

Collaborative study for the establishment of the WHO 3rd International Standard for Endotoxin, the Ph. Eur. endotoxin biological reference preparation batch 5 and the USP Reference Standard for Endotoxin Lot H0K354

L. Findlay¹, T. Desai¹, A. Heath¹, S. Poole¹, M. Crivellone², W. Hauck², M. Ambrose², T. Morris², A. Daas³, G. Rautmann⁴, K.H. Buchheit³, J.M. Spieser^{†3}, E. Terao⁵

ABSTRACT

An international collaborative study was organised jointly by the World Health Organization (WHO)/National Institute for Biological Standards and Control (NIBSC), the United States Pharmacopeia (USP) and the European Directorate for the Quality of Medicines & HealthCare (EDQM/Council of Europe) for the establishment of harmonised replacement endotoxin standards for these 3 organisations. Thirty-five laboratories worldwide, including Official Medicines Control Laboratories (OMCLs) and manufacturers enrolled in the study. Three candidate preparations (10/178, 10/190 and 10/196) were produced with the same material and same formulation as the current reference standards with the objective of generating a new (3rd) International Standard (IS) with the same potency (10 000 IU/vial) as the current (2nd) IS, as well as new European Pharmacopoeia (Ph. Eur.) and USP standards. The suitability of the candidate preparations to act as the reference standard in assays for endotoxin performed according to compendial methods was evaluated. Their potency was calibrated against the WHO 2nd IS for Endotoxin (94/580). Gelation and photometric methods produced similar results for each of the candidate preparations. The overall potency estimates for the 3 batches were comparable. Given the intrinsic assay precision, the observed differences between the batches may be considered unimportant for the intended use of these materials. Overall, these results were in line with those generated for the establishment of the current preparations of reference standards. Accelerated degradation testing of vials stored at elevated temperatures supported the long-term stability of the 3 candidate preparations.

It was agreed between the 3 organisations that batch 10/178 be shared between WHO and EDQM and that batches 10/190 and 10/196 be allocated to USP, with a common assigned value of 10 000 IU/vial. This value maintains the continuity of the global harmonisation of reference materials and unitage for the testing of endotoxins in parenteral pharmaceutical products.

1 L. Findlay, T. Desai, A. Heath, S. Poole, National Institute for Biological Standards and Control (NIBSC), Potters Bar, Hertfordshire, EN6 3QG, UK.

2 M. Crivellone, W. Hauck, M. Ambrose, T. Morris, United States Pharmacopeial Convention, Rockville MD 20852-1790, USA.

3 A. Daas, K.H. Buchheit, J.M. Spieser[†], European Directorate for the Quality of Medicines & HealthCare, Department of Biological Standardisation, OMCL Network & HealthCare (DBO), Council of Europe, Strasbourg, France.

4 G. Rautmann, European Directorate for the Quality of Medicines & HealthCare, Laboratory Department (DLab), Council of Europe, Strasbourg, France

5 E. Terao (corresponding author's e-mail: eriko.terao@edqm.eu), European Directorate for the Quality of Medicines & HealthCare, Department of Biological Standardisation, OMCL Network & HealthCare (DBO), Council of Europe, F-67081 Strasbourg, France

[†] Deceased 2013

Based on the results of the collaborative study, batch 10/178 was established by the European Pharmacopoeia Commission as the Ph. Eur. Endotoxin Biological Reference Preparation (BRP) batch 5. The same batch was also established by the Expert Committee on Biological Standardisation (ECBS) of WHO as the WHO 3rd IS for Endotoxin. Batch 10/190 was adopted as the USP Endotoxin Reference Standard, lot H0K354 and vials from this same batch (10/190) will serve as the United States Food and Drug Administration (USFDA) Endotoxin Standard, EC-7.

KEYWORDS

Biological Reference Preparation, Biological Standardisation Programme, Collaborative study, EDQM, Endotoxin, European Pharmacopoeia, International Standard, USP, WHO.

1. INTRODUCTION

The control of parenteral pharmaceutical products for bacterial endotoxins is a fully harmonised procedure between the Ph. Eur., the USP and the Japanese Pharmacopoeia (JP) [1,2,3]. Reference preparations used in these assays are, since the collaborative study for the establishment of the 2nd IS for Endotoxin in 1996, one of the truly harmonised standards prepared from a common starting material, evaluated in an international collaborative study and adopted as the International Standard and as compendial standards. The present study, initiated in 2007, aimed at establishing replacement preparations for the current WHO IS, Ph. Eur. BRP and USP reference standard, stocks of which were dwindling. An international collaborative study was carried out to calibrate 3 candidate preparations against the WHO 2nd IS for Endotoxin (94/580) using official pharmacopoeial methods (gelation and photometric assays).

2. PARTICIPANTS

Thirty-five worldwide spread laboratories from 18 countries (1 Australia, 1 Austria, 1 Belgium, 1 Brazil, 1 Canada, 1 China, 2 Denmark, 1 France, 4 Germany, 1 Italy, 5 Japan, 2 Korea, 1 The Netherlands, 1 Norway, 1 Portugal, 1 Sweden, 2 Switzerland, 7 USA and the Council of Europe/EDQM) took part in the study. These laboratories included OMCLs/regulatory institutes (19 laboratories) and manufacturers (therapeutics: 8; reagents: 8). A list of participating laboratories (and their representatives) is given in Section 9. Each laboratory is identified in this article by a number between 1 and 35 that is not related to the order of listing.

3. MATERIAL

3.1. Candidate material

The starting material for the candidate standards was kindly donated by the USFDA-Center for Biologics Evaluation and Research (CBER). It comprised endotoxin taken from an endotoxin bulk extracted from *Escherichia coli* (Braude strain) group O113:H10:K negative prepared by Westphal extraction [4]. The same endotoxin bulk had been the source of the current and previous endotoxin standards: the WHO 1st and 2nd IS for Endotoxin (84/650 and 94/580, respectively), the Ph. Eur. Endotoxin BRP batches 3 and 4, the USP Reference Standard lots F and G series (G, G-1, G2B274 and G3E069) as well as the FDA reference lots EC-1 through EC-6 [5,6].

3.2. Production of the candidate batches

The production fill for the candidate batches was performed at NIBSC. In order to maintain continuity in the amount of endotoxin per vial between the consecutive batches of IS, multiple pilot productions and full-scale trial fills were conducted in 2009-2010 to determine the amount of endotoxin needed to yield 10 000 IU/vial. The formulation of the current standards was maintained for the candidate batches as it has been used successfully, without stability issues, for the 2nd IS for Endotoxin (94/580), the USP Lot F/FDA EC-5 and the Ph. Eur. BRP batches 3 and 4.

Three candidate batches (10/178, 10/190 and 10/196) were produced in autumn 2010 from a common bulk solution of endotoxin. For each candidate batch, an aliquot of the endotoxin bulk solution and the excipients concentrate were combined and stirred for 1 hour at 2-8°C. The solution was brought to the final concentration with cold (2-8°C) water for injection and stirred overnight at room temperature. Filling of the vials was performed at room temperature, under continuous stirring of the solution. Freeze-drying was performed over 4 days for each lot. After freeze drying, each vial contains the residue of 1.0 mL of a solution that contained 1.2 µg E. coli O113:H10:K negative endotoxin, 10 mg lactose and 1 mg polyethylene glycol 8000. The main specifics of the batches are shown in Table 1.

3.3. Stability studies

Accelerated thermal degradation (ATD) studies for the candidate preparations are in progress at NIBSC. Twenty vials of each fill were stored at each of the following temperatures: + 70°C, -20°C, +4°C, +20°C, +37°C, +45°C and +56°C. Samples were tested in a photometric chromogenic assay. Potencies were calculated relative to the current IS using a parallel line analysis. For each fill, two plates were used, reversing the order the test samples were placed on the plate to minimise plate-positional effects. Table 2 shows the potencies (in IU) of accelerated degradation samples of the 3 candidate preparations relative to the standard Endotoxin curve (current IS), and the potencies (in per cent) of the samples stored at +56°C relative to those stored at -20°C for 10 months. The mean potency estimates of the fill samples stored at -20°C and +56°C relative to the standard endotoxin curve ranged from around 10 000 to 10 500 IU for the -20°C samples and 10 400 to 10 700 IU for the +56°C samples for each of the 3 preparations, there was no observed drop in potency between the samples stored at +56°C and those stored at -20°C after storage for 10 months.

Table 3 shows the ATD study results for samples of the 3 endotoxin candidates preparations stored for 15 months at -20°C and at +56°C, and the relative potencies of the candidate preparations stored at + 56°C, expressed as per cent of those stored at -20°C. There was some evidence that the outer columns of the plate were giving faster reaction times than those from the replicates of the same samples in more central columns. The results of the repeat of parallel line analysis (15 months) excluding the 2 outer columns are presented in Table 4. Results of the ATD study after 17 months storage are shown in Table 5 (all data) and in Table 6 (excluding results from the outer 2 columns).

Table 7 shows the estimated endotoxin potency for samples stored at +56°C as a percentage of that at -20°C, for periods from 10 to 17 months. There was no detectable degradation after 10 months at +56°C. There was an apparent drop in activity for preparation C (10/196) after 17 months. However, the pattern of results suggests that it is unlikely that there would be such a genuine drop between 15 and 17 months for a material that had proved highly stable at +56°C for 15 months, intrinsic variability of the assay may have been a major contributor to

the observed value. Moreover, applying the “rule of thumb” that degradation rates will double with every 10°C increase in storage temperature, 10 months at +56°C with no detectable degradation would be equivalent to around 150 years at -20°C. Even if there were a genuine drop to 95% after 17 months, this would be equivalent to over 250 years at -20°C before an equivalent loss was apparent for samples stored at -20°C.

The data in Tables 2 to 6 and summarised in Table 7 indicate that all three preparations are highly stable. Assessment of the stability of samples stored at temperatures ranging from +4°C to +45°C will continue.

3.4. Preliminary studies

3.4.1. Comparison with current standards

A preliminary study established that the current pharmacopoeial standards (USP reference lot G and Ph. Eur. BRP batch 4) and the WHO 2nd IS still yield equivalent results in testing, allowing for a simplified study protocol, in which the current WHO 2nd IS is the single comparator for the test samples.

3.4.2. Pre-testing of the candidate batches

Upon completion of the fills in 2010, preliminary collaborative testing was performed at NIBSC, USP and EDQM (Laboratory Department and 2 European OMCLs) to evaluate the suitability of the candidate batches. Vials from the 3 candidate batches were tested using the gelation and photometric (both chromogenic and turbidimetric) assays, with the 2nd IS as the reference standard. Results showed similarity across the 3 fills, with the average vial content about 10 000 IU/vial.

4. COLLABORATIVE STUDY

4.1. Distributed samples

Each participant was provided with 15 vials for each assay method (gel-clot or photometric) performed:

- 3 vials of the 2nd IS for Endotoxin (94/580; assigned potency: 10 000 IU/vial);
- 3 vials of each of the 3 candidate preparations (10/178, 10/190, 10/196) coded as sample A, B, C respectively;
- 3 vials of sample D, a coded duplicate of preparation (10/190; sample B).

The participants were informed that the candidate vials were filled at a nominal 10 000 IU/vial.

4.2. Study design and data reporting

The study protocol was based on that used for the evaluation of the WHO 2nd IS for Endotoxin in 1994-95 [4,5]. Participants were requested to perform semi-quantitative Limulus Amoebocyte Lysate (LAL) gelation assays and/or quantitative LAL photometric assays (chromogenic and/or turbidimetric). Assays were to be conducted according to published pharmacopoeial methods (e.g. Ph. Eur. General Chapter 2.6.14., USP general test <85> or JP general test 4.01 [1,2,3]). The assays were to be performed with the LAL reagent routinely used by the laboratory. For gelation assays, the sensitivity of the LAL reagent had to be of 0.03 or 0.06 IU/mL. Due to international improvements in the quality, comparability, and reliability of LAL reagent since 1994, a common, single LAL reagent was not provided to collaborators (as had been done for the calibration study of the 2nd IS). The reference standard for all assays was the WHO 2nd IS for Endotoxin (94/580; 10 000 IU/vial). Participants were asked to reconstitute 1 vial of 2nd IS

and 1 of each candidate preparation to 2000 IU/mL (assuming 10 000 IU/vial for the candidate preparations). Each assay was to include dilutions of reconstituted vials of the IS and all 4 test samples. The candidates were to be tested at the same nominal concentrations and at the same number of replicates as the IS, i.e. the candidate preparations were to be treated exactly as if they were the IS itself.

For gelation assays, datasheets were provided for up to 8 dilutions of each stock solution and up to 4 replicates per assay, to be used as needed in accordance with the participants' protocol.

For photometric assays, datasheets were provided for up to 5 dilutions of each stock solution and up to 3 replicates per assay. The objective was to perform a sufficient number of dilutions (3 or more) to allow a parallel line analysis of the data. Each vial had to be assayed twice: once using freshly reconstituted material and a second time on the same vials within 2 weeks after reconstitution. The reconstituted vials were to be stored at + 4°C in the interim. This assay set was to be repeated 2 more times, each time using unopened vials of 2nd IS, samples A, B, C and D. Thus, 15 vials in total (3 vials of IS and 3 vials of each of the 4 test preparations) were each assayed twice in total using each assay method.

The study protocol and reporting sheets were provided electronically to all participants.

5. STATISTICAL ANALYSIS

All reported raw data were analysed by the USP using the SAS® software.

For gelation data:

The relative potency was determined for each of the laboratory-sample-vial-day combinations. These data were examined for unusual values corresponding to a relative potency outside 50-200 percent.

For photometric data:

Linear regression of $\ln(\text{results})$ vs. $\ln(\text{concentration})$ for all combinations of laboratory, assay (when a laboratory provided results for multiple photometric assays), sample or standard, say and vial, were computed. The results were examined for unusual residual values. The remaining R^2 values were then examined for outliers and data failing the suitability criterion specified in the USP Chapter <85> that the absolute value of the correlation coefficient (R) of the standard curve should be at least 0.98, corresponding to $R^2 > 0.96$ [2] were excluded.

The calculated potencies per assay were later provided to the EDQM as SAS® datasets to prepare supplementary tables and figures. The raw data were not re-analysed but some additional statistics were calculated on the basis of the tabled potencies: geometric means across laboratories, Huber's robust means (with $k=1.5$) that reduce the influence of extreme values and other supplementary statistics.

6. RESULTS

6.1. Assay data returned

Thirty-one laboratories in 17 countries contributed data by the deadline. Four laboratories reported results after the deadline. These results were not included in the statistical evaluation and the assignment of the potency to the candidate preparations but were evaluated later for comparison with results from the collaborative study (see Section 8: Addenda). Thus, for the calibration of the candidate batches, 24 laboratories reported data using the gelation method; 23 laboratories returned photometric assay results, using one or more photometric

(chromogenic and/or turbidimetric) methods; 17 laboratories returned chromogenic assay results, 13 laboratories returned turbidimetric assay results and 8 laboratories provided results from more than 1 photometric assay for a total of 31 photometric assays. Table 8 summarises the methods used by each participating laboratory.

6.2. Assay validity: data excluded from analysis

Gelation data from 1 laboratory (Lab 27) were invalid and not included in the analysis. One laboratory (Lab 14) provided gelation results from freshly reconstituted material only and 1 (Lab 3) provided data for 4 days, all but the first of which were treated as “later” days assays. Two laboratories (Lab 10 and 12) had gelation assay data that appeared to be entered as the transposition of what was intended; this was corrected in the statistical analysis data file.

Two laboratories (Lab 17 and 26) marked some photometric results as outliers in the data sheets submitted. As the values were evidently out of any reasonable range, the laboratory’s judgement was accepted and these few points were not included in the analyses.

Photometric results with undetermined endpoints reported as “greater (>) than some value” were treated as missing. One set of photometric results (Lab 22) was conducted more than 2 weeks after reconstitution and was not used. One laboratory (Lab 18) used only 2 dilutions for the photometric assay. As the pharmacopoeial method calls for at least 3 dilutions and 2 dilutions does not permit assessment of lack of fit, this laboratory’s photometric data were not used. Two laboratories (Lab 16 and 17) performed a non-pharmacopoeial photometric assay using recombinant LAL (factor C). These data were not included in the overall analysis and were dealt with separately. This left 23 laboratories with usable gelation data and 22 with usable photometric assay data.

6.3. Potency estimates

6.3.1. Gelation assays

The relative potency was determined for each of the 564 laboratory-sample-vial-day combinations. After examination of these data for unusual values, 558 log relative potencies were further analysed. Seventy per cent of results correspond exactly to a determined potency of 10 000 IU/vial, 7% to values less than 10 000 and 24% to values greater than 10 000. Four values corresponded to an absolute log relative potency greater than 0.7 (corresponding approximately to a relative potency outside the 50-200% interval) and these data were not used in further analyses. Examination of those 4 values showed that their log-relative potency values would either become negative on the same dilution or at most 1 dilution later. The extreme relative potencies correspond to results differing by 2 or more dilutions.

Table 9 provides a complete overview of the potency estimates in IU/vial for each laboratory-day-sample-vial combination. The values vary from 2500 IU/vial (Lab 23, sample B) to 38 750 IU/vial (Lab 21, sample C). The two-sided paired t-test of ln-transformed potencies (mean of 3 vials) showed no significant differences ($\alpha=0.05$) between values obtained on day 1 and on day 2 or later ($p=0.528$).

Table 10 shows the geometric mean per laboratory and sample. Histograms of these values are provided in Figure 1. The values range from 8476 IU/vial (Lab 21, sample B) to 17 311 IU/vial (Lab 5, sample C). The geometric mean across laboratories was 10 414, 10 739, 10 768 and 10 937 IU/vial for the respective samples. It was noted that the somewhat high results from Lab 5 may have resulted in a slight overestimation of the potencies for samples B, C and D. To reduce the influence of extreme values Huber’s robust mean ($k=1.5$) was also calculated. This gave 10 250, 10 598, 10 509 and 10 648 IU/vial for the respective samples.

6.3.2. Photometric assays

Linear regression of ln-transformed results versus ln-transformed concentration for each laboratory-day-sample-vial combination, were computed. A total of 1019 regressions were examined for unusual residual values. There were no standardised residuals greater than 4.0 in magnitude, compared to the 1% point for Grubb's test of 4.9. Thus, no values were dropped as outliers based on this analysis. Next, the 1019 R^2 values were examined. There was 1 value inferior to 0.88 compared with a next lowest value of 0.92. This was for one sample and these data were omitted from further analyses. Two sets of data failed the condition that the R value was required to be at least 0.98 (or $R^2 > 0.96$) for the IS and were excluded.

When the data were reanalysed with and without the assumption of parallelism (equal slopes), 2 values (of 806 regressions) of the ratio of slopes (sample/standard) was found to be outside 0.8-1.25. No data were excluded. Eight values (of 806) of the estimated relative potency (assuming parallelism) fell outside 50-200%. Of these 8 values, 4 were from 1 laboratory, 3 from a second laboratory, while the last value came from a third laboratory. All 8 values were excluded from the final calculations.

Table 11 provides a complete overview of the potency estimates in IU/vial for each laboratory-day-sample-vial combination. Also shown is whether a chromogenic or turbidimetric method was used. The values vary from 4203 IU/vial (Lab 22, sample A, chromogenic) to 24 310 IU/vial (Lab 32, sample D, turbidimetric). The two-sided paired t-test ($\alpha=0.05$) of ln-transformed potencies (mean of 3 vials) showed no significant differences ($\alpha=0.05$) between values obtained on Day 1 and on Day 2 or later ($p=0.304$).

Table 12 shows the geometric mean per laboratory and sample. Histograms of these values are provided in Figure 2. Results obtained with the chromogenic method are displayed in light-grey boxes and the turbidimetric method in dark-grey boxes. The values ranged from 7389 IU/vial (Lab 15, sample D, turbidimetric) to 17 702 IU/vial (Lab 32, sample D, turbidimetric). The geometric mean across laboratories using the chromogenic method was 9798, 10 333, 10 426 and 10 696 IU/vial for the respective samples. For the turbidimetric method this was 10 292, 10 641, 10 986 and 11 229 IU/vial respectively for samples A-D. No significant difference ($\alpha=0.05$) between the chromogenic and turbidimetric method is found with the unpaired two-sided t-test ($p=0.246$, 0.482, 0.301, 0.326 respectively) so it was considered appropriate to pool both methods for the overall mean which yields 10 017, 10 470, 10 673 and 10 904 IU/vial respectively. To reduce the effect of extreme values, notably from Lab 1 and 32, Huber's robust mean ($k=1.5$) was also calculated. This gave 10 120, 10 404, 10 449 and 10 730 IU/vial respectively.

6.3.3. Gelation versus photometric assays

An analysis of variance of the pooled set of ln-transformed potencies (4 samples times 52 determinations) showed no significant difference ($\alpha=0.05$) between samples ($p=0.06$) or the 2 methods ($p=0.30$). It is therefore considered justified to calculate the mean potency per sample/batch based on the pooled set of 52 results per sample. The overall potencies of the 3 batches are shown in Table 13.

6.3.4. Two-way comparisons between batches

The mean values per laboratory were plotted against each other in Figure 3. Each plot shows 1 of the 6 possible pairs. The two-sided paired t-test ($\alpha=0.05$), as well as the sign test show a significant difference between sample A and the other batches ($p<0.05$ for each pair). No significant difference is observed between samples B, C or between samples C and D, but the difference between the identical batches B and D is significant ($p=0.04$). This slight difference may not be important, however, as the confidence limits of the final potency estimates ($\alpha=0.05$)

do overlap. Although samples B, C and D appear to be slightly more potent than sample A, the difference may be considered unimportant for the intended use.

7. DISCUSSION AND CONCLUSIONS

This collaborative study aimed at calibrating and assigning the endotoxin potency values to replacement batches for the WHO Endotoxin IS, Ph. Eur. Endotoxin BRP batch 4 and USP standard (lot G series – G3E069). In order to ensure the continuity of unitage, the potency values of 3 candidate batches were evaluated against the current WHO 2nd IS for Endotoxin (94/580), using compendial gelation or photometric assays.

Preliminary results from the accelerated thermal degradation study showed that the 3 candidate batches were stable after storage for 10 months at high temperature (56°C). It is expected that the long-term stability of the 3 batches would be suitable for the proposed use of this material.

There was no significant difference between potencies obtained on day 1 (the day of reconstitution) and day 2 (up to 2 weeks after reconstitution) of either assay, indicating that the reconstituted solution is stable for up to 2 weeks in the refrigerator. This also allowed the data of the 2 days to be pooled for the calibration of the endotoxin potency of the candidate batches.

Seventy per cent of the gelation assay results corresponded exactly to a potency of 10 000 IU/vial. The mean values (Huber's robust mean) for the 3 batches (samples A, B = D, C) were slightly higher, at 10 250, 10 598 and 10 509 IU/vial respectively with gelation assays.

The 2 photometric methods (chromogenic and turbidimetric assays) gave similar results. This allowed the calculation of an overall potency with the photometric assays for each of the 3 batches (10 120, 10 404 and 10 449 respectively for samples A, B = D, C).

The comparison of gelation and photometric assays was unbiased by the number of laboratories performing the test: 23 laboratories provided useable gelation data and 22 laboratories provided useable photometric assay data. The semi-quantitative gelation assay values trended higher for the 3 batches than the quantitative photometric assays. This was most likely reflective of the method itself, and could result from an insufficient number of dilutions surrounding the end point (2-fold dilution steps) with the gelation assay. The difference between gelation and photometric assay results was however not statistically significant, so that an overall potency value for all assays could be calculated for each batch (10 190, 10 588, 10 743 IU/vial respectively). This showed that the 3 candidate batches are suitable for all applications (gelation and photometric assays).

All batches shared the same endotoxin starting solution. The statistical comparisons presented in this report indicate that the potency of the candidate batch A is slightly closer to 10 000 IU/vial than the 2 other batches. However, the confidence intervals for the 3 batches overlapped and the ranges of values were similar. Given the assay precision, the 3 batches may be considered equivalent for their intended use and be assigned a common value of 10 000 IU/vial. This potency assignment, to one significant value, follows the precedence of the assignment for the WHO 2nd IS in 1996 [5, 6]. In that former study, the geometric mean of gelation assays was 9600 (CL: 9300-10 300) IU/vial. The geometric mean for all photometric assays was 11 700 (CL: 11 000-12 400) IU/vial, and the combined mean of all assays was 10 400 (CL: 9900-10 900) IU/vial. The potency assignment endorsed by the WHO for the 2nd IS was 10 000 IU/vial.

In order, as much as possible, to avoid drift during the calibration of future replacement batches, the WHO/NIBSC, the EDQM and the USP recommended to establish the batch with the potency closest to 10 000 IU/vial (i.e., batch 10/178, sample A) as the new WHO IS (3rd IS).

The WHO, EDQM and USP agreed:

- to assign the 3 candidate batches with the common potency value of 10 000 IU/vial. This assigned value maintains the harmonisation of reference material and unitage among the WHO IS for Endotoxin, the Ph. Eur. Endotoxin BRP and the USP Reference Standard for Endotoxin;
- that sample A (10/178) be shared between WHO and EDQM to be established as the WHO 3rd IS for Endotoxin and the Ph. Eur. Endotoxin BRP batch 5 respectively;
- that samples B (10/190) and C (10/196) be allocated to USP for distribution as replacement batches of the USP Endotoxin Reference.

On the basis of the results of this collaborative study and on the preliminary results of the stability study, the European Pharmacopoeia Commission adopted sample A (10/178) as the Ph. Eur. Endotoxin Biological Reference Preparation batch 5 with an assigned value of 10 000 IU/vial in June 2012. It was recommended to store the preparation at -20°C and to ship it at ambient temperature.

Sample A (10/178) was adopted in October 2012 by the ECBS as the WHO 3rd IS for Endotoxin. Batch (10/190) was established in December 2011 as the USP Endotoxin RS (Lot H0K354) with an assigned content of 10 000 IU/vial. Vials of this batch (10/190) were presented to the USFDA to be established as the Endotoxin EC-7. Batch 10/196 will be kept at USP for future use.

8. ADDENDA

8.1. Data from additional laboratories

Data were received from an additional four participants after the study deadline had passed. These results were not available for the above analysis. They are presented here as an addendum for completeness.

Three of the laboratories provided data from gelation assays, and four from photometric assays, with one laboratory providing data from both chromogenic and turbidimetric methods. The results from the individual gelation assays are shown in Table 14.

Laboratory 20 used 2 replicates per dilution while the other two laboratories used 4 replicates. All used doubling dilution series. Laboratory 28 provided two sets of results, one using tubes (28T), the other using microtitre plates (28P). Laboratory 28 obtained identical gelation results for the IS and all samples, resulting in estimates of 10,000 IU/vial for all samples, with both the tube and plate methods. Laboratory 20 also had identical results (10 000 IU/vial) except for sample A in one assay that was one dilution step lower (5000 IU/vial). The results from laboratory 33, based on their own calculations, were a little more variable. The laboratory geometric means are shown in Table 15.

The photometric assays were analysed as parallel line assays, relating the log-transformed assay response to log-concentration, using the CombiStats statistical software package [7]. Laboratory 33 returned raw assay response data for sample A–D, but only details of fitted standard curves for the standard were provided. The results provided from their own calculations were therefore used. The results from individual photometric assays are shown in Table 16. The results from laboratory 6 were highly variable. That laboratory used 10-fold dilution steps, which are not ideal for quantitative estimation. Also, an identical plate layout for each assay was used. It is possible that there were plate-positional effects affecting the assay results. Sample A, which had estimates closest to 10 000 IU/vial, was closest to the IS on the

plate. The results from laboratory 20 were also highly variable. That laboratory did not use consistent dilution series across the assays, and often samples were tested at only one or two dilutions. This is not ideal for quantitative estimation using the parallel line method. Laboratory 28 used two-fold dilutions, and the calculated results were all highly consistent, and close to 10 000 IU/vial for all samples in all assays. Laboratory 33 used ten-fold dilutions, and had more variable results. The laboratory geometric means are shown in Table 17. Apart from laboratory 28, and the gelation assays from laboratory 20, the results from the additional participants were quite variable. However, overall, there is no evidence that would indicate a need to modify the consensus values obtained from the main collaborative study reports.

8.2. Assays using recombinant LAL/Factor C

Two participants in the study used a photometric method using recombinant LAL/Factor C (rFc). Because this method is currently not compendial, these data were not included in the overall analysis for the assignment of potency to the candidate preparations. The datasets from the rFc assay method were analysed at the EDQM as parallel line assays, relating the log transformed assay response to log dose, using the CombiStats statistical software package [7].

Both laboratories carried out 3 assays on freshly reconstituted vials and 3 assays on solutions stored at 4°C for not more than 2 weeks. Laboratory 16 used 2 ten-fold dilution series at 4 doses. Laboratory 17 used 3 ten-fold dilution series at 3 doses.

The results expressed in IU/vial are displayed in Table 18. Also shown is the geometric mean (GM) and geometric coefficient of variation (GCV) of the 3 assays (within-laboratory variation). The overall GM across both laboratories is shown per sample and per fresh/stored solution.

The individual values range from 8134 to 11 003 IU/vial. The GM (n=3) range from 8929 to 9928 IU/vial. The GCV (n=3) range from 0.5% to 11.7%. Overall, the results are lower than the assigned potency of 10 000 IU/vial, for all samples.

The intra-laboratory variation was acceptable for most reported assays. Overall, the potencies of all samples obtained in both laboratories were lower than their assigned value of 10 000 IU/vial.

An rFc cloned from a different horseshoe crab species from the one specified in the current pharmacopoeial LAL method (*Carcinoscorpius rotundicauda*; this rFc shares 90.5% sequence identity with *Limulus polyphemus* LAL) was developed at the National University of Singapore [8]. Two commercial kits are based on the use of this reagent (PyroGene® by Lonza and EndoDtech® by BioDtech Inc.). Another alternative technology for endotoxin test is based on an ELISA-format using plates coated with a bacteriophage receptor protein targeting the conserved core region of lipopolysaccharide and a detection system with rFc cloned from *Tachypleus tridentatus* (EndoLISA kit, Hyglos GmbH). This method is claimed to reduce the effect of interacting factors by capturing endotoxin recognised by the bacteriophage receptor protein. The 2 datasets analysed in this report were generated using the same source of rFc (i.e. PyroGene kit from Lonza).

The very limited data available from this study do not allow comparison of the photometric assay using rFc with the current pharmacopoeial methods. Additional data is required to draw clearer conclusions on the validity of assays using rFc as alternative pharmacopoeial methods. Method validation could include data from various kit manufacturers and users as well as results from a collaborative trial involving several laboratories testing a common sample panel and various available kits.

9. PARTICIPANTS (in alphabetical order of country)

- [1] C. Rolls, A. Smith, Therapeutic Goods Administration (TGA), Australia
- [2] D. Pullirsch, Agency for Health and Food Safety (AGES), Austria
- [3] O. Carabin, G. Waeterloos, Scientific Institute of Public Health (IPH), Belgium
- [4] S. Dalmora, Universidade Federal de Santa Maria, Brazil
- [5] N. Fortin, Health Canada, Canada
- [6] H. Gao, National Institutes for Food and Drug Control (NIFDC), China
- [7] G. Rautmann, M. Fernandez, European Directorate for the Quality of Medicines & HealthCare (EDQM), Council of Europe
- [8] K. Vorup, Leo Pharma A/S, Denmark
- [9] U.B. Westergaard, Statens Serum Institut (SSI), Denmark
- [10] Y. Cortez, Agence nationale de sécurité du médicament et des produits de santé (ANSM), France
- [11] S. Deutschmann, H. Kavermann, Roche Diagnostics GmbH, Germany
- [12] A. Ruland, Pyroquant Diagnostik GmbH, Germany
- [13] I. Spreitzer, Paul Ehrlich Institut (PEI), Germany
- [14] P. Weidner, Acila AG, Germany
- [15] C. von Hunolstein, M. Boccanera, L. Campitelli, Istituto Superiore di Sanità (ISS), Italy
- [16] K. Sugiyama, National Institute of Health Sciences (NIHS), Japan
- [17] T. Nakatani, Lonza Japan Ltd, Japan
- [18] Y. Nakagawa, Japanese Pharmacopoeia Reference Standards Laboratory, Pharmaceutical and Medical Device Regulatory Science Society of Japan (PMRJ), Japan
- [19] T. Oda, Seikagaku Biobusiness Corporation, Japan
- [20] A. Takaoka, Wako Pure Chemical Industries Ltd, Japan
- [21] H. Min, Charles River Laboratories, Korea
- [22] J. Joung, Korea Food and Drug Administration (KFDA), Korea
- [23] S. R. Andersen, Norwegian Medicines Agency (NoMA), Norway
- [24] L. Soares, Infarmed IP, Portugal
- [25] K. Erlandsson-Persson, Medical Products Agency (MPA), Sweden
- [26] P. Lang, H. Rockstroh, Hoffmann-La Roche AG, Switzerland
- [27] P. Bruegger, Novartis Pharma AG, Switzerland
- [28] B. de Vries, J. Berger, National Institute for Public Health and the Environment (RIVM), The Netherlands

- [29] M. Dawson, B. Markley, Associates of Cape Cod Inc., USA
- [30] R. Deschenes, Amgen Inc., USA
- [31] J. Schultz, Charles River Laboratories Inc., USA
- [32] J.L. Kenney, US Food and Drug Administration (FDA), USA
- [33] G. Johnson, Lonza Walkersville Inc., USA
- [34] J. Mudrick, Medimmune Inc., USA
- [35] M. Ambrose, M. Crivellone, T. Morris, Unites States Pharmacopeial Convention (USP), USA

10. ABBREVIATIONS

ATD: Accelerated Thermal Degradation; BRP: Biological Reference Preparation; BSP: Biological Standardisation Programme; CBER: Center for Biologics Evaluation and Research; Chrom: Chromogenic; CL: Confidence Limits; DBO: Department of Biological Standardisation, OMCL Network & HealthCare; ECBS: Expert Committee on Biological Standardization; EDQM: European Directorate for the Quality of Medicines & HealthCare; EU: Endotoxin Unit (1EU = 1 IU); FDA: US Food and Drug Administration; GCV: Geometric Coefficient of Variation⁶; GM: Geometric Mean; NIBSC: National Institute for Biological Standards and Control; IS: International Standard; IU: International Unit; JP: Japanese Pharmacopoeia; LAL: Limulus Amoebocyte Lysate; OMCL: Official Medicines Control Laboratory; Ph. Eur.: European Pharmacopoeia; USP: United States Pharmacopoeia; Turb.: Turbidimetry; WHO: World Health Organization

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6 There co-exist 2 different definitions of the GCV in the published literature. They are not equivalent and caution is advised when comparing the GCV's from different studies. The definition used in this report is $GCV = (10^v - 1)^{0.5} \cdot 100\%$, whereas some other publications use the definition $GCV = (10^s - 1) \cdot 100\%$. In these equations $s^2 = v$ is the sample variance of the $\log_{10}$ -transformed potencies.

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12. ACKNOWLEDGEMENTS

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This article is dedicated to the memory of Mr Jean-Marc Spieser, Head of EDQM/DBO, who passed away in April 2013. Jean-Marc devoted his life to promoting cooperation for improved and harmonised global quality standards for medicines and healthcare. This study is an example among many others of his important contribution to this endeavour.

13. TABLES AND FIGURES

Table 1 – Specifics of the candidate batches

	Batch 10/178 (Sample A)	Batch 10/190 (Samples B & D)	Batch 10/196 (Sample C)
Date of fill	September 16, 2010	October 7, 2010	October 21, 2010
N. of vials filled	25 651	25 644	26 026
Mean fill mass	0.9995 g CV=0.1506 % N=260	0.9996 g CV=0.1920 % N=264	0.9990 g CV=0.1790 % N=268
Mean residual moisture	0.3659% CV=23.55% N=30	0.5246% CV=20.07% N=30	0.3060% CV=16.72% N=30
Mean oxygen headspace	0.55% CV=17.94% N=30	0.71% CV=15.95% N=30	0.66% CV=14.25% N=30
Microbial analysis	Pre-filled = 0	Pre-filled = 0	Pre-filled = 0
Bacterial Colony Count	Post-filled = 0	Post-filled = 0	Post-filled = 0
(Cfu/mL, N=4 vials)	Post-Freeze-Dried = 0	Post-Freeze-Dried = 0	Post-Freeze-Dried = 0
Microbial analysis	Pre-filled = 0	Pre-filled = 0	Pre-filled = 0
Mold/Yeast Colony Count	Post-filled = 0	Post-filled = 0	Post-filled = 0
(Cfu/mL, N=4 vials)	Post-Freeze-Dried = 0	Post-Freeze-Dried = 0	Post-Freeze-Dried = 0

Table 2 – ATD for Endotoxin Candidate Standards: potencies (in IU) of accelerated degradation samples relative to the standard Endotoxin curve, and the potencies (in %) of the samples stored at +56°C relative to those stored at -20°C for 10 months

Assay	Fill	-20°C (10 months)	+56°C (10 months)	+56 as % of -20°C (10 months)
1	A (10/178)	10223	10313	100.9
2		9872	10736	108.8
GM		10046	10522	104.7
3	B, D (10/190)	11102	10457	94.2
4		9984	10868	108.9
GM		10528	10660	101.3
5	C (10/196)	10798	10001	92.6
6		10057	10911	108.5
GM		10421	10446	100.2

Table 3 – ATD for Endotoxin Candidate Standards: potencies (in IU) of accelerated degradation samples relative to the standard Endotoxin curve, and the potencies (in %) of the samples stored at +56°C relative to those stored at -20°C for 15 months. All data (see text)

Assay	Fill	-20°C (15 months)	+56°C (15 months)	+56 as % of -20°C (15 months)
1	10/178	12064	10444	92.0
2		10141	10300	
GM		11061	10171	
3	10/190	10126	10003	106.3
4		10073	11515	
GM		10099	10732	
5	10/196	11099	9854	98.8
6		10376	11402	
GM		10732	10600	

Table 4 – ATD for Endotoxin Candidate Standards: potencies (in IU) of accelerated degradation samples relative to the standard Endotoxin curve, and the potencies (in %) of the samples stored at +56°C relative to those stored at -20°C for 15 months. Repeat of parallel line analysis excluding the two outer columns (see text)

Assay	Fill	-20°C (15 months)	+56°C (15 months)	+56 as % of -20°C (15 months)
1	10/178	11317	10834	93.1
2		10761	9737	
GM		11035	10271	
3	10/190	9641	10816	103.9
4		10926	10506	
GM		10263	10660	
5	10/196	10680	10424	97.3
6		11239	10896	
GM		10956	10657	

Table 5 – ATD for Endotoxin Candidate Standards: potencies (in IU) of accelerated degradation samples relative to the standard Endotoxin curve, and the potencies (in %) of the samples stored at +56°C relative to those stored at -20°C for 17 months. All data (see text)

Assay	Fill	-20°C (17 months)	+56°C (17 months)	+56 as % of -20°C (17 months)
1	10/178	10840	9413	94.7
2		9663	9986	
GM		10235	9696	
3	10/190	11058	9733	95.0
4		9609	9858	
GM		10308	9795	
5	10/196	11256	8265	81.8
6		9155	8333	
GM		10151	8299	

Table 6 – ATD for Endotoxin Candidate Standards: potencies (in IU) of accelerated degradation samples relative to the standard Endotoxin curve, and the potencies (in %) of the samples stored at +56°C relative to those stored at -20°C for 17 months. Repeat of parallel line analysis excluding the two outer columns

Assay	Fill	-20°C (17 months)	+56°C (17 months)	+56 as % of -20°C (17 months)
1	10/178	10252	10126	94.4
2		10727	9682	
GM		10487	9902	
3	10/190	10553	10484	99.9
4		10527	10585	
GM		10540	10534	
5	10/196	10645	8765	81.7
6		9662	7842	
GM		10142	8291	

Table 7 – Summary of ATD results: relative potencies at +56°C expressed as % of -20°C

Fill	+56°C as % of -20°C		
	10 months	15 months	17 months
A (10/178)	104.7	93.1	94.4
B, D (10/190)	101.3	103.9	99.9
C (10/196)	100.2	97.3	81.7

Table 8 – Methods used by laboratories

Lab. code	Gelation	Photometric	
		Chromogenic	Turbidimetric
1	X	X	X
2	X		X
3	X	X	X
4	X	X	X
5	X		
6		X*	
7	X		
8		X	
9	X		
10	X		X
11	X	X	X
12	X	X	
13	X		X
14	X	X	
15			X
16	X	(x)	
17		(x)	X
18	X	X	
19	X		
20	X*		X*
21	X		
22		X	
23	X	X	
24	X	X	
25	X		
26		X	
27	X	X	
28	X*	X*	
29	X		
30		X	X
31		X	
32	X		X
33	X*	X*	X*
34	X	X	X
35	X	X	X

(x): recombinant LAL method (not included in overall analysis – see text)

*: data not included in the statistical evaluation and potency value assignment due to late data reporting

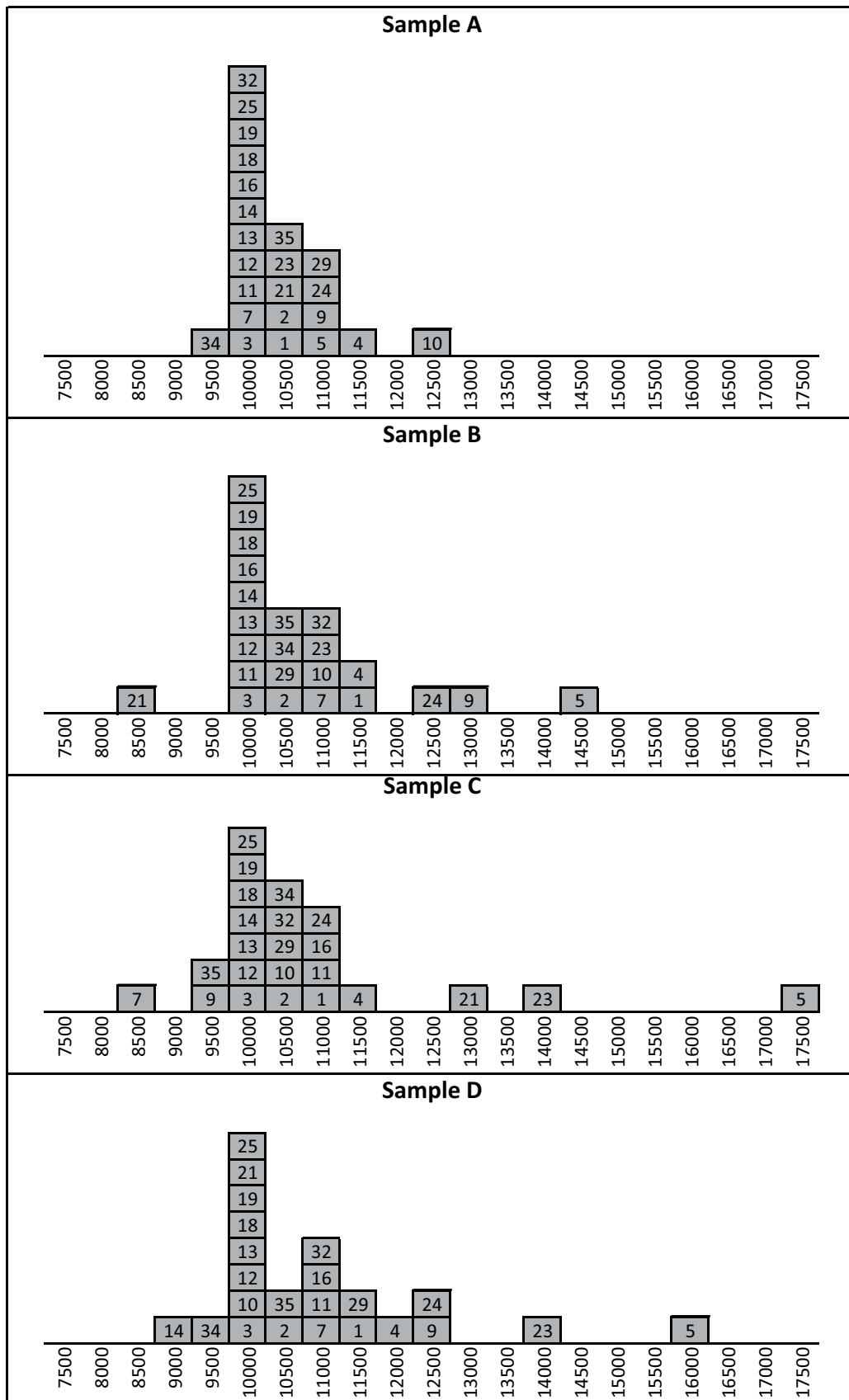
Table 9 – Overview of results per laboratory (gelation assays; IU/vial)

Lab	Day	Sample A			Sample B			Sample C			Sample D		
		Vial 1	Vial 2	Vial 3	Vial 1	Vial 2	Vial 3	Vial 1	Vial 2	Vial 3	Vial 1	Vial 2	Vial 3
1	1	10000	11892	10000	10000	10000	20000	11892	11892	10000	11892	10000	14142
	2	10000	10000	10000	11892	10000	10000	11892	10000	10000	10000	10000	14142
2	1	11892	10000	10000	10000	10574	11180	10574	10574	10000	10000	10000	10574
	2	10574	10000	10000	10574	10000	10574	11180	10574	10574	10000	10574	10574
3	1	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
	2	10000	10000	10000	10000	10000	10000	10000	8412	10000	10000	10000	10000
	3	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
	4	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
4	1	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
	2	14142	10000	16818	14142	8409	20000	14142	8409	20000	14142	10000	20000
5	1	10000	10000	10000	10000	16818	16818	20000	20000	20000	11892	20000	20000
	2	16818	10000	10000	16818	20000	10000	16818	20000	10000	16818	20000	10000
7	1	10000	10000	8409	10000	11892	8409	11892	14142	4204	10000	14142	11892
	2	10000	10000	11892	10000	10000	20000	11892	10000	5000	10000	11892	10000
9	1	10000	12910	11362	14669	14669	12910	10000	10000	11362	10000	16667	20000
	2	10000	10000	11362	10000	10000	16667	6000	10000	10000	10000	7692	16667
10	1	10000	10000	10000	10000	10000	10000	10000	4984	10000	10000	4984	10000
	2	20064	20064	10000	10000	14165	14165	20064	14165	10000	10000	14165	14165
11	1	8409	14142	8409	10000	11892	10000	10000	14142	10000	8409	16818	10000
	2	10000	10000	10000	10000	8409	10000	11892	10000	10000	11892	10000	10000
12	1	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
	2	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
13	1	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
	2	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
14	1	7071	14142	10000	7071	14142	10000	7071	14142	10000	7071	10000	10000
16	1	10000	10000	10000	10000	10000	10000	20000	10000	10000	20000	10000	10000
	2	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
18	1	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
	2	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
19	1	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
	2	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
21	1	13919	16685	13919	10000	6089	13919	38750	8476	13919	10000	11798	10000
	2	8476	10000	5161	8476	5161	10000	11798	10000	10000	8476	10000	10000
23	1	14142	3536	10000	20000	10000	2500	5000	14142	20000	20000	14142	5000
	2	20000	7071	20000	20000	14142	14142	20000	14142	20000	20000	14142	20000
24	1	10000	10000	10000	10000	10000	20000	10000	5000	20000	10000	10000	20000
	2	10000	10000	20000	10000	10000	20000	10000	10000	20000	10000	10000	20000
25	1	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
	2	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
29	1	11892	10000	14142	10000	10000	14142	11892	10000	20000	11892	10000	20000
	2	10000	10000	10000	10000	10000	10000	5000	10000	10000	10000	10000	10000
32	1	10000	10000	10000	20000	10000	10000	14142	10000	10000	14142	11892	10000
	2	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
34	1	10000	10000	10000	10000	10000	10000	10000	10000	10000	7071	10000	10000
	2	8409	10000	10000	10000	10000	14142	10000	10000	14142	10000	8409	14142
35	1	10000	10000	10000	10000	10000	10000	7071	10000	10000	10000	10000	10000
	2	11892	10000	10000	11892	10000	10000	10000	10000	10000	11892	10000	10000

Table 10 – Overview of mean results per laboratory (gelation assays; IU/vial)

Lab	Sample A	Sample B	Sample C	Sample D
1	10293	11554	10905	11554
2	10389	10476	10574	10283
3	10000	10000	9857	10000
4	11554	11554	11554	11892
5	10905	14557	17311	15874
7	10000	11225	8655	11225
9	10889	12910	9381	12739
10	12613	11231	10597	10000
11	10000	10000	10905	10905
12	10000	10000	10000	10000
13	10000	10000	10000	10000
14	10000	10000	10000	8909
16	10000	10000	11225	11225
18	10000	10000	10000	10000
19	10000	10000	10000	10000
21	10595	8476	13243	10000
23	10595	11225	14142	14142
24	11225	12599	11225	12599
25	10000	10000	10000	10000
29	10905	10595	10293	11554
32	10000	11225	10595	10905
34	9715	10595	10595	9715
35	10293	10293	9439	10293
GM	10414	10739	10768	10937
GCV*	6.2	11.2	14.8	13.4
Huber's robust mean (K=15)	10250	10598	10509	10648

*: see note in Section 10.



Numbers in the boxes are the laboratory codes.

Figure 1 – Histograms of mean results per laboratory (gelation assays; IU/vial, Huber's robust means)

Table 11 – Overview of the mean results per laboratory (photometric assays; IU/vial)

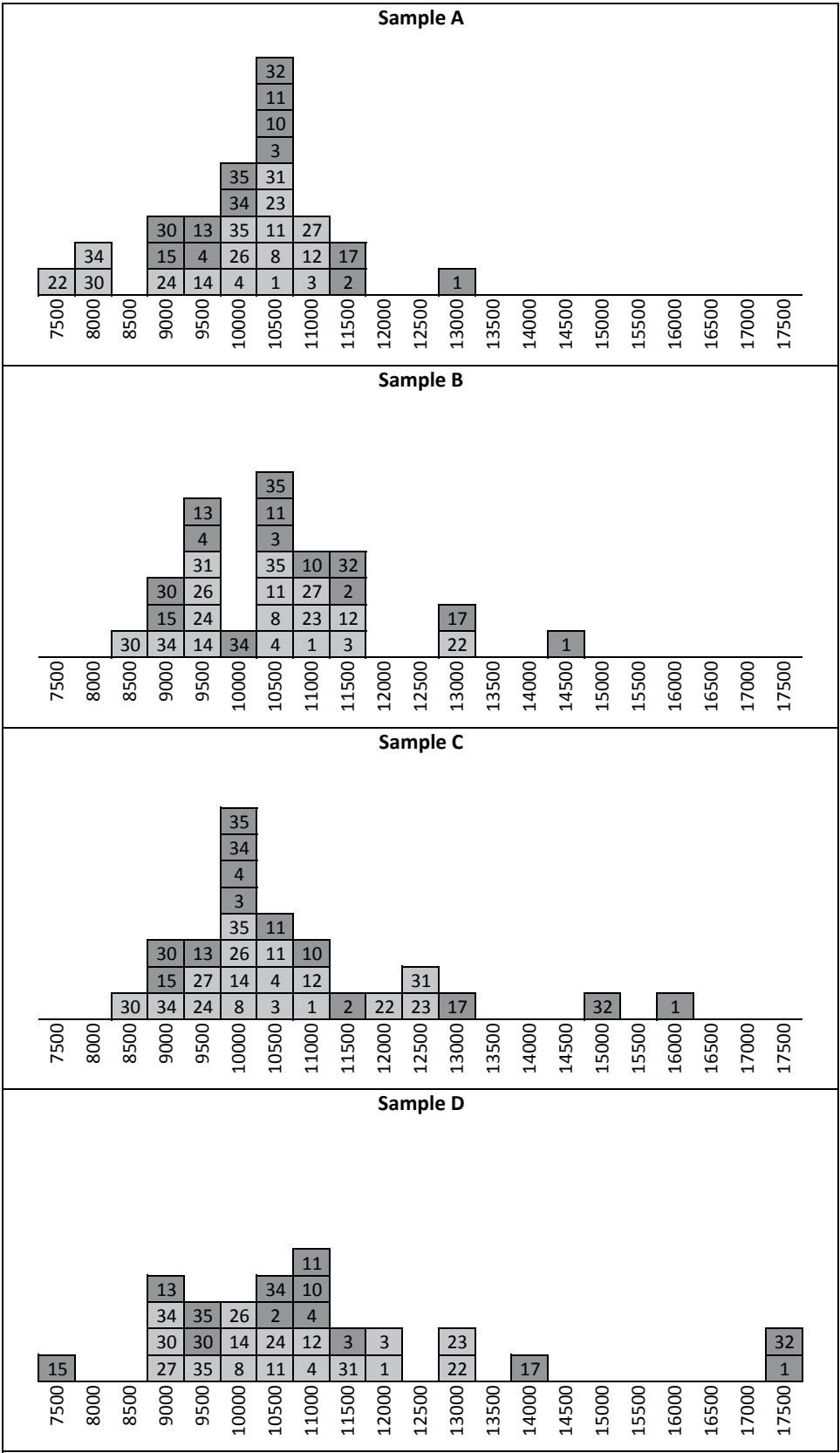
Lab	Method	Day	Sample A			Sample B			Sample C			Sample D		
			Vial 1	Vial 2	Vial 3	Vial 1	Vial 2	Vial 3	Vial 1	Vial 2	Vial 3	Vial 1	Vial 2	Vial 3
1	Chrom	1	9267	10356	12357	10181	10385	13768	10172	11405	11473	12658	12332	13023
		2	9913	10159	11525	10974	10032	11412	11178	11205	11747	11842	11623	11583
	Turb	1	13167	21914	8092	16419	14209	9812	17933	16054	12527	20679	16072	13791
		2	10691	20343	9676	14942	14308	17316	16248	15793	17723	17599	19195	19114
2	Turb	1	9863	11062	12629	11197	10471	12722	10775	10469	11185	10229	10175	11982
		2	10571	10793	12990	11169	10995	13134	11162	10580	14250	9631	10268	
3	Chrom	1	10055	10062	9985	10068	10538	9290	10007	9147	8768	11095	10634	9884
		2	10820	9867	11709	10526	10697	12186	10606	8958	12075	10550	12230	13292
		3	12131	12151	12019	13781	12197	12405	11240	10213	10681	12361	13362	12327
		4	11424	10732	12345	10616	10839	13511	11039	10165	13566	14296	13506	12737
	Turb	1	9795	9844	10026	9404	10304	10639	9461	8990	11100	10010	10475	11263
		2	9913	9718	10437	9625	10432	10621	9929	9084	10868	10656	11093	11549
		3	10786	10338	11176	12745	10388	12199	10065	9507	12104	11329	12154	12640
		4	10530	10507	10408	9971	10212	10156	11781	9626	10981	12562	12015	12009
4	Chrom	1	9947	9985	9906	10506	10458	10449	10924	11014	9398	11028	11060	11183
		2	10380	9294	10492	11210	9314	11282	11778	9893	10127	12503	8256	11742
	Turb	1	8766	9483	9560	8982	9271	11155	9464	10009	11176	11090	10550	13669
		2	9094	10045	9039	9307	9747	9005	9313	10297	8940	10704	11393	10091
8	Chrom	1	8752	9580	11546	9139	10775	9524	10948	9996	9016	10270	10338	8912
		2	9123	11218	12250	10564	11378	11462	10470	10187	10445	9272	10274	10867
10	Turb	1	10830	10125	10878	10806	11145	11517	11371	10766	11598	11295	11288	11106
		2	10568	10678	10632	9266	11480	11645	11297	11512	10520	11156	11466	10901
11	Chrom	1	9680	10660	10323	9340	11617	9628	9716	10698	10718	10621	10473	9888
		1	10188	10220	10548	10362	10803	9660	11146	10774	10250	11630	10901	10421
		2	10299	10497	10940	10326	10305	10911	10866	10709	10799	10258	10814	10941
		2	10951	10372	10250	10493	11246	10204	10344	11093	10862	10584	10555	10710
	Turb	1	9982	10423	10054	9831	10904	9504	10632	10271	9722	11014	10570	10171
		2	10263	11135	10581	10489	10471	11026	11277	10678	10220	11483	11145	10208
12	Chrom	1	10400	11450	10922	11999	10968	11547	10956	10392	11167	9991	11126	10963
		1	10448	11247	11102	11806	10741	11469	11227	10503	11060	10569	11395	10681
		2	9773	11814	10881	10597	11665	11834	10309	11487	12075	10555	11727	11930
		2	10105	11506	11463	10866	11636	12649	10775	11956	11878	10855	11945	12123
13	Turb	1	9984	9341	9149	9181	9706	9495	9613	9080	10291	8281	8285	9580
		2	10179	9956	9682	9157	9750	10184	9032	9696	10913	8500	9253	10434
14	Chrom	1	8815	9210	9730	8215	8946	9663	9773	9214	9362	8377	9273	9802
		2	10118	10222	9555	10203	10405	9628	10444	10006	9990	10479	11192	9857
15	Turb	1	8568	9003	8785	8351	7727	9077	9335	10158	6586	6089	6402	7691
		2	8812	9085	10513	7901	10027	10116	8703	11072	8427	7008	8477	9135
17	Turb	1		10286	11753	12738	12525	13370	12721	12316	13105	13483	12710	14944
		2	11584	10418	12881	12367	11782	14649	13315	12213	15565	13727	12326	16560
22	Chrom	1	10224	5444		12006	10316		10884	10957		12738	10255	
		2	10518	4203	11068	14353	14482	13224	11421	14005	13036	13040	14180	14074
23	Chrom	1	9629	9616	9208	10176	11242	9793	10244	12961	11053	10490	13475	11084
		2	10967	12012	11323	11895	12768	11153	13437	14792	13118	12227	15883	15680
24	Chrom	1	9524	10449	10194	10024	11037	9509	9708	11172	11538	8532	17847	10731
		2	6830	8606	8773	8509	9503	8406	7378	8676	9222	9067	10684	9015
26	Chrom	1	9704	9529	10783	11320	8963	10093	13832	9308	10716	10619	9604	10407
		2	11173	8875	9517	8717	8457	10140	9269	8204	10740	9477	9398	10515
27	Chrom	1	10802	11913	10811	11277	11050	10303	10671	7842	10746	11323	9541	9070
		2	10734	11820	11441	12324	11554	10873	10867	8195	10392	9696	9175	6811
30	Chrom	1	8723	7784	7729	9124	8458	8325	9264	8137	8151	10442	8842	8558
		2	8139	6581	9123	8840	7350	8998	8867	7268	9623	9341	7675	9971
	Turb	1	8040	8018	8974	8860	8050	9518	8139	7870	9102	9007	8942	9909
		2	10024	8914	8936	10951	8679	8956	13683	8667	8853	11616	9044	9789
31	Chrom	1	10897		10328	9357		11644	13306		13252	10339		16091
		2	11715	7872	11042	10323	10477	7331	12873	12052	11940	10149	10381	11704
32	Turb	1	16683	7662	17184	21900	7595	17182	17802	13627	23848	23665	18067	24310
		2	8288	9097	7648	9568	8449	11077	12427	12225	12775	16755	13113	13475
34	Chrom	1	8609	8215	7293	8856	8880	8008	8548	9194	8127	8636	9016	8227
		2	8791	8196	8284	9729	9094	9114	9812	8826	8328	9673	11489	8369
	Turb	1	10293	11028	9732	9831	10820	8940	10086	11718	10062	10677	10501	10344
		2	10476	9686	9862	10433	9916	9524	10916	10004	8784	10914	12115	9604
35	Chrom	1	9939	10120	10564	9076	10729	10656	8251	11765	11018	9289	10297	9817
		2	10274	9737	10515	10874	10091	10256	8437	10176	11252	10407	8260	8998
	Turb	1	10442	9864	10141	9833	10219	10120	8137	10713	11052	8642	9868	8888
		2	9740	9812	10223	10330	12006	9997	7925	11376	10736	10041	10494	8769

Chrom: chromogenic assay. Turb: turbidimetric assay

Table 12 – Overview of the mean results per assay (photometric assays; IU/vial)

Lab	Method	Sample A	Sample B	Sample C	Sample D
1	Chromogenic	10547	11059	11185	12165
3	Chromogenic	11070	11311	10461	12117
4	Chromogenic	9993	10516	10492	10874
8	Chromogenic	10328	10436	10159	9966
11	Chromogenic	10405	10388	10658	10642
12	Chromogenic	10909	11467	11134	11136
14	Chromogenic	9596	9480	9789	9790
22	Chromogenic	7709	12776	11998	12773
23	Chromogenic	10409	11127	12506	12975
24	Chromogenic	8976	9456	9508	10611
26	Chromogenic	9899	9564	10201	9990
27	Chromogenic	11243	11213	9699	9168
30	Chromogenic	7971	8494	8515	9092
31	Chromogenic	10277	9712	12671	11546
34	Chromogenic	8217	8932	8788	9173
35	Chromogenic	10187	10262	10055	9481
1	Turbidimetric	13038	14278	15940	17587
2	Turbidimetric	11264	11576	11337	10428
3	Turbidimetric	10281	10520	10243	11451
4	Turbidimetric	9322	9550	9840	11195
10	Turbidimetric	10615	10944	11170	11201
11	Turbidimetric	10399	10356	10456	10754
13	Turbidimetric	9708	9572	9749	9022
15	Turbidimetric	9106	8816	8930	7389
17	Turbidimetric	11345	12874	13161	13888
30	Turbidimetric	8792	9126	9214	9676
32	Turbidimetric	10402	11695	14964	17702
34	Turbidimetric	10168	9892	10222	10666
35	Turbidimetric	10034	10394	9884	9424
GM	Chromogenic	9798	10333	10426	10646
GCV	(N= 16)	11.7	10.6	11.3	12.0
GM	Turbidimetric	10292	10641	10986	11229
GCV	(N= 13)	10.4	13.9	18.2	25.2
GM	Pooled (N= 29)	10017	10470	10673	10904
GCV		11.2	12.0	14.7	18.8
Huber's robust mean (k=1.5)		10120	10404	10449	10730

GM: geometric mean; GCV: geometric coefficient of variation (see also note in section 10)



Numbers in the boxes are the laboratory codes.
Chromogenic assays are shown in light-grey. Turbidimetric assays are shown in dark-grey.

Figure 2 – Histograms of mean results per laboratory (photometric assays; IU/vial, Huber's robust means)

Table 13 – Overall potency of the 3 candidate batches (gelation and photometric assays)

Sample	Potency (IU/vial)	
	Estimate	95% CL
A	10190	(9927 - 10461)
B	10588	(10252 - 10935)
C	10715	(10289 - 11159)
D	10900	(10414 - 11410)
B & D	10743	(10356 - 11145)

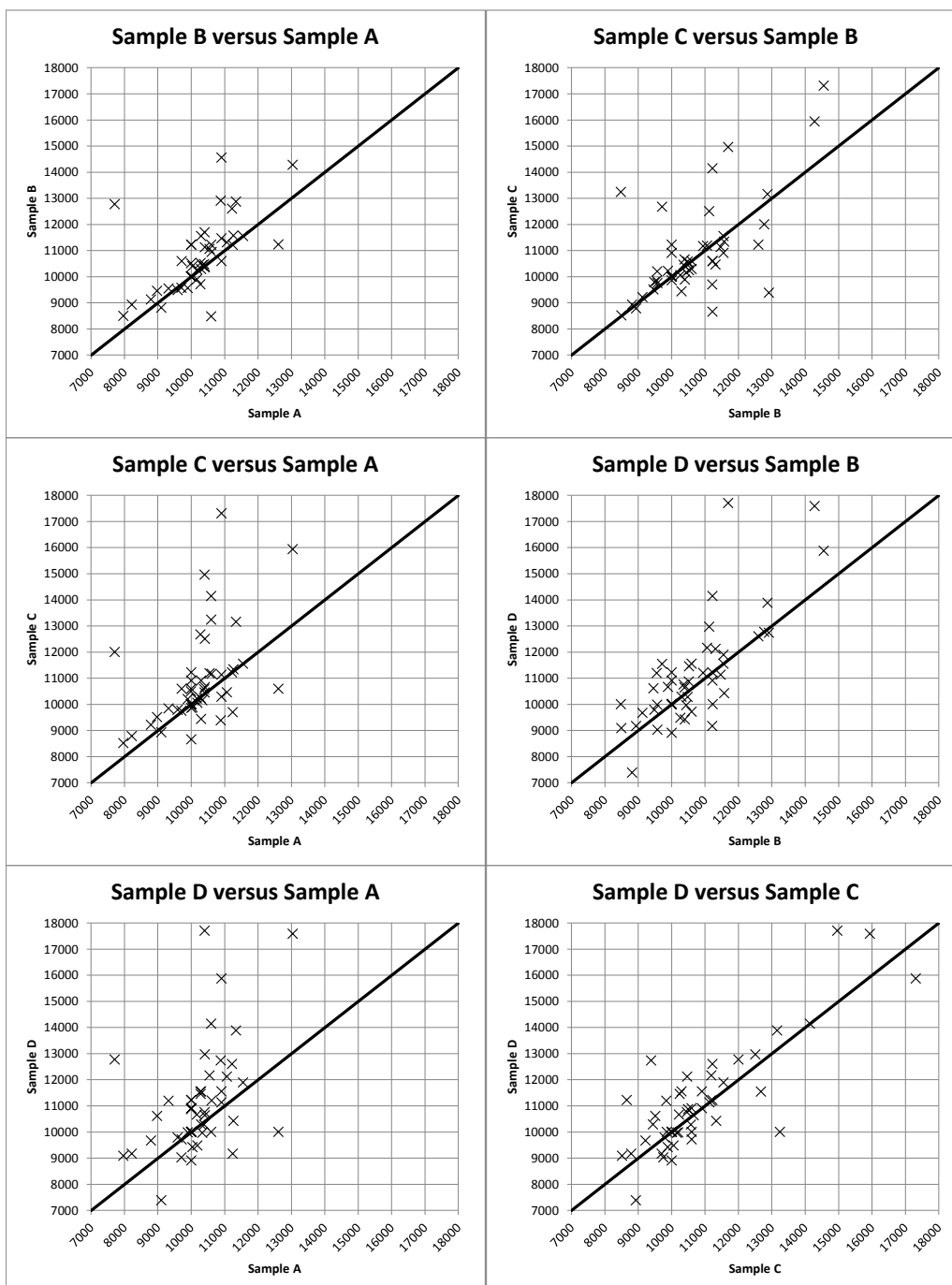


Figure 3 – Two-way comparison between batches (IU/vial)

Table 14 – Addendum: Gelation assays: IU/vial - Individual assay results

Lab	Day	Sample A			Sample B			Sample C			Sample D		
		Vial 1	Vial 2	Vial 3	Vial 1	Vial 2	Vial 3	Vial 1	Vial 2	Vial 3	Vial 1	Vial 2	Vial 3
20	1	10000	5000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
	2	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
28P	1	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
	2	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
28T	1	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
	2	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
33	1	6000	10000	5000	7000	10000	7000	8000	10000	10000	6000	8000	10000
	2	10000	10000	10000	10000	10000	10000	10000	10000	12000	5000	7000	12000

Table 15 – Addendum: Gelation assays: IU/vial – Geometric mean results by laboratory

Lab	Sample A	Sample B	Sample C	Sample D
20	8910	10000	1 000	10000
28P	10000	10000	10000	10000
28T	10000	10000	10000	10000
33	8370	9490	9980	8000

Table 16 – Addendum: Photometric assays: IU/vial - Individual assay results

Lab	Method	Day	Sample A			Sample B			Sample C			Sample D		
			Vial 1	Vial 2	Vial 3	Vial 1	Vial 2	Vial 3	Vial 1	Vial 2	Vial 3	Vial 1	Vial 2	Vial 3
6	Chrom	1	9365	8455	9874	4580	5095	5178	7645	7316	7957	5813	6025	6656
		2	7392	9569	12611	3763	5594	6698	6629	7607	10418	4765	6243	8053
20	Turb	1	10994	12862	22738	11460	9785	20526	16004	9710	31708	14105	12344	18260
		2	-	4883	8973	12632	6448	7705	31062	12472	13260	30928	6511	8521
28	Chrom	1	10109	10036	10061	10248	10097	10013	10168	10088	10071	10091	10033	9984
		2	10178	9991	9958	10154	10098	10132	10141	10028	10037	9983	9940	9990
33	Turb	1	14680	12120	7660	7660	9390	11280	10510	14440	15280	10140	23250	12670
		2	16070	14480	12860	6870	13880	11810	8540	21690	15670	8400	19380	13420
	Chrom	1	9530	9570	9450	10600	11280	11370	10710	10750	10750	10850	10690	11150
		2	10090	6380	7990	11600	10120	11390	10290	9580	7770	11870	9960	10250

Note: Lab 20 labelled assays 1 – 6, but did not specify vial or day. Results above assume 1-6 represents vial 1 days 1, 2 etc.

Table 17 – Addendum: Photometric assays: IU/vial – *Geometric mean results by laboratory*

Lab	Method	Sample A	Sample B	Sample C	Sample D
6	Chrom	9417	5073	7849	6183
20	Turb	10709	10632	17135	13268
28	Chrom	10055	10124	10089	10003
33	Turb	13782	10122	14319	14525
	Chrom	8826	11050	9782	10800

Table 18 – Addendum: Potency estimates in IU/vial, with a chromogenic assay using recombinant LAL/Factor C

Lab. code	Assay	Sample A		Sample B		Sample C		Sample D	
		Fresh	Stored	Fresh	Stored	Fresh	Stored	Fresh	Stored
16	1	9865	9356	9635	9053	9444	9028	9181	8853
	2	9365	10091	8134	9185	9123	10575	8874	11003
	3	9487	9245	9083	8693	9145	9830	9218	9550
	GM	9570	9557	8929	8974	9236	9791	9090	9762
	GCV	2.7%	4.9%	9.0%	2.9%	1.9%	8.2%	2.1%	11.7%
17	1	9302	9219	9705	9190	9310	9440	9266	9108
	2	9644	9661	10671	9728	10246	9519	10537	9376
	3	9332	9716	9450	9855	9935	9517	9275	10149
	GM	9425	9530	9928	9587	9822	9492	9675	9534
	GCV	2.0%	2.9%	6.6%	3.8%	5.0%	0.5%	7.7%	5.8%
Overall GM		9497	9543	9415	9276	9525	9640	9378	9647
Overall GM		9520		9345		9582		9512	

Establishment of the Ph. Eur. erythropoietin chemical reference substance batch 1

C. Burns¹, A.F. Bristow¹, K.H. Buchheit², A. Daas², M. Wierer²,
A. Costanzo²

ABSTRACT

The Erythropoietin (EPO) European Pharmacopoeia (Ph. Eur.) Biological Reference Preparation (BRP) batch 3 was calibrated in 2006 by in vivo bioassay and was used as a reference preparation for these assays as well as for the physicochemical methods in the Ph. Eur. monograph Erythropoietin concentrated solution (1316). In order to avoid the frequent replacement of this standard and thus reduce the use of animals, a new EPO Chemical Reference Substance (CRS) was established to be used solely for the physicochemical methods. Here we report the outcome of a collaborative study aimed at demonstrating the suitability of the candidate CRS (cCRS) as a reference for the physicochemical methods in the Ph. Eur. monograph. Results from the study demonstrated that for the physicochemical methods currently required in the monograph (capillary zone electrophoresis (CZE), polyacrylamide gel electrophoresis (PAGE)/immunoblotting and peptide mapping), the cCRS is essentially identical to the existing BRP. However, data also indicated that, for the physicochemical methods under consideration for inclusion in a revised monograph (test for oxidised forms and glycan mapping), the suitability of the cCRS as a reference needs to be confirmed with additional work. Further to completion of the study, the Ph. Eur. Commission adopted the cCRS as "Erythropoietin for physicochemical tests CRS batch 1" to be used for CZE, PAGE/immunoblotting and peptide mapping.

KEYWORDS

Erythropoietin, CRS, biological standardisation, collaborative study, European Pharmacopoeia, Chemical Reference Substance, capillary zone electrophoresis, CZE, peptide mapping, polyacrylamide gel electrophoresis, PAGE, immunoblotting, glycan mapping, oxidised form.

1. INTRODUCTION

The European Pharmacopoeia (Ph. Eur.) Biological Reference Preparation for Erythropoietin (EPO) batch 3 (BRP3) was prepared in 2006 [1]. The BRP3 was calibrated by *in vivo* bioassay (normocythaemic and polycythaemic mouse bioassays) for use as a reference preparation for these bioassays. In addition, it was also characterised for use for the physicochemical methods prescribed in the Ph. Eur. monograph *Erythropoietin concentrated solution (1316)* [2] namely capillary zone electrophoresis (CZE), polyacrylamide gel electrophoresis (PAGE) and immunoblotting, and peptide mapping. As a result of its use for both bioassay and physicochemical analysis, this standard has been heavily used. To reduce the need to produce a replacement BRP calibrated by *in vivo* bioassay, the Steering Committee of the Biological Standardisation Programme (BSP) endorsed a project, BSP081, to establish a new EPO

1 C. Burns, A.F. Bristow, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Herts, UK.

2 K.H. Buchheit, A. Daas, M. Wierer, A. Costanzo (corresponding author's e-mail: angele.costanzo@edqm.eu), European Directorate for the Quality of Medicines & HealthCare (EDQM), Department of Biological Standardisation, OMCL Network & HealthCare (DBO), Council of Europe, Strasbourg, France.

Chemical Reference Substance (CRS) to be used for physicochemical analyses only, thus saving stocks of the BRP for use exclusively as a bioassay reference preparation.

In most cases, the physicochemical tests of the quoted Ph. Eur. monograph use lower amounts of materials and are often performed in different quality control laboratories. It was therefore decided to produce a candidate material containing 100 µg of EPO per vial, instead of 250 µg/vial as is the case for the current BRP. A trial fill was performed and a prequalification study was carried out in 2 laboratories to assess the suitability of the pilot material for its intended use. As the outcome of the prequalification was satisfactory, the batch of candidate CRS (cCRS) was manufactured using the same procedure, the starting materials, the formulation buffer and the lyophilisation process being essentially identical to those used for the production of the current BRP. The cCRS was then tested, in parallel with BRP3, during the course of an international collaborative study to assess its suitability as a reference standard for some of the physicochemical methods prescribed in the monograph. Since a revision of the monograph is being discussed to include methods for glycan mapping and for testing of oxidised forms of EPO, adequacy of the candidate material for the new methods was also investigated during the same study.

The BRP3 and its predecessors have proved to be very stable over time [1]. However, the cCRS contains lower amounts of materials and the formulation buffer used for the lyophilisation resulted in a residual water content (1.74% on average) which was slightly higher than usual. Therefore, an accelerated degradation study was performed to ensure that the CRS would be stable enough for its intended use and to verify that the current recommended storage and distribution conditions were still adequate. This manuscript describes the results of the collaborative study and of the stability assessment.

2. AIM

The aim of BSP081 was to establish a CRS for EPO to be used for the physicochemical tests required in the existing monograph *Erythropoietin concentrated solution (1316)* i.e. CZE, PAGE/immunoblotting and peptide mapping. In addition, the study also provided the opportunity to assess the suitability of the cCRS for use in new methods that are planned for inclusion in the proposed revision of the monograph i.e. glycan mapping and oxidised form.

3. PARTICIPANTS

Five laboratories took part in the study. They are listed in section 9 hereunder. Laboratories have been coded for the study and their code numbers do not necessarily relate to the order of listing.

4. MATERIALS, METHODS AND STUDY DESIGN

4.1. Candidate CRS

The cCRS was produced in November 2012 at NIBSC (National Institute for Biological Standards and Control, UK) through blending, formulation and lyophilisation of two bulk products, one containing recombinant human (rhu) EPO-alpha (α) and the second rhuEPO-beta (β), from two different manufacturers. The EPO- α bulk is a similar material (from a different batch) from the same manufacturer as the EPO- α used for the preparation of BRP3. For EPO- β , the same bulk material was used for BRP3 and cCRS. The final content of the cCRS is 100 µg of EPO/vial, consisting of equal proportions of each form (W/W). The composition of the

protein-free formulation buffer is identical and the lyophilisation cycle very similar (see section 4.1.1 hereunder for more details) to those used for the production of BRP3.

4.1.1. Starting materials and buffers

Erythropoietin bulks

The bulks of rhuEPO- α and rhuEPO- β were presented as frozen solutions each originating from a single batch of production. The EPO concentrations, i.e. 3.34 g/L and 2.46 g/L respectively, were confirmed just before use by measuring the UV absorbance spectrum from 240 nm to 340 nm and calculating the corresponding concentration at the maximum absorption wavelength i.e. 280 nm.

The solutions were thawed at room temperature and volumes of each bulk corresponding to 848 mg, i.e. 254 mL and 345 mL respectively, were combined.

Formulation buffer

The formulation ingredients were tested prior to the definitive fill in order to avoid any contamination. The carrier (formulation buffer) was prepared as a 2-fold concentrated solution containing 300 g D-(+)-trehalose, 30 g arginine, 1.0 g Tween-20, 45 g NaCl and 31.2 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$. These reagents were dissolved in Milli-Q grade water to a final volume of 5 L. The pH was measured as pH 7.3 and the solution was then filtered for sterilisation through a 0.2 μm Nalgene cellulose nitrate vacuum filter unit (cat. no. 450-0200).

4.1.2. Filling and lyophilisation

The vials were produced under conditions required for the preparation of International Standards [3]. The fill solution was prepared by combining 3393 mL of formulation buffer, 254 mL of EPO- α , 345 mL of EPO- β and 2795 mL of Milli-Q grade water under appropriate conditions to ensure sterility.

After thorough mixing to obtain a homogeneous solution, a volume of 0.4 mL, corresponding to 100 μg of EPO, was dispensed per vial (sterile, 5 mL white-glass screw-cap), using an automated dispensing system. The vials were subsequently frozen and lyophilised using cycling parameters defined during a pilot phase. At the end of the lyophilisation cycle, vials were stoppered under nitrogen atmosphere. In-process and post-manufacturing quality controls of the filled material, including microbiology testing (filled products and environmental monitoring), showed that the cCRS was suitable for its intended use. The mean filling weight was 0.410 g (range 0.400 g to 0.423 g, $n=462$) and mean dry weight 0.016 g ($n=6$), with coefficients of variation (CV) of 0.41 % and 0.36 %, respectively. Mean headspace oxygen was determined as 0.78 % (CV 11.1 %, $n=12$) and the mean residual moisture after freeze-drying was 1.74 % (CV 4.55 %, $n=12$). To confirm storage and shipping conditions, the cCRS was subjected to an accelerated degradation study (see section 6 for more details).

4.2. Collaborative study materials

A sufficient number of vials were distributed to the participants in order for them to perform at least 3 independent assays for each method considered. For each independent day of testing, fresh vials of BRP3 and cCRS were to be used. Reconstituted materials could be stored at +4°C for a short time, if necessary, but any unused material was to be discarded at the end of the day.

Sample A: EPO cCRS batch 1

Each participant was instructed to store the vials of cCRS at -20°C upon receipt and until use. New vials of cCRS were to be reconstituted freshly just before use on each independent day of assay following the instructions given in the study protocol, buffer and concentrations varying depending on the method for which the vials were to be used. In some cases, one vial of cCRS was used in two different assays performed on the same day.

Sample B: EPO BRP batch 3

EPO BRP batch 3 [1] contains 250 µg of EPO-α and -β (1:1) per vial. Vials of BRP3 were to be stored at -20°C upon receipt and until use. For each day of assay one new vial of BRP3, sufficient for all physicochemical tests, was to be reconstituted just before use, as described in the study protocol. Participants were requested, where possible, to perform all tests in parallel in order to reduce the number of vials of BRP3 used. If this was not possible, additional samples were provided on request.

4.3. Methods

Where applicable, tests were performed following the instructions of the Ph. Eur. monograph *Erythropoietin concentrated solution (1316)* [2]. For the methods not yet present in the monograph, detailed instructions were given in the study protocol. However, as outlined in Table 1, some participants only carried out a subset of the tests.

Table 1 – Tests performed by participants

Lab	SDS-PAGE/ Immunoblotting	CZE	Peptide mapping	Glycan mapping	Oxidised form
1	-	X	X	X	-
2	X	X	X	X	X
3	X	X	X	X	X
4	-	X	X	-	-
5	X	X	X	X	-
Total	3	5	5	4	2

CZE: capillary zone electrophoresis; Lab: laboratory; PAGE: polyacrylamide gel electrophoresis.

4.3.1. CZE

CZE was performed for isoform analysis. After reconstitution of the samples in 100 µL or 250 µL of water, for the cCRS and BRP3 respectively, and before desalting, 150 µL of the BRP3 solution were taken out for use in the peptide mapping test. Desalting, preconditioning, rinsing and electrophoresis were then performed with 100 µL each of cCRS and BRP3 as prescribed in the monograph. Participants were instructed to carry out triplicate injections. A summary of the instrumentation and methodological details used in each laboratory is given in Table 2. The remainder of the desalted samples was used for the PAGE test (section 4.3.2).