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Proposed 1st WHO International Standard for insulin, human

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Summary

Stocks of the 1st IRP, 66/304, are exhausted, and there is currently no provision of a reference material for the standardisation of human insulin immunoassays. A candidate preparation of pure human insulin was prepared in ampoules coded 11/212. A collaborative study was organised with 19 laboratories in 11 countries in two phases. In Phase 1, the candidate preparation was value assigned in SI units using a mass balance approach, with confirmatory data provided by HPLC and total nitrogen analysis. Phase 2 was designed to provide confirmatory data on the immunoreactivity of the candidate standard and its suitability to serve as an International Standard for the calibration of immunoassays of human insulin. This phase was also designed to assess the commutability of the candidate preparation, 11/212, in insulin immunoassays.

Participants were sent sub-portions of the active pharmaceutical ingredient (API) used to prepare the candidate standard, 11/212, and were requested to perform the mass balance tests using these sub-portions. The data obtained was combined with the 11/212 fill data in order to assign an insulin content to 11/212. Upon initial data analysis from Phase 1 of the study, it became apparent that smaller weights of active pharmaceutical ingredient (API) had been subject to a drying effect during sub-portioning, resulting in lower estimates of the insulin content of the candidate standard by HPLC assay compared with the expected content determined by mass balance. Participants were sent additional sub-portioned bulk API in a repeat of Phase 1. Data from the Phase 1 repeat mass balance assessment of the bulk API was used in conjunction with 11/212 fill data to assign a final insulin content to the candidate 11/212 of 9.19 mg/ampoule, with expanded uncertainty of 9.14 – 9.24 mg/ampoule ($k=2$). This value assignment was in good agreement with the estimated content of insulin observed using orthogonal methods of total nitrogen analysis (9.14 mg/ampoule (expanded uncertainty 8.97 – 9.30 mg/ampoule ($k=2$)) and by HPLC assay (9.19 mg/ampoule (expanded uncertainty 9.07 – 9.31mg/ampoule ($k=2$)).

The data provided in Phase 2 of the study found 11/212 to be immunoreactive, and to behave in a similar manner to the 1st International Reference Preparation (1st IRP) 66/304 in the immunoassays included in the study. This phase of the study also included an assessment of the impact of the new standard on the routine measurement of insulin in human serum and plasma samples. All laboratories measured fourteen human serum samples and five human plasma samples in parallel with dilutions of 11/212 and 66/304. The two standards behaved in a similar manner in all immunoassays. The geometric mean of laboratory immunoassay estimates was 3.18 IU/amp for the 1st IRP 66/304 and 267.1 IU/amp for the candidate standard 11/212, both of which are in very close agreement with the assigned value of 3 IU/amp for the 1st IRP 66/304 and 9.19 mg/amp for 11/212, after application of the accepted conversion factor of insulin activity (1 IU = 0.0347 mg insulin) to give 264.8 IU/amp.

The immunoassay results were also analysed to assess the commutability of the standard with patient samples using a difference in bias approach. Of the 14 laboratories which demonstrated a consistent patient sample bias, the candidate standard 11/212 was shown to be fully commutable with patient samples in 8 laboratories. For the remaining 6 laboratories in which 11/212 was found non commutable, 2 of these used the same methods in which the candidate standard was shown to be commutable by other laboratories, and in a further 2 methods the candidate standard had a

comparable response to the 1st IRP 66/304. The remaining 2 laboratories (the same method on different platforms) were outside the statistically-defined limits of commtability used in this study.

Taken together, the results from Phase 2 immunoassay estimates and commutability assessment suggest that the candidate standard is suitable as to act as a replacement for the 1st IRP 66/304 for the continued calibration of immunoassay methods for the measurement of insulin.

A thermally accelerated degradation study was also performed. The data from HPLC and immunoassay estimates of accelerated thermal degradation samples of 11/212 indicate that the candidate standard is sufficiently stable when stored at -20ºC to serve as an International Standard.

Therefore it is proposed that the candidate preparation in ampoules coded 11/212 is established as the **1st International Standard for insulin, human,** with an assigned content of **9.19 mg/ampoule (expanded uncertainty of 9.14 – 9.24 mg/ampoule; *k=2*)**. This value can be converted into IU using the internationally recognised specific activity of pure insulin (1IU=0.0347 mg).

Introduction

Insulin is a 51 amino acid, 5808 Da protein that is synthesised from its precursors preproinsulin and proinsulin in the beta cells of the pancreatic islets. In addition to its role as a therapeutic, measurement of insulin by immunoassay plays a key role in the assessment of beta cell secretion, and may improve the classification and management of diabetes in addition to monitoring for the development of insulin resistance [1-3].

The majority of insulin immunoassays are traceable to the 1st WHO International Reference Preparation (IRP) for human insulin, 66/304, stocks of which are now exhausted. This standard was produced from insulin that had been isolated and purified from human pancreas, and was assigned a potency by in vivo bioassay (IU/ampoule). The current WHO standard for human insulin (coded 83/500) was established in the mid-1980s, and was produced through the enzymatic modification of porcine insulin. The potency was also defined in IU (per mg), based on a multi-method collaborative study by in vivo bioassay. Lack of a globally-accepted International Standard of high purity has been identified as a limiting factor in improving the consistency of clinical assays for human insulin. In addition, therapeutic grade insulin is now often produced using recombinant DNA technology (biosynthetic), and its control and potency assignment is performed by HPLC in SI units of mass rather than units of bioactivity. Thus, the current WHO International Standard, 83/500, is no longer suitable for the control and potency assignment of therapeutic preparations of insulin.

As a result, there is now a requirement for a single global standard containing high purity insulin to reflect the status of insulin as a well-characterized, mass-balance assigned molecule [3-4]. It is anticipated that a highly pure insulin standard will aid in improving the consistency and accuracy of clinical diagnostic tests [5-6]. Also, this will enable universal adoption of the internationally-recognised specific activity conversion factor for pure insulin where 1 IU of activity is contained in 0.0347 mg insulin (as stated in the PhEur and USP) [4].

With these points in mind, a preparation of highly purified, therapeutic grade insulin has been filled into ampoules (NIBSC code 11/212) following procedures recommended by WHO [7], to produce the proposed 1st International Standard for insulin, human. This batch of ampoules has been evaluated in a two-phase collaborative study. First, to assign a value to the candidate standard using a mass-balance approach, and secondly, to determine its immunoreactivity using current immunoassay methods, and to assess the suitability of the candidate standard to calibrate immunoassays used to measure the insulin content of human serum and plasma samples.

The aims for this study were therefore:

Phase 1

1. To assign an insulin content to the candidate standard, 11/212, using a mass-balance assessment of the insulin content of the bulk active pharmaceutical ingredient (API) used to prepare the candidate standard and the processing data from the preparation of the batch of ampoules coded 11/212.

2. To provide confirmatory data for the assigned insulin content of the candidate standard using the orthogonal methods of total nitrogen content analysis and HPLC assay to determine the insulin content of the candidate standard in terms of mass-balanced insulin API.

Phase 2

1. To demonstrate the suitability of the candidate standard, 11/212, to serve as an International Standard to calibrate human insulin immunoassays by examining its behaviour in immunoassays used for the measurement of patient samples.

2. To assess the relationships among the 1st IRR for insulin immunoassay, 66/304, local standards and 11/212.

3. To assess the stability of the candidate preparation, 11/212, after accelerated thermal degradation.

Participants

19 laboratories in 11 countries took part in either the Phase 1 or Phase 2 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	Dr Stefaan Marivoet Tosoh Europe N.V., Transportstraat, 3940 Tessenderlo
CHINA	Li Bo and Liang Chenggang National Institutes for Food and Drug Control, 2 Tiantan Xili, Beijing 100050
DENMARK	Ulla Riber, Lene Adamczewski, Signe Baerentzen and Therese Linden Novo Nordisk A/S, Novo Allé, DK-2880, Bagsvaerd
DENMARK	Lars Husager, Neils Fabricus and Elisabeth Huusom Danish Health and Medicines Agency
FRANCE	Jean-Bernard Graff Eli Lilly and Company, Building 400, BP 10, 2 Rue du Colonel Lilly, Fergersheim, 67640
GERMANY	Dr Stefan Hutzler Roche Diagnositcs GmbH, Building 751/Room 125, Nonnenwald 2, 82377, Penzberg
INDIA	Dr Renu Jain, Ms Shalini Tewari, Mr Tara Chand, Dr Gaurav Pratap Singh, Ms Sudha V. Gopinath, Dr J.P. Prasad, Ms Gurminder Bindra, Dr Meena Kumari, Ms Shruti Dixit and Ms Vandana Saklani, National Institute of Biologicals – Ministry of Health and Family Welfare, Government of India, A32, Sector 62, Noida-201309
ITALY	Luca Pallavicini, Alberto Campani, Martino Grisorio and Iris Kutschera DiaSorin, Via Cresentino snc, 13040 Saluggia (VC)
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USA	Ryan Masica Beckman Coulter, 322 Lake Hazeltine Drive, Chaska, MN 55318
USA	Kim Dancheck and Matt Borer

	Eli Lilly, Lilly Research Labs, 1400 West Raymond Street, Dock 314, IN 46221
USA	Holly Groth and Qian Ding Ortho Clinical Diagnostics, 130 Indigo Creek Drive, NY 1426-5103
USA	C. Randy Reamer, Mary Walczak and Omar Quraishi. Siemens Healthineers, 511 Benedict Avenue, Tarrytown, NY 10591
USA	Tina Morris, Mary Crivallone, Edith Chang, C. Nie, S. Shrestha, Luba Parris and Mary Waddell United States Pharmacopoeia, Reference Standards Laboratory, Rockvile, MD

Materials and Methods

Bulk materials and processing

A bulk preparation of highly purified, recombinant human insulin (insulin API) was generously donated to the WHO by Novo Nordisk. The material was provided as 100g of crystalline insulin API (batch AM0H22101).
All processing was performed at the National Institute for Biological Standards and Control (NIBSC, Blanche Lane, Potters Bar, EN6 3QG, UK).

Insulin API for mass balance:
Approximately 25 g of the insulin API was removed in a dry box and further distributed under controlled relative humidity (3%) into smaller vials containing, either 100, 200, 250, 300 or 500 mg insulin API for the tests required for the mass balance characterisation and nitrogen analysis. Vials containing approximately 50 mg were prepared for the HPLC assay. API sub-portions were prepared on 23rd November 2011. As described in the results section, the use of the dry box may have inadvertently dried the small quantities of sub-portioned insulin API. Thus, a further set of sub-portions were prepared on 17th July 2015 at ambient temperature and humidity for a repeat of elements of the Phase 1 study (Phase 1 Repeat study).

Candidate standard for insulin, 11/212
55.028 g of the bulk API was removed in a dry box under controlled humidity, dissolved in ddH_2O and acidified with 0.2 M HCl until the solution was clear. The acidified solution was neutralised to pH 7.4 with 0.2 M NaOH and the final volume was made up gravimetrically to 5503 g to give a concentration of insulin API of 10 mg/g final solution. This solution was dispensed in 1 g aliquots into glass ampoules, lyophilised and sealed on 23rd November 2011.
In total, 5000 ampoules were filled and are available for use. Ampoules are stored at -20ºC at NIBSC. The mean fill mass was 1.0002 g (CV 0.125%) with a mean dry weight of 0.0100 g (CV 1.94%). The mean residual moisture was 1.3657% (CV 23.3%) and mean oxygen head space 0.63% (CV 32.34%).

Phase 1 and Phase 1 Repeat - value assignment by mass balance

Methods contributed

The Phase 1 and Phase 1 Repeat collaborative studies were organised by NIBSC to determine the insulin content of the bulk API in order to define the content of the candidate standard by a mass balance approach. Both studies included the estimation of the insulin content of 11/212 by total nitrogen analysis of the insulin API and by HPLC assay of 11/212 in terms of the insulin API to provide orthogonal estimates.

Participants were provided with vials of the insulin API, sub-portioned as described above, with which to perform the mass balance assessment tests. A summary of the methods used and the portioned insulin API required for each test are described in Table 2. Participants were requested to follow the appropriate pharmacopeial methods where possible, and to report their data using the data return sheet (see Appendix 1 – Phase 1 study protocol and Table 2 below). Further details of the methods are described in Appendix 1 – Phase 1 study protocol.

Ampoules of the candidate standard, 11/212 were provided for the assessment of insulin content and related impurities by reverse phase HPLC. Participants were requested to follow pharmacopeial method EP 2.2.29 and to report their data using the data return sheets provided (Appendix 1 – Phase 1 study protocol). A vial of EDQM insulin (porcine) reference material (European Pharmacopoeial Reference Standard, Insulin (porcine) I0320000, batch 3.3) was also provided, to be used for the system suitability tests for the HPLC methods.

Table 2: Mass balance and orthogonal tests to value assign the candidate, 11/212

Test - mass balance	**Insulin API provided per test (mg)**	**Laboratories performing tests in Phase 1**	**Laboratories performing tests in Phase 1 Repeat**
Water content (EP 2.2.32 – Loss on Drying (LOD))	200	Labs 1, 2, 3, 4, 5, 8,	Labs 1, 2, 5, 8,
Zinc (EP 2.2.23 - Atomic absorption spectrometry - AAS)	50	Labs 1, 2, 3, 4, 5,	Lab 1
Sodium (EP 2.2.23 – AAS) Chloride and Acetate (Ion chromatography)	250 100	Labs 1, 5	Lab 1
Test - orthogonal methods	**Insulin API provided pertest (mg)**	**Laboratories performing tests in Phase 1**	**Laboratories performing tests in Phase 1 Repeat**
Nitrogen (Elemental or Kjeldahl)	200	Labs 1, 5, 8	Labs 1, 3, 5, 8
HPLC assay (EP 2.2.29 - RP-HPLC)	50	Labs 1-8	Labs 1, 2, 5, 8, 9

Stability assessment of 11/212

Thermally-accelerated degradation samples (ATD samples) stored at temperatures of +4°C, +20°C, +37°C and +45°C for 78 months were available in limited numbers. Samples were analysed for insulin content by a HPLC assay in comparison with a reference sample stored at -20°C. Remaining ATD samples were distributed to participants in Phase 2 for assessment by immunoassay based on assay capacity and sample availability. Samples were provided coded A-E and participating laboratories received one to three sets of all five coded ATD samples for analysis.

Collaborative study for the evaluation of 11/212 by immunoassay – Phase 2

The collaborative study was organised by NIBSC. All participants were provided with ampoules of the candidate standard, 11/212, 1 ampoule of the 1st IRP 66/304 and a panel of 14 human serum and 5 human plasma samples coded InsSerum1 to InsSerum14 and InsPlasma1 to InsPlasma5, as described in Table 3. Human serum and plasma samples were kindly collected by Dr Gwen Wark (UKNEQAS) or purchased from First Link UK Ltd and TCS Biosciences.

Table 3. Material provided for Phase 2 of the Insulin collaborative study

Preparation	**Contents**
1st IRR for human insulin immunoassay, coded 66/304	3 IU/ampoule
Candidate standard, labelled CS613-11/212	9.19 mg/ampoule (equivalent to 264.8 IU)[1]
ATD samples of 11/212, labelled CS613 Sample A to E	Assumed to be equivalent to 11/212
Human serum samples (n=14) labelled InsSerum1 to 14	0.5 – 1.0 ml human serum, volume dependent on assay requirements
Human plasma samples (n=5) labelled InsPlasma1 to 5	0.5 – 1.0 ml human plasma, volume dependent on assay requirements

[1]Conversion factor used: 1IU = 0.0347 mg (PhEur)

Participants were requested to prepare dilutions of the ampouled preparations and to measure, in duplicate or triplicate, the insulin concentration of these dilutions and the serum/plasma samples using the immunoassay(s) normally in use in their laboratory. Participants were requested to perform at least three independent assays, using fresh ampoules and including all the preparations allocated in each assay. As it was important that each participant prepared the same dilutions of each ampouled preparation in order to aid calculations of commutability, participants were requested to prepare and measure the insulin concentration in a minimum of 7 core dilutions of 66/304 and 11/212 that were common to all participants (and that covered a minimum of five dose points in the linear part of their standard curve). These core dilutions were 288, 144, 72, 36, 18, 9 and 4.5 µIU/ml. Further details are provided in Appendix 2 – Phase 2 study protocol. In total, 15 insulin immunoassay methods were contributed, and included: Abbott ARCHITECT; Abbott Alinity I; Beckman Access2; Beckman DxI; Diasorin Liaison; Diasorin Liaison XL; Invitron

ELISA; Roche Elecsys; Mercodia ELISA; Siemens ADVIA Centaur; Ortho Clinical Diagnostics VITROS; TOSOH AIA and TOSOH AIA-CL.

Data and Statistical Analysis

Value assignment of 11/212 by mass balance

The mass balance assignment to the candidate standard was made according to the following formula:

Insulin plus related impurities = 100% - [%LOD + %zinc + %salts (Na^+, Cl^-, $Acet^-$)]

(where related impurities were assessed by RP-HPLC on the candidate standard)

Data generated from the mass balance assessment of the insulin content of the insulin API (corrected for related impurities using data from HPLC on the candidate standard) was used in conjunction with data from the filling of the candidate standard to assign the final insulin content to the candidate standard. The insulin solution used to fill the candidate standard was prepared to give a concentration of insulin API “as is” of 10.00 mg/g final solution. This solution was dispensed in 1g aliquots into glass ampoules, lyophilised and sealed. Data from the fill was as follows:

Mean filling weight = 1.0002 g per ampoule (RSD of 0.12%)

The data from the insulin API mass balance and this mean filling weight, was combined to give the insulin plus A21 desamido content of the candidate standard as follows:

Insulin plus A21 desamido = API mass balance value/100 x 10 x 1.0002

Uncertainty

Standard uncertainty of the insulin content of candidate standard was calculated using the following:

$$\sqrt{u_{LOD}^2 + u_{Zn}^2 + u_{salts}^2 + u_{rel\ imp}^2 + u_{fill\ wt}^2}$$

where u_{LOD}, u_{Zn}, u_{salts}, $u_{rel\ imp}$, denote standard uncertainties due to tests for loss on drying, zinc content, salt content and related impurities and $u_{fill\ wt}$ denotes the standard uncertainty for the filling weight. The individual standard uncertainties were calculated using a variance components analysis.

Total Nitrogen analysis
Given the MW of insulin (5807.58), the atomic weight of nitrogen (14.0067) and the number of nitrogen (N) atoms per human insulin molecule (65) the theoretical insulin (plus related substances) content was determined from an analysis of total N as follows:

Theoretical insulin = [(65 x 14.0067)/5807.58] x 100% = 15.68%

Using the experimentally derived measurement of the N content (%) of the bulk API, the insulin content of the insulin API was calculated:

% N (bulk API) / % N (theoretical) = human insulin plus related impurities per 100mg insulin API

Assessment of the insulin content of 11/212 by HPLC
Content of human insulin plus A21 desamido insulin per ampoule of the candidate standard is given by:

$$\frac{\text{A x mass (mg) of HCl added to ampoule}}{\text{B x mass (mg) HCl added to ref (b)/mass insulin API in ref (b)}} \times \text{insulin content of bulk material (mg /mg “as is”)}$$

where A is the sum of human insulin and A21 desamido insulin peak area (mean of three injections) for the reconstituted candidate standard, B is sum of human insulin and A21 desamido insulin peak area (mean of three injections) for the dissolved insulin API and ref (b) is the reference solution prepared from the insulin API as described in Appendix 1a (Phase 1 study protocol, HPLC assay section).

Assessment of the immunoreactivity of 66/304 and 11/212
Analysis was performed with insulin concentrations as reported by the participants, using results from the nominal concentration range of 2.25 to 144 µIU/mL only. To determine if an assay showed acceptable dilutional linearity, linear regression analysis was applied to each sample in each assay run to estimate the slope of $\log_{10}$ reported concentration against $\log_{10}$ nominal concentration. The r^2 value was confirmed to exceed 0.98 in all cases. Estimates for 66/304 and 11/212 were considered invalid if the slope was outside the range [0.91, 1.10].

Results from all valid assays were corrected for dilution factor and combined to generate unweighted geometric mean (GM) estimates for each laboratory and these laboratory means were used to calculate overall unweighted geometric mean estimates. Variability between laboratories has been expressed using geometric coefficients of variation (GCV = $\{10^s-1\}\times 100\%$ where s is the standard deviation of the $\log_{10}$ transformed estimates).

Assessment of commutability
Analysis of difference in bias. All reported results were $\log_{10}$ transformed for analysis in order to achieve approximately constant scatter over the range of concentrations used. A consensus value for each sample, shown in Appendix 4, Table 4a, was calculated as Huber's robust mean of

laboratory means using the R package ‘WRS2’ (8). Bias values were then calculated for all reported results as the difference between the reported value and the study consensus value for that sample. The standard deviation of the bias values for plasma and serum samples only was calculated within each laboratory and a pooled value, s_P, was calculated across all laboratories. Commutability criteria representing the maximum acceptable difference in bias were then set as $\pm 3s_P$. Reference standards were to be concluded as commutable if the observed difference in bias was within the commutability criteria. Confidence intervals for the difference in bias between each reference standard (66/304 or 11/212) and plasma/serum samples were calculated using a mixed linear model (with random factor assay run) for each laboratory. For this commutability assessment, the bias for plasma and serum samples has been assumed to be constant over the concentration range used. In order to meet this assumption, results for the reference standards from the nominal concentration range of 4.5 to 144 µIU/mL only were used.

Assessment of stability
The relative content from HPLC analysis, or relative immunoreactivitives from immunoassay data, of the accelerated thermal degradation samples were used to fit an Arrhenius equation relating degradation rate to absolute temperature assuming first-order decay (9), and hence predict the degradation rates when stored at a range of temperatures.

Results

Phase 1 and Phase 1 Repeat - value assignment by mass balance

Data returned for analysis – Phase 1
As shown in Table 2, two laboratories performed the tests for the sodium, chloride and acetate ions and five laboratories measured the zinc content of the insulin API. Six laboratories performed loss on drying (LOD) and three laboratories performed total nitrogen analysis. All laboratories (n=8) in Phase 1 performed the HPLC assay for insulin content and related impurities of the candidate standard, 11/212.

Data returned for analysis – Phase 1 Repeat
Laboratories were asked to repeat certain elements of the Phase 1 study using freshly sub-portioned insulin API. Samples were allocated based on assay capacity and sub-portion availability. As shown in Table 2, one laboratory (Lab 1) repeated the assessment of zinc, sodium, chloride and acetate thereby confirming the data from the Phase 1 study. The Phase 1 data for zinc, sodium, chloride and acetate was therefore used throughout the subsequent mass balance calculations. Four laboratories repeated the assessment of LOD and nitrogen analysis and five laboratories provided HPLC assay data. The Phase 1 data for related impurities was used for subsequent mass balance calculations.

Mass balance assignment and orthogonal data – Phase 1
A summary of the mass balance and orthogonal data from Phase 1 of the study is shown in Table 4. Laboratory mean estimates are shown in Appendix 3 (Tables A3a-A3c).

As shown in Table 4, the insulin content of 11/212 as calculated by the mass balance approach is 9.2021 mg/amp with an expanded uncertainty ($k=2$) of 9.1624 – 9.2419 mg/amp. This estimate was supported by analyses of the nitrogen content of the insulin API which provided an estimate of the insulin content of 11/212 of 9.2211 mg/amp with an expanded uncertainty ($k=2$) of 9.1586 – 9.2836 mg/amp. However, the results of the Phase 1 study indicated that estimates of the insulin content of the candidate standard as determined by HPLC were 1-2% lower than the content assigned by mass balance. By HPLC, the insulin content of 11/212 as calculated by the mass balance approach was 9.0579 mg/amp with an expanded uncertainty ($k=2$) of 8.9816 – 9.1342 mg/amp.

A series of in-house assessments, outlined in Figure 1, were performed which confirmed that the apparent loss of insulin content in 11/212 was not due to heterogeneity of the fill or through the process of freeze-drying, but instead, indicated that the sub-portions of insulin API provided to each participant for testing, may have been inadvertently dried during preparation. Loss of water from the insulin API sub-portions could have greatest impact on measurements of water content by loss on drying (LOD), nitrogen content and the HPLC assay of insulin content. The drying did not occur in the handling of the material used to prepare the candidate standard, and this inconsistency is likely to have caused the observed discrepancy in the content estimates. In the Phase 1 Repeat study, participants were asked to repeat elements of Phase 1 using insulin API which had been sub-portioned at ambient temperature and humidity.

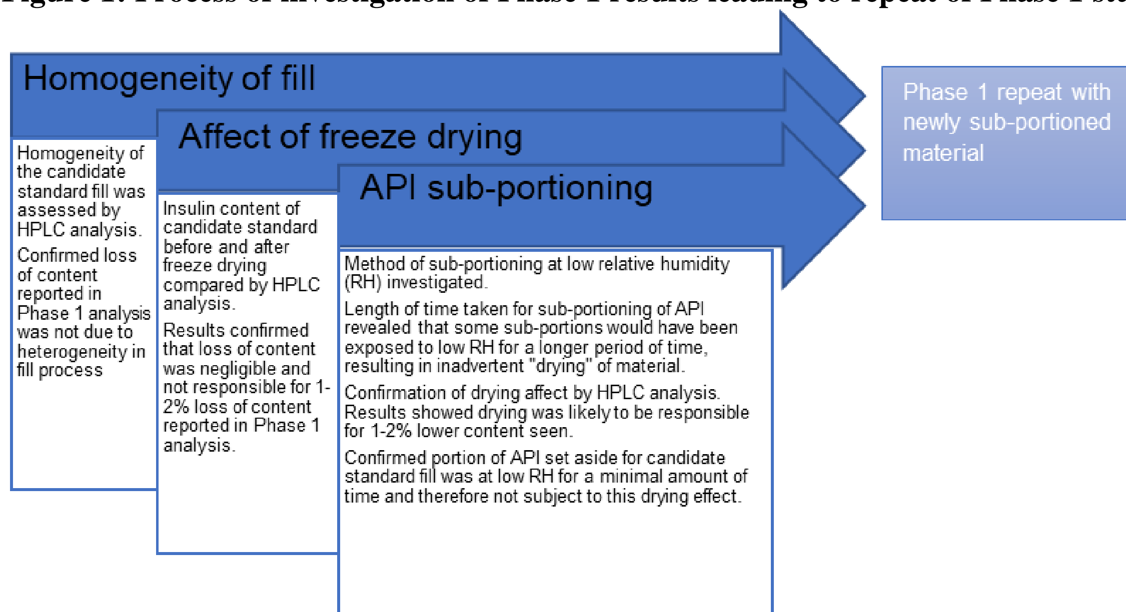
Table 4: Mass value assignment using data from the Phase 1 study and content estimates from the orthogonal tests of nitrogen analysis and HPLC assay.

Phase 1 Tests – Mass Balance	Phase 1 Study		
	Estimate mg/100 mg API	Std Error	n
LOD	6.9222	0.1563	6
Zinc	0.3632	0.0032	5
Sodium	0.0244	0.0005	2
Acetate	0.2383	0.0040	1
Chloride	0.1600	0.0000	1
Related Impurities	**Percentage**	**Std Error**	**n**
% related impurities by HPLC	0.3130	0.0466	8
Phase 1 – Mass Balance Calculation	**Insulin content**	**Std Error**	**Rel. Std. Error**
Insulin content of API including related impurities	92.2919 mg/100 mg API	0.1564	0.1694%
Insulin content or API corrected for related impurities	92.0030 mg/100 mg API	0.1617	0.1758%
Insulin content of 11/212	**9.2021 mg/amp**	0.0199	0.2159%
Approximate expanded uncertainty (k=2)	*9.1624 – 9.2419*		

Phase 1 Test – orthogonal	%age N	Std Error	n
Nitrogen	14.5012	0.0452	3
Phase 1 – Content Calculation (Nitrogen)	**Insulin content**	**Std Error**	**Rel. Std. Error**
Insulin content of API including related impurities	92.4820 mg/100 mg API	0.2880	0.3114%
Insulin content or API corrected for related impurities	92.1925 mg/100 mg API	0.2903	0.3149%
Insulin content of 11/212	**9.2211 mg/amp**	0.0313	0.3389%
Approximate expanded uncertainty (k=2)	*9.1586 – 9.2836*		

Phase 1 Test – orthogonal	Insulin content	Std Error	Rel. Std. Error
HPLC assay - uncorrected estimate of content of 11/212 in terms of insulin API (n=8)	9.8452	0.0356	0.3618%
Insulin content (corrected) for mass balance	**9.0579 mg/amp**	0.382	0.4213%
Approximate expanded uncertainty (k=2)	*8.9816 – 9.1342*		

Figure 1: Process of investigation of Phase 1 results leading to repeat of Phase 1 study



Mass balance assignment and orthogonal data – Phase 1 Repeat
A summary of the mass balance and orthogonal data from Phase 1 Repeat study is shown in Table 5. Laboratory mean estimates are shown in Appendix 3 (Tables A3d-A3f). An overall summary of the final content estimates as determined by mass balance, nitrogen analysis or HPLC assay is shown in Table 5 and Figure 2.

As shown in Table 5, the insulin content of 11/212 as calculated in the Phase 1 Repeat study by the mass balance approach is 9.1921 mg/amp with an expanded uncertainty (k=2) of 9.1419 – 9.2422 mg/amp which was comparable to the Phase 1 estimate. Analyses of the nitrogen content of the insulin API gave an estimate of 9.1354 mg/amp with an expanded uncertainty (k=2) of 8.9731 – 9.2978 mg/amp, which is in broad agreement with the mass balance assigned value. Of the four laboratories that determined the nitrogen content of the insulin API using newly prepared sub-portions of 200 mg insulin API, three laboratories determined nitrogen content by elemental analysis and one laboratory performed Kjeldhal analysis. The mean percentage nitrogen of 14.3665% was lower than the mean estimate determined in the Phase 1 study (15.5102%) which is consistent with the 200 mg sub-portions of the Phase 1 study having been dried and thereby contain more insulin per milligram of API. However, the data from this study are more variable, as demonstrated by the larger expanded uncertainty for the mean estimate of the content of the candidate standard shown in Table 5 below and Figure 2.

By HPLC, the insulin content of 11/212 as calculated in the Phase 1 Repeat study by five laboratories is 9.1872 mg/amp with an expanded uncertainty (k=2) of 9.0669 – 9.3075 which, as shown in Figure 2 is higher than the estimates obtained in Phase 1. As the majority of the HPLC assays used sub-portions of 50 mg, it is likely that these smaller sub-portions were more affected by drying during the sub-portioning procedure in the dry box which resulted in a higher insulin content in the HPLC calibrant.

Table 5: Mass value assignment using data from the Phase 1 Repeat study and content estimates from the orthogonal test of nitrogen analysis and HPLC assay.

Phase 1 Repeat Tests – Mass Balance	Phase 1 Study		
	Estimate mg/100 mg API	**Std Error**	**n**
LOD	7.0232	0.2190	4
Zinc (used to confirm Phase 1 data)	0.3703	0.000	1
Sodium (used to confirm Phase 1 data)	0.0207	0.0004	1
Acetate (used to confirm Phase 1 data)	0.1650	0.0022	1
Chloride (used to confirm Phase 1 data)	0.2600	0.0037	1
Related Impurities	**Percentage**	**Std Error**	**n**
% related impurities by HPLC - *Phase 1 data*	0.3130	0.0466	8
Phase 1 Repeat – Mass Balance Calculation*	**Insulin content**	**Std Error**	**Rel. Std. Error**
Insulin content of API including related impurities	92.1909 mg/100 mg API	0.2191	0.2376%
Insulin content or API corrected for related impurities*	91.9023 mg/100 mg API	0.2226	0.2422%
Insulin content of 11/212	**9.1921 mg/amp**	0.0251	0.2727%
Approximate expanded uncertainty (k=2)	*9.1419 – 9.2422*		

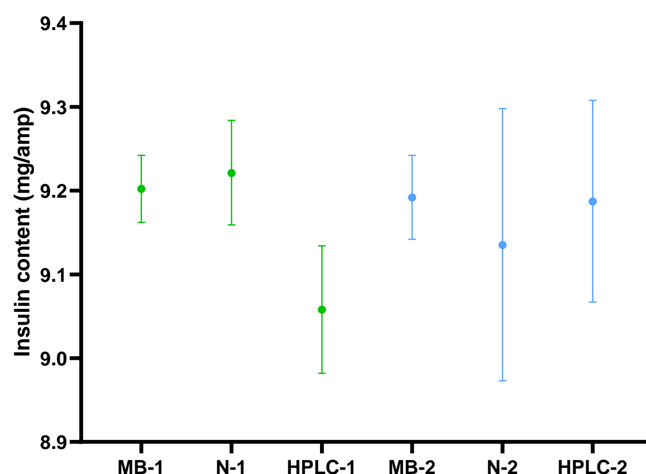
*Using Phase 1 data for salt, zinc and related impurities

Phase 1 Repeat Test – orthogonal	%age N	Std Error	n
Nitrogen	14.3665	0.1262	4
Phase 1 Repeat – Content Calculation (Nitrogen)	**Insulin content**	**Std Error**	**Rel. Std. Error**
Insulin content of API including related impurities	91.6229 mg/100 mg API	0.8049	0.8785%
Insulin content or API corrected for related impurities*	91.3361 mg/100 mg API	0.8036	0.8798%
Insulin content of 11/212	**9.1354 mg/amp**	0.0812	0.8887%
Approximate expanded uncertainty (k=2)	*8.9731 – 9.2978*		

*Using Phase 1 data for related impurities

Phase 1 Repeat Test – orthogonal	Insulin content	Std Error	Rel. Std. Error
HPLC assay - uncorrected estimate of content of 11/212 in terms of insulin API (n=5)	9.9967	0.0595	0.5951%
Insulin content (corrected) for mass balance	**9.1872 mg/amp**	0.0601	0.6546%
Approximate expanded uncertainty (k=2)	*9.0669 – 9.3075*		

Figure 2: Summary of content estimates for the candidate standard by mass balance, nitrogen analysis and HPLC assay from the original Phase 1 study (MB-1, N-1, HPLC-1) and the Phase 1 Repeat Study (MB-2, N-2, HPLC-2)



Results summary – value assignment of 11/212 by mass balance

An overall summary of the Phase 1 and Phase 1 repeat results of the mass balance assessment and orthogonal tests of nitrogen analysis and HPLC assay is provided in Table 6. The data suggest that smaller sub-portions containing 50-100 mg insulin API used in the Phase 1 study for the HPLC assay were likely to have been subject to variable drying resulting in an underestimation of the content of the candidate standard by some laboratories. The results presented here (Table 6) show that the HPLC data of the Phase 1 Repeat study supports an assignment of content to the candidate standard, 11/212, by mass-balance using the Phase 1 Repeat study assignment of **9.19 mg/ampoule** with expanded uncertainty of **9.14 to 9.24 mg/ampoule (*k=2*)**.

Table 6: Overall summary of estimates of the insulin content of 11/212 by mass balance, nitrogen analysis and HPLC assay

Data set	Method	11/212 Insulin content (mg)	Expanded Uncertainty (*k=2*)	
			Lower	Upper
Phase 1	Mass Balance	9.202	9.162	9.242
	Nitrogen	9.221	9.159	9.284
	HPLC	9.058	8.982	9.134
Phase 1 Repeat	Mass Balance	9.192	9.142	9.242
	Nitrogen	9.135	8.973	9.298
	HPLC	9.187	9.067	9.308

Results Phase 2

Assay validity
In total, participants in the collaborative study performed 41 runs of the 15 method/platform combinations listed in Materials and Methods Phase 2 above. All immunoassays reported insulin concentration in mIU/L or µIU/mL.The following exclusions were made as the slopes of the fitted regression lines (Appendix 4, Table 4b) did not meet the validity criteria described above: run 1 (66/304 and 11/212) and run 2 (11/212) by laboratory 10, run 3 (11/212) by laboratory 17a and run 2 (66/304) by laboratory 17b.

Estimates of the immunoreactivity of 66/304 and 11/212
Participants were requested to run a set of core dilutions of each standard prepared from an initial concentration (dilution 1) of 576 µIU/mL, as described in Appendix 2 – Phase 2 study protocol. Using all assay runs that met the validity criteria, geometric mean laboratory estimates of the insulin concentrations of dilution 1 of 66/304 and 11/212 were determined (Table 7 and Figure 3). Estimates from individual runs are shown in Appendix 4, Table 4c. As summarized in Table 7, estimates ranged from 437.4 µIU/mL to 811.6 µIU/mL with a **geometric mean estimate of 609.8 µIU/mL (95% CI: 560.8 – 663.0, n=15, GCV 16.3%) for 66/304** and from 436.6 µIU/mL to 861.0 µIU/mL with a **geometric mean estimate of 581.3 µIU/mL (95% CI: 525.7 – 642.7, n=15, GCV 19.9%) for 11/212**.

The estimate ranges for both 11/212 and 66/304 are broadly in agreement with each other, and the geometric means for each standard are both in good agreement with the expected concentration of 576 µIU/mL. Intra-assay variability was acceptable for the measurement of both 66/304 and 11/212 with reported GCV% values of laboratory estimates being less than 5% in the majority of assays (Table 7), and importantly, both standards appear to be behave in a similar manner in each assay.

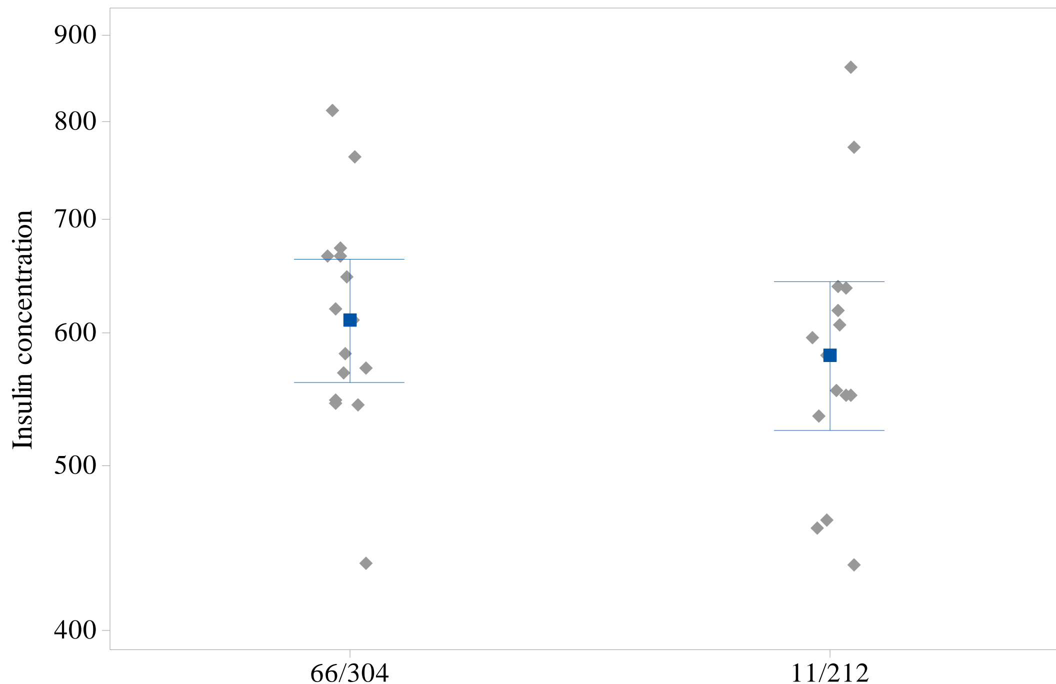
After taking into account the dilution factor from ampoule reconstitution, these laboratory geometric mean estimates are equivalent to **3.18 IU/amp** for 66/304 and **267.1 IU/amp** for 11/212, which are in very good agreement with the assigned 3 IU/amp for 66/304 and 9.19 mg/amp for 11/212, which is equivalent to 264.8 IU/amp after application of the activity conversion factor for pure insulin, 1IU = 0.0347 mg insulin.

Table 7. Laboratory geometric mean estimates of insulin concentration in µIU/mL for dilution 1 of 66/304 and 11/212.

Lab	66/304		11/212	
	GM	**GCV**	**GM**	**GCV**
10	582.7	12.3%	581.6	n/c
11a	567.3	1.9%	606.3	15.0%
11b	672.5	n/c	637.4	n/c
12	544.2	2.7%	553.7	3.1%
13	437.4	3.4%	436.6	2.0%
14a	546.6	3.1%	464.4	5.2%
14b	542.7	1.4%	459.1	2.9%
15	609.6	5.7%	594.8	8.2%
16a	664.9	4.4%	550.0	5.0%
16b	665.2	1.3%	550.1	5.2%
17a	811.6	13.0%	771.4	1.8%
17b	761.7	0.7%	861.0	13.5%
18a	619.1	1.3%	638.5	1.3%
18b	647.1	0.8%	618.0	1.5%
19	571.6	0.8%	534.9	6.2%
GM	609.8		581.3	
95% CI	560.8 – 663.0		525.7 – 642.7	
GCV	16.3%		19.9%	

GM: Geometric Mean
GCV: Geometric Coefficient of Variation
CI: Confidence Interval
n/c: Not calculated (n<3)

Figure 3. Laboratory geometric mean estimates of the insulin concentration of dilution 1 of 66/304 and 11/212. Overall geometric mean and 95% CI are also shown.



Assessment of reference standard commutability

The commutability of 66/304 and 11/212 with plasma and serum samples was assessed for all methods included in the study. The limits for acceptable bias difference are defined as described above, excluding data from laboratory 10 which had significantly higher variability in bias values than for all other laboratories, giving ±0.070, or 0.851 to 1.175 on the untransformed scale, i.e. the bias for a reference standard must be demonstrated to be not less than 85.1% and not more than 117.5% of the bias observed for plasma/serum samples.

The limits were applied to the mean plasma/serum sample bias in each laboratory as shown in Figure 4. For each laboratory, Figure 4 shows individual data points for the bias from the consensus values for each plasma/serum sample and each nominal reference material concentration between 4.5 to 144 µIU/mL. The mean bias for serum and plasma samples for each method and the upper and lower limts defining acceptable bias difference using limits of commutability are also shown in Table 8a and the bias values for each reference material dilution are shown in Table 8b. Values in red indicate where the bias for the dilution is outside the acceptable bias difference from the mean value observed for serum and plasma samples. Confidence intervals (90%) are also shown and highlighted in green where they are fully within the commutability criteria range (±0.070) and in red where they are fully outside this range. Table 8b also shows the slopes of $\log_{10}$ reported

concentration against $\log_{10}$ consensus concentration which are expected to have a value of 1 (i.e. constant bias) for this difference in bias approach to be appropriate. All slopes were in the range [0.91, 1.10] with the exception of laboratory 10 (plasma and serum sample slope of 1.112).

The results (Figure 4 and Table 8) after applying the difference in bias approach can be summarized as follows:

- *Methods in which 66/304 and 11/212 are both within the the limits of commutability at 3 or more dilutions:* ***11a, 11b, 14a, 18b, 19***

- *Methods in which 11/212 is within the limits of commutability but 66/304 is not:* ***12, 16a, 16b***

- *Methods in which 66/304 is within the limits of commutability but 11/212 is not:* ***14b*** *(same method and platform as 18b (commutable),* ***18a*** *(same method and platform as 14a (commutable)*

- *Methods in which the biases of 66/304 and 11/212 are comparable but outside the limits of commutability:* ***13, 15***

- *Methods in which the biases of 66/304 and 11/212 are not comparable and both are outside the limits of commutability:* ***17a, 17b*** *(same method on different platforms)*

Fourteen laboratories showed consistent patient sample bias (11a, 11b, 12, 13, 14a, 14b, 15, 16a, 16b, 17a, 17b, 18a, 18b and 19). Of these, 66/304 was found to be commutable with patient samples in 6 cases (11b, 14a, 14b, 18a, 18b and 19), and commutable in another (11a) at 3 dilutions (144, 72 and 36 µIU/mL), with 3 dilutions (18, 9 and 4.5 µIU/mL) outside the lower limits of commutability of -0.030 with values of 0.037, 0.033 and -0.055 respectively (Table 8a; confidence interval also giving an inconclusive result). 66/304 was non commutable with patient samples in 7 cases (12, 13, 15, 16a, 16b, 17a and 17b).

The candidate standard 11/212 was found to be commutable with patient samples in 8 of these 14 cases (11a, 11b, 12, 14a, 16a, 16b, 18b and 19) and non commutable with patient samples in 6 cases (13, 14b, 15, 17a, 17b and 18a), although for lab 14b this was unclear for all dilutions where the bias for 11/212 was within the limits of commutability at 2 dilutions (144 and 4.5 µIU/mL) (Table 8a and 8b; confidence interval also giving inconclusive result). Of the cases in which 11/212 was non commutable, it is interesting to note that the method used in lab 18a is the same as that used by lab 14a, in which 11/212 was commutable. Similarly, where 11/212 was found non-commutable by lab 14b, this was the same method and platform as that used in lab 18b in which 11/212 was commutable.

For the majority of laboratories, the bias values of the two reference preparations 11/212 and 66/304 were aligned. For example, although non commutable in labs 13 and 15, the bias values of 11/212 and 66/304 were comparable, suggesting that 11/212 behaves in a similar manner in these assays in comparison to the 1st IRP, 66/304, to which they are calibrated. In three cases (12, 16a

and 16b) 11/212 was found commutable but 66/304 was not, indicating that 11/212 may show an improvement in commutability with these methods compared with 66/304.

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 directly related to the bias seen in patient samples in each assay. For these immunoassays, where the mean bias seen in patient serum and plasma samples in each laboratory is quite small, the resulting limits of commutability may be too strict to fully define acceptable commutability. There is the potential for intra-assay variability between methods to therefore have had an impact on the statistically-derived definitions of commutability/non commutability. This variability may be influenced, for example, by experimental variation caused by a number of external factors such as assay procedures, dilution buffers, different operators etc. 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 this.

In summary, of the 14 laboratories which demonstrated a consistent patient sample bias, the candidate standard 11/212 was shown to be fully commutable with patient samples in 8 laboratories. For the remaining 6 laboratories in which 11/212 was found non commutable, 2 of these used the same methods in which the candidate standard had been shown to be commutable by other laboratories, and in a further 2 methods the candidate standard had a comparable response to the 1st IRP 66/304. For the remaining 2 laboratories (the same method on different platforms), both 11/212 and 66/304 were outside the statistically-defined limits of commutability used in this study. Taken together with the results of the immunoassay estimates, which were in good agreement with the assigned values of the standards, the results indicate that the introduction of the candidate standard, 11/212, as a replacement for the 1st IRP 66/304, is suitable for the continued calibration of immunoassays for human insulin.

Figure 4. For each laboratory, comparison of the bias for serum and plasma samples (●) with the bias observed for each dilution of 66/304 (●) and 11/212 (●). Dashed line indicates no bias from study consensus values.

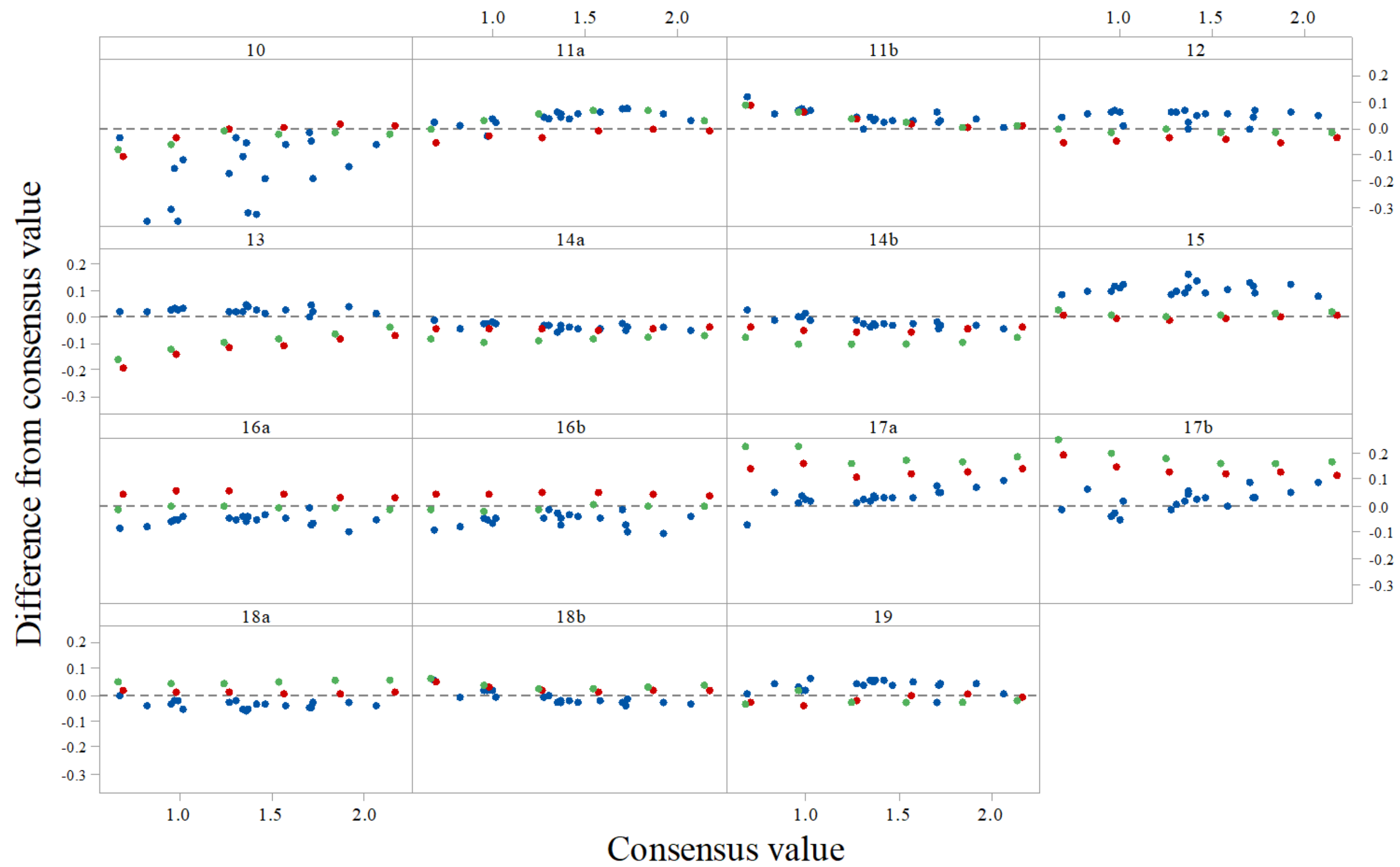


Table 8. Mean bias values obtained for 66/304 and 11/212.

(a) The mean bias for serum and plasma samples for each method and the upper and lower limts defining acceptable bias difference

(b) Mean bias values obtained for 66/304 and 11/212. Values in red indicate where the bias for the dilution is outside the acceptable bias difference from the mean value observed for serum and plasma samples.

(a)

Laboratory	Mean Serum Bias	Lower Limit	Upper Limit
10	-0.163	-0.234	-0.093
11a	0.040	-0.030	0.110
11b	0.042	-0.028	0.113
12	0.045	-0.025	0.115
13	0.024	-0.046	0.095
14a	-0.039	-0.109	0.031
14b	-0.023	-0.093	0.047
15	0.108	0.038	0.179
16a	-0.058	-0.129	0.012
16b	-0.060	-0.130	0.011
17a	0.029	-0.041	0.100
17b	0.016	-0.054	0.087
18a	-0.040	-0.110	0.031
18b	-0.014	-0.084	0.056
19	0.032	-0.039	0.102

(b)

Sample	Nominal µIU/mL	Laboratory														
		10	**11a**	**11b**	**12**	**13**	**14a**	**14b**	**15**	**16a**	**16b**	**17a**	**17b**	**18a**	**18b**	**19**
66/304	144	0.009	-0.013	0.008	-0.041	-0.076	-0.040	-0.041	0.004	0.025	0.034	0.138	0.109	0.009	0.015	-0.015
	72	0.015	-0.005	0.000	-0.055	-0.089	-0.048	-0.047	-0.003	0.030	0.040	0.126	0.127	0.004	0.012	0.000
	36	0.001	-0.013	0.013	-0.047	-0.115	-0.052	-0.060	-0.011	0.042	0.046	0.121	0.119	0.000	0.007	-0.006
	18	-0.007	-0.037	0.034	-0.041	-0.122	-0.049	-0.060	-0.015	0.055	0.046	0.106	0.124	0.006	0.015	-0.023
	9	-0.037	-0.033	0.062	-0.051	-0.143	-0.046	-0.053	-0.010	0.056	0.042	0.160	0.147	0.008	0.027	-0.043
	4.5	-0.112	-0.055	0.086	-0.054	-0.200	-0.050	-0.039	0.004	0.041	0.043	0.140	0.193	0.015	0.044	-0.035
11/212	144	-0.024	0.027	0.007	-0.019	-0.041	-0.076	-0.081	0.019	-0.019	-0.009	0.183	0.164	0.051	0.032	-0.025
	72	-0.019	0.065	0.003	-0.019	-0.065	-0.083	-0.098	0.009	-0.013	-0.008	0.164	0.159	0.052	0.025	-0.030
	36	-0.024	0.067	0.018	-0.019	-0.090	-0.088	-0.106	0.006	-0.014	-0.001	0.170	0.159	0.045	0.018	-0.029
	18	-0.010	0.052	0.037	-0.007	-0.103	-0.095	-0.108	-0.001	-0.005	-0.017	0.160	0.176	0.041	0.019	-0.029
	9	-0.063	0.028	0.061	-0.017	-0.129	-0.099	-0.107	0.003	-0.008	-0.023	0.219	0.198	0.040	0.031	0.017
	4.5	-0.083	-0.007	0.088	-0.003	-0.164	-0.088	-0.082	0.026	-0.019	-0.021	0.224	0.248	0.049	0.057	-0.040
66/304 slope		1.078	1.019	0.935	0.999	1.092	0.995	0.995	0.982	0.992	1.000	0.991	0.954	0.991	0.966	0.994
11/212 slope		1.049	1.017	0.924	0.983	1.085	1.001	0.987	0.972	0.997	1.005	0.965	0.947	0.988	0.972	1.023
Serum slope		1.112	1.053	0.946	0.997	0.998	0.984	0.959	1.003	1.006	1.002	1.066	1.065	0.988	0.950	0.999
90% CI for Bias Diff (66/304)		**0.070 – 0.188**	-0.093 – -0.048	**-0.031 – 0.036**	**-0.102 – -0.081**	**-0.185 – -0.147**	**-0.014 – 0.003**	**-0.034 – -0.015**	**-0.119 – -0.092**	**0.089 – 0.109**	**0.086 – 0.114**	**0.080 – 0.125**	**0.090 – 0.150**	**0.041 – 0.057**	**0.031 – 0.055**	**-0.068 – -0.036**
90% CI for Bias Diff (11/212)		0.057 – 0.177	**-0.032 – 0.012**	**-0.024 – 0.042**	**-0.065 – -0.044**	**-0.156 – -0.118**	**-0.056 – -0.039**	-0.078 – -0.058	**-0.101 – -0.073**	**0.036 – 0.056**	**0.033 – 0.061**	**0.135 – 0.180**	**0.138 – 0.198**	**0.084 – 0.100**	**0.040 – 0.064**	**-0.070 – -0.038**

Stability of 11/212

Stability of 11/212 was assessed by HPLC (Lab 9) and immunoassay (Labs 11b, 15, 17 and 19) analysis of coded accelerated thermal degradation (ATD) samples of 11/212, stored at elevated temperatures of 4°C, 20°C, 37°C and 45°C for 78 months, in comparison with a reference ampoule of 11/212 stored at -20°C. Data from Lab 17 were excluded, as dilutions of the ATD samples were not tested. Table 9 shows laboratory geometric mean estimates of insulin concentration for accelerated thermal degradation samples of 11/212 relative to sample C (-20°C) for both HPLC and immunoassay estimates. The data from Table 9 shows that by HPLC (Lab 9), there is very little reduction in relative potency at either +4 or +20ºC, and indeed, the predicted loss using HPLC estimates alone indicate 11/212 is highly stable, with no loss in potency predicted when stored at -20°C, and a predicted loss of only 0.156% per year when stored at +20ºC. For immunoassay data (Labs 11b, 15 and 19), there was a more noticeable loss of immunoreactivity at elevated temperatures, although it is noted that the ATD samples were at elevated temperatures for over 6 years. Combining the relative potencies from all laboratories, a loss of 0.298% per year is predicted for 11/212 when stored at -20°C. This data indicates that the candidate standard 11/212 shows acceptable stablility and is therefore suitable to serve as an International Standard.

Table 9. Laboratory geometric mean estimates of insulin concentration for accelerated thermal degradation samples of 11/212 relative to sample C(-20ºC).

Sample (Storage Temp)	Lab 9	Lab 11b	Lab 15	Lab 19	GM	GCV	95% CI
E (+4 ºC)	1.01	0.95	0.94	0.98	0.97	3.1%	0.92 – 1.01
D (+20 ºC)	0.99	0.87	0.99	0.96	0.95	6.3%	0.86 – 1.05
B (+37 ºC)	0.94	0.82	0.93	0.95	0.91	7.1%	0.81 – 1.01
A (+45 ºC)	0.89	0.88	0.85	0.83	0.86	3.4%	0.82 – 0.91

GM: Geometric Mean

GCV: Geometric Coefficient of Variation

CI: Confidence Interval

Discussion

There currently is no International Standard or Reference Preparation for human insulin that reflects the purity and biosynthetic nature of insulin material that is now manufactured internationally and used clinically for treatement of diabetes. Stocks of the 1st International Reference Preparation for human insulin, 66/304, widely used for the calibration of immunoassays for human insulin, are exhausted, and the current International Standard for human insulin, 83/500, is prepared from modified porcine insulin. In addition to the type of material used to prepare previous standards, both of these reference preparations were assigned a potency by bioassay in IU, a unitage that no longer reflects the international transition to a mass-assigned molecule.

A new candidate International Standard for human insulin, coded 11/212, was produced, and has been assessed by International collaborative study in two phases. In the first phase of this study the candidate standard, 11/212, was assigned a value of 9.19 mg/ampoule with expanded uncertainty (k=2) of 9.14 to 9.24 mg/ampoule by mass balance methods. This value assignment was supported by HPLC estimates of 9.19 mg/ampoule and 9.14 mg/ampoule by total Nitrogen analysis.

The immunoreactivity of the candidate standard, 11/212, in current insulin immunoassays was assessed in the second phase of the study. The overall laboratory estimates for dilution 1 of the 1st IRP 66/304 and 11/212 were in close agreement, with 437.4 µIU/mL to 811.6 µIU/mL and a geometric mean of 609.8 µIU/mL for 66/304 and 436.6 µIU/mL to 861µIU/mL with a geometric mean of 581.3 µIU/mL for 11/212. The geometric mean estimate of 609.8 µIU/mL for the 1st IRP 66/304 is equivalent to 3.18 IU/amp after taking into account initial ampoule dilutions, and the geometric mean estimate of 581.3 µIU/mL for the candidate standard 11/212 is equivalent to 267.1 IU/amp. Both are in very close agreement with the respective assigned values of 3 IU/amp for 66/304 and 9.19 mg/amp for 11/212, which is equivalent to 264.8 IU/amp after application of the internationally recognised specific activity conversion factor for pure insulin (1 IU of activity is contained in 0.0347 mg insulin). Alongside HPLC and total nitrogen analysis, this immunoassay estimate further supports the mass assigned value of 9.19 mg/ampoule to the candidate 11/212.

Both the candidate standard 11/212 and the 1st IRP 66/304 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. In the 14 laboratories that demonstrated constant patient sample bias, the 1st IRP 66/304 was commutable in 7 laboratories at a minimum of 3 dilutions, and non commutable with 7 laboratories. The candidate standard 11/212 was fully commutable in 8 laboratories and non commutable in 6. Of these 6 laboratories, there were only 2 laboratories (17a and 17b, representing 1 method on 2 platforms) in which the bias values for all dilutions of 11/212 were further outside the limits of commutability than 66/304, a further 2 methods (14b and 18a) in which 11/212 was demonstrated to be non commutable, but had been shown by other laboratories (labs 18b and 14a respectively) to exhibit commutability, and 2 methods (labs 13 and 15) in which the bias values for 66/304 and 11/212 were well aligned despite being outside the limits of commutability.

Experimental variation, such as that casued by external factors (e.g. different dilution buffers, operators and procedures) may have had an impact on the commutability as assessed by the difference in bias approach. It is noted that participants were required to freeze stocks of 66/304 due to limited availability of ampoules, and that 66/304 and 11/212 were prepared using different excipient formuations. It is also important to note that within the confines of a collaborative study, where each assay is performed only two to three times, it is not possible to assess how experimental variation may impact commutability. It therefore should be recommended that each manufacturer performs their own assessment of the behaviour of the candidate standard 11/212 in their assays in comparison with native samples.

Stocks of the 1st IRP, 66/304, are exhausted, and there is currently no provision of a reference material for the standardisation of human insulin immunoassays. The candidate standard 11/212 and the 1st IRP 66/304 were shown to behave in a very similar manner in the immunoassays in this

study, indicating that the introduction of the candidate standard 11/212 as the 1st International Standard for insulin will not negatively impact the current calibration of insulin immunoassays. A thermally accelerated degradation study was also performed. The data from HPLC and immunoassay estimates of accelerated thermal degradation samples of 11/212 indicate that the candidate standard is sufficiently stable when stored at -20ºC to serve as an International Standard.

The availability of 11/212 as the 1st International Standard for human insulin, prepared from therapeutic pure insulin, is likely to aid in improving the consistency and accuracy of clinical diagnostic tests [5-6]. In addition, the data in this study supports the use of the accepted pure insulin activity conversion factor, 1IU = 0.0347 mg. Global use of this conversion will facilitate harmonisation initiatives which have historically been hampered by the use of various conversion factors by different assay kits [1, 2, 10, 11]. One example of the initiatives underway to harmonise immunoassays for human insulin uses secondary reference materials, such as pooled native serum samples, that have been value assigned using isotope dilution-liquid chromatography/tandem mass spectrometry (IDMS) measurement, calibrated to a pure recombinant human insulin preparation [1]. Indeed, there is evidence that these serum based calibrators may out-perform a pure recombinant insulin preparation in the calibration of insulin immunoassays [1]. The availability of 11/212 would enable the additional use of this standard as the primary calibrator in these efforts as it fulfils the requirements of a primary calibrator (a stable, pure recombinant human insulin preparation that has been mass assigned using a primary reference procedure).

In addition to providing a well characterised, stable, SI traceable International Standard based on pure human insulin for the calibration of insulin immunoassays, the provision of a mass-balance assigned International Standard for human insulin will also be of particular relevance for the standardisation of therapeutic preparations of human insulin. The transition to a mass assigned, biosynthetic, product has resulted in a move away from the current International Standard, value assigned in IU by bioactivity. Regional Pharmacopoeias have established their own reference standards for their respective monographs, and discontinuity between different pharmacopoeias and also between batches of reference standard from the same agency have since been observed [4]. To reduce these differences, the availability of a single, global, human insulin standard that could be used to calibrate regional/compendial standards using HPLC methods would improve the consistency between these standards, ideally resulting in consistent dosing to patients worldwide and more straight forward quality control for global manufacturers [4]. It is anticipated that additional data will be produced to support the use of 11/212 as a global standard for the calibration of therapeutic preparations of insulin.

Proposal

It is proposed that the candidate preparation in ampoules coded 11/212 is established as the **1st International Standard for insulin, human,** with an assigned content of **9.19 mg/ampoule (expanded uncertainty of 9.14 – 9.24 mg/ampoule; *k=2*)**. This value can be converted into IU using the internationally recognised specific activity of pure insulin (1IU=0.0347 mg).

Comments from Participants

A copy of this report was sent to all participants involved in both the Phase 1 and Phase 2 of the collaborative study, with responses received from approximately half the participants. All those who replied were in agreement with the proposal. Some minor amendments to text were suggested either to clarify names and addresses of participants, or to clarify statistical methods used for Phase 1 (explanatory notes added to Methods section and Appendix 4 raw data).

Acknowledgements

We gratefully acknowledge the important contributions of all participants of the collaborative study phases, Novo Nordisk who kindly donated the human insulin material, the Standardisation Science Group at NIBSC for preparation of the trial materials and sub-portions, the Standards Processing Division at NIBSC for the preparation and dispatch of ampouled materials and the Biostatistics group at NIBSC for analysis of the collaborative study data.

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Appendix 1a – Phase 1 study protocol

Appendix 1b – Phase 1 repeat study protocol

Appendix 2 – Phase 2 study protocol

(for Appendices 1a, 1b and 2, please see separate word document)

Appendix 3

Laboratory mean estimates for the mass balance tests from Phase 1 and Phase 1 repeat are summarised in the tables below. Note the overall laboratory mean and standard error of the mean have been calculated using individual laboratory estimates using a variance components analysis.

Table A3a: Laboratory mean estimates for the loss on drying (LOD) in mg/100 mg insulin API in Phase 1

Lab	LOD (Phase 1)
1	7.0553
2	6.8911
3	6.2319
4	7.2299
5	7.2986
8	6.8267
MEAN	6.9222
Std Error	0.1563

Table A3b: Mean laboratory estimates of the Nitrogen content of insulin API in the Phase 1 study

Lab	%N (Phase 1)
1	14.421
5	14.508
8	14.575
MEAN	14.5012
Std Error	0.0452

Table A3c: Mean laboratory estimates of the HPLC content of 11/212 in terms of the insulin API in the Phase 1 study.

Lab	HPLC (Phase 1) 11/212 content before mass balance correction (mg)
1	9.7801
2	9.8408
3	9.8909
4	9.8862
5	9.9420
6	9.6310
7	9.8589
8	9.9321
ESTIMATE	9.8452
SE	0.0356
Relative SE (%)	0.3618

Table A3d: Laboratory mean estimates for the loss on drying (LOD) in mg/100 mg insulin API in the Phase 1 Repeat study

Lab	LOD (Phase 1 Repeat)
1	7.3885
2	6.4208
5	6.9820
8	7.3018
MEAN	7.0232
Std Error	0.2190

Table A3e: Mean laboratory estimates of the Nitrogen content of insulin API in the Phase 1 Repeat study

Lab	%N (Phase 1 Repeat)
1	14.2940
3	14.3747
5	14.1378
8	14.6594
MEAN	14.3665
Std Error	0.1262

Table A3f: Mean laboratory estimates of the HPLC content of 11/212 in terms of the insulin API in the Phase 1 Repeat study.

Lab	HPLC (Phase 1 Repeat) 11/212 content before mass balance correction (mg)
1	9.896
2	10.045
5	10.208
8	9.896
9	9.937
ESTIMATE	9.9967
SE	0.0595
Relative SE (%)	0.5951

Appendix 4

Table 4a. Consensus values obtained for all samples used for the assessment of the commutability of 66/304 and 11/212.

Sample	Dilution	Expected µIU/mL	Robust mean $\log_{10}$ µIU/mL	Robust GM µIU/mL
11/212	4	144	2.143	139.02
11/212	8	72	1.841	69.31
11/212	16	36	1.541	34.72
11/212	32	18	1.241	17.41
11/212	64	9	0.952	8.95
11/212	128	4.5	0.660	4.57
66/304	4	144	2.171	148.26
66/304	8	72	1.868	73.78
66/304	16	36	1.566	36.83
66/304	32	18	1.266	18.47
66/304	64	9	0.974	9.43
66/304	128	4.5	0.691	4.90
InsSerum1	-	-	0.992	9.81
InsSerum2	-	-	0.951	8.93
InsSerum3	-	-	1.413	25.85
InsSerum4	-	-	0.970	9.34
InsSerum5	-	-	1.270	18.61
InsSerum6	-	-	1.302	20.03
InsSerum7	-	-	1.724	53.00
InsSerum8	-	-	1.915	82.29
InsSerum9	-	-	1.574	37.54
InsSerum10	-	-	1.345	22.14
InsSerum11	-	-	1.459	28.78
InsSerum12	-	-	2.068	116.96
InsSerum13	-	-	1.716	51.94
InsSerum14	-	-	0.816	6.55
InsPlasma1	-	-	1.364	23.12
InsPlasma2	-	-	1.367	23.31
InsPlasma3	-	-	0.676	4.74
InsPlasma4	-	-	1.013	10.30
InsPlasma5	-	-	1.700	50.14

Table 4b. Fitted linear regression slopes for log_{10} reported concentration against log_{10} nominal concentration for 66/304 and 11/212 for each run of each method.

Lab	Sample	Slope (Run 1)	Slope (Run 2)	Slope (Run 3)
10	11/212	1.171*	1.103*	0.927
10	66/304	1.103*	1.095	0.969
11a	11/212	1.054	0.997	
11a	66/304	0.965	1.043	
11b	11/212	0.911		
11b	66/304	0.915		
12	11/212	0.975	0.952	0.981
12	66/304	0.986	0.963	0.983
13	11/212	1.064	1.077	1.069
13	66/304	1.061	1.083	1.062
14a	11/212	0.999	0.978	0.983
14a	66/304	1.005	0.953	0.964
14b	11/212	0.969	0.965	0.983
14b	66/304	0.971	0.962	0.989
15	11/212	0.948	0.946	0.978
15	66/304	0.953	0.952	0.977
16a	11/212	1.004	0.974	0.971
16a	66/304	0.996	0.968	0.950
16b	11/212	1.002	1.003	0.968
16b	66/304	1.000	0.976	0.960
17a	11/212	0.993	0.960	0.900*
17a	66/304	1.006	0.981	0.941
17b	11/212	0.951	0.929	0.918
17b	66/304	0.958	0.866*	0.995
18a	11/212	0.970	0.964	0.987
18a	66/304	0.965	0.966	0.979
18b	11/212	0.966	0.959	0.948
18b	66/304	0.955	0.945	0.936
19	11/212	0.999	0.959	
19	66/304	1.014	1.003	

*Estimates were considered invalid if the slope was outside the range [0.91, 1.10]

Table 4c. Individual run estimates of the insulin concentration in µIU/mL for dilution 1 of 66/304 and 11/212.

Lab	Sample	Run 1	Run 2	Run 3
10	11/212	-	-	581.6
10	66/304	-	536.7	632.5
11a	11/212	549.2	669.3	
11a	66/304	574.9	559.8	
11b	11/212	637.4		
11b	66/304	672.5		
12	11/212	559.6	567.2	534.7
12	66/304	543.7	559.1	530.2
13	11/212	444.4	427.2	438.6
13	66/304	452.6	436.7	423.4
14a	11/212	492.5	450.4	451.6
14a	66/304	529.1	562.4	548.7
14b	11/212	471.3	461.4	445.1
14b	66/304	544.6	549.5	534.3
15	11/212	645.2	592.2	550.9
15	66/304	644.5	609.5	576.8
16a	11/212	522.7	553.0	575.5
16a	66/304	643.2	654.5	698.3
16b	11/212	519.6	572.7	559.6
16b	66/304	655.8	671.0	668.9
17a	11/212	781.2	761.6	-
17a	66/304	718.1	812.3	916.7
17b	11/212	821.2	782.3	993.5
17b	66/304	765.4	-	758.1
18a	11/212	636.9	647.5	631.3
18a	66/304	615.6	628.6	613.2
18b	11/212	609.1	627.5	617.7
18b	66/304	641.8	651.9	647.7
19	11/212	512.8	558.1	
19	66/304	574.8	568.4	

Appendix 5 – Draft IFU

1st WHO International Standard for Insulin, human
11/212
(version 1, dated XX/XX/XXXX)

1. INTRODUCTION

The 1st International Reference Reagent (IRR) for human insulin, in ampoules coded 66/304, has been widely used for the calibration of immunoassays for human insulin. However, stocks of the 1st IRR are exhausted, and the lack of a globally accepted International Standard of high purity has been identified as a limiting factor in improving the consistency of clinical assays for human insulin. In addition, the 1st IRP 66/304 and the current WHO International Standard for human insulin, 83/500, are both assigned in IU, a untiage that no longer reflects the international transition to a mass assigned molecule. It has therefore been acknowledged that there is a requirement for a single global standard containing high purity insulin to reflect the status of insulin as a well-characterized, mass-balance assigned molecule.
With these points in mind, a preparation of highly purified, therapeutic grade insulin was filled into ampoules (NIBSC code 11/212) following procedures recommended by WHO [1], to produce the 1st International Standard for insulin, human, which was assigned a content of 9.19 mg/ampoule (with expanded uncertainty 9.14 – 9.24 mg/ampoule) by mass balance methods. The 1st International Standard was established at the [70th Meeting of the WHO ECBS (2019)]. This material replaces the 1st IRP for human insulin, 66/304.

2. CAUTION

THIS PREPARATION IS NOT FOR ADMINISTRATION TO HUMANS OR ANIMALS IN TH HUMAN FOOD CHAIN:
The preparation does not contain material of human origin.

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 preocedures 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 **9.19 mg/ampoule** (with expanded uncertainty of 9.14 – 9.24 mg/ampoule, k=2) of lyophilized human insulin**.** This value can be converted into IU using the internationally recognised specific activity of pure insulin (1IU=0.0347 mg).

4. 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.

5. 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

6. 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.

7. PREPARATION OF AMPOULES AND COLLABORATIVE STUDY

A bulk portion of the insulin active pharmaceutical ingredient (API) was removed in a dry box under controlled humidity, dissolved in ddH_2O and acidified with 0.2 M HCl until the solution was clear. The acidified solution was neutralised to pH 7.4 with 0.2 M NaOH and the final volume was made up gravimetrically to give a concentration of insulin API of ~10 mg/g final solution. The solution was dispensed as 1 g aliquots into glass ampoules, lyophilised and sealed. Ampoules were stored at -20ºC.

This batch of ampoules, coded 11/212, was evaluated in a two-phase collaborative study to 1) value assign the standard by mass balance methods, and 2) to assess its immunoreactivity and suitability to serve as an International Standard by immunoassay in comparison with the 1st IRP 66/304 and a panel of human serum and plasma samples.

The results of the Phase 1 study gave an assigned content for 11/212 of 9.19 mg/ampoule (with expanded uncertainty 9.14 – 9.24 mg/amp) by mass balance methods, with good agreement from HPLC estimates of 9.19 mg/amp (9.07 – 9.31 mg/amp) and total nitrogen analysis of 9.14 mg/amp (8.97 – 9.30 mg/amp).

In Phase 2, the laboratory geometric mean immunoassay estimates for 11/212 of 267.1 IU/amp were in very good agreement with the assigned contents of 9.19 mg/amp to 11/212, after application of the activity conversion factor for pure insulin of 1IU = 0.0347 mg insulin to give 264.8 IU/amp. Both 11/212 and 1st IRP 66/304 had acceptable GCVs and behaved in a similar manner in the immunoassays used.

A thermally accelerated degradation study was also performed. The data from HPLC and immunoassay estimates of accelerated thermal degradation samples of 11/212 indicated that the candidate is sufficiently stable when stored at -20ºC to serve as an International Standard.

The commutability of 11/212 with patient samples in the immunoassay methods used in the Phase 2 collaborative study was assessed using a difference in bias approach. In the 14 laboratories that demonstrated constant patient sample bias, the 1st IRP 66/304 was commutable with 7 laboratories at a minimum of 3 dilution and non commutable with 7 laboratories. The candidate standard 11/212 was commutable in 8 laboratories and non commutable in 6. Of these 6 laboratories, there were only 2 laboratories (representing 1 method on 2 platforms) in which the bias values for all dilutions of 11/212 were further outside the limits of commutability than 66/304, a further 2 methods in which 11/212 was demonstrated to be non commutable, but had been shown by other laboratories (same method and platform) to exhibit commutability, and 2 methods in which the bias values for 66/304 and 11/212 were well aligned despite being outside the limits of commutability.

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 standard 11/212 in all immunoassay methods. It is therefore recommended that manufacturers make their own assessment of the commutability of the reference standard 11/212 with their assay method.

In conclusion, the candidate standard 11/212 was deemed to represent a well characterised, mass assigned standard for pure human insulin, that was shown to behave in a very similar manner to the 1st IRP 66/304 in immunoassays of human insulin in terms of immunoreactivity and commutability, and is therefore suitable as a replacement of 66/304 for the continued calibration of these immunoassays.

8. 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.

9. 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.

10. REFERENCES

[1] WHO Tech Rep Ser No 800, 1990, 181-214
[2] ECBS WHO report to be referenced

11. 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)	N/A		
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.			