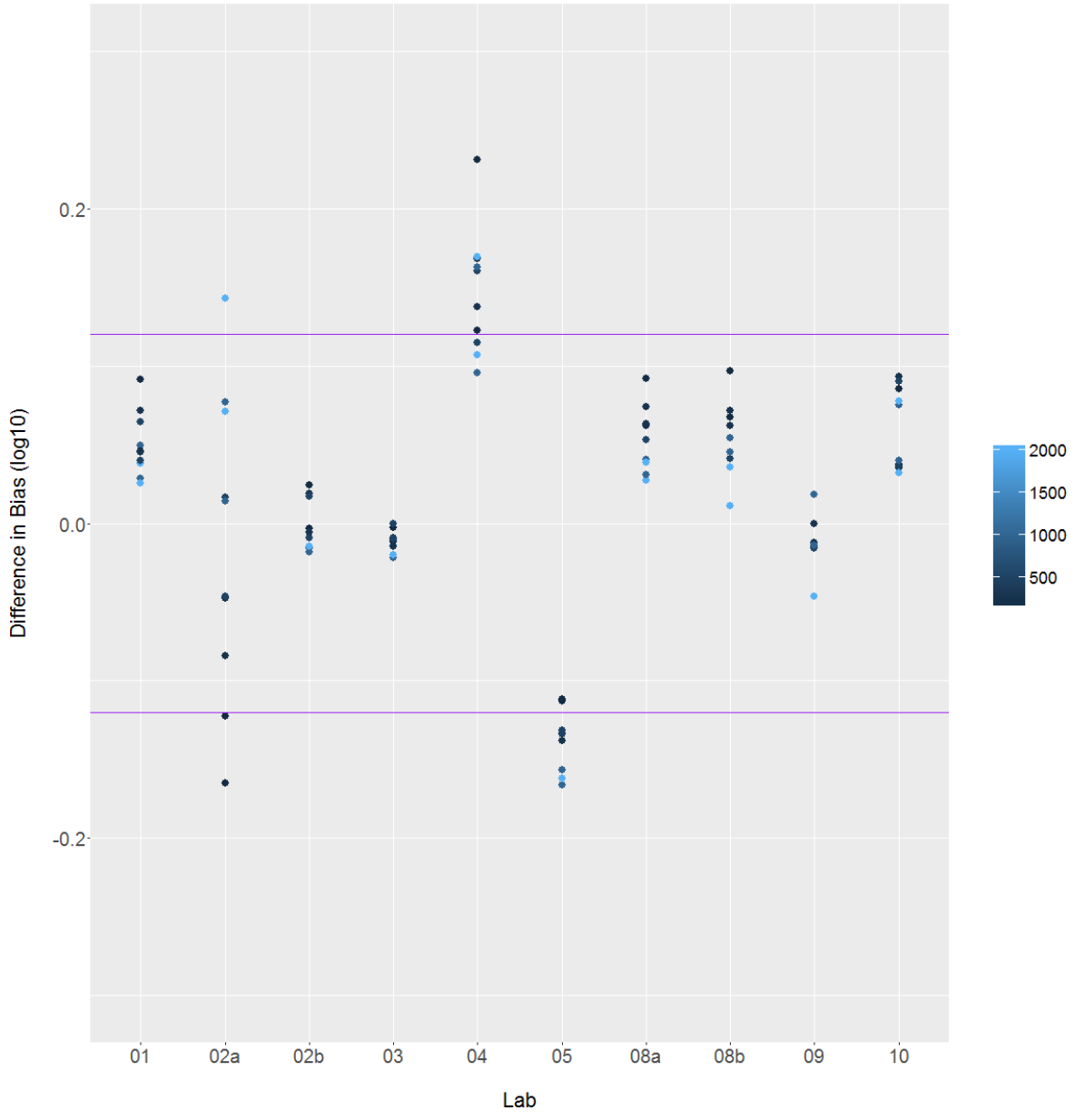


Figure 3iii: Estimated differences in bias between serum samples and the WHO Reference Reagent for recombinant, human prolactin, 97/714. Blue shading in the key represents the different concentrations of the reference material.



Appendix 1

Table A1.1 Individual assay estimates for the prolactin immunoreactivity in mIU/amp expressed in terms of kit standards (reported prolactin concentration) of the current standard, 84/500, the candidate standard 83/573 (Sample C) and the WHO Reference Reagent for human, recombinant Prolactin 97/714.

Lab	84/500				83/573 (Sample C)				97/714			
	Assay 1	Assay 2	Assay 3	Assay 4	Assay 1	Assay 2	Assay 3	Assay 4	Assay 1	Assay 2	Assay 3	Assay 4
01	55.1	46.9			67.5	64.9			1461.8	1376.5		
02a	np	np			np	42.3			np	np		
02b	62.6	64.9			92.9	93.3			1563.7	1612.3		
03	70.7	63.0			89.8	91.7			1530.1	1501.0		
04	52.4	54.6			62.7	66.3			1535.2	1756.2		
05	60.0	61.0	59.5	62.2	70.9	71.1	70.1	70.7	1127.5		1125.0	
07a	54.2	50.0			63.5	64.2			2020.8	2185.7		
07b	53.1	52.7			64.6	65.1			1290.6	1279.2		
07c	49.6	49.2			60.3	57.4			1141.1	1158.0		
07d	58.5	52.7			74.4	67.1			1332.9	1239.4		
07e	58.3	53.5			76.7	71.1			1441.3	1306.2		
07f	56.0	50.9			62.1	60.2			1049.5	1062.5		
07g	47.9	56.0	52.9	47.9	61.1	68.1	67.6	60.1	1108.8	1255.1	1176.2	1131.3
07h	np	nl			np	np			nl	nl		
07i	56.2	55.8			63.6	64.8			1579.4	1541.0		
08a	50.6	48.9			70.8	67.5			1156.7	1145.1		
08b	53.1	53.8			62.5	67.9			1242.0	1195.1		
09	50.6	53.7			81.6	74.5			1728.1	1676.8		
10	67.5	66.8			93.5	79.7	71.5		1557.2	1723.2		

Table A1.2 Fitted slopes (log_{10} reported concentration using kit standard against log_{10} dilution) for the current standard, 84/500, the candidate standard, 83/573 (Sample C), and the WHO Reference Reagent for human, recombinant Prolactin, 97/714.

	84/500				83/573 (Sample C)				97/714			
Lab	**Assay 1**	**Assay 2**	**Assay 3**	**Assay 4**	**Assay 1**	**Assay 2**	**Assay 3**	**Assay 4**	**Assay 1**	**Assay 2**	**Assay 3**	**Assay 4**
01	1.00	1.01			0.98	0.99			0.98	1.01		
02a	1.23	1.18			1.11	1.04			1.17	1.16		
02b	1.01	0.99			0.99	0.97			1.01	1.00		
03	1.01	1.02			1.01	1.02			1.01	1.02		
04	1.01	1.03			0.96	0.96			0.96	0.96		
05	1.01	1.01	1.01	1.01	1.01	1.00	1.00	1.00	0.99		0.99	
07a	0.97	0.99			0.96	0.98			0.93	0.95		
07b	1.00	1.00			0.97	0.97			1.01	1.00		
07c	1.02	1.03			1.00	1.01			1.01	1.03		
07d	1.04	1.05			1.02	1.03			1.00	1.02		
07e	1.01	1.01			1.03	1.05			1.04	1.05		
07f	0.96	1.01			1.02	1.04			1.03	1.02		
07g	1.00	0.95	0.93	0.97	1.03	1.00	0.93	1.00	0.99	0.96	0.92	0.98
07h	0.66				0.67	0.75						
07i	1.01	1.02			1.01	1.02			1.02	1.02		
08a	1.01	1.03			0.96	0.96			0.97	1.00		
08b	0.96	0.98			0.97	0.99			0.98	0.98		
09	1.05	1.00			1.04	0.99			1.03	0.99		
10	1.04	1.01			1.00	1.03	0.96		1.02	1.01		

Table A1.3 Individual assay estimates of prolactin immunoreactivity in mIU/amp for the current IS 84/500, the candidate standard, 83/573 and the WHO Reference Reagent for recombinant human prolactin 97/714 calculated relative to the current IS 84/500 or the candidate IS 83/573*.

*assuming 63 mIU/amp

Lab	83/573 (Sample C) vs 84/500				97/714 vs 84/500				97/714 vs vs 83/573.C			
	Assay 1	Assay 2	Assay 3	Assay 4	Assay 1	Assay 2	Assay 3	Assay 4	Assay 1	Assay 2	Assay 3	Assay 4
01	64.9	73.3			1406.1	1554.5			1364.1	1336.6		
02a	np	np			1078.9	1045.4			1318.5	np		
02b	78.7	76.1			1324.3	1316.1			1060.6	1089.1		
03	67.3	77.1			1146.8	1262.3			1073.3	1031.3		
04	63.4	64.3			1571.9	1716.5			1538.6	1660.0		
05	62.6	61.7	62.4	60.2	995.8		1001.8		1001.6		1011.2	
07a	62.1	68.1			1977.6	2318.1			2005.5	2144.6		
07b	64.4	65.5			1288.2	1287.5			1259.5	1238.1		
07c	64.5	62.9			1219.7	1248.4			1192.2	1254.5		
07d	67.4	67.5			1208.3	1245.5			1128.9	1163.2		
07e	69.7	70.4			1311.0	1294.2			1184.4	1158.0		
07f	58.4	62.7			992.6	1106.3			1043.3	1112.3		
07g	67.7	64.5	67.7	66.5	1227.9	1188.6	1177.6	1251.1	1142.7	1161.2	1096.1	1185.9
07h	64.2	Standard nl			nl	Standard nl			nl	nl		
07i	60.1	61.6			1490.6	1464.5			1563.4	1497.7		
08a	74.1	73.2			1211.2	1241.4			1029.6	1068.5		
08b	62.4	66.9			1240.7	1176.3			1252.5	1108.3		
09	85.4	73.5			1809.3	1656.0			1334.1	1418.9		
10	73.4	63.3			1222.6	1368.1			1048.9	1362.3		

Table A1.4: Slope ratios for the candidate standard, 83/573 (Sample C) and the WHO Reference Reagent for human, recombinant Prolactin, 97/714, relative to the current standard, 84/500.

Lab	83/573 (Sample C) vs 84/500				97/714 vs 84/500				97/714 vs vs 83/573.C			
	Assay 1	Assay 2	Assay 3	Assay 4	Assay 1	Assay 2	Assay 3	Assay 4	Assay 1	Assay 2	Assay 3	Assay 4
01	0.99	0.99			0.99	1.00			1.00	1.01		
02a	0.91	0.88			0.95	0.98			1.05	1.12		
02b	0.98	0.98			1.00	1.00			1.03	1.02		
03	1.00	1.00			1.00	1.00			1.00	1.00		
04	0.95	0.94			0.95	0.94			1.00	1.00		
05	1.00	0.99	0.99	0.99	0.98		0.97		0.99		0.99	
07a	0.99	0.99			0.96	0.95			0.97	0.96		
07b	0.97	0.96			1.01	1.00			1.04	1.04		
07c	0.98	0.98			0.99	1.00			1.00	1.01		
07d	0.98	0.98			0.97	0.97			0.98	0.99		
07e	1.02	1.04			1.03	1.03			1.01	0.99		
07f	1.06	1.03			1.07	1.01			1.01	0.98		
07g	1.03	1.05	1.00	1.03	0.99	1.01	0.99	1.01	0.96	0.96	0.98	0.98
07h	1.02											
07i	1.00	1.00			1.01	1.00			1.01	1.00		
08a	0.95	0.93			0.97	0.97			1.01	1.05		
08b	1.01	1.01			1.02	1.01			1.01	1.00		
09	0.99	0.99			0.99	0.99			0.99	1.00		
10	0.96	1.02			0.98	1.00			1.02	0.98		

Table A1.5: Geometric mean prolactin concentration in mIU/L of the human serum samples coded S1 to S16 reported relative to kit standards for each laboratory method

Lab	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16
01	371.0	799.3	272.0	209.7	1510.0	3202.9	446.5	314.7	104.7	238.9	148.4	2344.7	1791.2	334.3	351.6	4160.5
02a*	211.4	606.8	137.9	80.4	1489.7	3898.6	293.6	177.5	38.8	119.6	59.7	2639.9	1888.3	193.2	217.3	5240.5
02b	452.9	1010.2	330.7	246.2	1971.2	4102.2	563.3	387.1	129.5	304.9	176.7	3069.1	2368.9	436.8	434.7	5360.2
03	443.1	995.8	316.6	233.8	1926.5	3922.5	561.1	379.8	128.8	301.1	174.2	3016.5	2325.0	420.6	430.4	5085.2
04	336.2	695.9	238.5	172.4	1424.1	3068.6	387.4	259.4	96.5	220.5	133.9	2144.1	1680.9	308.0	313.4	3903.5
05	444.5	910.5	322.9	264.5	1878.5	3898.0	539.4	397.9	139.7	327.2	182.3	2852.7	2277.8	454.1	445.6	5107.4
08a	296.1	659.9	181.4	166.5	1224.8	2570.2	351.6	280.1	88.2	206.7	112.3	1889.5	1385.0	294.4	292.7	3225.7
08b	314.0	671.6	200.9	181.6	1267.4	2717.8	340.5	288.5	94.0	223.9	116.5	2068.0	1496.8	313.9	316.5	3607.9
09	519.4	1090.7	377.4	247.2	2009.4	4021.0	751.0	397.7	174.5	342.9	191.9	3108.6	2380.9	486.1	533.3	or
10	406.2	897.1	300.7	223.5	1638.6	3653.6	503.3	356.4	124.2	271.7	167.9	2575.4	2082.4	378.1	408.5	4621.7

or: outside range of assay

*excluded from determination of overall mean values and between-laboratory variability

Table A1.6: Individual assay estimates by an automated chemiluminescent immunoassay by Participant 10 for the prolactin immunoreactivity in mIU/amp of twelve ampoules coded H1-H12 of the candidate standard 83/573 expressed in terms of 84/500

83/573 Ampoule Code	Content in terms of 84/500 (mIU/amp)
H1	70.88
H2	76.68
H3	75.91
H4	76.34
H5	72.45
H6	64.58
H7	76.25
H8	72.37
H9	74.31
H10	71.77
H11	74.40
H12	67.50
GM	72.70
GCV	5.4
N	12

Appendix 2
Proposed International Collaborative Study to Establish the 4th International Standard for Prolactin, human, pituitary

Study Protocol
Introduction
The 3rd International Standard (IS) for Prolactin (PRL), human, pituitary, in ampoules coded 84/500, was established by the Expert Committee on Biological Standardization (ECBS) in 1988 and has been widely used for the calibration of immunoassays to measure serum PRL concentration (WHO, ECBS 39th Report, Technical Report Series (TRS) No. 786, pg 24). Determinations of serum PRL are widely applied in clinical diagnostics, contributing to investigations of reproductive disorders, pituitary dysfunction and in the diagnosis and monitoring of treatment of prolactinomas. Stocks of the 3rd IS, 84/500, are low and there is a requirement to replace this standard.

In recent years, it has become difficult to obtain pituitary derived material and this was noted in 2000 when the WHO Reference Reagent for PRL, human, recombinant, coded 97/714 was established ((WHO, ECBS 52nd Report, TRS No. 924, pg 32). However, the use of recombinant, human PRL to calibrate PRL immunoassays has not been adopted and the calibration of immunoassays remains to the 3rd IS.

Ideally, the provision of bulk PRL of pituitary origin to NIBSC would allow a programme of development in order to identify the optimum formulation using current knowledge and technology. We continue to investigate if a source of pituitary PRL is available. However, to ensure the continued provision of a standard, we are initiating a project to evaluate a batch of ampoules, coded 83/573, which was prepared at the same time as the current IS with a view to this batch being established as the 4th WHO IS for PRL, human, pituitary. This preparation was included in the collaborative study to establish the WHO Reference Reagent for PRL, human, recombinant, 97/714, and evaluation in terms of the current IS suggests that it may be suitable as an replacement. Therefore, we intend to organise a collaborative study with expert laboratories to aid in the value assignment of the candidate standard.

The aims of the study would be:

1. to calibrate the candidate standard, 83/573, in terms of the current IS, 84/500
2. to assess the suitability of 83/573 to calibrate PRL immunoassays
3. to assess the stability of 83/573 after accelerated thermal degradation

Materials
The materials to be provided to collaborators are listed in Table 1. Each participant will be allocated a set of preparations based on assay capacity and sample availability.

Table 1: Preparations for inclusion in the collaborative study

Preparation	Contents
3rd IS for PRL, human, pituitary, 84/500	53 mIU per ampoule
Candidate IS for PRL, human, pituitary, 83/573	Nominally, 63 mIU per ampoule
Accelerated thermal degradation samples of 83/573 stored at +4°C, +20°C, +37°C, +45°C	Nominally, 63 mIU per ampoule
WHO Reference Reagent for PRL, human, recombinant, 97/714	1400 mU per ampoule
Human serum samples (n=16)	0.5 – 1.0 ml volume

3rd IS for PRL, human, pituitary, 84/500
The 3rd IS contains the residue, after freeze-drying, of 1 ml of a solution which contained PRL extract, 1 mg human serum albumin, 5 mg lactose and 0.63 mg ammonium formate. The 3rd IS has been tested and found to be negative for HBsAg, anti-HIV and HCV NAT.

Candidate IS for PRL, human, pituitary, 83/573
The candidate IS contains the residue, after freeze-drying, of 1 ml of a solution which contained PRL extract, 1 mg human serum albumin, 5 mg lactose and 0.63 mg ammonium formate. The candidate IS has been tested and found to be negative for HBsAg, anti-HIV and HCV NAT. Ampoules will be identified by a code letter.

Accelerated thermal degradation (ATD) samples of the candidate IS, 83/573
Ampoules of the candidate IS which have been incubated at +4°C, +20°C, +37°C and +45°C for 10 months will be included in the study to assess the stability of the candidate standard. Ampoules will be identified by a code letter.

WHO Reference Reagent for PRL, human, recombinant, 97/714
The WHO Reference Reagent for PRL, human, recombinant contains the residue, after freeze drying, of 1 ml of a solution which contained purified human, recombinant PRL, 4 mg Na_2PO_4, 30 mg trehalose, 3 mg arginine, 4.5 mg NaCl, 0.1 mg Tween 20. The material is not of human origin.

Human serum samples
Individual, human serum samples coded S1-S16 contain 0.5 – 1.0 ml volume of human serum obtained from two commercial sources. Serum samples from First Link (UK) Ltd. (Birmingham, UK) have been tested by an FDA approved method and found to be non-reactive for HIV I/II Ab, HIV p24, HBsag, anti-HCV and Syphilis TP. Serum samples from Equitech Enterprises Inc (Kerryville, USA), obtained through Europa Bioproducts Ltd (Ely, UK), have been tested and found negative or non-reactive at the donor level for RPR, HBsAg, HBcAB, HCV, HIV I/II Ab, HBV, HCV and HIV PCR (NAT) and HTLV I/II by FDA approved methods.

The concentration of PRL in the human serum samples ranges from 150 - 5000 mIU/L.

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

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

Handling of the Preparations
On receipt, ampoules should be stored at -20°C or below until use. Before opening, ampoules should be brought to room temperature to minimise moisture uptake.

Ampoules should be reconstituted in a suitable assay diluent or PBS containing 0.1% BSA to prepare a stock solution. Test dilutions are prepared from each stock solution. The diluent should include protein to prevent surface adsorption (typically 0.1 % (w/v) bovine serum albumin or 0.1 % (w/v) human serum albumin).

Where a fresh ampoule will not be available for subsequent assays, the stock solution may be aliquotted and snap frozen for use in subsequent assays. Where this is the case, participants are requested to provide details of freeze-thaw steps.

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

Serum samples should be thawed at room temperature prior to each assay and mixed thoroughly by inversion. As the stability of each serum sample is not known, participants are requested to assay immediately. An aliquot of frozen serum is provided for each run of each method.

Tests Requested
Participants are requested to carry out the assay method(s) normally in use in their laboratory and to perform **two independent runs**. An independent run uses test sample dilutions prepared specifically for that run from new ampoules or from a frozen stock solution where fresh ampoules are not available.

If assay capacity allows, we kindly request for statistical purposes, that triplicate measurements are made of each sample in each run. This may not be possible, particularly in a manual assay format, and duplicate measurements will be acceptable.

Participants are requested to dilute the ampouled preparations using their standard assay diluent or PBS containing 0.1% BSA such that five or more concentrations which are expected to provide a response in the linear part of the dose response curve are measured. In order to compare different immunoassay methods, it is essential that some of the measured dilutions of ampouled materials are common to all participants. **We kindly request that dilutions of 2000, 1000, 500, 250, 125 mIU/L are included in each assay**. Further details, including a suggested

dilution scheme for each ampouled preparation, is included in Appendix 1. Please provide details of the assay diluent used and all volumes used in preparing the dilutions.

In the same runs as the ampouled preparations, participants are requested to include the set of serum samples that are provided for each independent assay.

Participants are also asked to ensure that all assays include their local standard and to provide details of the assay method used, together with all raw assay data in electronic format (Excel) in the form of clearly annotated optical densities, counts or RLU for central computation at NIBSC. An example of a reporting table is shown in Appendix 1.

Participants' estimates of the PRL concentration of the ampouled preparations are requested, as calculated by the method normally used in their laboratory. However, please note that the analysis of data from all participating laboratories at NIBSC may result in small differences between participant's estimates and the values reported after central computation.

Use of Samples
The use of all candidate standard(s) and serum samples is restricted to this study.

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

Report
A preliminary report will be prepared and circulated to all participants for comment before submission to the Expert Committee on Biological Standardization 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.

For further information, please contact Dr Jackie Ferguson (e-mail: jackie.ferguson@nibsc.org)
National Institute for Biological Standards and Control (http://www.nibsc.org)
Tel: 44 (0) 1707 641135; Fax: 44 (0) 1707 641057

Study Protocol Appendix 1:

A: Reconstitution and dilution of the 3rd WHO IS for PRL, human pituitary, 84/500, (53 mIU/amp)

1. Before opening, ampoules should be brought to room temperature to minimize moisture uptake. Tap the ampoule to ensure all contents are in the base of the ampoule.
2. Reconstitute the ampoule of 84/500 in a final volume of 1.656 ml of suitable assay diluent or PBS containing 0.1% BSA to prepare a 32,000 mIU/L solution. Snap freeze aliquots of this solution for future assays.
3. Dilute the solution in step 2, 1/4 in assay diluent or PBS containing 0.1% BSA to provide an 8000 mIU/L solution. For methods with a wide range, this may also be a test sample.

4. Dilute the solution in step 3, 1/2 in assay diluent or PBS containing 0.1% BSA to provide an 4000 mIU/L solution. For methods with a wide range, this may also be a test sample.
5. Prepare a further five, two-fold dilutions such that dilutions of **2000, 1000, 500, 250 and 125 mIU/L** are prepared.
6. Prepare any intermediate dilutions required for your method from the 8000 mIU/L solution.

B: Reconstitution and dilution of the candidate standard for PRL, human, pituitary standard, 83/573, and accelerated degradation samples of 83/573 (63 mIU/amp)

1. Before opening, ampoules should be brought to room temperature to minimize moisture uptake. Tap the ampoule to ensure all contents are in the base of the ampoule.
2. Reconstitute the ampoule of 83/573 in a final volume of 1.969 ml of suitable assay diluent or PBS containing 0.1% BSA to prepare a 32,000 mIU/L solution.
3. Dilute the solution in step 2, 1/4 in assay diluent or PBS containing 0.1% BSA to provide an 8000 mIU/L solution. For methods with a wide range, this may also be a test sample.
4. Dilute the solution in step 3, 1/2 in assay diluent or PBS containing 0.1% BSA to provide an 4000 mIU/L solution. For methods with a wide range, this may also be a test sample.
5. Prepare a further five, two-fold dilutions such that dilutions of **2000, 1000, 500, 250 and 125 mIU/L** are prepared.
6. Prepare any intermediate dilutions required for your method from the 8000 mIU/L solution.

C: Reconstitution and dilution of the WHO 1st Reference reagent for recombinant PRL, 97/714 (1400 mU/amp)

1. Before opening, ampoules should be brought to room temperature to minimize moisture uptake.Tap the ampoule to ensure all contents are in the base of the ampoule.
2. Reconstitute the ampoule of 97/714 in a final volume of 4.375 ml of suitable assay diluent or PBS containing 0.1% BSA to prepare a 320,000 mIU/L solution
3. Dilute the solution in step 2, 1/10 in assay diluent or PBS containing 0.1% BSA to provide an 32,000 mIU/L solution.
4. Dilute the solution in step 3, 1/4 in assay diluent or PBS containing 0.1% BSA to provide an 8000 mIU/L solution. For methods with a wide range, this may also be a test sample.
5. Dilute the solution in step 4, 1/2 in assay diluent or PBS containing 0.1% BSA to provide an 4000 mIU/L solution. For methods with a wide range, this may also be a test sample.
6. Prepare a further five, two-fold dilutions such that dilutions of **2000, 1000, 500, 250 and 125 mIU/L** are prepared.

To enable comparison across different immunoassays at the same dilution point, participants are asked to include the 5 core concentrations (underlined) in all assays if they are within the method range. If assay space permits, additional concentrations should be included.

D. Data reporting

Participants are requested to provide details of the assay method used, including dilution steps, together with all raw data. Participants' own calculated estimates of prolactin concentration are also requested. A sample table for reporting the data from a single run of a method is provided below in Table A1.

Table A1 Sample data reporting table for one run of an assay method

Method: _____		**RLU/Absorbance Units/Counts**			**Prolactin concentration (mIU/L) in terms of KIT**
Run: _____					
Sample	**Dilution (or Kit Std conc^{n})**	**Replicate 1**	**Replicate 2**	**Replicate 3**	
Baselines					
Kit standard 1					
Kit standard 2					
Kit standard 3					
Kit standard 4					
Kit standard 5					
84/500 dil^{n} 1					
84/500 dil^{n} 2					
84/500 dil^{n} 3					
84/500 dil^{n} 4					
84/500 dil^{n} 5					
84/500 dil^{n} 6					
83/573 dil^{n} 1					
83/573 dil^{n} 2					
83/573 dil^{n} 3					
83/573 dil^{n} 4					
83/573 dil^{n} 5					
83/573 dil^{n} 6					
97/714 dil^{n} 1					
97/714 dil^{n} 2					
97/714 dil^{n} 3					
97/714 dil^{n} 4					
97/714 dil^{n} 5					
97/714 dil^{n} 6					
Insert measurements for any other coded samples of 83/573					
Serum S1					
Serum S2					
Serum S3					
Serum S4					
Serum S5					
Serum S6					
Serum S7					
Serum S8					
Serum S9					
Serum S10					
Serum S11					
Serum S12					
Serum S13					
Serum S14					
Serum S15					
Serum S16					

Appendix 3: Draft Instructions for use

WHO International Standard

4th International Standard for Prolactin, human, pituitary

NIBSC Code: 83/573

Instructions for use

(Version 5.0, Dated 01/12/2016)

1. INTENDED USE

The 3rd International Standard (IS) for Prolactin, human, in ampoules coded 84/500, has been widely used for the calibration of immunoassays for prolactin. Stocks of the 3rd IS are exhausted and the WHO Expert Committee on Biological Standardization (ECBS) has recognized (2015) the need for a replacement IS. The 4th IS for Prolactin, human, pituitary, in ampoules coded 83/573 contains highly purified Prolactin of human, pituitary origin and was established at the 67th Meeting of WHO ECBS (2016). This material replaces the 3rd IS for Prolactin, human, 84/500, which is discontinued.

2. CAUTION

This preparation is not for administration to humans.

The preparation contains material of human origin, and either the final product or the source materials, from which it is derived, have been tested and found negative for HBsAg, anti-HIV and HCV RNA. However, as with all materials of biological origin, the preparation should be regards as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials to avoid cuts.

3. UNITAGE

Each ampoule of the International Standard contains 67 MILLI INTERNATIONAL UNITS.

4. CONTENTS

Country of origin of biological material: Sweden

Each ampoule contains the residue, after freeze-drying, of 1.0 ml of a solution which contained:

Human prolactin	approximately 3.2 μg
Human albumin	1 mg
Lactose	5 mg
Ammonium formate	0.63 mg

Nitrogen gas at slightly less than atmospheric pressure.

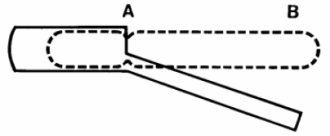
5. STORAGE

Unopened ampoules should be stored at -20°C.

Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

Tap the ampoule gently to collect the material at the bottom (labelled) end. Ensure ampoule is scored all round at the narrow part of the neck with a diamond or tungsten carbide tipped glass knife file or other suitable implement before attempting to open. Place the ampoule in the ampoule opener, positioning the score at position 'A'; shown in the diagram below. Surround the ampoule with cloth or layers of tissue paper. Grip the ampoule and holder in the hand and squeeze at point 'B'. The ampoule will snap open. Take care to avoid cuts and projectile glass fragments that enter eyes. Take care that no material is lost from the ampoule and that no glass falls into the ampoule.



Side view of ampoule opening device containing an ampoule positioned ready to open. 'A' is the score mark and 'B' the point of applied pressure.

7. USE OF MATERIAL

For all practical purposes each ampoule contains the same quantity of the substances listed above. Dissolve the total contents of the ampoule in a known volume of a suitable solvent with carrier protein (0.05 - 0.1% w/v BSA or HSA). Rinse the ampoule several times using the known volume of solvent to ensure recovery of ampoule contents returning the rinses to the known volume. No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution. For economy of use, it is recommended that the solution be sub-divided into several small containers and stored at -40°C, or below. The ampoules do not contain bacteriostat and a solutions of the material should not be assumed to be sterile.

8. PREPARATION OF AMPOULES

A quantity of highly purified extract was donated by KabiVitrum, Sweden through the good offices of Dr L. Fryklund. Preparation of the batch of ampoules coded 83/573 is described by Schulster et al., 1989 (1). The batch of ampoules, coded 83/573, was evaluated in a collaborative study in which ten laboratories in seven countries participated, with the aims being:

1. To calibrate the candidate standard, 83/573, in terms of the 3rd IS, 84/500, by immunoassay.
2. To assess the suitability of the candidate standard, 83/573, to serve as the 4th IS for the calibration of immunoassays of prolactin.
3. To determine the stability of the candidate standard, 83/573, by comparison with ampoules stored at elevated temperatures as part of an accelerated degradation stability study.

From this study, the geometric mean estimate of immunoreactivity of 83/573 in terms of 84/500 was 67 mIU per amp (n=19; GCV 8%). Preparation 83/573 is sufficiently stable to serve as an IS. Analysis of thermally-accelerated degradation samples gave a predicted loss of immunoreactivity per year of 0.007% when stored at -20°C.

9. STABILITY

NIBSC follows the policy of WHO with respect to its reference materials.
It is the policy of WHO not to assign an expiry date to their international reference materials. They remain valid with the assigned potency and status until withdrawn or amended. Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference materials should be stored on receipt as indicated on the label. In addition, once reconstituted, diluted or aliquoted, users should determine the stability of the material according to their own method of preparation, storage and use. Users who have data supporting any deterioration in the characteristics of any reference preparation are encouraged to contact NIBSC.

10. REFERENCES

(1) Schulster D., Gaines Das R.E. and Jeffcoate S.L., International Standards for human Prolactin:calibration by international collaborative study. J. Endocrinol. (1989) 121, 157-166

11. ACKNOWLEDGEMENTS

We gratefully acknowledge the important contributions of all the participants in the collaborative study and KabiVitrum and Dr L., Frykland who kindly donated the human, pituitary extract.

12. FURTHER INFORMATION

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

13. CUSTOMER FEEDBACK

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

14. CITATION

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

15. MATERIAL SAFETY SHEET

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

16. LIABILITY AND LOSS

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

17. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 7 mg
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable. Attached: No

18. CERTIFICATE OF ANALYSIS

NIBSC does not provide a Certificate of Analysis for WHO Biological Reference Materials because they are internationally recognised primary reference materials fully described in the instructions for use. The reference materials are established according to the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_biolefstandardsrev2004.pdf (revised 2004). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.