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Report on a Collaborative Study for Proposed 1st WHO International Standard for Ranibizumab

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NOTE:
This document has been prepared for the purpose of inviting comments and suggestions on the proposal(s) contained therein, Written comments on the proposal(s) MUST be received in English by **19 September 2025** and should be addressed to:

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Comments may also be submitted electronically to **Dr Ivana Knezevic** at email: knezevici@who.int.

The distribution of this document is intended to provide information to a broad audience of potential stakeholders and to improve the transparency of the consultation process. Following consideration of all comments received, the proposal(s) will then be considered by the WHO Expert Committee on Biological Standardization (ECBS) prior to a final decision being made and published in the WHO Technical Report Series.

Summary

The present multi-centre collaborative study was aimed at developing and assessing the suitability of a candidate ranibizumab preparation to serve as the WHO International Standard for in-vitro biological activities of ranibizumab. For this, a candidate ranibizumab preparation and a comparator preparation were formulated, filled into ampoules and lyophilized at MHRA. Both preparations and a coded duplicate were evaluated for their bioactivity in diverse cell-based VEGF neutralization bioassays and binding assays and compared with participant's in-house reference standards where available.

Eighteen participants from twelve countries contributed study data. Study results showed that all ranibizumab preparations elicited VEGF binding and VEGF neutralizing effect consistent with the mechanism of action of the molecule. Importantly, the use of the candidate standard (coded 23/124) when compared with the in-house reference standards where available improved variability in potency estimates and allowed for a close agreement between laboratories for each of the bioactivities tested.

The study results and the limited data on stability available at present confirm that the preparation coded 23/124 is suitable and sufficiently stable to serve as the 1st International Standard for Ranibizumab for the bioactivities attributed to this antibody. Therefore, it is proposed to the ECBS that the candidate standard (coded 23/124) be established as the 1st WHO International Standard for Ranibizumab with assigned values per ampoule of 1,000 International Units (IU) of VEGF165 neutralizing activity and 1,000 IU of VEGF165 binding activity.

The International Standard, with its defined IU of bioactivity, is intended for controlling bioassay performance and for calibration of in-vitro bioassays. Given its role in calibration of secondary standards (manufacturers, regional), the ranibizumab IS will facilitate global

harmonization and consistency in potency assessment of different ranibizumab products, ensuring they are safe and effective. Importantly, users should note that the International Standard cannot be used as the reference product for biosimilarity determination. Furthermore, the International Standard is not intended for defining the product's specific activity or to revise the product labelling or for changing the therapeutic dose.

Responses from study participants

All study participants were requested to review the draft report carefully and comment on the proposal relating to the establishment of the proposed candidate 23/124 as the 1st International Standard for Ranibizumab for in vitro biological activities assessed in this study and the independent assignment of 1000 IU for each of the different bioactivities attributed to this antibody namely VEGF165 neutralisation and VEGF165 binding.

All eighteen participants commented on the draft report.

Response forms relating to the establishment of the proposed candidate 23/124 as the 1st International Standard for Ranibizumab were received from seventeen participants (94%). All agreed unanimously on the establishment of the proposed candidate 23/124 as the 1st International Standard for Ranibizumab with agreement from thirteen responders on the proposed unitage for VEGF165 neutralisation and VEGF165 binding activity. Four agreed solely with the proposed unitage for VEGF165 neutralisation activity (since these participants only performed neutralization assays).

Participants were requested to check their assay details and comment on the correctness of their study data reported. Sixteen participants confirmed on the accuracy of the report in terms of assay details and their data. One participant was unable to confirm on the calculated results due to differences in methods of analysis between their laboratory and MHRA, however, despite these differences, the participant confirmed their agreement with the outcome of the final report. Another participant requested re-examination of their laboratory data - the laboratory mislabelled the identity of some samples in the plate layout while reporting data for one of the assays which unfortunately was overlooked during statistical analysis. In view of this issue, all data for this single laboratory was rechecked which revealed sample mislabelling in a plate of another assay. Statistical analysis was repeated for data from this laboratory and the issue resolved. However, re-analysis resulted in slightly revised potency estimates for the individual laboratory and also for the overall participants' data. All revised data are reported in the final report.

One participant provided details of the reagents (previously missing) used in the binding assay and this was updated as necessary in the report. Finally, minor comments relating to typographical errors, names and address details in the participant listing for different laboratories were also received. All minor corrections were rectified in the final report.

Introduction

Over two decades ago, the recognition that vascular endothelial growth factor (VEGF), in particular VEGF-A, is overexpressed in cancers and eye neovascular disorders and predominantly drives pathological angiogenesis as well as vascular leakage in these diseases prompted intense development of molecules targeting VEGF (1-7). Since then, several molecules targeting VEGF/VEGF receptor 2 have been approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA). These include monoclonal antibodies, namely Bevacizumab (Avastin®, Genentech/Roche), Ranibizumab (Lucentis®, Genentech/Novartis), Brolucizumab (Beovu®, Novartis), Ramucirumab (Cyramza®, Eli Lilly) and the Fc-fusion protein Aflibercept (Eylea®/ Zaltrap®, Regeneron) (Table 1A). The first product to be approved was Avastin® followed a couple of years later by Lucentis® and then the others (8-14). These medicines have transformed management paradigms for treatment of certain cancers and ocular disorders and in some cases even become the accepted standard of care (8-19). Avastin® achieved global annual sales exceeding 7 billion US dollars in 2019 (20). Such high sales combined with expiry of patent protection stimulated fierce biosimilar development in the anti-VEGF space. Today several anti-VEGF biosimilars are approved with a view to improving patient access to treatment (Table 1B). Nevertheless, development of novel molecules continues as shown by the recent approval of Faricimab (Vabsymo®, Roche), a bispecific antibody targeting VEGF and Angiopoietin for treatment of age-related macular degeneration and diabetic retinopathy (21) (Table 1A)

Ranibizumab (Lucentis®- originator product) is the Fab moiety derived from bevacizumab (a recombinant humanised monoclonal antibody directed against human VEGF-A) and was generated by site directed mutagenesis of a human IgG1 framework (95%) with murine complementarity-determining regions (5%). It has a higher affinity for VEGF-A compared to the parental antibody (5, 11, 19, 22, 23). Produced in Escherichia coli (E.coli), the fragment consists of a 214-residue light-chain linked by a disulphide bond at its C-terminus extremity to the 231-residue N-terminal segment of the heavy chain (11). It has an approximate molecular weight of 48 kDa (23 kDa and 25 kDa for the light and heavy chain, respectively) and does not possess any post-translational modifications (11). Ranibizumab binds with high affinity to VEGF-A isoforms generated by alternative mRNA splicing, e.g. VEGF121, VEGF165, and their biologically active proteolytic cleavage product VEGF110, however, the VEGF 165 isoform is the main determinant of the mechanism of action of ranibizumab. Binding of ranibizumab to VEGF165 inhibits the interaction of VEGF165 with its receptors (Flt-1 or VEGFR1 and kinase insert domain receptor, KDR or VEGFR2), on the surface of endothelial cells and prevents VEGF165 signalling resulting in inhibition of functional responses such as proliferation, migration and angiogenesis.

Approved initially for neovascular age-related macular degeneration (nAMD or wet AMD) in USA (2006) and Europe (2007), ranibizumab is currently indicated in Europe for adults with proliferative diabetic retinopathy, treatment of visual impairment due to macular oedema caused by diabetes or retinal vein occlusion or sight problems associated with choroidal neovascularisation. It is also used in preterm infants to treat retinopathy of prematurity but is only used for specific disease stages and for aggressive posterior retinopathy disease (11).

Patent expiry for Lucentis® (US-June'22; Europe-July'22) along with the unmet medical need arising from the high cost of the mAb has led to a surge of approvals for many biosimilar ranibizumab products worldwide (same molecules marketed under different brand names in

different regions) with others currently in different phases of development (24-31). In some regions, there is possibly also a prevalence of approved products (copy versions) that do not conform with the widely accepted biosimilar principles effective in highly regulated countries or those defined in the revised WHO guidelines for biosimilars (32). Given the expansion in the number of ranibizumab products and the changes (including site transfers, scale-up etc) that frequently occur post-approval, the risk of a shift in bioactivity and product divergence with potential clinical impact remains (33-35). Therefore, the availability of an international standard (IS) with defined international units of bioactivity will facilitate the calibration of secondary (pharmacopeial, regional) and manufacturers' in-house bioactivity reference standards and harmonize bioactivity across different products. Furthermore, the IS can help to understand and manage any drift in bioactivity among marketed products as they evolve post-authorization. This is critical in view of the product interchangeability between the originator and a biosimilar product but also between biosimilar products allowed in certain jurisdictions (36,37). Therefore, in recognition of the need to ensure patient access to safe and effective products (World Health Assembly resolution, WHA 67.14, 2014) and the expansion of the WHO standardization programme for monoclonal antibodies (38), work was initiated on the ranibizumab IS, the 2nd IS in the anti-VEGF product class.

Herein, we have developed a candidate ranibizumab IS and assessed its suitability to serve as a 'reference standard' for bioactivity determination in a multi-centre international collaborative study using a range of methods in current use. This report describes the strategy for IS development, the results of the studies as well as data on the stability of the proposed IS and the units assigned. It is anticipated that the IS, when established, will serve as a primary standard and calibrant for qualifying in-house reference standards for bioassays and aid global harmonization where possible. In addition, it will assist in the evaluation of bioactivity of different products throughout their life cycle.

Based on its categorization as a VEGF antagonist, this project was endorsed by the WHO Expert Committee on Biological Standardization in October 2016.

Aims and Study Design

The purpose of this study was to characterize a candidate standard for its suitability as the 1st WHO IS in cell-based bioassays and binding assays for ranibizumab and assign a unitage for activity. The study sought

- To evaluate and assess the suitability of a candidate lyophilised ranibizumab preparation to serve as the 1st IS for ranibizumab bioassays by assaying its biological activity in a range of routine, 'in-house' bioassays,
- To assess the relative activity of the ranibizumab preparations in assays in current use (e.g. bioassays, binding assays) and determine, where possible, the concentrations of ranibizumab required to neutralise specific amounts of VEGF IS.
- To compare the candidate with characterised 'in-house' laboratory standards where these are available.

The study also included an additional lyophilised preparation containing a 20% lower amount of ranibizumab in comparison with the candidate preparation to assess the ability of the assays to detect differences in activity.

Participants

Nineteen participants from twelve different countries (from 4 WHO regions) were dispatched study samples. Participants included four biotech manufacturers, two contract research organizations (CRO), eight control laboratories, two pharmacopoeias and three commercial suppliers (cell-lines/reagents). Eighteen participants returned data which contributed to the study (Table 2). Participating laboratories have been assigned code numbers allocated at random, and not representing the order of listing in Table 2 to retain confidentiality in the report.

Materials and Processing

A preparation of recombinant ranibizumab from a single batch of bulk drug substance was kindly donated to WHO (see Acknowledgement). A commercial batch of Lucentis® (innovator product, Novartis) was purchased through a supplier to serve as a comparator.

Initially, a trial fill using the bulk drug substance at a predicted protein content of 50 μg/ml was conducted using a formulation buffer containing 25 mM sodium citrate tribasic dihydrate, 150 mM sodium chloride, 1% Human serum albumin, pH 6.5. For testing, the biological activity of the lyophilized preparation was compared with the bulk material and the frozen baseline using the conventional primary human umbilical vein endothelial cells (HUVECs) based assay in which the mAb fragment blocks VEGF165 stimulated cell proliferation (2, 6). The formulation was found to be suitable (data not shown) as seen previously with ISs for other mAbs (e.g., bevacizumab, an anti-VEGF mAb; anti-TNF mAbs) and so it was selected for the final lyophilization of the candidate preparation.

Table 3 provides information on the candidate preparation, the protein content, the number of ampoules and the study code. For the candidate preparation (23/124, study codes A & C), lyophilized at MHRA using ECBS guidelines (39), a solution of ranibizumab at a theoretical protein concentration predicted to be 50 μg/ml was distributed in 1 ml aliquots into 5 ml ampoules. The approximate mass content of the protein in the ampoules, given as 'predicted μg' in Table 3 has been calculated from the dilution of the bulk material of known protein mass content as provided by the manufacturer.

In addition to the candidate fill, two small scale fills, both containing the same excipients and dispensed in 1 ml volumes as for the candidate preparation were also performed at the same facility. These included i) the comparator preparation (SS939, study code D) using the commercial product, Lucentis® and ii) an additional preparation (SS939, study code B) using the same bulk drug substance as the candidate standard. The former contained a similar protein content (50 μg/ml predicted) as the candidate standard while the latter had a slightly lower mAb content (40 μg/ml predicted).

For all fills, buffers and excipients (final compositions as shown in Table 3), were prepared using nonpyrogenic water and depyrogenated glassware and solutions filtered using sterile nonpyrogenic filters (0.22μM Stericup filter system, Millipore, USA) where appropriate. All preparations were lyophilised under optimised and controlled conditions as per WHO

recommendations (39), the glass ampoules sealed under dry nitrogen by heat fusion and stored at -20°C in the dark until shipment at ambient temperature.

Characterisation of the lyophilised preparations

For each fill, a percentage of ampoules were weighed, residual moisture of each preparation was measured by the coulometric Karl-Fischer method (Mitsubishi CA100) and the headspace oxygen content was determined by frequency modulated spectroscopy using the Lighthouse FMS-760 Instrument (Lighthouse Instruments, LLC). The mean fill weights, moisture content and headspace oxygen content, which is a measure of ampoule integrity, are reported in Table 3. Testing for microbial contamination using the total viable count method did not show any evidence of microbial contamination.

Study Design

Participating laboratories were provided with a sample pack of 5 ampoules each of the study samples A-D, for each assay type being undertaken. In addition, participants performing VEGF165 neutralization assays were provided with 5 ampoules of the 1st VEGF IS (coded 19/246) to serve as a common source for the bioassays to reduce assay variability arising from the use of human VEGF165 from different suppliers.

Prior to performing assays for the study, participants were advised to perform a pilot assay using the study samples for each of the assay types they intended to undertake to ensure appropriate assay conditions and optimal dose response curves would be generated. For neutralization bioassays, participants were also advised to select a suitable dose of the 1st VEGF IS (coded 19/246).

After establishing suitable conditions, participants were asked to assay all samples concurrently on a minimum of three separate occasions using their routine methods, within a layout which allocated the samples across 3 plates or an alternative layout which used 4 plates and allowed testing of replicates as per the study protocol (Appendix). It was requested that participants assess all test samples, A-D and their in-house (IH) standard where available in duplicate on each plate and perform at least 8 dilutions for each sample using freshly reconstituted ampoules for each assay. If using the alternative layout (since inclusion of all samples on each plate was not possible), participants were asked to test samples A, C, D on 3 plates and A-C on an additional plate along with IH standard on each plate. Participants were requested to return their raw assay data, using the spreadsheet templates provided, and their own calculations of potency of the study samples relative to preparation A and/or their own in-house standard.

For binding assays, participants were requested to use their proprietary assay kits or in-house assays to assess the binding of serially diluted samples and their in-house standard to human VEGF165 (sourced by participant since the VEGF IS contains 0.5% human serum albumin as an excipient and is unsuitable for use in such assays). As for cell-based assays, participants were requested to perform three independent assays on three separate occasions and return raw data in a format appropriate for the assay technique used.

Statistical analysis

An independent statistical analysis of all bioassay data was performed at MHRA. Analysis of dose-response curve data was performed using a four-parameter logistic (sigmoid curve) model

$$y = \alpha - \frac{\delta}{1 + 10^{\beta(\log_{10}x - \log_{10}\gamma)}}$$

where y denotes the assay response, x is the concentration, α is the upper asymptote, δ is the difference between upper and lower asymptotes, β is the slope factor and γ is the EC_{50} (50% effective concentration). Assay responses were log transformed for all assay methods. Models were fitted using the R package 'drc' (40, 41). Parallelism (similarity) for a pair of dose-response curves was concluded by demonstrating equivalence of the parameters α, β and δ. Equivalence bound values and the methods for determining them are described in the Results section of this report. Laboratory 4 submitted data that did not meet upper and lower bounds for samples A, B, C and D and this method could not be used. In this case, a parallel line model fitting log response to log-dose for this linear section of the assay response range was used instead (42).

Where satisfactory parallelism was concluded for a sample, the model was fitted to both the sample and the standard, with common values of α, β and δ to directly estimate its relative potency. All relative potency estimates were combined to generate an unweighted geometric mean (GM) potency for each laboratory and these laboratory means were used to calculate overall unweighted geometric mean potencies. Variability between assays and laboratories has been expressed using geometric coefficients of variation (GCV = $\{10^s\text{-}1\} \times 100\%$ where s is the standard deviation of the $\log_{10}$ transformed potencies).

Stability studies

All stability studies were undertaken at MHRA. Accelerated degradation studies were performed to predict the long-term stability of the candidate standard. Ampoules of the lyophilised preparation were stored at different temperatures, namely 45°C, 37°C, 20°C and 4°C and tested at indicated time points in a reporter gene assay (RGA) together with ampoules stored at the recommended temperature of -20°C and -70°C as baseline reference temperature. Where possible, relative bioactivities of the accelerated thermal degradation samples were used to fit an Arrhenius equation relating degradation rate to absolute temperature assuming first-order decay (43) and hence predict the degradation rate when stored at -20 °C.

For stability assessment after reconstitution, samples of the candidate standard 23/124 were reconstituted and left at 4°C or room temperature for either 1 day or 1 week. The reconstitutions were timed to allow all samples to be assayed concurrently against a freshly reconstituted ampoule. For freeze-thaw assays, samples of the candidate standard 23/124 were reconstituted, aliquoted and subjected to a series of freeze-thaw cycles (up to 4 cycles) at -30°C. They were then assayed concurrently with a freshly reconstituted ampoule. For both conditions, the RGA was used and the assay carried out twice, with three plates within each assay.

Results

Data Returned for Analysis

Results were received from 18 laboratories. All participants provided raw data from the assays so a global analysis of assay validity could be applied to allow data to be treated equally and to allow comparison of data among different laboratories.

Assay Methods

A summary of the assay methods used in the study is shown in Tables 4 and 5. Seventeen laboratories performed VEGF neutralization assays and five laboratories performed VEGF binding assays. All study participants used their own qualified bioassays with their own assay acceptance criteria, and IH reference standards where available. Four laboratories used proprietary IH reference standards representative of their therapeutic product (innovator Lucentis®/biosimilar) while six participants used either the innovator or an approved biosimilar product as the IH standard. One laboratory used a research grade Bevacizumab. Seven laboratories did not use any IH reference standard in their assays (Tables 4, 5).

Three different types of bioassays reflecting the mechanism of action of ranibizumab were used (2, 6). Besides the conventional assay using primary HUVECs in which the mAb blocks VEGF165 stimulated endothelial cell (EC) proliferation, reporter gene assays (RGAs) using a stable human embryonic kidney (HEK293) cell line transfected with KDR (VEGFR2) and the VEGF165 responsive element nuclear factor of activated T cells (NFAT) upstream of a luciferase reporter gene were employed (44, 45). The latter mostly employed commercially available thaw and ready-to-use cells. An additional approach was the enzyme-fragment complementation (EFC) assay using the HEK293 cell line transfected with KDR and two β-galactosidase fragments which through dimerization of VEGFR2 leads to EFC-mediated β-galactosidase activation (46, 47). Table 4 shows that a similar number of participants performed the reporter gene and HUVEC assays (n=8 in both cases) in comparison with the EFC (n=2) assays. One participant used both RGA and HUVEC assays for testing the bioactivity.

Binding assays were performed by five participating laboratories (Table 5). Four participants employed the direct enzyme-linked immunosorbent assay (ELISA) format in which the mAb was captured using VEGF165 immobilised onto the assay plate and detected using horseradish peroxidase (HRP)-conjugated antibody. One participant assessed binding by surface plasmon resonance (SPR) using the Biacore 8000 instrument (Cytiva) but since no other laboratory used SPR, no analysis of data was undertaken.

Assay Validity

Equivalence bounds for each model parameter (α, β and δ) were determined separately for neutr alization (HUVEC/RGA & EFC) assays and binding assays. For the purposes of the analysis presented in this report, these bounds were set using data returned for coded duplicate samples A and C from all laboratories. As the model parameters are expected to be equivalent when testing the same sample against itself, absolute differences in $\log_{10}\alpha$, $\log_{10}\beta$ and $\log_{10}\delta$ parameters for samples A and C were calculated for each plate and upper equivalence bounds set as the 90^{th} percentile of these values, using values obtained across the study. Data from

laboratory 4 were excluded from this calculation since the doses tested did not achieve upper and lower response limits, while data from laboratories 6 and 13 were excluded due to high intra-assay residual error. All 3 laboratories performed HUVEC assays.

For HUVEC assays, the calculations gave upper bounds of 0.037, 0.217 and 0.111 for the absolute difference in $\log_{10}\alpha$, $\log_{10}\beta$ and $\log_{10}\delta$ parameters respectively. The upper bound for $\log_{10}\beta$ corresponded to a slope factor ratio of 1.647. For RGA and EFC assays, upper bound values were 0.049, 0.075 and 0.051 for the absolute difference in $\log_{10}\alpha$, $\log_{10}\beta$ and $\log_{10}\delta$ parameters respectively. The upper bound for $\log_{10}\beta$ corresponded to a slope factor ratio of 1.190. For binding assays, upper bound values were 0.025, 0.046 and 0.028 for the absolute difference in $\log_{10}\alpha$, $\log_{10}\beta$ and $\log_{10}\delta$ parameters respectively with the upper bound for $\log_{10}\beta$ corresponding to a slope factor ratio of 1.111. For two dose-response curves to be concluded as parallel, equivalence had to be demonstrated for all three parameters (α, β and δ). It should be noted that the equivalence bounds were intended for use in the analysis of data from this study only, in order to apply consistent criteria to all laboratories and assess their relative performance. For laboratory 4, a threshold of 0.95 for the R^2 value was used to confirm acceptable linearity of the samples and the slope factor ratio bound calculated for other HUVEC assays was used to assess parallelism. Further to this, an equivalence bound set using the 90% percentile of the relative potency estimates of the coded duplicate sample C relative to A was calculated, resulting in limits of 0.817 to 1.223, and any plate with an estimate outside of these values was excluded from further summary calculations. The bounds should not be interpreted as suitable values for routine use in the assessment of assay validity and may be overly stringent or lenient in the case of some laboratories.

Appendix 1 Table 1 shows the percentage of analysis results across plates per laboratory not meeting the defined equivalence criteria and Appendix 1 Table 2 indicates the percentage of invalid cases per laboratory. No invalid cases relative to Sample A were noted in a single laboratory (1/21 cases) and in nine others, total invalidity rates were ≤25%. Four laboratories had total invalidity rates of >50%.

Potency Estimates Relative to Sample A or In-House Reference Standards

Geometric mean potency estimates calculated relative to candidate standard sample A or relative to in-house reference standards where available are summarised for each laboratory performing neutralisation assays in Table 6A, and binding assays in Table 6B. Individual assay potency estimates relative to A are shown in Appendix 1 Table 3. Overall summary of relative potency estimates for each assay type relative to A and to in-house reference excluding laboratory 10 (due to the difference in reference used) are shown in Table 7 and in Appendix 1 Table 4. Boxplots of individual laboratory potency estimates are shown in Figure 1.

Intra-laboratory GCV values for the potencies of samples B, C and D relative to A ranged from 1% to 61% in neutralisation assays, with a median value of 9%. For neutralization assays, when the in-house standards were used, GCV values ranged from 3% to 30 % with a median value of 7%. As expected, higher variability was evident with the HUVEC assays regardless of the standard used with GCV values $\geq$ 20% in 5 of the 8 labs in comparison with the RGA and EFC assays where this was noted in only 1 of the 8 labs performing the RGA and none of the 2 labs with EFC assays.

Inter-laboratory GCV values for samples B, C and D relative to A in neutralisation assays were 9%, 3% and 7% respectively and slightly lower than when in-house standards were used (12%, 11% and 10% respectively), although there was limited neutralisation assay data contributed for comparison with in-house standards (n=8 to 10) as opposed to A (n=17). RGA assays showed low GCV values for B, C and D relative to A of 3%, 4% and 5% (n=7) respectively and slightly higher GCVs of 14%, 14% and 8% respectively relative to in-house standards (n=5). HUVEC assays showed GCV values for B, C and D relative to A of 11%, 2% and 8% (n=8) respectively and values relative to in-house standards of 6%, 8% and 11% respectively (n=3 to 5).

For binding assays (ELISAs, n=4), intra-laboratory GCV values ranged from 4% to 20% (median 6%) relative to sample A and 2% to 6% (median 5%) in comparison with in-house standards. A similar level of inter-laboratory variability as for neutralisation assays relative to sample A was evident, with GCV values of 9%, 3% and 6% for samples B, C and D respectively. There were only two laboratory GM values for analysis when in-house standards were used and thus no GCV values were calculated. Overall, the levels of variability in cell-based neutralisation assays were comparable with those seen in binding assays regardless of the standard used.

The study data show that the use of sample A as a reference standard to calculate the relative potency of samples B, C or D allows a close agreement between laboratories for each of the bioactivities tested in comparison with in-house standards.

Potency estimates of B relative to sample A

Seventeen laboratories tested sample B in the neutralisation assays. The overall GM potency of sample B relative to sample A was 0.86 with a GCV of 9% and 95% confidence limits of 0.82 to 0.90 (Table 7). The laboratory GM potency values ranged from 0.75 to 1.05. Four laboratories tested sample B in binding assays, with an overall GM potency relative to sample A of 0.85, a GCV of 9% and 95% confidence limits of 0.75 to 0.97 (Table 7). The laboratory GM potency values ranged from 0.78 to 0.95 (Table 6B). As noted above, potency estimates and GCV for sample B were comparable across cell-based neutralisation assays as well as binding assays and consistent with the lower amount of the mAb present in this sample.

Potency estimates of D relative to sample A

Seventeen laboratories tested sample D containing the originator mAb in the neutralisation assays. The overall GM potency of sample D relative to sample A was 0.93 with a GCV of 7% and 95% confidence limits of 0.90 to 0.96 (Table 7). The laboratory GM potency values ranged from 0.79 to 1.01 (Table 6A). Four laboratories tested sample D in binding assays, with an overall GM potency relative to sample A of 0.93, a GCV of 6% and 95% confidence limits of 0.85 to 1.02 (Table 7). The laboratory GM potency values in these assays ranged from 0.88 to 0.99 (Table 6B).

Estimates of EC_{50} Derived from Neutralization Assays

Laboratory geometric mean EC_{50} estimates based on the assumed content of 50 µg for the samples together with the VEGF concentration used by the laboratories in their neutralization

assays are shown in Table 8. There was a correlation between the EC50 value and the concentration of VEGF used by participants as shown in Figure 3 (R=0.872 for HUVEC and R=0.976 for RGA). The intercepts for linear regression line in HUVEC assays were also different from RGA/EFC assays, suggesting different dilutions of the candidate standard would be needed to neutralize a fixed amount of VEGF depending on the assay type (HUVEC or RGA/EFC). A summary of EC_{50} estimates for HUVEC assays using a fixed VEGF concentration of 22.5 IU (WHO VEGF IS, 19/246) is shown in Table 9.

Stability Studies

Accelerated Temperature Degradation Assays

Samples of the candidate standard (23/124) stored at elevated temperatures (4°C, 20°C, 37°C and 45°C) were assayed at MHRA concurrently with those stored at the recommended temperature of -20°C, and the baseline reference temperature of -70°C using the RGA. Data from ampoules stored up to 708 days indicate no significant loss in candidate bioactivity. The potencies of all samples were expressed relative to the appropriate -70°C baseline samples and the results are summarised in Table 10. Since no loss in activity was evident following storage at any of the elevated temperatures, no predicted loss in activity can be calculated at the present time.

Stability after Reconstitution and on Freeze-Thaw

Reconstituted ampoules of the candidate standard 23/124 were stored at 4°C or 22°C (room temperature) for either 1 day or 1 week and assayed concurrently against a freshly reconstituted ampoule in the RGA method. Data indicate that the potency of the reconstituted candidate standard is not diminished after a week of storage at either of the temperatures. The potencies of all samples were expressed relative to the freshly reconstituted samples and the results are summarised in Table 11.

For freeze-thaw assays, samples of the candidate standard 23/124 were reconstituted, aliquoted and subjected to a series of freeze-thaw cycles (up to 4 cycles) at -30°C. They were then assayed concurrently with a freshly reconstituted ampoule using the RGA. The assay was carried out twice, with three plates within each assay. Results indicate that the potency of the candidate standard is retained with no decrease in activity despite repeated freeze-thaw cycles (up to 4 cycles). The potencies of all samples were expressed relative to the freshly reconstituted samples and the results are summarised in Table 12.

Discussion

The availability of several structurally different anti-VEGF biotherapeutics in recent years is gradually transforming clinical practice and management of eye diseases in an increasingly ageing, obese and diabetic population worldwide (6, 10-14, 18, 21, 22). This is complemented by the recent upsurge in approvals of ranibizumab biosimilars (23-30) which provide an opportunity for selection of cost-effective options for increased patient access across different ophthalmic indications (30).

Ranibizumab elicits its clinical effects by binding with high affinity to VEGF isoforms via its Fab region to prevent the interaction of VEGF with its receptors, VEGFR1 and KDR (VEGFR2), located on the endothelial cell surface and their consequent activation. This blockade interrupts the downstream signaling process required for EC proliferation and migration which leads to angiogenesis (2, 4, 5, 6). Such modulation of VEGF bioactivity reduces the pathological angiogenesis and vascular hyper-permeability associated with aberrant VEGF expression and visual impairment in intraocular diseases e.g., neovascular age-related macular degeneration and diabetic macular edema (11). Since ranibizumab lacks the Fc region, it does not exert any Fc-mediated effector functions.

Consistent with the antibody's mechanism of action, a range of bioassays representative of the functional activity of this molecule contributed to the collaborative study. Among these is the conventional bioassay employing primary human endothelial cells (ECs) or HUVECs, a well-established and commonly used cellular model for investigating VEGF endothelial functions (24,29, 48, 49) based on their expression of KDR (VEGFR2), the key receptor for VEGF signalling but also VEGFR1 and co-receptors, neuropilin-1 (NRP1) and NRP2 which interact with VEGF165 (2, 6). Binding of VEGF165 to KDR causes receptor dimerization and phosphorylation which, in turn, induces a cascade of events and ultimately cell proliferation. As a result, HUVEC assays have been in use in laboratories to assess the bioactivity of VEGF and its inhibitors. In the latter case, the HUVEC assay measures the inhibitor's dose-dependent neutralising activity in response to VEGF induced cell stimulation. This assay type is used by some manufacturers for batch release testing. However, considering the limitations of such assays - the need for primary ECs (low passage number), the high degree of variability associated with use of primary ECs and the assay duration (4-5 days), modern approaches using early endpoints are increasingly being applied in some laboratories. Typical examples are assays based on reporter gene construct (RGA) and enzyme-fragment complementation (EFC) technology - both are early endpoint assays which allow rapid readout (6-24 hours), have low variability and are easy to perform (24, 30, 44-47). The RGA employs the KDR transfected HEK293 (the stable embryonic human kidney cell-line) cells engineered to express the VEGF165 responsive element nuclear factor of activated T cells (NFAT) upstream of a luciferase reporter. Binding of VEGF to KDR transduces intracellular signalling and results in NFAT-RE-mediated luminescence, while in the EFC assay, the engineered KDR transfected HEK293 cells express two β-galactosidase fragments which undergo complementation to form the functional enzyme which hydrolyses the substrate to generate a chemiluminescent signal after VEGF binding. Importantly, both assays recapitulate the mechanism of action of ranibizumab which makes them suitable options for bioactivity determination (24, 44-47). For ease of use, ready-to-use cell-lines (available from commercial suppliers e.g., Promega, Eurofins, DiscoverX etc) for both assay systems have been used here. However, the RGA appears to be the preferred choice of several laboratories (including manufacturers and contract research organisations). Indeed, this is borne out also with this study data showing a similar number of laboratories performing RGA and HUVEC assays and a higher variability in HUVEC assays (n=8) as opposed to RGA (n=8) and EFC (n=2) assays.

Geometric mean estimates for the different samples tested were similar between HUVEC assays and RGA/EFC assays relative to candidate standard sample A. Importantly, the overall inter-laboratory GCV values for the samples B, C and D relative to candidate standard sample A in neutralisation assays were low at 9%, 3% and 7% respectively, as opposed to 12%, 11% and 10% relative to suitable in-house reference standards. In HUVEC assays, the GCV was

11%, 2% and 8% relative to A and 6%, 8% and 11% relative to in-house reference standards - all used ranibizumab. However, GCV increased to 150%, 8% and 11% when 1 lab used the whole mAb bevacizumab as the in-house reference standard instead of the ranibizumab. For RGA assays, values were 3%, 4% and 5% relative to A and 14%, 14% and 8% when in-house reference standards were used. For binding assays, a similar level of inter-laboratory variability relative to sample A was seen with GCV values of 9%, 3% and 6% for samples B, C and D (n=4). GCV values were not calculated when in-house standards were used due to limited data (n=2). The study data indicated that use of the common standard sample A compared with in-house reference standards allowed for a close agreement between laboratories for each of the bioactivities tested.

To assess the capability of assays to detect differences, sample B containing an expected 20% lower protein content than sample A was included. Overall using valid neutralisation assays (n=17), the laboratory GM potency relative to sample A was 0.86. Similarly, the overall binding activity of sample B was 15% lower than A with GM relative potency of 0.85. Interestingly, the neutralising activities of sample B relative to sample A in HUVEC assays and RGA assays revealed GM relative potency values of 0.86 and 0.84, respectively, indicating potential superiority of RGA assays over HUVEC assays for detecting differences. All assays detected lower activity resulting from reduced ranibizumab content in sample B.

In the case of Sample D (lyophilised from the comparator product), it was noted that regardless of the assay system, cell-based bioassays or binding assays, the overall GM potency estimates were 6% lower than sample C (coded duplicate of candidate standard) when sample A was used for calculation. The GM potency estimates for sample D were also lower than sample A or C when in-house reference standards were used. This small difference in relative potency between samples A and D may possibly be due to a slight difference in protein content estimates between the two samples.

To assess the inhibitory effect of Ranibizumab, EC_{50} estimates were derived for each laboratory performing VEGF neutralization assays. For the proposed IS, the inhibitory activity was determined for HUVEC assays by using the following equation:

Amount of Ranibizumab (IU) inhibiting a fixed amount of VEGF (IU) =

$$\frac{\text{potency of preparation (IU)} \times EC_{50}\text{ (ng)}}{\text{Assumed mass content (ng)}}$$

where EC_{50} values are derived from assays performed by selected laboratories using 22.5 IU of VEGF165 IS, an assumed mass content for the Ranibizumab candidate standard is 50,000 ng and the proposed arbitrary unitage of 1000 IU for the Ranibizumab candidate standard. In this case, an EC_{50} value of 25.397 was used (GM of EC_{50} of A, C combined) and based on this value, 0.508 IU of Ranibizumab inhibits 22.5IU of VEGF165.

Data from the present multi-centre collaborative study has demonstrated that the candidate standard sample A (coded 23/124) provided valid relative potency estimates for different samples across different assay types performed by participants. Potency estimates for the coded duplicate sample C showed no difference from that obtained with sample A. Use of sample A 23/124 as a common standard for calculating the relative potency of samples reduced the inter-

laboratory variability and enabled closer agreement in potencies between the participating laboratories.

At the present time, stability data for the candidate preparation A is limited to results obtained with storage up to 1.92 years (over 23 months). No discernible loss in potency was noted, a similar situation to that seen with ISs for other mAbs (e.g., Adalimumab stored for 15 months, Infliximab stored for 4 years 10 months) lyophilized using the same formulation and assessed after storage at elevated temperatures. However, studies on stability monitoring of 23/124 will continue over time to assess stability and any predicted loss in bioactivity. Further stability studies of the candidate preparation 23/124 undertaken post-reconstitution have shown that the potency is not reduced after 1 week of storage at either 4°C or room temperature or after repeated freeze-thaw (up to 4 cycles). However, it should be noted that this information does not provide a recommendation for local storage of ranibizumab, which should be internally validated.

Based on the study results and the limited stability data available at the current time, it can be concluded that candidate preparation coded 23/124 is suitable and sufficiently stable to serve as the 1st IS for Ranibizumab. Considering the two different activities of Ranibizumab studied here and the rationale for the replacement standard in future, it is best to assign independent arbitrary unitage values for the individual activities. It is therefore proposed that the candidate preparation coded 23/124 be assigned an arbitrary unitage of 1,000 IU per ampoule for VEGF165 neutralizing activity and 1,000 IU per ampoule for VEGF165 binding activity. The IS will help in controlling bioassay performance, support calibration of secondary or local standards and facilitate global harmonization and consistency in potency assessment of different Ranibizumab products. It should be noted that the IS is not intended for defining the specific activity or for use as the reference product for biosimilarity determination. Furthermore, it is not intended for revising the product labelling or changing the therapeutic dosage.

Conclusions and Proposal

Data from the multi-centre collaborative study demonstrated the suitability of the ranibizumab candidate preparation (23/124) in a range of in vitro bioactivity assays that were performed. Stability data also confirmed the suitability of the ranibizumab candidate preparation (code 23/124) to serve as the 1st WHO International Standard for the *in vitro* biological activities of ranibizumab.

It is therefore proposed to the WHO ECBS that the candidate preparation code **23/124** be accepted as the **WHO 1st IS for Ranibizumab** with assigned values per ampoule of 1000 IU for VEGF165 neutralising activity and 1000 IU for VEGF165 binding activity.

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Table 1A: Anti-VEGF/VEGFR2 biologics and their year of approval

Trade name	INN	Manufacturer	Product type	Target	US approval	EU approval	Indication
Avastin®	Bevacizumab	Genentech/Roche	Humanised antibody	VEGF	2004	2005	Oncology
Lucentis®	Ranibizumab	Genentech/Novartis	Humanised Fab fragment	VEGF	2006	2007	Ophthalmology
Eylea®/Zaltrap®	Aflibercept	Regeneron Pharmaceuticals	Fc-receptor fusion protein	VEGF	2011	2012	Ophthalmology
Cyramza®	Ramucirumab	Eli Lilly and Company	Fully human antibody	VEGFR2	2014	2014	Oncology
Beovu®	Brolucizumab	Novartis Pharm Corp	Single-chain antibody fragment	VEGF	2019	2020	Ophthalmology
Vabsymo®	Faricimab	Genentech/Roche	Bispecific antibody	VEGF + Angiopoietin	2022	2022	Ophthalmology

Table 1B: Ranibizumab biosimilar products (originator and biosimilar) currently approved in Europe, UK and USA

EU			UK			USA			
Trade name	**Approval Year**	**MAH**	**Trade name**	**Approval Year**	**MAH**	**Trade name**	**Approval Year**	**MAH**	**INN[1]**
Byooviz®	Aug'21	Samsung Bioepis	Byooviz®	Aug'21	Samsung Bioepis	Byooviz®	Sep'21	Samsung Bioepis/Biogen	Ranibizumab-nuna
Ranivisio ®[2]	Aug'22	BioeqAG / Polpharma Biologic	Ongavia®[1]	May'22	Midas Pharma (BioeqAG/ Teva)	Cimerli ®[1] (interchangeable)	Aug'22	Coherus Biosciences	Ranibizumab-eqrn
Ximluci ®	Nov'22	STADA / Xbrane	Ximluci ®	Jan'23	Genus Pharmaceuticals (STADA)	N/A			
Rimmyrah ®	Jan'24	Qilu Pharma	Rimmyrah ®	Feb'24	Qilu Pharma	N/A			
Epruvy/Ranibizumab Midas®	Sep'24*	Midas Pharma							

1 – In USA, the INN is based on the WHO INN with addition of a 4-letter suffix which is unique to a product. In the EU and UK, however, the WHO INN is adopted which in this case is Ranibizumab. 2- same product commercialised by different marketing authorisation holders (MAH).

Table 2: List of participants

Participants	Laboratory address	Country	Stakeholder
Shubrata Khedkar, Sujata Savant, Nagaraja HM and Dipankar Das	United States Pharmacopeia - India (P) Ltd, Plot D6 and D8, IKP Knowledge Park, Genome valley, Shameerpet, Turkapally village, Medchal, Telangana, Hyderabad, 500101	India	Pharmacopeia
Hirotaka Nishimura, Takuo Suzuki and Akiko Ishii-Watabe	National Institute of Health Sciences, Division of Biological Chemistry and Biologicals, 3-25-26, Tonomachi, Kawasaki-ku, Kawasaki, Kanagawa, 210-9501	Japan	Control/ Regulator
Joanna Guan and Christian Erickson	Bio-Techne, Bioassay, 614 McKinley Place NE, Minneapolis, MN55413	USA	Supplier
Gangling Xu, Feng Zhang and Lan Wang	National Institutes for Food and Drug Control (NIFDC), Division of Monoclonal Antibodies, No.31 Huatuo Road, Daxing District, Beijing, 102629	China	Control
Francesca Luciani, Federico Gonnella and Agnese D'Angiò	ISS, Biologicals and Biotechnologicals Unit, National Centre for the Control and Evaluation of Medicines (CNCF), Istituto Superiore di Sanità, Viale Regina Elena 299, Rome, 161	Italy	Control/ Regulator
Verena Sonnenberg and Ulrike Herbrand	Charles River, Max Planck Str.15a, Erkrath, 40669	Germany	CRO
Ruchi Singhal and Jane Lamerdin	Eurofins DiscoverX, 42501 Albrae Street, Fremont, CA94538	USA	Supplier
Chan-Wook Park, Kyumin Han and Yunjung Chae	Samsung Bioepis, 76 Songdogyoyuk-ro, Yeonsu-gu, Incheon, 21987	Republic of Korea	Pharma
Sylvie Jorajuria and Anna Niewiadomska	European Directorate for the Quality of Medicines & HealthCare (EDQM) Council of Europe, 7 Allee Kastner, CS 30026, Strasbourg, 67081	France	Pharmacopeia
Hyungok Chun and Jihwa Kim	National Institute of Food and Drug Safety Evaluation, Biologics Research Division, 187, Osongsaengmyeong 2-ro, Osong-eup, Heungdeok-gu, Cheongju-si, 28159	Republic of Korea	Control/ Regulator
Lingling Mu and Jinjin Wen	Jecho Biopharmaceuticals Co Ltd, No.2633 Zhongbin Road, Sino-Singapore Eco-City, Binhai New District, Tianjin, 300467	China	Pharma
Yongbao Sun, Hongyan Ye and Jiulin Wang	Qilu Pharmaceutical Co Ltd, No.8888 Lvyou Road, High-Tech Zone, Shandong Province, Jinan, 250100	China	Pharma
Jamison Grailer and Denise Garvin	Promega, Kornberg Center, Promega Corp, 5430 E Cheryl Parkway, Madison, WI 53711	USA	Supplier
John Hogwood and Haiyan Jia	Science and Research, MHRA, Blanche Lane, South Mimms, Potters Bar, Herts, EN6 3QG	UK	Control/ Regulator
Roger Tam and Lioudmila Tepliakova	Health Canada, BRDD, 251 Sir Frederik Banting Driveway, Ottawa, Ontario, K1A OK9	Canada	Control/ Regulator
Stefanie Siebert, Florent Beyriere and Joseph Fanous	Novartis, Pharma AG, Lichtstrasse 35, 4056, Basel.	Switzerland	Pharma
Scott Craig and Ann-Maree Catanzariti	Therapeutic Goods Administration, TGA Laboratories, 1 Tindal Lane, Canberra ACT, 2609	Australia	Control/ Regulator
Subhash Chand, Ajay Kumar Ade, Nidhi Solanki, Sabiha Aaysha, Charu M. Kamal and Harish Chander	National Institute of Biologicals, A-32, Sector-62, Noida, 201309	India	Control
Stuart Dunn and Yunhe Xu	Labcorp Early Development Laboratories, Otley Road, Harrogate, HG3 1PY	UK	CRO

Table 3: Fill details and characteristics of Ranibizumab preparations

Ampoule Code	Fill Date	Study Code	No of Ampoules in stock	Protein Content (μg)	Fill Weight		Residual Moisture		Headspace Oxygen	
					Mean g (n)	CV[1] %	Mean % (n)	CV[1] %	Mean % (n)	CV[1] %
23/124[2]	11/5/23	A, C	5,500	50	1.010 (222)	0.149	1.142 (12)	39.90	0.27 (12)	33.77
SS939	11/5/23	D	100	50	1.009 (3)	0.20	0.20 (3)	84.8	2.63 (3)	9.9
SS939[3]	11/5/23	B	100	40	1.010 (3)	0.10	0.22 (3)	46.8	2.30 (3)	22.4

Excipients used for lyophilization: 25mM Sodium citrate tribasic dihydrate, 150mM Sodium chloride pH 6.5 and 1% Human serum albumin

[1]CV: Coefficient of Variation; n : number of estimates;
[2]The candidate preparation was expressed in E. coli. The comparator (code D) is also E. coli expressed.
[3] This preparation was produced from the same bulk drug substance/material as used for 23/124 and was included for assessing the capability of the assays to detect differences.

All ampoules of the candidate preparation are intended for use as WHO International standard and will be stored at -20ºC at MHRA as the custodian laboratory.

Table 4: Summarised information on VEGF165 neutralisation assays contributed to the study

Lab code	Assay type	Cell line	Final VEGF 165 concentration (IU/ml)	IH standard	Assay length (hrs)	Assay readout	Readout reagent
1	Reporter Gene	KDR/NFAT-RE-luc2P HEK293[1]	50.4	I	6.5	Luminescence	Bio-Glo™ luciferase
2	Reporter Gene	KDR/NFAT-RE-luc2P HEK293[1]	4	None	6.5	Luminescence	Bio-Glo™ luciferase
3	Reporter Gene	KDR/NFAT-RE-luc2P HEK293[1]	22.5	None	6.5	Luminescence	Bio-Glo™ luciferase
7	Reporter Gene	KDR/NFAT-RE-luc2P HEK293[1]	22.5	B	6.5	Luminescence	Bio-Glo™ luciferase
9	Reporter Gene	KDR/ NFAT-RE-luc2P HEK293[2]	45	B[$]	7.0	Luminescence	Steady Glo™ luciferase
11a	Reporter Gene	KDR/NFAT-RE-luc2P HEK293[1]	22.5	B	6.0	Luminescence	Bio-Glo™ luciferase
12	Reporter Gene	KDR/NFAT-RE-luc2P HEK293[1]	22.5	None	6.0	Luminescence	Bio-Glo™ luciferase
17	Reporter Gene	NFAT-RE-luc2P HEK293[3]	13.5	B	6.0	Luminescence	Bio-Glo™ luciferase
4	Anti- proliferation	HUVEC[4]	40	I[$]	90-98	Fluorescence	AlamarBlue™
6	Anti- proliferation	HUVEC[5]	56.3	None	97.5-103.5	Fluorescence	AlamarBlue™
10	Anti- proliferation	HUVEC[5]	22.5	O	77-79	Fluorescence	Resazurin
11b	Anti- proliferation	HUVEC[6]	22.5	B	72	Fluorescence	AlamarBlue™

13	Anti- proliferation	HUVEC[7]	22.5	I	96 - 105	Fluorescence	AlamarBlue™
15	Anti- proliferation	HUVEC[8]	22.5	None	72	Absorbance	CellTiter 96® AQueous One
16	Anti- proliferation	HUVEC	18.0	B[$]	89	Absorbance	MTS
18	Anti- proliferation	HUVEC[9]	66.67	B[$]	65-70	Fluorescence	AlamarBlue™
5	Enzyme fragment complementation	HEK 293 KDR/KDR[10]	30.0	None	20	Luminescence	PathHunter® bioassay detection
14	Enzyme fragment complementation	HEK 293 KDR/KDR[10]	7.11	None	19-21	Luminescence	PathHunter® bioassay detection

IH: in-house standard used; B: biosimilar product; I: innovator product Lucentis (Novartis/Roche Inc); O: Other human VEGF antibody (research grade Bevacizumab biosimilar; BioTechne); Own manufactured product[$], Thaw and ready reporter cells[1]or cell-line[2] (subcultured in-house until use in assay) from Promega or engineered in-house[3] or sourced from DiscoverX[10] or Eurofins[10]; HUVEC sourced from various suppliers e.g., Thermofisher[4], Lonza[5], TCS[6], GIBCO[7], Cell applications[8] and Sciencell[9]

Table 5: Summarised information on binding assays contributed to the study

Lab code	Assay type	IH standard	Immobilization reagent	Detection reagent	Assay readout	Readout reagent
8	ELISA	None	VEGF165[11]	Anti human IgG (Fab specific) peroxidase labelled antibody	Absorbance	TMB substrate
9	ELISA	B[$]	VEGF165[11]	Anti human IgG (Fab specific) peroxidase labelled antibody	Absorbance	TMB substrate
15	ELISA	None	VEGF165[11]	Goat anti human kappa light chain-peroxidase labelled antibody	Absorbance	Ultra TMB substrate
18	ELISA	B[$]	VEGF165[12]	Chicken anti human IgG (Fab specific) peroxidase labelled antibody	Absorbance	TMB substrate
12	SPR	None	VEGF165[13, 14]	N/A	Response units	N/A

IH: in-house standard; B: biosimilar product; Own manufactured product[$]; VEGF sourced from Biotechne[11,] Sino[12] and Peprotech[13]; immobilized on a CM5 sensor chip[14]

Table 6A: Laboratory geometric mean relative potency estimates for neutralization assays

Method	Lab	Potencies relative to sample A									Potencies relative to in-house reference											
		Sample B			Sample C			Sample D			Sample A			Sample B			Sample C			Sample D		
		GM	GCV (%)	N	GM	GCV (%)	N	GM	GCV (%)	N	GM	GCV (%)	N	GM	GCV (%)	N	GM	GCV (%)	N	GM	GCV (%)	N
HUVEC	04	0.82	3	3	0.99	2	3	0.94	n/a	2	0.99	n/a	1	n/a	n/a	n/a	-	-	-	0.90	n/a	2
HUVEC	06	0.75	n/a	1	0.99	n/a	1	0.98	61	5	-	-	-	-	-	-	-	-	-	-	-	-
HUVEC	10	0.81	11	8	0.95	12	8	0.94	26	8	3.59	n/a	1	7.07	n/a	1	n/a	n/a	n/a	n/a	n/a	n/a
HUVEC	11	0.78	15	3	1.02	11	6	0.79	11	4	1.00	21	6	0.87	18	4	1.13	30	5	0.82	25	5
HUVEC	13	0.95	14	3	1.01	n/a	2	0.92	n/a	2	0.77	n/a	1	0.88	n/a	2	-	-	-	0.78	n/a	1
HUVEC	15	0.92	n/a	2	0.99	9	7	0.90	19	7	-	-	-	-	-	-	-	-	-	-	-	-
HUVEC	16	0.98	4	3	1.00	9	10	0.94	5	6	0.99	6	12	0.97	7	3	0.98	8	12	0.95	8	9
HUVEC	18	0.92	n/a	1	1.00	11	7	1.01	20	5	1.01	18	7	0.96	n/a	1	1.00	24	6	1.00	6	6
RGA	01	-	-	-	-	-	-	-	-	-	1.01	n/a	2	0.78	n/a	2	0.89	3	3	0.87	6	3
RGA	02	0.83	11	8	1.00	9	9	0.92	11	8	-	-	-	-	-	-	-	-	-	-	-	-
RGA	03	0.86	2	8	1.09	5	5	0.99	4	8	-	-	-	-	-	-	-	-	-	-	-	-
RGA	07	0.81	n/a	2	0.97	12	10	0.96	18	6	0.95	18	6	0.76	n/a	1	1.02	4	4	1.03	22	3
RGA	09	0.83	3	6	0.99	5	8	0.96	5	6	1.07	7	7	0.90	5	6	1.08	4	8	1.01	3	8
RGA	11	0.88	16	5	0.97	1	5	0.85	8	6	1.13	12	9	1.03	26	5	1.21	3	4	0.95	19	6
RGA	12	0.84	7	6	1.00	9	8	0.96	3	5	-	-	-	-	-	-	-	-	-	-	-	-
RGA	17	0.81	n/a	2	0.97	6	10	0.97	8	6	0.91	5	10	0.76	n/a	2	0.90	6	11	0.87	8	7
EFC	05	0.83	13	7	0.97	9	8	0.87	7	3	-	-	-	-	-	-	-	-	-	-	-	-
EFC	14	1.05	n/a	1	1.02	14	7	0.99	10	5	-	-	-	-	-	-	-	-	-	-	-	-

- indicates no data for analysis; GM: Geometric Mean; GCV: Intra-lab Geometric Coefficient of Variation (%), not calculated if N < 3; N: Number of valid estimates; n/a: not calculated or no valid estimates obtained.

Table 6B: Laboratory geometric mean relative potency estimates for binding assays

Lab	Potencies relative to sample A									Potencies relative to in-house reference (in mg/ml)											
	Sample B			Sample C			Sample D			Sample A			Sample B			Sample C			Sample D		
	GM	GCV (%)	N	GM	GCV (%)	N	GM	GCV (%)	N	GM	GCV (%)	N	GM	GCV (%)	N	GM	GCV (%)	N	GM	GCV (%)	N
08	0.86	17	4	0.99	8	6	0.90	20	6	-	-	-	-	-	-	-	-	-	-	-	-
09	0.83	6	9	1.00	5	8	0.96	5	9	0.92	4	9	0.77	6	9	0.92	5	8	0.89	4	9
15	0.78	14	7	0.95	8	8	0.88	8	8	-	-	-	-	-	-	-	-	-	-	-	-
18	0.95	7	3	1.02	4	12	0.99	5	9	0.96	6	12	0.87	2	3	0.98	5	11	0.96	4	9

- indicates no data for analysis; GM: Geometric Mean; GCV: Intra-lab Geometric Coefficient of Variation (%), not calculated if N < 3; N: Number of valid estimates; n/a: not calculated or no valid estimates obtained.

Table 7: Summary of relative potency estimates, calculated relative to Sample A or in-house reference standards

Method	Sample	Potencies relative to A					Potencies relative to in-house reference standards				
		GM	LCL	UCL	GCV(%)	N	GM	LCL	UCL	GCV(%)	N
Neutralization (all)	A						0.98	0.91	1.05	11	10
	B	0.86	0.82	0.90	9	17	0.87	0.80	0.95	12	9
	C	0.99	0.98	1.01	3	17	1.02	0.93	1.11	11	8
	D	0.93	0.90	0.96	7	17	0.92	0.86	0.98	10	10
Neutralization (HUVEC)	A	-	-	-	-	-	0.95	0.82	1.10	13	5
	B	0.86	0.79	0.94	11	8	0.92	0.84	1.00	6	4
	C	0.99	0.97	1.01	2	8	1.03	0.85	1.25	8	3
	D	0.92	0.87	0.99	8	8	0.89	0.78	1.01	11	5
Neutralization (RGA)	A	-	-	-	-	-	1.01	0.90	1.13	9	5
	B	0.84	0.81	0.86	3	7	0.84	0.71	0.99	14	5
	C	1.00	0.97	1.03	4	7	1.01	0.86	1.18	14	5
	D	0.94	0.90	0.99	5	7	0.95	0.86	1.05	8	5
Neutralization (RGA/EFC)	A	-	-	-	-	-	1.01	0.90	1.13	9	5
	B	0.86	0.81	0.91	8	9	0.84	0.71	0.99	14	5
	C	0.99	0.96	1.02	4	9	1.01	0.86	1.18	14	5
	D	0.94	0.90	0.98	6	9	0.95	0.86	1.05	8	5
Binding	A	-	-	-	-	-	0.94	0.75	1.18	n/a	2
	B	0.85	0.75	0.97	9	4	0.81	0.38	1.75	n/a	2
	C	0.99	0.94	1.05	3	4	0.95	0.62	1.45	n/a	2
	D	0.93	0.85	1.02	6	4	0.92	0.57	1.49	n/a	2

Data from all Laboratories (excluding lab 10). - indicates no data for analysis; GM: Geometric Mean; LCL: Lower 95% Confidence Limit; UCL: Upper 95% Confidence Limit; GCV: Inter-lab Geometric Coefficient of Variation (%); N: Number of labs.

Table 8: Laboratory geometric mean EC_{50} estimates (ng) and final VEGF concentrations in neutralisation assays

Method	Lab	VEGF conc in assay (IU/mL)	GM					GCV (%)				
			A	B	C	D	IH	A	B	C	D	IH
HUVEC	04	36	-	-	-	-	-	-	-	-	-	-
HUVEC	06	56.3	50.0	74.4	64.5	74.7	-	69	82	25	72	-
HUVEC	10	22.5	28.2	33.1	27.3	31.6	74.1	23	20	20	21	47
HUVEC	11b	22.5	46.9	51.0	52.7	56.5	51.3	202	109	176	121	159
HUVEC	13	22.5	17.4	18.0	16.4	15.8	13.6	57	49	52	65	41
HUVEC	15	22.5	17.0	27.6	18.5	20.1	-	35	34	70	33	-
HUVEC	16	18	7.5	7.7	7.7	7.8	7.5	17	21	13	14	10
HUVEC	18	66.67	82.2	88.4	78.5	82.4	85.4	14	21	22	18	10
RGA	01	50.4	122.1	139.7	116.0	118.1	108.1	3	17	13	13	11
RGA	02	4	18.2	22.1	18.1	19.3	-	4	11	7	9	-
RGA	03	22.5	69.3	79.3	63.9	69.6	-	6	4	6	5	-
RGA	07	22.5	71.8	82.6	73.7	75.5	67.7	9	14	13	14	21
RGA	09	45	97.7	117.6	96.6	102.7	103.5	1	10	9	10	10
RGA	11a	22.5	64.4	78.9	64.8	73.1	71.6	17	13	8	24	14
RGA	12	22.5	61.1	72.2	62.6	64.5	-	7	9	7	6	-
RGA	17	13.5	40.7	47.0	41.4	44.1	36.0	14	14	14	20	15
EFC	05	30	15.2	17.9	15.5	16.8	-	14	20	13	17	-
EFC	14	7.11	5.0	5.3	5.0	5.0	-	48	50	60	52	-

- indicates no data for analysis; GM: Geometric Mean; GCV: Inter-lab Geometric Coefficient of Variation (%); N: Number of labs.

Table 9: Summary of EC_{50} estimates (ng)[1] for selected neutralisation assays using a fixed amount of VEGF (22.5 IU)

All Assays					
Sample	**GM**	**LCL**	**UCL**	**GCV**	**N**
A	30.3	13.5	68.3	188%	9
B	36.4	16.5	80.1	179%	9
C	30.9	13.9	68.7	183%	9
D	32.9	14.7	73.8	186%	9
IH	47.8	19.6	116.5	105%	5
HUVEC Assays					
A	25.0	11.7	53.7	62%	4
B	28.9	14.0	59.5	57%	4
C	25.8	11.2	59.3	69%	4
D	27.5	11.3	66.9	75%	4
IH	37.3	4.1	340.7	144%	3
RGA Assays					
A	66.5	59.2	74.7	8%	4
B	78.1	71.4	85.5	6%	4
C	66.1	58.8	74.3	8%	4
D	70.5	63.2	78.7	7%	4
IH	69.6	48.7	99.3	n/a	2

[1]EC_{50} estimates (ng) calculated on the basis of the assumed protein content of 50 µg for the samples
GM: Geometric Mean; LCL: Lower 95% Confidence Limit; UCL: Upper 95% Confidence Limit; GCV: Inter-lab Geometric Coefficient of Variation (%); n/a: not calculated or no valid estimates obtained; N: Number of labs

Table 10: Summary of results from accelerated temperature degradation testing of the candidate preparation using the RGA-based neutralization assay

Time stored (years)	Storage Temperature (°C)	95% Lower Confidence Limit	Relative Potency to -70°C	95% Upper Confidence Limit
1	**-20**	**0.82**	**1.00**	**1.22**
1	**+4**	**0.84**	**0.99**	**1.17**
1	**+20**	**0.78**	**0.96**	**1.06**
1	**+37**	**0.84**	**0.99**	**1.12**
1	**+45**	**0.84**	**0.97**	**1.12**
1.92	**-20**	**1.01**	**1.02**	**1.02**
1.92	**+4**	**0.93**	**1.02**	**1.12**
1.92	**+20**	**0.89**	**0.94**	**1.01**
1.92	**+37**	**0.93**	**0.98**	**1.03**
1.92	**+45**	**0.99**	**1.02**	**1.05**

Table 11: Summary of results from reconstitution stability studies of candidate preparation 23/124 tested using the RGA-based neutralization assay

Time stored (Days)	Storage Temperature (°C)	95% Lower Confidence Limit	Relative Potency to a freshly reconstituted ampoule	95% Upper Confidence Limit
1	+4	1.00	1.02	1.04
1	+22	1.09	1.12	1.14
7	+4	0.94	0.98	1.02
7	+22	0.71	0.87	1.06

Table 12: Summary of results from freeze-thaw stability studies of candidate preparation 23/124 tested using the RGA-based neutralization assay

Cycles	95% Lower Confidence Limit	Relative Potency	95% Upper Confidence Limit
1	0.97	1.02	1.08
2	1.04	1.09	1.15
3	0.96	1.06	1.16
4	0.89	1.02	1.18

Figure 1: Boxplot of laboratory GM relative potency estimates for Neutralisation and Binding assays

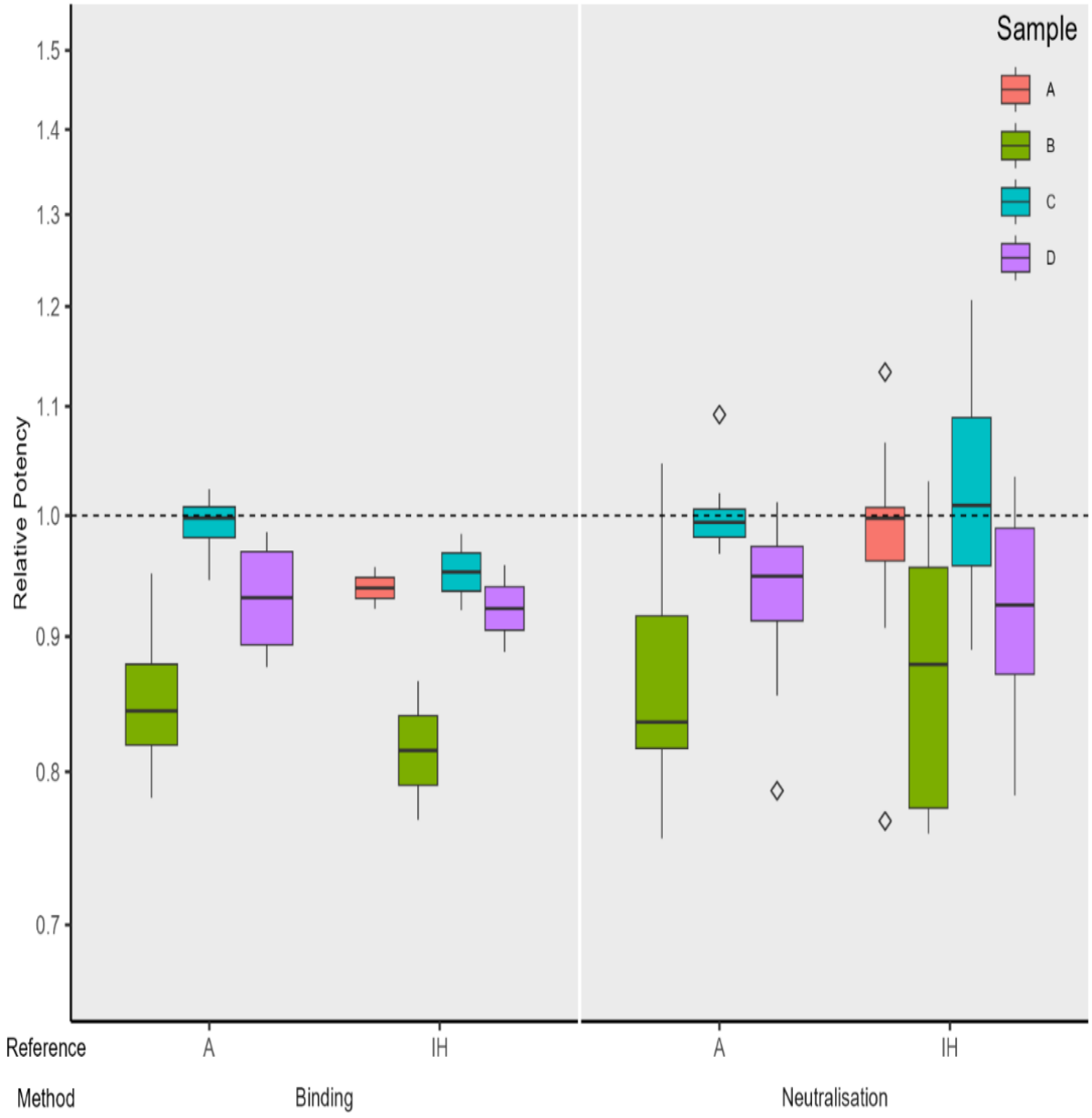


Figure 2: Boxplot of laboratory GM relative potency estimates for Neutralisation assays

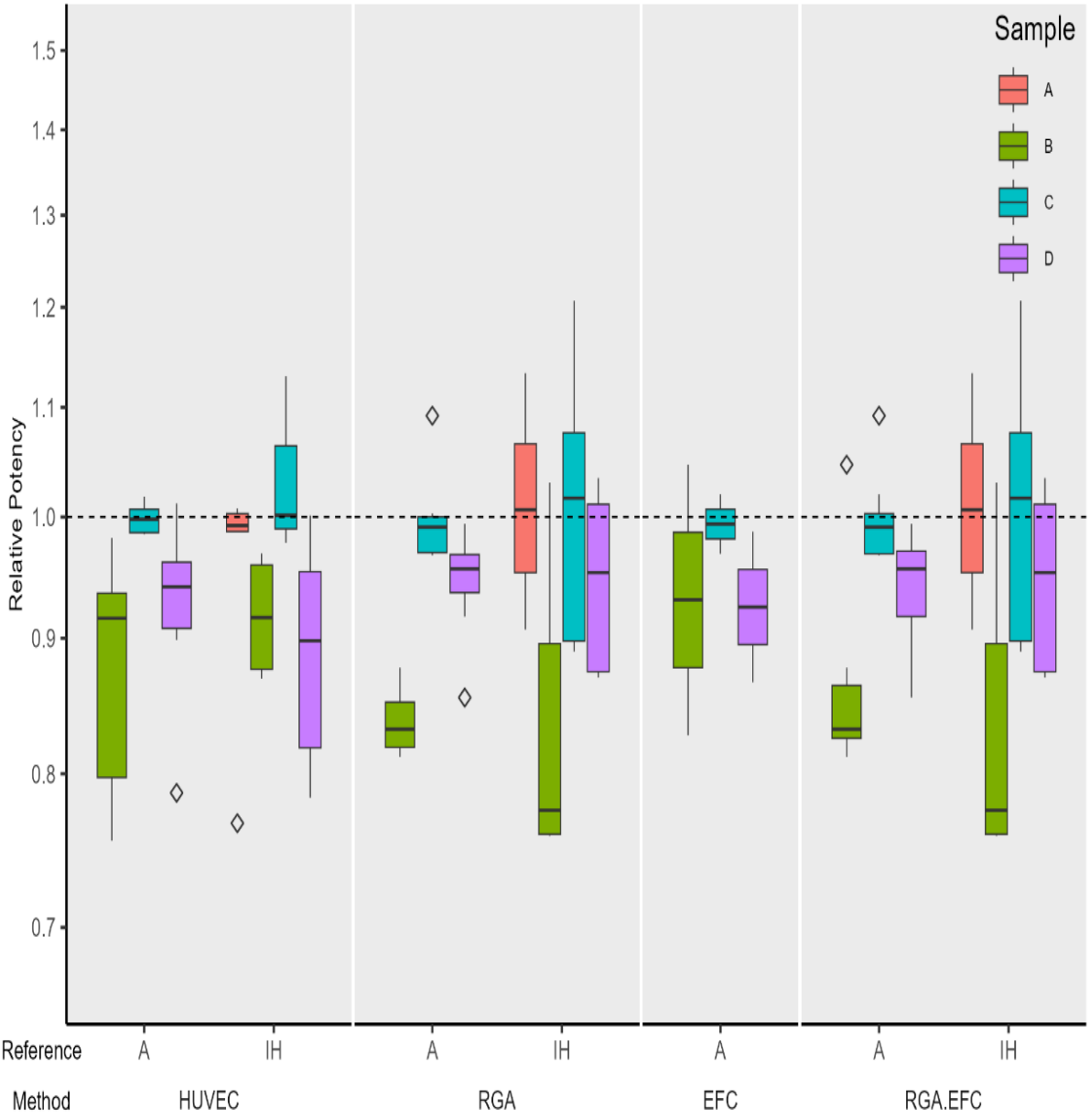
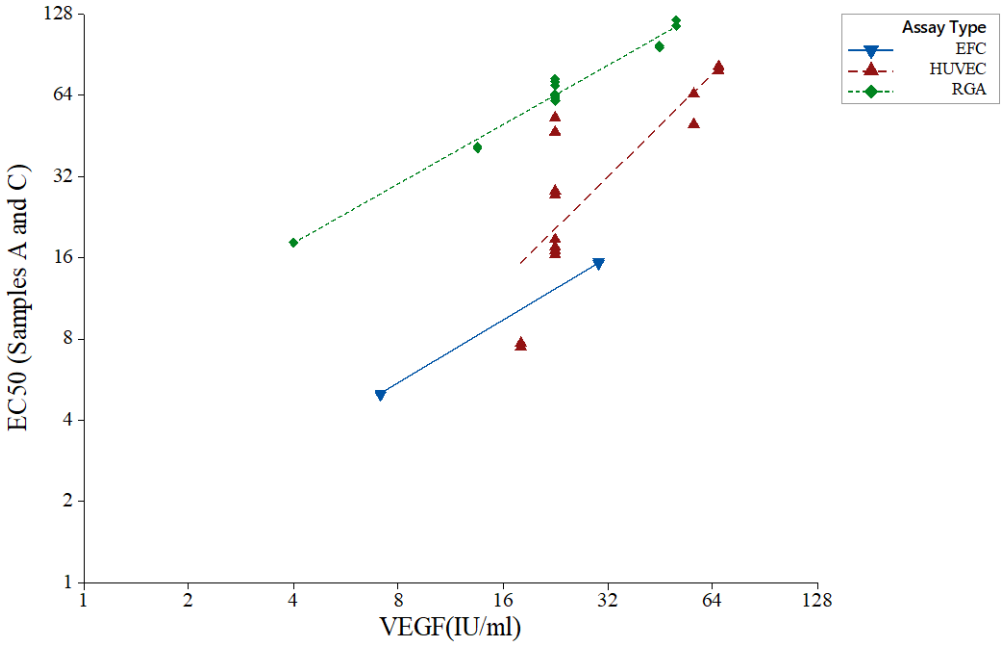


Figure 3: Scatter plot of EC50 (A, C) versus VEGF (IU/ml)



Appendix 1 Table 1: Percentage of invalid plates per laboratory

Assay	Lab	% of plates invalid *vs* sample A			% of plates invalid vs in-house reference			
		B	**C**	**D**	**A**	**B**	**C**	**D**
HUVEC	04	0%	0%	33%	75%	100%	100%	33%
HUVEC	06	89%	89%	44%	-	-	-	-
HUVEC	10	33%	33%	33%	92%	92%	100%	100%
HUVEC	11	70%	54%	69%	60%	64%	64%	64%
HUVEC	13	67%	78%	78%	89%	78%	100%	89%
HUVEC	15	33%	42%	29%	-	-	-	-
HUVEC	16	0%	17%	33%	0%	0%	0%	0%
HUVEC	18	67%	42%	44%	42%	67%	50%	33%
RGA	01	-	-	-	33%	33%	0%	0%
RGA	02	11%	0%	11%	-	-	-	-
RGA	03	11%	44%	11%	-	-	-	-
RGA	07	33%	17%	33%	50%	67%	67%	67%
RGA	09	33%	11%	33%	22%	33%	11%	11%
RGA	11	58%	58%	50%	25%	58%	67%	50%
RGA	12	33%	11%	44%	-	-	-	-
RGA	17	33%	17%	33%	17%	33%	8%	22%
EFC	05	22%	11%	67%	-	-	-	-
EFC	14	67%	42%	44%	-	-	-	-
Binding	08	56%	33%	33%	-	-	-	-
Binding	09	0%	11%	0%	0%	0%	11%	0%
Binding	15	22%	11%	11%	-	-	-	-
Binding	18	0%	0%	0%	0%	0%	8%	0%

- indicates no data for analysis.

Appendix 1 Table 2: Percentage of assays per laboratory not meeting equivalence criteria for different curve parameters (α: upper asymptote, δ: range between upper and lower asymptotes, β: slope factor ratio)

Assay	Lab	Relative to Sample A											
		Sample B			Sample C			Sample D			In-house reference		
		α	β	δ	α	β	δ	α	β	δ	α	β	δ
HUVEC	04	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	-	-	-
HUVEC	06	33%	78%	67%	44%	67%	44%	33%	44%	33%	-	-	-
HUVEC	10	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	17%	58%
HUVEC	11	60%	30%	50%	38%	38%	31%	38%	31%	46%	47%	20%	40%
HUVEC	13	44%	44%	22%	44%	56%	44%	67%	44%	56%	67%	67%	44%
HUVEC	15	0%	0%	0%	0%	17%	17%	11%	0%	11%	-	-	-
HUVEC	16	0%	0%	0%	17%	0%	0%	33%	0%	0%	0%	0%	0%
HUVEC	18	0%	0%	0%	0%	0%	0%	0%	11%	0%	8%	0%	8%
RGA	02	0%	11%	0%	0%	0%	0%	0%	11%	0%	-	-	-
RGA	03	0%	11%	0%	22%	22%	22%	0%	11%	0%	-	-	-
RGA	07	33%	0%	0%	8%	8%	8%	22%	11%	22%	42%	25%	42%
RGA	09	0%	33%	0%	11%	11%	11%	0%	33%	11%	11%	22%	11%
RGA	11	25%	42%	25%	33%	17%	33%	33%	25%	33%	8%	17%	8%
RGA	12	22%	11%	22%	0%	11%	0%	0%	44%	0%	-	-	-
RGA	17	33%	0%	33%	17%	0%	17%	33%	0%	33%	8%	8%	8%
EFC	05	0%	22%	0%	0%	11%	0%	0%	67%	11%	-	-	-
EFC	14	0%	33%	0%	0%	17%	0%	0%	22%	11%	-	-	-
Binding	08	56%	33%	33%	22%	33%	22%	22%	33%	22%	-	-	-
Binding	09	0%	0%	0%	11%	11%	11%	0%	0%	0%	0%	0%	0%
Binding	15	22%	11%	22%	11%	0%	11%	11%	11%	11%	-	-	-
Binding	18	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

- indicates no data for analysis;

Appendix 1 Table 3: Individual assay relative potency estimates relative to Sample A

Lab	Assay	Assay	Plate	Sample B	Sample C	Sample D	In-house reference
4	HUVEC	1	1	0.84	0.97	0.94	-
4	HUVEC	2	1	0.79	0.97	0.94	-
4	HUVEC	3	1	0.82	1.01	0.90NL	-
6	HUVEC	1	1	0.77NP	0.35NP	0.65NP	-
6	HUVEC	1	2	1.66NP	1.25NP	0.74NP	-
6	HUVEC	1	3	0.71NP	0.47NP	0.65NP	-
6	HUVEC	2	1	0.82NP	0.85NP	0.87	-
6	HUVEC	2	2	0.78NP	0.81NP	0.87	-
6	HUVEC	2	3	0.75	0.99	0.98	-
6	HUVEC	3	1	0.62NP	0.85NP	0.69NP	-
6	HUVEC	3	2	1.78NP	1.09NP	2.15	-
6	HUVEC	3	3	0.82NP	0.75NP	0.58	-
10	HUVEC	1	1	0.80	0.85	0.78	0.28
10	HUVEC	1	2	1.57CD	1.27CD	1.09CD	0.12CD
10	HUVEC	1	3	0.79	0.95	1.36	0.14NP
10	HUVEC	2	1	0.91	1.04	0.73	0.17NP
10	HUVEC	2	2	1.12CD	1.33CD	1.01CD	0.12CD
10	HUVEC	2	3	0.74	0.90	1.25	0.09NP
10	HUVEC	3	1	0.76	1.02	0.77	0.12NP
10	HUVEC	3	2	1.00CD	1.33CD	0.89CD	0.12NP
10	HUVEC	3	3	0.70CD	0.82	1.07CD	0.17NP
10	HUVEC	4	1	0.66CD	0.79CD	0.70CD	0.16NP
10	HUVEC	4	2	0.96	1.16	0.84	0.13NP
10	HUVEC	4	3	0.82	0.89	0.92	0.13NP
11	HUVEC	1	1	0.62NP	0.96NP	1.70NP	1.42NP
11	HUVEC	1	2	1.18NP	1.27NP	0.82NP	0.70NP
11	HUVEC	1	3	0.68	1.06NP	1.08NP	0.73NP
11	HUVEC	2	2	0.90	n/a	0.87NP	0.90
11	HUVEC	2	3	0.77	n/a	1.09NP	0.94
11	HUVEC	3	1	0.60NP	0.66NP	0.68NP	0.69NP
11	HUVEC	3	2	0.77NP	0.90	0.75NP	0.94NP
11	HUVEC	3	3	0.89NP	0.99NP	0.77NP	0.92NP
11	HUVEC	4	1	-	0.95	0.83	1.30NP
11	HUVEC	4	2	-	1.08	0.74	0.85
11	HUVEC	4	3	1.36NP	0.89NP	-	1.44NP
11	HUVEC	5	1	-	1.14	0.88	1.44
11	HUVEC	5	2	-	0.98NP	0.71	1.00
11	HUVEC	5	3	-	1.13	1.20NP	0.94
11	HUVEC	5	4	1.07NP	0.93	-	0.90NP
13	HUVEC	1	1	0.77	0.92NP	0.97NP	1.04NP
13	HUVEC	1	2	0.84	0.96NP	0.89NP	0.99NP
13	HUVEC	1	3	0.83NP	0.94NP	0.83NP	0.98NP
13	HUVEC	2	1	0.80NP	1.02NP	1.20NP	1.05NP

13	HUVEC	2	2	0.80NP	1.01NP	1.03NP	1.05NP
13	HUVEC	2	3	0.82	0.99NP	0.99NP	1.16NP
13	HUVEC	3	1	1.02	1.01	0.92	1.07NP
13	HUVEC	3	2	0.75NP	0.80NP	0.98NP	1.18NP
13	HUVEC	3	3	1.03	1.01	0.91	1.30
15	HUVEC	1	1	-	0.73CD	0.37NP	-
15	HUVEC	1	2	-	0.97	0.86	-
15	HUVEC	1	3	-	1.03	0.68	-
15	HUVEC	1	4	0.86	1.70CD	-	-
15	HUVEC	2	1	-	1.09NP	1.17	-
15	HUVEC	2	2	-	0.92	1.05	-
15	HUVEC	2	3	-	0.96	0.93	-
15	HUVEC	2	4	0.94	1.07NP	-	-
15	HUVEC	3	1	-	1.06	0.83	-
15	HUVEC	3	2	-	1.37CD	0.86	-
15	HUVEC	3	3	-	0.87	0.86	-
15	HUVEC	3	4	0.90	1.13	-	-
16	HUVEC	1	1	-	1.03	1.03	1.00
16	HUVEC	1	2	-	1.00	0.92	0.95
16	HUVEC	1	3	-	0.95	0.93	0.90
16	HUVEC	1	4	0.98	0.93	-	0.96
16	HUVEC	2	1	-	0.92	0.92	0.99
16	HUVEC	2	2	-	0.93	0.84NP	1.04
16	HUVEC	2	3	-	0.92	0.89	1.01
16	HUVEC	2	4	0.94	0.93NP	-	1.05
16	HUVEC	3	1	-	1.15	1.16NP	1.15
16	HUVEC	3	2	-	1.16	0.99NP	1.05
16	HUVEC	3	3	-	1.03	0.97	1.00
16	HUVEC	3	4	1.02	0.91NP	-	1.03
18	HUVEC	1	1	-	0.90	1.19	1.30
18	HUVEC	1	2	-	0.82CD	0.77CD	0.79CD
18	HUVEC	1	3	-	1.23CD	0.90CD	0.97CD
18	HUVEC	1	4	1.00CD	1.39CD	-	1.07NP
18	HUVEC	2	1	-	1.18	1.27	1.17
18	HUVEC	2	2	-	1.12	0.84	0.82
18	HUVEC	2	3	-	1.26CD	1.41CD	0.97CD
18	HUVEC	2	4	1.05CD	0.80CD	-	0.76CD
18	HUVEC	3	1	-	0.91	0.89	0.85
18	HUVEC	3	2	-	0.91	0.94NP	0.97
18	HUVEC	3	3	-	1.02	0.94	0.96
18	HUVEC	3	4	0.92	1.00	-	0.96
1	RGA	1	1	-	-	-	1.09NP
1	RGA	1	2	-	-	-	-
1	RGA	1	3	-	-	-	-
1	RGA	1	4	-	-	-	-
1	RGA	2	1	-	-	-	0.90
1	RGA	2	2	-	-	-	-

1	RGA	2	3	-	-	-	-
1	RGA	2	4	-	-	-	-
1	RGA	3	1	-	-	-	1.10
1	RGA	3	2	-	-	-	-
1	RGA	3	3	-	-	-	-
1	RGA	3	4	-	-	-	-
2	RGA	1	1	0.84	1.00	0.98	-
2	RGA	1	2	0.71	1.00	0.82	-
2	RGA	1	3	0.86	0.94	0.93	-
2	RGA	2	1	0.88	1.10	1.02	-
2	RGA	2	2	0.75NP	1.01	0.85NP	-
2	RGA	2	3	0.74	0.96	0.83	-
2	RGA	3	1	0.79	0.85	0.85	-
2	RGA	3	2	0.91	1.10	1.01	-
2	RGA	3	3	0.97	1.10	1.11	-
3	RGA	1	1	0.87	1.03	0.98	-
3	RGA	1	2	0.88	1.08	1.02NP	-
3	RGA	1	3	0.91NP	1.11	1.06	-
3	RGA	2	1	0.87	1.07	1.00	-
3	RGA	2	2	0.87	1.07NP	1.00	-
3	RGA	2	3	0.86	1.11NP	1.00	-
3	RGA	3	1	0.89	1.09NP	0.99	-
3	RGA	3	2	0.82	1.01NP	0.93	-
3	RGA	3	3	0.85	1.17	1.00	-
7	RGA	1	1	-	0.87	0.89NP	1.20
7	RGA	1	2	-	1.03	0.93	1.39NP
7	RGA	1	3	-	1.01	1.14NP	1.26NP
7	RGA	1	4	0.78NP	0.86NP	-	1.33
7	RGA	2	1	-	0.96	0.90	0.98
7	RGA	2	2	-	1.16	1.01	1.19NP
7	RGA	2	3	-	0.86	0.94NP	1.07NP
7	RGA	2	4	0.88	0.83	-	1.21NP
7	RGA	3	1	-	0.87	0.79	0.83
7	RGA	3	2	-	1.02NP	1.28	0.99
7	RGA	3	3	-	1.05	0.90	1.06NP
7	RGA	3	4	0.75	1.10	-	1.03
9	RGA	1	1	0.81	0.98	0.98	0.96
9	RGA	1	2	0.82	0.93	0.81NP	0.85
9	RGA	1	3	0.89NP	1.13NP	1.02NP	0.99NP
9	RGA	2	1	0.83NP	1.03	1.05	0.99
9	RGA	2	2	0.81	0.93	0.90NP	0.87
9	RGA	2	3	0.85NP	1.02	0.91	0.92NP
9	RGA	3	1	0.85	1.02	0.95	0.96
9	RGA	3	2	0.86	1.04	0.98	1.00
9	RGA	3	3	0.81	0.99	0.92	0.95
11	RGA	1	1	0.73	0.94NP	0.98	0.96NP
11	RGA	1	2	0.77NP	0.98	0.83	0.83

11	RGA	1	3	0.76NP	1.22NP	1.13NP	1.09
11	RGA	2	1	1.08	0.97	0.89	0.77
11	RGA	2	2	0.93	0.98	0.87	0.83
11	RGA	2	3	0.79	0.96	0.79	0.80
11	RGA	3	1	0.64NP	0.70CD	1.14NP	0.72CD
11	RGA	3	2	0.73NP	0.96	0.68NP	0.98
11	RGA	3	3	1.10NP	1.41CD	0.97NP	1.30NP
11	RGA	4	1	0.90	1.03NP	1.09NP	0.89
11	RGA	4	2	0.63NP	0.72NP	0.77NP	0.92
11	RGA	4	3	0.91NP	1.21NP	0.79	0.87
12	RGA	1	1	0.89	1.03	1.02NP	-
12	RGA	1	2	0.93	0.83	0.98	-
12	RGA	1	3	0.83	0.93	0.92	-
12	RGA	2	1	0.85NP	1.08	0.94	-
12	RGA	2	2	0.81	1.04	1.00NP	-
12	RGA	2	3	0.81NP	0.86NP	0.88NP	-
12	RGA	3	1	0.89NP	1.06	0.97	-
12	RGA	3	2	0.81	1.02	0.98	-
12	RGA	3	3	0.79	1.00	0.94NP	-
17	RGA	1	1	-	0.97	0.87NP	1.16
17	RGA	1	2	-	1.15NP	1.09NP	1.24NP
17	RGA	1	3	-	1.01	1.02	1.12
17	RGA	1	4	0.85	0.99	-	1.14
17	RGA	2	1	-	0.99	0.90	1.16
17	RGA	2	2	-	1.12NP	1.02	1.11
17	RGA	2	3	-	0.99	0.86	1.06
17	RGA	2	4	0.91NP	1.08	-	1.15NP
17	RGA	3	1	-	0.91	0.81NP	1.06
17	RGA	3	2	-	0.95	1.04	1.08
17	RGA	3	3	-	0.89	0.99	1.12
17	RGA	3	4	0.78	0.91	-	1.00
5	EFC	1	1	0.79	1.01	0.86NP	-
5	EFC	1	2	0.79	0.97	0.89	-
5	EFC	1	3	0.83	1.04	0.89NP	-
5	EFC	2	1	1.07	1.11	1.14NP	-
5	EFC	2	2	0.85	0.87	0.87NP	-
5	EFC	2	3	0.99NP	0.91	0.83NP	-
5	EFC	3	1	0.76NP	0.97NP	0.77NP	-
5	EFC	3	2	0.74	0.88	0.80	-
5	EFC	3	3	0.76	0.98	0.91	-
14	EFC	1	1	-	1.39CD	1.22CD	-
14	EFC	1	2	-	1.06	1.07	-
14	EFC	1	3	-	1.04	0.93	-
14	EFC	1	4	0.96CD	0.80CD	-	-
14	EFC	2	1	-	1.22	1.20NP	-
14	EFC	2	2	-	0.91NP	0.89	-
14	EFC	2	3	-	0.97	1.11	-

14	EFC	2	4	1.05	1.16	-	-
14	EFC	3	1	-	1.24CD	0.99CD	-
14	EFC	3	2	-	0.87	1.20NP	-
14	EFC	3	3	-	0.88	0.95	-
14	EFC	3	4	0.97NP	1.10NP	-	-
8	Binding	1	1	1.06	1.57NP	1.46NP	-
8	Binding	1	2	0.79	0.88	0.80	-
8	Binding	1	3	0.96NP	1.11	0.92	-
8	Binding	2	1	0.89NP	1.21NP	1.24	-
8	Binding	2	2	0.78NP	1.00	0.97	-
8	Binding	2	3	0.86	0.98	0.99NP	-
8	Binding	3	1	0.73NP	1.12NP	0.77	-
8	Binding	3	2	1.07NP	1.02	0.74NP	-
8	Binding	3	3	0.75	0.98	0.77	-
9	Binding	1	1	0.80	0.95	0.91	1.05
9	Binding	1	2	0.88	1.05	0.97	1.06
9	Binding	1	3	0.80	0.93	0.94	1.11
9	Binding	2	1	0.92	1.07	1.08	1.16
9	Binding	2	2	0.77	1.01	0.99	1.12
9	Binding	2	3	0.78	1.53NP	0.91	1.05
9	Binding	3	1	0.83	1.01	0.96	1.02
9	Binding	3	2	0.87	1.01	0.98	1.14
9	Binding	3	3	0.84	0.99	0.94	1.06
15	Binding	1	1	0.89	1.01NP	0.90	-
15	Binding	1	2	0.79NP	0.97	0.81	-
15	Binding	1	3	0.76	0.86	0.83	-
15	Binding	2	1	0.67NP	1.05	0.76NP	-
15	Binding	2	2	0.82	0.95	0.96	-
15	Binding	2	3	0.60	0.85	0.86	-
15	Binding	3	1	0.78	0.92	0.93	-
15	Binding	3	2	0.85	1.03	0.96	-
15	Binding	3	3	0.82	0.97	0.80	-
18	Binding	1	1	-	0.99	1.00	1.09
18	Binding	1	2	-	0.98	0.94	0.99
18	Binding	1	3	-	1.03	0.95	1.04
18	Binding	1	4	0.96	1.02	-	1.11
18	Binding	2	1	-	1.00	1.01	1.09
18	Binding	2	2	-	1.03	1.00	1.02
18	Binding	2	3	-	1.05	1.09	1.09
18	Binding	2	4	0.89	1.01	-	1.05
18	Binding	3	1	-	0.98	0.95	1.02
18	Binding	3	2	-	1.02	0.95	0.94
18	Binding	3	3	-	1.04	0.99	1.00
18	Binding	3	4	1.01	1.14	-	1.14

-indicates no data for analysis NL: Non-Linear; NP: Non-Parallel; CD: Coded Duplicates outside thresholds; n/a: not calculated or no valid estimates obtained.

Appendix 1 Table 4: Summary of relative potency estimates, calculated relative to in-house reference

Method	Sample	All laboratories[1]					Excluding laboratory 10				
		GM	LCL	UCL	GCV(%)	N	GM	LCL	UCL	GCV(%)	N
Neutralization (all)	A	1.10	0.84	1.44	50	11	0.98	0.91	1.05	11	10
	B	1.07	0.67	1.74	95	10	0.87	0.80	0.95	12	9
	C	1.02	0.93	1.11	11	8	1.02	0.93	1.11	11	8
	D	0.92	0.86	0.98	10	10	0.92	0.86	0.98	10	10
Neutralization (HUVEC)	A	1.18	0.66	2.11	74	6	0.95	0.82	1.10	13	5
	B	1.38	0.44	4.30	150	5	0.92	0.84	1.00	6	4
	C	1.03	0.85	1.25	8	3	1.03	0.85	1.25	8	3
	D	0.98	0.78	1.01	11	5	0.89	0.78	1.01	11	5
Neutralization (RGA)	A	1.01	0.90	1.13	9	5	1.01	0.90	1.13	9	5
	B	0.84	0.71	0.99	14	5	0.84	0.71	0.99	14	5
	C	1.01	0.86	1.18	14	5	1.01	0.86	1.18	14	5
	D	0.95	0.86	1.05	8	5	0.95	0.86	1.05	8	5
Neutralization (RGA/EFC)	A	1.01	0.90	1.13	9	5	1.01	0.90	1.13	9	5
	B	0.84	0.71	0.99	14	5	0.84	0.71	0.99	14	5
	C	1.01	0.86	1.18	14	5	1.01	0.86	1.18	14	5
	D	0.95	0.86	1.05	8	5	0.95	0.86	1.05	8	5
Binding	A	0.94	0.75	1.18	n/a	2	0.94	0.75	1.18	n/a	2
	B	0.81	0.38	1.75	n/a	2	0.81	0.38	1.75	n/a	2
	C	0.95	0.62	1.45	n/a	2	0.95	0.62	1.45	n/a	2
	D	0.92	0.57	1.49	n/a	2	0.92	0.57	1.49	n/a	2

[1]All Laboratories (includes lab 10). GM: Geometric Mean; LCL: Lower 95% Confidence Limit; UCL: Upper 95% Confidence Limit; GCV: Inter-lab Geometric Coefficient of Variation (%); N: Number of labs

Appendix 1: Study Protocol

COLLABORATIVE STUDY OF 1ST WHO INTERNATIONAL STANDARD FOR RANIBIZUMAB BIOACTIVITY
CS726 Study Protocol
Project Leaders: Haiyan Jia and Meenu Wadhwa

1. BACKGROUND

The advent of innovative therapeutic monoclonal antibodies has transformed the treatment of numerous diseases, including neovascular intraocular diseases and cancers. With patent expiry on some of these originators, the number of approved biosimilars for monoclonal antibodies is increasing rapidly worldwide improving their cost effectiveness and availability. The WHO has recognised a global need for standardisation of biotechnology products following requests for advice on appropriate control measures to ensure safety, quality and efficacy (WHO Technical Report Series, 56th Report, 941:12-13, 2007). As a result, International Standards (IS) have been established for several therapeutic monoclonal antibodies. The use and role of such ISs in assurance of product consistency and international harmonization has been explained recently in the WHO guideline on evaluation of biosimilars (Replacement of Annex 2 of WHO Technical Report Series, No. 977).
Biosimilars for ranibizumab are currently at different stages of development with several products already approved by FDA, EMA and MHRA. Therefore, there is an urgent need for an IS to facilitate global harmonisation of bioactivity of ranibizumab products.

2. AIMS OF THE STUDY

a) To assess the suitability of ampouled preparations of ranibizumab to serve as the 1st WHO IS for ranibizumab by assaying their biological activity in a range of bioassays and binding assays.

b) To evaluate the relative activity of the ampouled preparations of ranibizumab in different bioassays and binding assays in current use, and to determine, if possible, the concentrations of ranibizumab required to neutralise specific amounts of the 1st WHO IS for VEGF165 (19/246).

c) To compare the ampouled preparations of ranibizumab with various individual established in-house reference standards where available.

3. MATERIALS PROVIDED FOR THE STUDY

All participants will be sent:

a) A set of samples coded **A, C and D** (5 ampoules for each preparation) for testing in ranibizumab assays. Each ampoule contains approximately 50 µg of ranibizumab.

b) An additional set of sample coded **B** for testing in the assays. This ampoule contains an unknown amount of ranibizumab, but should be treated/diluted in the same way to samples **A, C and D**.

c) Five ampoules of the 1st WHO IS for VEGF165 (19/246) to be used in cell-based bioassays only, containing **9,000 International Units (IU)** of VEGF165 per ampoule.

4. STORAGE AND RECONSTITUTION OF PREPARATIONS

Prior to initiating the study, please read the Instructions for Use provided with the collaborative study. Please note the statements regarding safety and that these preparations are not for human use.
Lyophilized preparations provided should be stored at -20°C or below until use.

a) Preparations A to D should be reconstituted with 1 ml of sterile distilled water. Mix GENTLY and ensure contents are completely dissolved prior to use. Use carrier protein (e.g. assay medium containing foetal bovine serum) where extensive dilution is required.

b) The 1st WHO IS for VEGF165 (19/246) should be reconstituted with 1 ml of sterile distilled water. Mix GENTLY and ensure contents are completely dissolved prior to use. This solution contains VEGF165 at a concentration of **9,000 IU/ml**. Use carrier protein (e.g. assay medium containing foetal bovine serum) where extensive dilution is required.

5. ASSAYS

Cell-based VEGF165 neutralisation assays

Since these assays are based on the inhibitory action of ranibizumab which neutralises the biological activity of VEGF165, participants are requested to use a fixed dose of the VEGF165 IS (19/246), which has been provided to mitigate any variability that may arise from use of VEGF165 from different suppliers. The dose of VEGF165 IS for use will need to be determined by participants, which should provide a biological response similar to the dose of VEGF165 routinely used by participants in their in-house cell-based neutralisation assays for ranibizumab. In our hands, the following concentrations of VEGF165 IS provide acceptable ranibizumab dose-response curves in the assays given below. However, since the assays may vary, please define suitable doses for your assay by conducting a pilot assay prior to the final runs.

- For HUVEC assays – a final dose of 22.5 IU/ml of VEGF165 IS.
- For reporter gene assays – a final dose of 22.5 IU/ml of VEGF165 IS.

For performing neutralisation assays, follow each of the steps a) – f) listed in Section 6 Assay Design as below.

Use validated in-house assays where possible. Note that the WHO IS for VEGF165 (19/246) contains **9,000 IU** of activity per ampoule. Remember to include appropriate controls in the assays - blank control (cells only no VEGF165 and no ranibizumab) and also VEGF165 control (cells with VEGF165 without ranibizumab) at the fixed concentration used for the assay.

Binding assays

These should be carried out using validated in-house methods or kits, **follow each of the steps a) – f) in Section 6 Assay Design as below.**

Please note: The WHO IS for VEGF165 (19/246) contains **0.5% human serum albumin** as an excipient and therefore is unsuitable for use in binding assays. Participants should use VEGF165 from a commercial supplier**.**

6. ASSAY DESIGN

Participants are requested to use their own validated in-house procedures and follow steps a) to f) as listed below:

a) Use a freshly reconstituted ampoule of each preparation A to D in each of the assays. An assay is considered independent if the assay is carried out on different days/occasions.

b) Participants are asked to perform a pilot assay for each assay method used, to ensure that all preparations (A to D) are diluted such that the concentration range falls within the working range of the assay.

c) Following suitable dose-response curves and an adequate signal above background ratio in the pilot assay, perform at least 3 independent assays for each of the preparations A to D (and in-house reference standard where available) using the most appropriate dilutions derived from the pilot assay for the different preparations tested.

d) For each independent assay each plate should include at least 1 independent dilution series for each preparation (A, B, C, D) in duplicate. This should be repeated 3 times across 3 different plates for each independent assay as illustrated in the example assay plate layouts of **Appendix 2** at the end of the protocol. This will provide a total of 9 estimates from 3 independent assays. Please try to vary the positions of the samples on different plates to ensure that estimates are not susceptible to systematic bias

due to positional effects. Include a dose response curve of the in-house reference standard on each plate if the **Example Plate Layout A** (dilutions down the plate) is employed.

e) Suggested assay plate layouts are given in **Appendix 2** of this protocol. The layouts can be amended to suit in-house methods if preferred, however, it is important to ensure that each plate includes at least 1 dilution series for each of the samples (A, B, C, D) plus in-house reference standard if available. If using the **Example Plate Layout B** (dilutions across the plate), sample B may need to be included on a separate plate (Plate 4), please also include sample A, sample C and in-house reference standard if available on this separate plate 4. Please vary the position of IH, A, B, C in different assays to ensure that estimates are not susceptible to systematic bias due to positional effects. See suggested the **Example Plate Layout B** for Plate 4 only in **Appendix 2**. If you have any queries about how to design your assay plate layouts, please contact us using the email address below.

f) Include appropriate controls in the assays.

7. INFORMATION TO BE SUPPLIED AND PRESENTATION OF RESULTS

We have included assay information sheets in **Appendix 1**, and Example Plate Layouts (A & B) for 96-well plate formats in **Appendix 2**.
Separate **Excel raw data templates** are provided for returning assay plate layouts, sample dilutions and assay results obtained from 3 independent assays for the samples tested. For binding assays, this will need to be amended as per the plate layout used for the assay. Please record the final dilutions and concentrations in the assay after all components and cells have been added.
Please provide all the details requested for EACH assay as indicated in the assay information sheets in **Appendix 1**
Information required for EACH assay should include:

a) Assay details, and how it was performed.

b) In-house ranibizumab standard.

c) VEGF165 – WHO IS for VEGF165 (19/246) for cell-based neutralisation assays and commercial supplied VEGF165 for binding assays.

d) Dilutions used – stock concentrations and final dilutions in EACH assay. Report all dilutions for ranibizumab candidate samples, in-house reference standards and VEGF165, and **the final dilutions and concentrations in the assay after all components and cells have been added.**

e) Please PROVIDE ALL RAW DATA (96-well plate readouts e.g., Absorbance, Fluorescence Units or Luminescence Units etc.) as direct analysis of the raw data provided by the assays permits data from all participants to be handled consistently, as far as possible.

f) We request participants to follow the examples provided and enter data as indicated in the **Excel raw data templates** (that has been provided separately).

g) Although MHRA will calculate relative potencies from the raw data provided by participants, participants are requested (if possible) to calculate the potencies of each preparation using their own in-house methods relative to their in-house reference standard. Please provide information of all methods used for calculating results.

h) Where participants do not have an in-house ranibizumab standard, please report all potencies relative to coded sample A.

8. DATA SUBMISSION

a) Please provide all information requested as this is needed for compilation of the study report following instructions given in the relevant sections of this report by using the assay information sheets in **Appendix 1** and provided data reporting spreadsheets of the separate Excel file.

b) Please return all completed data spreadsheets of the Excel file and assay information sheets for all the replicates of all the assays electronically.

Please kindly submit results together with the completed assay information sheets in Appendix 1 by 8th January 2024 at the latest.

For submission of study results and the assay information, and for any further information please email:

Dr Haiyan Jia
Email: haiyan.jia@mhra.gov.uk

Dr Meenu Wadhwa
Email: meenu.wadhwa@mhra.gov.uk

9. REPORTING OF RESULTS

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

10. CONDITIONS OF PARTICIPATION

Participants in the collaborative study should note the following conditions:

a) They participate in the study with the understanding that they agree not to publish or circulate information concerning the materials sent to them without the prior consent of the MHRA study organisers.

b) The participants results may be shared anonymously with not-for-profit public health bodies in the interests of global harmonisation.

c) It is normal practice to acknowledge participants as contributors of data rather than co-authors in publications describing the establishment of the standard.

d) Individual participants' data will be reported and coded blind to other participants during the preparation of the study report and supporting publications.

e) All participants will receive a copy of the study report and proposed conclusions for comment before final establishment of the international standard

APPENDIX 1
Collaborative Study for Ranibizumab 1st WHO IS – Assay Information

Company name:

1. What is the nature of your in-house ranibizumab standard?
   a) Expression host:
   b) Form:
   c) Stock concentration:

2. How did you obtain the ranibizumab standard?
   a) Commercial supplier name:
   b) Manufactured in-house (please give reference if available):

3. What measures do you use with the ranibizumab standard?

a) Mass:
b) Units (please provide information on how they were derived):

4. Assay methodology information
Please note: If more than one assay type is performed then please provide the details for EACH assay type.
Please record all information on concentrations and dilutions of assay components relating to EACH assay on the **Results Excel Spreadsheets** provided as separate **Excel templates**.
Briefly outline the assay methods used (provide full protocol on separate sheets if available):

Cell-based neutralisation assay

a) Please report details of the cell line, source, seeding density (how many cells per well of 96-well plates) and passage number of cells used in each assay, if applicable:
b) Any pre-treatment of the cells before the assay:
c) Details on ampoule reconstitution, dilution steps and dilution buffer used:
VEGF165 IS (19/246)
Sample A
Sample B
Sample C
Sample D
In-house ranibizumab standard:
Please also provide details of any blanks if applicable:
d) Serial dilutions of ranibizumab in the plate layout and final dose range of ranibizumab used in the assay (please also provide separately in the **Results Excel Spreadsheets**):
e) Report details of the readout of the assay (e.g. Absorbance, Fluorescence, Luminescence), reagent and equipment used to obtain this readout:
f) Additional comments:
g) For VEGF165 neutralisation assays indicate the concentration of VEGF165 IS used in the assay: the concentration of VEGF165 IS working solution, the concentration used in neutralization of ranibizumab samples, and also the final concentration in the assay after addition of the cells as appropriate for the assay.For example, in some assays – VEGF165 IS working solution = 90 IU/ml; diluted 1:1 with ranibizumab samples = 45 U/ml; further diluted 1:1 with cells = 22.5 U/ml.
h) Length of incubation of the cells with ranibizumab + VEGF165:

Binding assay

Please note: The WHO IS for VEGF (19/246) contains **0.5% human serum albumin** as an excipient and therefore is unsuitable for use in binding assays; participants should use VEGF165 from a commercial supplier**.**

a) Details on ampoule reconstitution, dilution steps and dilution buffer used:
Sample A
Sample B
Sample C
Sample D
In-house ranibizumab standard:
Please also provide details of any blanks if applicable:
b) Serial dilutions of ranibizumab in the plate layout and final dose range of ranibizumab used in the assay (please also provide separately in the **Results Excel Spreadsheets**):
c) Source/Supplier of VEGF165, dilution buffer and final coating concentration used in the assay:
d) Please report details of the readout of the assay (e.g. Absorbance, Fluorescence, Luminescence), reagent and equipment used to obtain this readout:
e) Additional comments:

APPENDIX 2
Collaborative Study for Ranibizumab 1st WHO IS - Suggested Plate Layouts

The following plate layouts are suggested for VEGF165 neutralisation assays.
Similar plate layouts should be adopted for binding assays.

Example Plate Layout A – Dilutions down the plate
Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	IH	IH	A	A	B	B	C	C	D	D	VEGF
B	Blank	IH	IH	A	A	B	B	C	C	D	D	VEGF
C	Blank	IH	IH	A	A	B	B	C	C	D	D	VEGF
D	Blank	IH	IH	A	A	B	B	C	C	D	D	VEGF
E	Blank	IH	IH	A	A	B	B	C	C	D	D	VEGF
F	Blank	IH	IH	A	A	B	B	C	C	D	D	VEGF
G	Blank	IH	IH	A	A	B	B	C	C	D	D	VEGF
H	Blank	IH	IH	A	A	B	B	C	C	D	D	VEGF

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	B	B	C	C	D	D	IH	IH	A	A	VEGF
B	Blank	B	B	C	C	D	D	IH	IH	A	A	VEGF
C	Blank	B	B	C	C	D	D	IH	IH	A	A	VEGF
D	Blank	B	B	C	C	D	D	IH	IH	A	A	VEGF
E	Blank	B	B	C	C	D	D	IH	IH	A	A	VEGF
F	Blank	B	B	C	C	D	D	IH	IH	A	A	VEGF
G	Blank	B	B	C	C	D	D	IH	IH	A	A	VEGF
H	Blank	B	B	C	C	D	D	IH	IH	A	A	VEGF

Plate 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	D	D	IH	IH	A	A	B	B	C	C	VEGF
B	Blank	D	D	IH	IH	A	A	B	B	C	C	VEGF
C	Blank	D	D	IH	IH	A	A	B	B	C	C	VEGF
D	Blank	D	D	IH	IH	A	A	B	B	C	C	VEGF
E	Blank	D	D	IH	IH	A	A	B	B	C	C	VEGF
F	Blank	D	D	IH	IH	A	A	B	B	C	C	VEGF
G	Blank	D	D	IH	IH	A	A	B	B	C	C	VEGF
H	Blank	D	D	IH	IH	A	A	B	B	C	C	VEGF

IH = In-house reference standard; Blank = Cells only control; VEGF = Cells+VEGF165 control

Example Plate Layout B – Dilutions across the plate

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	Blank
B	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	Blank
C	A	A	A	A	A	A	A	A	A	A	A	Blank
D	A	A	A	A	A	A	A	A	A	A	A	Blank
E	C	C	C	C	C	C	C	C	C	C	C	VEGF
F	C	C	C	C	C	C	C	C	C	C	C	VEGF
G	D	D	D	D	D	D	D	D	D	D	D	VEGF
H	D	D	D	D	D	D	D	D	D	D	D	VEGF

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	C	C	C	C	C	C	C	C	C	C	C	Blank
B	C	C	C	C	C	C	C	C	C	C	C	Blank
C	D	D	D	D	D	D	D	D	D	D	D	Blank
D	D	D	D	D	D	D	D	D	D	D	D	Blank
E	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	VEGF
F	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	VEGF
G	A	A	A	A	A	A	A	A	A	A	A	VEGF
H	A	A	A	A	A	A	A	A	A	A	A	VEGF

Plate 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	D	D	D	D	D	D	D	D	D	D	D	Blank
B	D	D	D	D	D	D	D	D	D	D	D	Blank
C	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	Blank
D	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	Blank
E	A	A	A	A	A	A	A	A	A	A	A	VEGF
F	A	A	A	A	A	A	A	A	A	A	A	VEGF
G	C	C	C	C	C	C	C	C	C	C	C	VEGF
H	C	C	C	C	C	C	C	C	C	C	C	VEGF

IH = In-house reference standard; Blank = Cells only control; VEGF = Cells+VEGF165 control

Plate 4: see next page

Example Layout B (Plate 4 only)– Dilutions across the plate
Please vary the order of IH, A, B on the plate in subsequent assays as shown below:

Assay 1: Plate 4

	1	2	3	4	5	6	7	8	9	10	11	12
A	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	Blank
B	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	Blank
C	A	A	A	A	A	A	A	A	A	A	A	Blank
D	A	A	A	A	A	A	A	A	A	A	A	Blank
E	B	B	B	B	B	B	B	B	B	B	B	VEGF
F	B	B	B	B	B	B	B	B	B	B	B	VEGF
G	C	C	C	C	C	C	C	C	C	C	C	VEGF
H	C	C	C	C	C	C	C	C	C	C	C	VEGF

Assay 2: Plate 4

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	B	B	B	B	B	B	B	B	B	B	Blank
B	B	B	B	B	B	B	B	B	B	B	B	Blank
C	C	C	C	C	C	C	C	C	C	C	C	Blank
D	C	C	C	C	C	C	C	C	C	C	C	Blank
E	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	VEGF
F	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	VEGF
G	A	A	A	A	A	A	A	A	A	A	A	VEGF
H	A	A	A	A	A	A	A	A	A	A	A	VEGF

Assay 3: Plate 4

	1	2	3	4	5	6	7	8	9	10	11	12
A	C	C	C	C	C	C	C	C	C	C	C	Blank
B	C	C	C	C	C	C	C	C	C	C	C	Blank
C	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	Blank
D	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	IH	Blank
E	A	A	A	A	A	A	A	A	A	A	A	VEGF
F	A	A	A	A	A	A	A	A	A	A	A	VEGF
G	B	B	B	B	B	B	B	B	B	B	B	VEGF
H	B	B	B	B	B	B	B	B	B	B	B	VEGF

IH = In-house standard; Blank = Cells

Medicines & Healthcare products Regulatory Agency

NIBSC
Confidence in Biological Medicines

WHO International Standard
1st International Standard for Ranibizumab
NIBSC code: 23/124
Instructions for use
(Version [Q-DOCS_Version], Dated [Q-DOCS_Date_Published])

§

1. INTENDED USE

The International Standard 23/124 is intended to support the calibration,characterisation and validation of assays used for assessing Ranibizumab and to support the establishment of inhouse standards.

The standard was assessed in an international collaborative study (described in section 3), for in vitro biological activities of ranibizumab.

2. CAUTION

This preparation is not for administration to humans or animals in the human food chain.
The preparation contains material of human origin, and either the final product or the source materials, from which it is derived, have been tested and found negative for HBsAg, anti-HIV and HCV RNA. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

Bioactivity: The preparation has been assigned the following arbitrary unitage per ampoule:

1,000 international units (IU)* of vascular endothelial growth factor 165 (VEGF165) neutralising activity.
1,000 IU of VEGF165 binding activity.

*These units are independent of the amount of VEGF165 used in various bioassays. For details regarding neutralising activity in terms of the 1st WHO IS for VEGF165 (coded 19/246), see report referenced in section 9. It should be noted that the neutralising activity may vary according to the assay format. Therefore, a relationship between the unitage of the WHO IS coded 23/124 and the activity assigned to in-house standards in the assay system in routine use should be established by the user.

Users should also note that the biological activity of VEGF165 is likely to vary between different suppliers and this should be controlled by use of an appropriate standard (e.g. WHO IS for VEGF165).

The Ranibizumab IS was tested in a multi-centre collaborative study involving 18 laboratories in 12 countries. Participants tested the IS using assays established in-house, and reported results for VEGF neutralisation (using endothelial cell proliferation, reporter gene and enzyme-fragment complementation) and binding assays (see reference in section 9).

It should be noted that the unitage or mass content of the standard should not be used to define the specific activity of Ranibizumab products for regulatory purposes nor to describe product labelling or dosage requirements. Furthermore, the standard and its unitage is not intended to serve any regulatory role in defining biosimilarity, and should not be inferred as serving this purpose.

4. CONTENTS

Country of origin of biological material: United Kingdom.
Each ampoule contains the residue after freeze-drying of 1.0 ml of a solution containing:

Ranibizumab, approximately 50 micrograms
25 mM tri-sodium citrate dihydrate
150 mM sodium chloride
1.0% human serum albumin

5. STORAGE

Unopened ampoules should be stored at -20°C, or below.
For economy of use, it is recommended that the solution be subdivided into aliquotes and stored at -40°C or below - avoiding repeated thawing/freezing.

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

6. DIRECTIONS FOR OPENING

DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body. Various types of ampoule breaker are available commercially. To open the ampoule, tap the ampoule gently to collect material at the bottom (labelled) end and follow manufactures instructions provided with the ampoule breaker.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution

Dissolve the total contents of the ampoule in 1.0 ml of sterile distilled water. Use carrier protein where extensive dilution is required. Users should note that in rare instances interference due to excipients may occur if the IS is used at high concentrations ($\geq$ 10 µg/ml).

8. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.

NIBSC follows the policy of WHO with respect to its reference materials.

Accelerated degradation studies have indicated that this material is suitably stable, when stored at -20°C or below, for the assigned values to remain valid until the material is withdrawn or replaced. These studies have also shown that the material is suitably stable for shipment at ambient temperature without any effect on the assigned values. Once reconstituted, diluted or aliquoted, users should determine the stability of the material according to their own method of preparation, storage and use. Users who have data supporting any deterioration in the characteristics of any reference preparation are encouraged to contact NIBSC.

9. REFERENCES

This standard was produced under WHO guidelines cited in the WHO Technical Reports Series, No. 932, 2006, Annex 2.

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Potters Bar, Hertfordshire, EN6 3QG. T +44 (0)1707 641000, nibsc.org
WHO International Laboratory for Biological Standards,
UK Official Medicines Control Laboratory

World Health Organization

10. ACKNOWLEDGEMENTS

We are thankful to Samsung Bioepis for their generous donation of the Ranibizumab material used in the collaborative study. We are grateful to all participants of the collaborative study for their contribution in evaluating the candidate preparations.

11. FURTHER INFORMATION

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

12. CUSTOMER FEEDBACK

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

13. CITATION

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

14. MATERIAL SAFETY SHEET

Classification in accordance with Directive 2000/54/EC, Regulation (EC) No 1272/2008: Not applicable or not classified

Physical and Chemical properties			
Physical appearance: Freeze dried powder		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	No	Irritant:	No
Flammable:	No	Handling: See caution, Section 2	
Other (specify):	Contains material of human origin		

Toxicological properties	
Effects of inhalation:	Not established, avoid inhalation
Effects of ingestion:	Not established, avoid ingestion
Effects of skin absorption:	Not established, avoid contact with skin

Suggested First Aid	
Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.

Action on Spillage and Method of Disposal
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.

15. LIABILITY AND LOSS

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

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 23.5mg
Toxicity Statement: Toxicity not assessed
Veterinary certificate or other statement if applicable. **Attached**: No

17. CERTIFICATE OF ANALYSIS

NIBSC does not provide a Certificate of Analysis for WHO Biological Reference Materials because they are internationally recognised primary reference materials fully described in the instructions for use. The reference materials are established according to the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards https://www.who.int/publications/m/item/annex2-trs932(revised 2004). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.