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International collaborative study to assess the suitability of the candidate 7th WHO International Standard for rabies vaccine

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**** See Appendix 1***

NOTE:
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Summary

The WHO International Standard (IS) for rabies vaccine is used in the standardisation of rabies vaccines in NIH mouse potency tests and in in vitro assays for glycoprotein content. A collaborative study was undertaken to assess the suitability of a candidate 7th IS (NIBSC code 16/204), which was prepared from a bulk of Vero cell-derived, Pitman Moore strain, rabies vaccine, and to calibrate it in International Units (IU).

Sixteen laboratories from 12 countries assayed the the candidate standard (in duplicate) against the 6th IS. Ten data sets were returned for the NIH mouse potency test, 9 data sets for enzyme-linked immunosorbent assay (ELISA) and 6 data sets for single radial immunodiffusion (SRD) assays. The overall geometric mean potency of the candidate 7th IS, as determined by Hubert's method for robust estimation, was 8.9 IU/ampoule in NIH tests. The mean rabies glycoprotein antigen content of the candidate in in vitro assays was 2.45 IU/ampoule in ELISA and 2.9 IU/ampoule in SRD. The inter-laboratory geometric coefficient of variation (GCV) for the candidate standard 16/204 in NIH tests was 51% and 10.5% and 8.2% in ELISA and SRD, respectively.

Ampoules of the candidate IS stored at elevated temperatures for ~13 months were assayed in ELISA. While there was an initial drop of 3.8% glycoprotein content for ampoules stored at -20°C compared to those at -70°C, there was no further decrease in the antigen content for samples stored at 4°C and 20°C. An accelerated degradation study is underway using the Arrhenius model to further predict the long-term degradation rate of the candidate and provide additional data on the stability of the material. The candidate 7th IS will be dispatched on dry ice until additional data is obtained for informing the stability of the material upon long-term storage and shipment at ambient temperatures.

The results of this collaborative study demonstrate that the performance of the candidate 7th IS is comparable to those observed in the previous collaborative study establishing the 6th IS. The implementation and use 16/204 as the WHO 7th IS Rabies Vaccine will enable the continued standardisation and control of the NIH mouse potency assay for rabies vaccine, as well as ELISA and SRD assays for glycoprotein content, performed during routine quality control by manufacturers or as part of the batch release of rabies vaccines as required by regulatory authorities. 16/204 will also facilitate the evaluation of the factors that contribute to assay sensitivity and variability and assist in the validation of assays under development to replace, reduce or refine the in NIH test. It is therefore proposed that 16/204 is established as the 7th WHO International Standard for Rabies vaccine with an assigned unitage of 8.9 IU/ampoule when assayed in in vivo NIH mouse potency tests. For measuring glycoprotein content in in vitro assays, it is proposed that the candidate standard 16/204 is established with an assigned unitage of 2.45 IU/ampoule in ELISA and 2.9 IU/ampoule in SRD.

Introduction

Quality control testing for vaccines is mandatory for batch release to ensure safety and potency of each batch. One of the most important tests for rabies vaccines is the potency test which gives an indication of the consistency of production of vaccine batches with respect to batches which have previously been shown to be efficacious in clinical trials. The currently required potency test for rabies vaccine for human use e.g. [1, 2] is the National Institutes of Health (NIH) mouse potency test [3]. This assay is also used to test product stability for establishing shelf-life.

The surface of the glycoprotein of the rabies virus is the immunogenic antigen included in rabies vaccines and is responsible of the protection against the rabies virus. The quantification of glycoprotein in rabies vaccines by e.g. single radial immunodiffusion (SRD) or enzyme-linked immunosorbent assay (ELISA) is used routinely during manufacture at different process stages and, in particular, at the final bulk stage allowing definition of the final antigen content per dose of vaccine. Only the native form of the rabies glycoprotein is able to induce protective neutralizing antibodies. Based on this and considering the 3Rs approach to animal testing, the EDQM and other organisations are developing an ELISA method using monoclonal antibodies against the trimeric form of the glycoprotein to replace the NIH test for determining rabies vaccine potency [4, 5].

The WHO's Expert Committee on Biological Standardization (ECBS) establishes reference standards for biological substances used in the prevention, treatment or diagnosis of human disease. WHO International Standards (IS) are recognized as the highest-order of references for biological substances and are assigned potencies in International Units (IU). Their primary purpose is to calibrate secondary references used in routine laboratory assays in terms of the IU thereby providing a uniform results-reporting system and traceability of measurements independent of the method used. International Standards are prepared in accordance with published WHO recommendations [6].

Rabies vaccines for human use are produced in many countries and the minimum potency requirements are expressed in IU. The WHO International Standard for rabies vaccine (inactivated) is used by manufacturers of human and veterinary vaccines and control testing laboratories for the standardisation of potency and glycoprotein assays e.g. [2-4].

The stock of the current International Standard (6^{th} IS NIBSC code 07/162) is essentially depleted and a candidate replacement has been prepared using bulk vaccine manufactured by the same process as the 6^{th} IS. The proposal to develop, assess and establish the candidate replacement IS for rabies vaccine was endorsed by ECBS in October 2016. In 2017/18, laboratories with established assays for rabies vaccine participated in a WHO international collaborative study to assess the suitability of a candidate standard (NIBSC code 16/204) to serve as the 7^{th} IS for Rabies vaccine. This report presents the findings of the collaborative study.

Aims of study

The aims of this WHO international collaborative study [6] are to assess the candidate IS against the 6^{th} IS in

- the NIH mouse potency assay,

in

- in vitro assays of rabies vaccines for glycoprotein content such as SRD and ELISA

and to

- assign a unitage to the candidate IS in International Units (IU) per ampoule. There is no international conventional reference measurement procedure for rabies vaccine potency and the IU is not readily traceable to the International System of Units (SI) of quantity.

It was originally intended that the study would be conducted in 2 phases with phase 1 involving the assessment and calibration of the candidate in assays of rabies vaccines in the NIH mouse test followed by phase 2 whereby the candidate would be tested in alternative in vivo potency assays such as modified mouse immunisation and challenge or serology assays under development or in use by laboratories as a 3Rs approach to in vivo potency testing. Participants of both study phases also were given the opportunity to assess the candidate in their in vitro assays (e.g. ELISA, SRD). However, due to low uptake of the alternative in vivo assays by prospective participants, the structure of the collaborative study was modified to involve the NIH test and in vitro potency assays only.

Materials and Methods

Table 1 lists the collaborative study samples. Sample sets were shipped on dry ice to participants under NIBSC dispatch reference CS605.

Formulation of the candidate 7th IS for Rabies vaccine (NIBSC code 16/204)

The bulk vaccine provided to NIBSC for formulating the candidate 7th IS is a batch produced by the same manufacturing process used to prepare the bulk that went into the formulation of the 6th IS [7]. The purified vaccine bulk was derived from a beta-propiolactone-inactivated Pitman-Moore (PM) strain of virus grown in Vero cells. Human albumin was added as a stabiliser and the batch used in the production of the rabies bulk was produced from a plasma pool which had been tested and found negative for HBsAg, HCV RNA and anti-HIV 1+2. The bulk vaccine was provided by the donor at a potency of 112 IU/mL according to their validated SRD assay performed during its manufacture.

Filling and freeze-drying

Table 2 lists an overview of the filling and freeze-drying procedure for the candidate 7th IS (NIBSC code 16/204). Using the same approach for preparing the 6th IS, the donated bulk vaccine was formulated to give a target potency of 7 IU/ml by SRD. The formulated candidate bulk was dispensed in 0.5mL aliquots into 5mL glass DIN ampoules and freeze dried under an atmosphere of nitrogen at NIBSC in September 2016 using documented procedures. This fill was 0.5g by liquid weight with a mean of 0.5238g (n=372) and coefficient of variation (%CV) 0.3255%. The mean dry weight of 6 ampoules of freeze-dried product was 0.0551g giving a %CV of 0.3478%. The variability of the fill and dry weight, as indicated by the %CVs, is acceptable with each parameter meeting the production target %CV of <1.0% [6]. Residual moisture and oxygen content were determined for 12 ampoules of 16/204 giving a mean of 0.4% (%CV, 20.5%) and 0.13% (%CV of 81.37%), respectively. These values are within NIBSC's working limits of <1% and <1.14%, respectively, indicating that exposure to atmospheric moisture and oxygen was kept to a minimum during freeze-drying and sealing. There is no formal pass or fail criterion for residual moisture content for WHO international Standards, but typically, biological preparations with a moisture content of less than 1% (w/w) have shown adequate long-term stability [6]. Also, from NIBSC's experience, it is not unusual to have relatively high %CVs for residual moisture

and oxygen content when measuring samples with contents approaching the sensitivity limits of the assays.

11,098 ampoules were filled and approximately 10,000 ampoules are available for issue as the International Standard. Ampoules are held at or below -20°C at NIBSC under controlled conditions.

Study samples: Blinded duplicates of the 7th IS, 6th IS for Rabies vaccine NIBSC code 07/162, former 5th IS for Rabies vaccine (NIBSC code RAV) and a liquid formulation of the 6th IS.

Blinded and coded duplicates of the candidate standard (Samples A & B) were prepared for use in the collaborative study. The current IS (07/162) [7] was provided in the study as calibrator for assigning unitage to the candidate 7th IS. Some laboratories performing the NIH assay were also provided with the 5th IS [8] which is available in limited amounts. Laboratories performing in vitro assays were provided with aliquots of liquid bulk 6th IS (Sample C) having been left over from the previous collaborative study and stored at -70°C in the interim. The 5th IS and Sample C were included in the study to provide additional information for assessing the expected potency of the 5th IS as well as intra-laboratory and inter-laboratory variability.

Study protocol

The final version of the study protocol is given in Appendix 2. In brief, participants were requested to:

- perform 3 independent assays
- reconstitute the freeze-dried samples as directed in the instructions for use. For the NIH assay, the reconstitution volume is 1mL distilled water. For in vitro assays such as ELISA and SRD, the volume is 0.5mL distilled water.
- prepare and test a series of dilutions for the 6th IS and each study sample and **test all samples concurrently**
- use a freshly opened vial or reconstituted ampoule for each assay. For the NIH test, a sufficient number of ampoules of the standard and freeze-dried study samples were supplied so that a fresh ampoule could be used for each of the two inoculations administered to mice.

Participants

Sixteen laboratories from 12 countries completed the study. The participants were from Argentina (2), Canada (1), France (2), Germany (2), India (2), Mexico (1) Russian Federation (1), Serbia (1), South Africa (1), Thailand (1) UK (1) and USA (1). All laboratories are referred to by code number allocated at random and not representing the order of listing in Appendix 1.

Assay methods

Assays used by participants are summarised in Table 3. Where laboratories performed multiple assay methods, laboratory codes are followed by a letter indicating the different methods e.g. lab 3a, 3b,3c.

NIH Mouse potency assay
Details of the NIH assay reported by each lab are shown in Table 4. Participants were requested to assay the study samples in the NIH mouse potency test as established in their laboratory.

In vitro assays for glycoprotein content
Participants were also requested to assay the study materials in any *in vitro* assays performed routinely in their laboratory. Details reported by each lab are shown for ELISA in Table 5 and SRD in Table 6. As some of the vaccine samples contain human albumin as stabiliser, participants were informed that the SRD gels should be soaked in PBS for 24 -48 h to wash out any unbound protein.

Cell-based in vitro antibody binding test (ABT)
One laboratory returned results for an in vitro ABT method whereby two-fold vaccine dilutions are incubated for one hour at 37°C with a fixed amount of rabies antibody (appropriate dilution of human rabies immunoglobulin). Subsequently, fixed amount of rabies strain CVS-11 is added, and mixtures are further incubated for one hour at 37°C. Finally, MNA cell suspension is added and the microplate is incubated for 19-23 hours at 37°C. The microplate is then fixed with 85% acetone, stained with FITC-anti-rabies conjugate and observed under the fluorescence microscope [9]. The raw data obtained represent ratios of fields with specific fluorescence/total of 20 field per dilution.

Statistical methods

An excel spreadsheet was provided so that all essential information could be recorded, including the raw data from each assay. Calculations were performed for NIH, ELISA and SRD results at NIBSC using the EDQM CombiStats software version 5.0 [10, 11]. For all assays, data for each preparation were analysed separately against the 6th IS. The potency estimates for each study sample are therefore based on direct pair-wise comparisons. Results for the ABT method were calculated and reported by the participant.

NIH mouse potency tests
Data from in vivo assays were analysed by probit parallel-line bioassay analysis [12] comparing transformed assay responses to log dose using the 6th IS as the reference with an assigned unitage of 8 IU/mL when reconstituted as directed in 1.0 mL of water. The data were checked to verify that all samples covered a range of responses, as any sample where 50% response was not covered by the range of doses used would be excluded from further analysis. Assays where non-linearity or non-parallelism was significant at the 1% were excluded from further analysis.

ELISA
ELISA data were analysed using a parallel line or sigmoid curve model with untransformed or log transformed responses. Model fit was assessed visually, and non-parallelism was assessed by calculation of the ratio of fitted slopes for the test and reference samples under consideration. The samples were concluded to be non-parallel when the ratio of fitted slopes (as calculated by CombiStats [10] according to the method in the European Pharmacopoeia chapter 5.3 [11]) was outside of the range 0.80 – 1.25 and no estimates are reported. Potencies of the duplicate candidate IS samples A and B along with Sample C are expressed relative to the 6th IS having an assigned unitage of 6.6 IU/ml when reconstituted as directed in 0.5 ml water i.e. 3.3 IU/ampoule.

Single radial immunodiffusion tests
SRD assays were analysed as slope-ratio assays. The statistical validity of the test in relation to linearity of dose responses and common intercept. Only assays at 1% level of significance for linearity and common intercept were accepted as valid. Potencies of the duplicate candidate IS samples A and B along with Sample C are expressed relative to the 6th IS, having an assigned unitage of 6.6 IU/ml when reconstituted as directed in 0.5 ml water i.e. 3.3 IU/ampoule.

Cell-based in vitro antibody binding test
For the in vitro ABT assay, the participant reconstituted the 6th IS, Sample A and Sample B in 1mL water. Potencies of the study samples were determined by the participant using the Spearman-Kaerber method.

Calculation of geometric mean potencies, intra-laboratory variability and inter-laboratory variability
Relative potency estimates from all valid assays were combined to generate a weighted or semi-weighted geometric mean (GM) for each laboratory and assay type [10, 11]. Overall mean potencies were calculated as the geometric means of the laboratory means. Variability between assays within laboratories and between laboratories has been expressed using geometric coefficients of variation ($GCV = [10^{s}-1]\times100\%$ where s is the standard deviation of the log10 transformed estimates) [10, 11]. Due to possible outliers and anomalous results, Huber's robust mean was also calculated using the R package 'WRS2'[13].

Results and data analysis

Validation of the Candidate 7th IS for Rabies vaccine (NIBSC code 16/204)

To assess whether the freeze-dried candidate material (16/204) was suitably potent for assessment in the collaborative study, it was tested by two reference laboratories prior to its distribution to participants.

Reference Laboratory 1 (RL1) tested the candidate in their validated NIH test and ELISA method (Tables 7 & 8). For the NIH test (Table 7), potencies were expressed relative to the 6th IS Rabies Vaccine (07/162). Combining the potencies obtained from 3 independent NIH assays gives a geometric mean (GM) of 7.09 IU/mL with a 95% confidence interval (CI) of 4.24 – 11.83 IU/mL.

For the ELISA assay (Table 8), RL1 reported potencies relative to the 6th IS Rabies Vaccine, and against an in-house standard calibrated against the 6th IS. For in vitro assays, the 6th IS has an assigned potency of 6.6 IU/ml when reconstituted in 0.5mL water which is equivalent to 3.3 IU/ampoule. RL1 stated that ampoules of the 6th IS and candidate 7th IS were reconstituted in 1 mL. Therefore, for this assessment, relative potencies are reported in Table 8 in UI/ampoule. RL1 concluded that the ELISA results are satisfactory in that the GM titres of the candidate 7th IS are identical (2.6 IU/ampoule) for the two references used (6th IS and in-house secondary standard) and the intra-laboratory variability as determined by the %CV is low (2.3% for the 6th IS and 6.2% for the in-house standard).

Reference Laboratory 2 (RL2) tested the candidate standard in their SRD utilizing 2 different commonly used monoclonal antibodies H26 MAb and H65 MAb (Table 9). For each SRD method, GM potencies were combined from 3 independent assays and expressed relative to the 6th IS (6.6 IU/mL when reconstituted in 0.5mL). The GM potency of the candidate in SRD:H26 Mab is 5.578 with a 95% CI of 4.904– 6.344 IU/mL (Table 9a) and in SRD:H65 Mab it is 7.031 with a 95% CI of 6.125– 8.072 IU/mL (Table 9b). These values for the freeze-dried candidate approximate the 7 IU/mL formulation target of the liquid bulk.

In conclusion, the validation assessment of the freeze-dried material indicated that the candidate 7th IS was suitable for further assessment in the international collaborative study.

Collaborative study data received

Sixteen laboratories returned data sets for 26 assays (Table 3). Ten labs (2, 3a, 5a, 6a, 7a, 9b, 10, 11 12b, 14) returned data for the NIH test. Eight labs reported results for 9 ELISAs (3b, 4, 6b1, 6b2, 7b, 9a, 12a, 15, 16). For laboratory 6, two technicians performed the ELISA test in parallel using the same reconstituted samples. For laboratory 6, the combined operator results (6b1 and 6b2) were therefore used to determine overall GM and %GCV. Assays for laboratory 9a were invalid due to confidence intervals for individual assay falling outside the 80%-120% range. Five labs returned data for 6 SRD assays (1, 3c, 7c, 8, 13a, 13b). For laboratory 13, SRD methods using different MAbs were performed. One laboratory (5b) calculated and provided results for their in vitro ABT method.

Intra-assay variability

Intra-assay variability was assessed by expressing the potency estimates for the coded duplicate samples A and B relative to each other within each assay i.e. as $M=Max(RP_A,RP_B)/min(RP_A,RP_B)$ where RP_A and RP_B denote the potency estimates obtained for samples A and B respectively. Geometric mean values of M were 1.48, 1.07 and 1.11 for the NIH, ELISA and SRD assays respectively, demonstrating that the highest level of intra-assay variability was for the NIH assays and the lowest level was for the ELISAs, as would be expected. Except for lab 2, assay 2 which gave a value of 5.19, the maximum observed coded duplicate difference in NIH assays was 3.22. For the ELISAs, only lab 16, assay 3 gave a value (1.47) greater than 1.21. For the SRD assays, only lab 1, assay 2 gave a value (1.73) that exceeded 1.37. Given that all the individual assay estimates had fully met the statistical criteria for validity that were applied, and that exclusion of the cases noted above resulted in negligible changes to the final proposed values (e.g. 9.1 IU/ampoule compared to 8.8 IU/ampoule for 16/204 in NIH assays, with even smaller differences in ELISA and SRD), all results were included for the subsequent analysis and final assignment of potencies.

NIH Test potency estimates relative to the 6th IS

The individual laboratory geometric mean potencies expressed relative to the 6th IS along with the intra-laboratory %GCVs are shown in Tables 10 & 11 for the candidate 7th IS blinded duplicate Samples A and B. For each sample, the overall geometric mean potency across laboratories, is also shown, along with the inter-laboratory %GCV, which measures between-laboratory variability.

Individual laboratory GM potencies for Sample A in the NIH test range from 5.696 IU/mL to 17.448 IU/mL with intra-laboratory variability ranging from 5% to 102% (Table 10). For Sample B, laboratory GM potencies range from 5.264 IU/mL to 17.47 IU/mL with intra-laboratory %GCV ranging from 3% to 103% (Table 11). The geometric mean potencies across laboratories, for Sample A and Sample B, are 8.803 IU/mL (95% CI; 6.587-11.764) and 8.777 IU/mL (95% CI 6.395-12.045), respectively. The inter-laboratory variability assessed in terms of the overall %GCV for sample A and sample B is 50% and 56%, respectively.

Combining the NIH test data across the duplicates of the candidate standard (Samples A and B) gives an overall GM potency of 8.790 IU/mL (95% CI 7.208-10.722) and %GCV of 51% (Table 12).
The inter-laboratory variability for the candidate 7th IS in the NIH test in this collaborative study is a slight improvement to the published %GCV of 61.6% reported in the collaborative study that established the 6th IS [7]. Taking into consideration the inherent variability of the NIH Test, the overall potency

estimate for the candidate 7th IS is in agreement with the estimated potency of 7.09 IU/mL (95% CI 4.24-11.83) reported by RL1 in the validation assessment (Table 7).

The four laboratories testing RAV in the NIH test returned an overall GM potency of 15.931 IU/mL (95% CI; 8.216-30.890). This value is in very good agreement with its assigned potency of 16 IU/mL providing added confidence that the continuity of the unitage has been maintained for the 6th IS. The overall %GCV of 52% for RAV in the NIH test in this collaborative study again reflects the inherent variability expected for this method (Table 13) [7].

ELISA potency estimates relative to the 6th IS

For ELISA, the individual laboratory geometric mean potencies expressed relative to the 6th IS along with the intra-laboratory %GCVs are shown in Tables 14 & 15 for the candidate 7th IS blinded duplicate Samples A and B. The overall geometric mean potency and %GCV across laboratories are also shown.

Individual laboratory GM potencies for Sample A in ELISA range from 4.333 IU/mL to 5.647 IU/mL with intra-laboratory variability ranging from 0.6% to 25% (Table 14). For Sample B, laboratory GM potencies range from 4.222 IU/mL to 5.635 IU/mL with intra-laboratory %GCV ranging from 2.9% to 10.1% (Table 15). The geometric mean potencies across laboratories, for Sample A and Sample B, are 4.954 IU/mL (95% CI; 4.528-5.421) and 4.915 IU/mL (95% CI 4.441-5.439), respectively. The inter-laboratory variability assessed in terms of the overall %GCV for sample A and sample B is 10.2% and 11.6%, respectively.

Combining the ELISA data across the duplicate samples gives an overall GM potency of 4.937 IU/mL (95% CI 4.650- 5.242) or 2.469 IU/ampoule with an overall combined %GCV of 10.5 % (Table 16). The overall potency estimate for the candidate 7th IS in ELISA is in agreement with the estimated potency of 2.6 IU/ampoule reported by RL1 in the validation assessment (Table 8).

For Sample C by ELISA, laboratories returned an overall GM potency of 7.388 IU/mL (95% CI; 2.744-11.235) and an overall %GCV of 11.2% (Table 17).

SRD potency estimates relative to the 6th IS

For SRD, the individual laboratory geometric mean potencies expressed relative to the 6th IS along with the intra-laboratory %GCVs are shown in Tables 18 & 19 for the candidate 7th IS blinded duplicate Samples A and B. The overall geometric mean potency and %GCV across laboratories are also shown.

Individual laboratory GM potencies for Sample A in SRD range from 5.212 IU/mL to 6.203 IU/mL with intra-laboratory variability ranging from 4.9% to 16.6% (Table 18). For Sample B, laboratory GM potencies range from 5.011 IU/mL to 6.349 IU/mL with intra-laboratory %GCV ranging from 0.6% to 8.4% (Table 19). The geometric mean potencies across laboratories, for Sample A and Sample B, are 5.710 IU/mL (95% CI; 5.307-6.144) and 5.882 IU/mL (95% CI 5.347-6.471), respectively. The inter-laboratory variability assessed in terms of the overall %GCV for sample A and sample B is 7.2% and 9.5%, respectively.

Combining the SRD data across the duplicate candidates gives an overall GM potency of 5.773 IU/mL (95% CI 5.490- 6.071) or 2.89 IU/ampoule with an overall combined %GCV of 8.2 % (Table 20).

For Sample C by SRD, laboratories returned an overall GM potency of 6.711 IU/mL (95% CI; 5.970-7.543) and an overall %GCV of 9.9% (Table 21).

In vitro ABT potency estimates relative to the 6th

The individual assay and overall GM potency estimates for Samples A, B and C reported by laboratory 5b in their in vitro ABT assay are shown in Table 22. The laboratory GM potencies for the duplicate samples are 6.7 and 6.6 IU/mL.

Stability studies

Table 23 lists the real-time results obtained for ampoules of 16/204 stored at -70°C, -20ºC, +4ºC, +20ºC and +37ºC for ~ 13 months and then placed at -70°C or less until assayed by RL1 in their validated ELISA. The glycoprotein antigen content of the samples was determined relative to an in-house standard calibrated against the 6th IS with results expressed as a percentage of the -70°C sample. While there is an initial drop of 3.8% for ampoules stored at -20°C compared to those at -70°C, there was no further decrease in the antigen content of the samples stored at 4°C and 20°C with estimates occurring within the range of assay variability (Table 23). An accelerated degradation study is underway using the Arrhenius model [14] to further predict the long-term degradation rate of the candidate and provide additional data on the stability of the material. The candidate 7th IS will be dispatched on dry ice until additional data is obtained for informing the stability of the material upon long-term storage and shipment at ambient temperatures.

Stability studies on reconstituted material have not been undertaken. It is anticipated that the contents of individual ampoules will be used on the day of reconstitution as the volumes required in both the in vivo potency tests and in vitro assays of glycoprotein antigen content mean that the contents would be used in a single assay. However, should users wish to store reconstituted material, they should determine the stability of reconstituted material according to their own method of preparation, storage and use. Multiple freeze/thaw cycles should be avoided.

Discussion

In this international collaborative study, the NIH, ELISA and SRD tests were used to evaluate the candidate 7th IS (16/204) in parallel to the current, 6th WHO IS (07/162). An in vitro ABT assay was also performed by one laboratory. Although informative, the limited amount of data obtained from the sole ABT method does not allow an adequate assessment of the suitability of the candidate 7th IS for standardising this assay method and is not considered further.

Serving as an additional benchmark, the 5th WHO IS (RAV) was included in the study for assay in the NIH test. A liquid bulk preparation retained from the production of the 6th IS (Sample C) was also included for testing in in vitro assays to allow supporting assessment of assay variability.

Both the candidate 7th IS and the 6th IS were prepared from purified vaccine bulks donated by the same manufacturer. The bulks were derived from a beta-propiolactone-inactivated Pitman-Moore (PM) virus strain grown in Vero cells. The manufacturer also provided the albumin-containing diluent for formulating the two standards. As the candidate 7th IS was to be calibrated directly against the 6th IS and to help maintain the continuity of the IU between the 6th IS and the new IS, the procedures at NIBSC for formulating, filling and freeze-drying were as similar as possible to those used in the development of the

6th IS. Furthermore, participants were instructed to reconstitute the candidate in the same volume(s) as previously established for the 6th IS (1 mL distilled water for NIH and 0.5 mL for ELISA or SRD).

When assayed against the 6th IS in the NIH test, the candidate 7th IS has a combined overall geometric mean potency of 8.8 IU/mL. With a %GCV of 51%, the interlaboratory agreement for the NIH test in this study is an improvement on the previous study that established the 6th IS (%GCV, 62%). This reduction in interlaboratory variability may be due to the like-for-like comparison between the candidate IS and the 6th IS as opposed to the 5th IS which was derived from a vaccine produced by a different manufacturer.
The corresponding values for the candidate are 4.9 IU/mL and 11%GCV in ELISA and 5.8 IU/mL and 8%GCV in SRD (Summarised in Table 24).

To account for possible outliers and anomalous results, Huber's robust means were also determined for the different assay methods (Table 24). The difference between the overall GM and the Huber's robust mean estimates for the candidate in each of the assay methods are minimal when assay variability is taken into consideration. Nevertheless, the proposal to the WHO ECBS is based on the robust mean values for each of the three assay methods. As for the previous study, the overall GM for the candidate 7th IS is greater than the means obtained by the ELISA and SRD assays. Furthermore, the inter-laboratory variability observed for the NIH, ELISA and SRD assays in this study are in line with those reported in the previous study with the NIH test having greater variability than the in vitro assays [7].

In summary, the results of this collaborative study demonstrate that the performance of the candidate 7th IS is comparable to those observed in the previous collaborative study establishing the 6th IS. The implementation and use 16/204 as the WHO 7th IS Rabies Vaccine will enable the continued standardisation and control of the NIH mouse potency assay for rabies vaccine as well as ELISA and SRD assays for glycoprotein content performed during routine quality control by manufacturers or as part of the batch release of rabies vaccines as required by regulatory authorities. The 7th IS will also facilitate the evaluation of the factors that contribute to assay sensitivity and variability and assist in the validation of assays under development to replace, reduce or refine the in NIH test.

Proposal

It is proposed that 16/204 is established as the 7th WHO International Standard for Rabies vaccine with an assigned unitage of 8.9 IU/ampoule when assayed in in vivo NIH mouse potency tests.
For measuring glycoprotein content in in vitro assays, it is proposed that the candidate standard 16/204 is established with an assigned unitage of 2.45 IU/ampoule in ELISA and 2.9 IU/ampoule in SRD.
The proposed unitage does not carry an uncertainty associated with its calibration. The only uncertainty is therefore derived from the variability of the dry fill weight of the ampoule content which had a coefficient of variation of 0.3478%.

The volume of distilled water recommended for reconstitution of 16/204 for use in the in vivo NIH tests is 1mL to give a potency of 8.9 IU/mL. For use in ELISA and SRD, the recommended volume for reconstitution is 0.5ml to give a glycoprotein content of 4.9 IU/mL for ELISA and 5.8 IU/mL for SRD.

The 7th IS will be shipped on dry ice until the stability study is completed and showing that the candidate is suitably stable for shipment at ambient temperature. The draft instructions for use is shown in Appendix 3.

Comments from participants

Ten participating laboratories responded to the report. There were no disagreements with the suitability of the candidate (NIBSC code 16/204) to serve as the 7th WHO IS for Rabies Vaccine. Some respondents had queries or suggestions for editorial changes and these have been addressed. One participant indicated that Huber's robust means should be calculated for the three assay methods to account for outliers. This has been added to the report.

Participants were asked for their opinion on the suitability and feasibility of assigning a single unitage to the candidate standard for use across the assay methods. One participating laboratory commented, "The practice of using International Units and providing a uniform results-reporting system can be useful however we must be cautious when interpreting the results and comparing potency estimates between assays. …It is not favorable to assign a single unitage for the 7th IS. The *in vivo* and the *in vitro* assays measure different biological parameters and have considerably different overall %GCV. The higher variability (*i*.e. 51.1% GCV) associated with the NIH mouse assay and its effect on the potency estimate when combined into a single unitage for the three assays, will influence potency assessments as measured by *in vitro* assays. This same rationale can be extended to combining the potency results for the ELISA and SRD assays. Therefore, in our opinion, it is preferable to assign three separate potencies for the 7th IS, one for each of the three assays studied.".

Three additional laboratories provided similar opinions in support of the proposal to assign three separate unitages for the candidate 7th IS for use in the different assay methods. No participant indicated a preference to assigning a single unitage to the candidate for the three assay methods or even a single unitage for use in ELISA and SRD methods combined and a separate unitage for the NIH assay.

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Tables

Table 1. Collaborative study samples shipped under NIBSC dispatch reference CS605.

Sample ID	Sample Description	sample volume filled per tube (mL)	Presentation	Assigned or target potency
07/162	6th IS for Rabies Vaccine	0.5	Freeze-dried	8 IU/ml in 1 mL (NIH) 6.6 IU/ml in 0.5 mL (ELISA and SRD)
RAV	5th IS for Rabies Vaccine	1.0	Freeze-dried	16 IU/mL in 1 mL (NIH test)
Sample A	Candidate 7th IS	0.5	Freeze-dried	Target formulation potency based on vaccine bulk antigen content. 7 IU/mL by SRD
Sample B	Candidate 7th IS	0.5	Freeze-dried	
Sample C	Bulk preparation remaining from 6th IS production	1.0	Liquid	Not determined

Table 2. Production summary for the candidate International Standard for 7th IS Rabies Vaccine

NIBSC code	16/204
Product name	7th IS Rabies Vaccine
Collaborative study code	CS605
Filling machine	Bausch &Stobel AFV5090
Date of filling	22 September 2016
No. of ampoules filled	11,098
Mean fill weight (g)	0.5238
CV of fill weight (%)	0.3255 n=372
Freeze dryer	CS100
Date of completion of lyophilization	27 September 2016
Mean dry weight (g)	0.0551
CV of dry weight (%)	0.3478 n=6
Mean residual moisture (%)	0.4
CV of residual moisture (%)	20.5 n=12
Mean oxygen content (%)	0.13
CV of oxygen content (%)	81.37 n=12
No. of ampoules available to WHO as of March 2018	10,298

Table 3. Laboratory codes and assay methods.

Laboratory code	Method
1	SRD
2	NIH
3a	NIH
3b	ELISA
3c	SRD
4	ELISA
5a	NIH
5b	ABT
6a	NIH
6b1	ELISA technician 1
6b2	ELISA technician 2
7a	NIH
7b	ELISA
7c	SRD

Laboratory code	Method
8	SRD
9a	ELISA
9b	NIH
10	NIH
11	NIH
12a	ELISA
12b	NIH
13a	SRD
13b	SRD
14	NIH
15	ELISA
16	ELISA

Abbreviations: ABT: Antibody binding test. This method tests the antigen content of a vaccine after neutralization with a standard antibody in competition with rabies strain CVS-11

Table 4. Details of the NIH assay reported by each lab.

Lab Code	Mouse strain	Sex	Age	Weight	Challenge virus strain
2	CF1	F	Information not provided	Information not provided	CVS-11
3a	Information not provided				
5a	NMRI	M&F	4-6 weeks	Information not provided	CVS-27
6a	NMRI	F	4 weeks	11-15g	Information not provided
7a	NIH	M&F	Information not provided	11-15g	CVS-11
9b	ICR	M	5 weeks	Information not provided	CVS
10	Balb/c	M&F	Information not provided	13-16g	Vnukovo-32
11	NIH	M&F	4-6 weeks	Information not provided	CVS-11
12b	Swiss albino	M&F	Information not provided	11-14g	CVS-11
14	NIH	M&F	1-1.5 months	14-16g	11032011

Table 5. Details of the ELISA method reported by each lab.

Lab Code	coating	primary	detection	Antigen target
3b	Mab 1112-1	Mab D1-25 biotin conjugated	Streptavidin peroxidase	Not specified
4	Mab D1	Mab D1		Glycoprotein trimeric form
6	Rabbit anti-rabies	Mab TW17	Rabbit anti-mouse IgG peroxidase conjugated	Not specified
7b	Equine anti-rabies IgG	Mab	Donkey anti-mouse IgG	Glycoprotein
12a	Rabbit anti-rabies	Mab	Anti-mouse peroxidase conjugated	Not specified
15	Rabbit anti-rabies	Mab-TW17	Rabbit anti-mouse IgG peroxidase conjugated	Not specified
16	Mab	polyclonal		Not specified

Table 6. Details of the SRD method reported by each lab.

Lab Code	Antibody	Concentration (µL antibody/mL gel)
1	Anti-glycoprotein serum # 1699	5
3c	Information not provided	
7c	In-house anti-glycoprotein	7.5
8	MAb H65	11
13a	MAb H26	3
13b	MAb H65	3

Table 7. Reference laboratory 1 validation testing of 16/204 in NIH Test. Potency was expressed relative to the 6th IS Rabies Vaccine, which has an assigned potency of 8IU/ml when reconstituted in 1 mL water as described in the Instructions for use for 07/162.

NIH

Assay number	Date of assay	Sample	Info	Lower limit	Estimate	Upper limit
1	18/11/2016	1	Candidtate 7th IS 16/204	2.73345	6.74406	16.4173
2	22/11/2016	1	Candidtate 7th IS 16/204	4.04686	8.98820	20.0338
3	30/11/2016	1	Candidtate 7th IS 16/204	1.83010	5.19064	13.5577

Weighted combination			
(IU/mL)	Lower limit	Estimate	Upper limit
Potency	4.24373	7.08558	11.8305

Table 8. Reference laboratory 1 validation testing of 16/204 in ELISA. Potency was expressed relative to the 6th IS Rabies Vaccine, and an in-house standard calibrated against the 6th IS. For in vitro assays, the 6th IS has an assigned potency of 6.6IU/ml when reconstituted in 0.5mL water as described in the Instructions for use for 07/162. This is equivalent to 3.3 IU/ampoule.

ELISA Assay	Volume of reconstitution (mL)	Theoretical potency (mL) based on the laboratory's reconstitution volume of 1 mL.	Potency relative to in-house standard (IU/ampoule)	Potency relative to 6th IS Rabies Vaccine (IU/ampoule)
1	1	3.5	2.839	2.681
2	1	3.5	2.590	2.659
3	1	3.5	2.529	2.568
GM potency (IU/ ampoule)			2.6	2.6
			6.2%	2.3%
Equivalent GM potency if reconstituted in 0.5mL (IU/ mL)			5.2	5.2

Abbreviations CV=Coefficient of variation; GM = Geometric Mean

Table 9. Reference laboratory 2 validation testing of 16/204 in SRD. Potency was expressed relative to the 6th IS Rabies Vaccine. For in vitro assays, the 6th IS has an assigned potency of 6.6IU/ml when reconstituted in 0.5mL water as described in the Instructions for use for 07/162. The estimates are expressed as IU/mL.

9a) SRD using H26 MAb

Date of assay	Sample	Info	Lower limit	Estimate	Upper limit
08/11/16	1	7th IS Filled vial	3.82210	5.08456	6.40450
12/10/16	1	7th IS Filled vial	5.25812	6.40066	7.93755
17/11/16	1	7th IS Filled vial	3.59290	5.03218	6.53564

Weighted combination			
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	4.90428	5.57812	6.34454

9b) SRD using H65 Mab

Date of assay	Sample	Info	Lower limit	Estimate	Upper limit
08/11/16	1	7th IS Filled vial	4.48597	6.29848	8.68599
12/10/16	1	7th IS Filled vial	5.77184	7.24827	9.48932
17/11/16	1	7th IS Filled vial	5.78136	7.29312	9.38875

Weighted combination			
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	6.12460	7.03112	8.07181

Table 10. Rabies vaccine potencies for Sample A expressed relative to the 6th IS (8 IU/mL in 1 mL) in the NIH Test.

Lab Code	Assay	LCL	Potency (IU/mL)	UCL	Intra-laboratory %GCV	LCL	Laboratory GM Potency (IU/mL)	UCL
2	1	~	NP	~				
2	2	7.665	19.865	55.043	n/a	3.976	7.595	14.508
2	3	1.530	3.665	8.514				
3a	1	2.539	6.307	15.454				
3a	2	0.750	2.261	6.026	102%	3.424	5.696	9.475
3a	3	4.146	8.704	19.113				
5a	1	3.146	8.982	25.980				
5a	2	1.998	5.262	13.412	32%	3.703	6.416	11.117
5a	3	2.500	6.022	14.359				
6a	1	7.416	26.306	114.504				
6a	2	6.757	20.125	67.315	46%	8.965	17.448	33.958
6a	3	4.556	12.426	34.924				
7a	1	3.835	7.359	13.956				
7a	2	4.546	7.974	13.932	8%	5.624	7.978	11.317
7a	3	4.644	8.600	16.076				
9b	1	1.688	7.523	33.242				
9b	2	2.400	9.582	40.120	18%	3.518	7.919	17.827
9b	3	1.819	6.958	26.087				
10	1	~	NP	~				
10	2	3.536	13.167	46.724	n/a	3.972	11.358	32.482
10	3	1.241	8.494	46.310				
11	1	3.089	9.306	28.454				
11	2	3.558	8.373	19.759	29%	4.357	7.532	13.020
11	3	2.153	5.746	13.723				
12b	1	2.850	7.123	17.452				
12b	2	2.047	5.401	13.271	15%	3.580	6.149	10.562
12b	3	2.189	5.976	15.344				
14	1	7.302	16.330	37.638				
14	2	7.799	17.448	40.095	5%	11.005	17.353	27.361
14	3	8.727	18.144	38.086				

		LCL		UCL
N=10	overall GM (IU/mL)	6.587	8.803	11.764
Sample A	Overall %GCV		50%	

Abbreviations
LCL= Lower 95% confidence limit; UCL= Upper 95% confidence limit; GCV=Geometric coefficient of variation; GM = Geometric Mean; NP= Non-parallel; n/a = Not applicable

Table 11. Rabies vaccine potencies for Sample B expressed relative to the 6th IS (8 IU/mL in 1 mL) in the NIH Test.

Lab Code	Assay	LCL	Potency (IU/mL)	UCL	Intra-laboratory %GCV	LCL	Laboratory GM Potency (IU/mL)	UCL
2	1	7.848	15.479	30.681				
2	2	6.434	16.923	47.894	11%	10.678	16.905	26.762
2	3	8.754	19.028	42.678				
3a	1	2.911	7.844	21.101				
3a	2	1.944	5.243	13.663	46%	4.952	8.265	13.796
3a	3	5.271	11.224	24.021				
5a	1	3.112	7.866	19.858				
5a	2	2.629	6.592	16.311	35%	3.593	6.163	10.571
5a	3	1.626	4.380	11.248				
6a	1	3.847	12.952	46.895				
6a	2	4.817	16.429	64.671	25%	6.412	12.658	24.990
6a	3	3.777	10.549	30.091				
7a	1	~	NL	~				
7a	2	4.079	7.042	11.991	n/a	4.511	6.759	10.128
7a	3	3.439	6.412	11.680				
9b	1	2.994	14.20	83.24				
9b	2	0.783	3.82	15.11	103%	2.510	5.833	13.554
9b	3	1.203	4.69	16.31				
10	1	1.257	5.323	19.064				
10	2	0.906	4.093	14.708	27%	2.389	5.264	11.596
10	3	1.585	6.597	23.670				
11	1	6.885	18.284	52.102				
11	2	2.530	6.838	18.238	67%	5.758	10.272	18.324
11	3	3.240	8.854	24.352				
12b	1	3.514	8.068	18.547				
12b	2	2.752	6.422	14.512	35%	3.891	6.681	11.471
12b	3	0.988	4.448	15.436				
14	1	8.122	17.158	36.904				
14	2	7.592	17.053	39.584	3%	11.199	17.474	27.267
14	3	8.709	18.130	38.069				

		LCL		UCL
N=10	overall GM Potency (IU/mL)	6.395	8.777	12.045
Sample B	Overall %GCV		56%	

Abbreviations
LCL= Lower 95% confidence limit; UCL= Upper 95% confidence limit; GCV=Geometric coefficient of variation; GM = Geometric Mean; NP= Non-parallel; n/a = Not applicable;

Table 12. Combined Overall GM Potencies for blinded duplicates of the Candidate 7th IS Rabies Vaccine (16/204) [Samples A and B] in NIH.

		LCL		UCL
N=20	overall GM Potency (IU/mL)	7.208	8.790	10.722
Samples A and B	Overall %GCV		51%	
	MAX		17.474	
	MIN		5.264	

Table 13. Rabies vaccine potencies for 5th IS Rabies Vaccine (RAV) expressed relative to the 6th IS (8 IU/mL in 1 mL) in the NIH Test.

Lab Code	Assay	LCL	Potency (IU/mL)	UCL	Intra-laboratory %GCV	LCL	Laboratory GM Potency (IU/mL)	UCL
2	1	4.155	8.822	18.886				
2	2	14.723	36.492	101.174	105%	9.474	16.187	27.656
2	3	6.705	21.350	78.096				
3a	1	9.200	21.822	54.569				
3a	2	2.608	7.726	22.689	87%	11.126	18.264	29.982
3a	3	11.663	23.720	48.776				
6a	1	8.147	32.449	152.439				
6a	2	7.860	24.004	85.065	38%	10.811	24.201	54.171
6a	3	3.687	16.965	99.305				
7a	1	4.243	7.989	14.964				
7a	2	4.762	9.026	17.484	12%	6.299	9.002	12.866
7a	3	5.632	9.945	18.009				

		LCL		UCL
N=4	overall GM Potency (IU/mL)	8.216	15.931	30.890
RAV	Overall %GCV		52%	

Note: Not all laboratories received RAV for testing.
Abbreviations
LCL= Lower 95% confidence limit
UCL= Upper 95% confidence limit
GCV=Geometric coefficient of variation
GM = Geometric Mean

Table 14. Rabies vaccine potencies for Sample A expressed relative to the 6th IS (6.6 IU/mL in 0.5 mL) in the ELISA Test.

Lab Code	Assay	LCL	Potency (IU/mL)	UCL	Intra-laboratory %GCV	LCL	Laboratory GM Potency (IU/mL)	UCL
3b	1	5.076	5.190	5.305				
3b	2	5.064	5.226	5.393	4.0%	5.079	5.166	5.256
3b	3	4.614	4.868	5.137				
4	1	5.323	5.535	5.754				
4	2	5.853	6.194	6.554	7.8%	5.343	5.647	5.969
4	3	5.255	5.369	5.486				
6b1	1	4.754	4.989	5.234				
6b1	2	4.881	5.056	5.238	12.0%	4.924	5.340	5.790
6b1	3	5.750	6.112	6.497				
6b2	1	5.077	5.188	5.302				
6b2	2	4.831	4.963	5.098	3.2%	5.032	5.156	5.284
6b2	3	5.202	5.276	5.352				
6b1 and 6b2 results on the same reconstituted material combined*					8.1%	5.056	5.209	5.366
7b	1	4.158	4.350	4.555				
7b	2	3.897	4.349	4.861	0.6%	4.210	4.333	4.459
7b	3	4.073	4.303	4.552				
9a**	1	~	invalid	~				
9a**	2	~	invalid	~	n/d	n/d	n/d	n/d
9a**	3	~	invalid	~				
12a	1	4.389	4.563	4.743				
12a	2	4.238	4.396	4.560	4.3%	4.320	4.426	4.535
12a	3	3.955	4.197	4.451				
15	1	5.368	5.644	5.932				
15	2	5.218	5.362	5.509	7.9%	4.978	5.257	5.552
15	3	4.734	4.864	4.996				
16	1	4.256	4.516	4.792				
16	2	3.808	3.962	4.122	25.2%	4.103	4.781	5.570
16	3	5.795	6.134	6.493				

		LCL		UCL
N=7	overall GM Potency (IU/mL)	4.528	4.954	5.421
Sample A	Overall %GCV		10.2%	

Abbreviations and notes
LCL= Lower 95% confidence limit
UCL= Upper 95% confidence limit
GCV=Geometric coefficient of variation
GM = Geometric Mean;
The grey boxes indicate the GM and %GCV for the same reconstituted material tested by different operators. These values were not included in the determination of the overall GM and %GCV.
*For laboratory 6b, the combined operator results were used to determine overall GM and %GCV.
** Assays for laboratory 9a invalid due to confidence intervals for individual assay falling outside the 80%-120% range.

Table 15. Rabies vaccine potencies for Sample B expressed relative to the 6th IS (6.6 IU/mL in 0.5 mL) in the ELISA Test.

Lab Code	Assay	LCL	Potency (IU/mL)	UCL	Intra-laboratory %GCV	LCL	Laboratory GM Potency (IU/mL)	UCL
3b	1	4.820	5.039	5.268	4.9%	4.973	5.170	5.374
3b	2	5.308	5.420	5.535				
3b	3	4.729	4.948	5.177				
4	1	5.236	5.463	5.700				
4	2	5.656	5.972	6.305	4.6%	5.545	5.635	5.727
4	3	5.528	5.631	5.735				
6b1	1	5.049	5.165	5.283				
6b1	2	5.291	5.544	5.810	5.0%	5.041	5.256	5.481
6b1	3	4.708	5.045	5.405				
6b2	1	5.132	5.216	5.302				
6b2	2	4.978	5.074	5.172	2.9%	5.109	5.222	5.336
6b2	3	5.279	5.373	5.468				
6b1 and 6b2 results on the same reconstituted material combined*					3.7%	5.144	5.229	5.316
7b	1	4.067	5.268	6.781				
7b	2	4.426	4.755	5.105	10.1%	4.283	4.400	4.520
7b	3	4.239	4.348	4.459				
9a**	1	~	invalid	~				
9a**	2	~	invalid	~	n/d	n/d	n/d	n/d
9a**	3	~	invalid	~				
12a	1	4.542	4.731	4.926				
12a	2	4.624	4.792	4.966	7.1%	4.355	4.586	4.830
12a	3	4.039	4.228	4.424				
15	1	5.111	5.370	5.64				
15	2	5.472	5.572	5.67	5.1%	5.136	5.338	5.547
15	3	4.891	5.049	5.21				
16	1	4.406	4.622	4.848				
16	2	3.732	3.914	4.104	8.8%	3.965	4.222	4.496
16	3	3.956	4.160	4.375				

		LCL		UCL
N=7	overall GM Potency (IU/mL)	4.441	4.915	5.439
Sample B	Overall %GCV		11.6%	

Abbreviations and notes
LCL= Lower 95% confidence limit
UCL= Upper 95% confidence limit
GCV=Geometric coefficient of variation
GM = Geometric Mean
The grey boxes indicate the GM and %GCV for the same reconstituted material tested by different operators. These values were not included in the determination of the overall GM and %GCV.
*For laboratory 6b, the combined operator results were used to determine overall GM and %GCV.
** Assays for laboratory 9a invalid due to confidence intervals for individual assay falling outside the 80%-120% range.

Table 16 Combined Overall GM Potencies for blinded duplicates of the Candidate 7th IS Rabies Vaccine (16/204) [Samples A and B] in ELISA.

		LCL		UCL
N=14	overall GM Potency (IU/mL)	4.650	4.937	5.242
Samples A and B	Overall %GCV		10.5%	
	MAX		5.647	
	MIN		4.222	

Table 17. Rabies vaccine potencies for Sample C expressed relative to the 6th IS (6.6 IU/mL in 0.5 mL) in the ELISA Test.

Lab Code	Assay	LCL	Potency (IU/mL)	UCL	Intra-laboratory %GCV	LCL	Laboratory GM Potency (IU/mL)	UCL
3b	1	7.610	7.907	8.215				
3b	2	7.999	8.226	8.459	5.6%	7.564	7.901	8.252
3b	3	6.960	7.391	7.849				
4	1	6.557	6.814	7.084				
4	2	6.985	7.432	7.907	4.5%	6.876	6.996	7.118
4	3	6.861	7.002	7.146				
6b1	1	7.085	7.254	7.427				
6b1	2	7.560	7.858	8.167	33.8%	7.309	8.892	10.819
6b1	3	11.692	12.449	13.260				
6b2	1	6.903	7.059	7.219				
6b2	2	6.732	6.948	7.172	49.5%	6.755	8.834	11.553
6b2	3	13.747	14.046	14.354				
6b1 and 6b2 results on the same reconstituted material combined*					36.9%	7.967	8.856	9.844
7b	1	~	NP/NL	~				
7b	2	5.565	6.091	6.666	n/a	5.750	7.055	8.658
7b	3	7.944	8.090	8.238				
9a**	1	~	invalid	~				
9a**	2	~	invalid	~	n/d	n/d	n/d	n/d
9a**	3	~	invalid	~				
12a	1	6.850	7.190	7.547				
12a	2	6.979	7.552	8.176	4.7%	6.917	7.157	7.405
12a	3	6.487	6.889	7.317				
15	1	ns	ns	ns				
15	2	ns	ns	ns	n/d	n/d	n/d	n/d
15	3	ns	ns	ns				
16	1	6.716	7.004	7.306				
16	2	5.457	5.713	5.980	13.2%	6.031	6.581	7.182
16	3	6.751	7.145	7.562				

N=7		LCL		UCL
Sample C	overall GM Potency (IU/mL)	2.744	7.388	11.235
	Overall %GCV		11.2%	

Abbreviations and notes
LCL= Lower 95% confidence limit
UCL= Upper 95% confidence limit
GCV=Geometric coefficient of variation
GM = Geometric Mean
NP= Non-parallel NL= Non-linear
ns = No sample received for testing
n/a = Not applicable n/d Not determined The grey boxes indicate the GM and %GCV for the same reconstituted material tested by different operators. These values were not included in the determination of the overall GM and %GCV.
*For laboratory 6b, the combined operator results were used to determine overall GM and %GCV.
** Assays for laboratory 9a invalid due to confidence intervals for individual assay falling outside the 80%-120% range.

Table 18. Rabies vaccine potencies for Sample A expressed relative to the 6th IS (6.6 IU/mL in 0.5 mL) in SRD.

Lab Code	Assay	LCL	Potency (IU/mL)	UCL	Intra-laboratory %GCV	LCL	Laboratory GM Potency (IU/mL)	UCL
1	1	5.794	6.164	6.550				
1	2	3.678	3.904	4.134	28.6%	4.395	5.212	6.181
1	3	5.442	5.895	6.368				
3c	1	0.000	4.786	6.076				
3c	2	5.245	6.113	6.721	16.6%	5.174	5.736	6.361
3c	3	3.282	4.610	5.271				
7c	1	5.056	5.559	6.077				
7c	2	4.576	5.069	5.561	4.9%	5.062	5.289	5.527
7c	3	4.684	5.212	5.744				
8	1	4.805	5.743	6.770				
8	2	5.527	6.763	8.309	8.5%	5.635	6.203	6.828
8	3	5.036	6.247	7.684				
13a	1	4.626	5.654	6.779				
13a	2	5.750	6.430	7.183	9.3%	5.562	5.970	6.408
13a	3	4.641	5.415	6.230				
13b	1	5.200	5.785	6.403				
13b	2	5.514	6.163	6.869	5.6%	5.555	5.918	6.305
13b	3	4.234	5.532	6.853				

		LCL		UCL
N=6	overall GM Potency (IU/mL)	5.307	5.710	6.144
Sample A	Overall %GCV		7.2%	

Abbreviations
LCL= Lower 95% confidence limit
UCL= Upper 95% confidence limit
GCV=Geometric coefficient of variation
GM = Geometric Mean

Table 19. Rabies vaccine potencies for Sample B expressed relative to the 6th IS (6.6 IU/mL in 0.5 mL) in SRD.

Lab Code	Assay	LCL	Potency (IU/mL)	UCL	Intra-laboratory %GCV	LCL	Laboratory GM Potency (IU/mL)	UCL
1	1	5.560	6.022	6.508				
1	2	6.453	6.739	7.052	8.1%	5.852	6.217	6.605
1	3	5.348	5.799	6.267				
3c	1	4.649	6.300	7.209				
3c	2	6.087	6.375	6.632	0.6%	6.172	6.349	6.532
3c	3	6.043	6.324	6.588				
7c	1	4.113	4.630	5.133				
7c	2	4.417	4.913	5.403	8.4%	4.783	5.011	5.250
7c	3	4.906	5.432	5.969				
8	1	5.312	6.346	7.549				
8	2	5.277	6.499	7.982	4.4%	5.709	6.267	6.879
8	3	4.959	5.981	7.133				
13a	1	4.598	5.640	6.781				
13a	2	5.093	5.894	6.768	5.6%	5.173	5.595	6.050
13a	3	4.563	5.292	6.050				
13b	1	5.305	6.188	7.182				
13b	2	4.843	6.150	7.707	6.8%	5.436	5.973	6.563
13b	3	4.399	5.506	6.625				

		LCL		UCL
N=6	overall GM Potency (IU/mL)	5.347	5.882	6.471
Sample B	Overall %GCV		9.5%	

Abbreviations
LCL= Lower 95% confidence limit
UCL= Upper 95% confidence limit
GCV=Geometric coefficient of variation
GM = Geometric Mean

Table 20. Combined Overall GM Potencies for blinded duplicates of the Candidate 7th IS Rabies Vaccine (16/204) [Samples A and B] in SRD.

		LCL		UCL
N=12	overall GM Potency (IU/mL)	5.490	5.773	6.071
Samples A and B	Overall %GCV		8.2%	
	MAX		6.349	
	MIN		5.011	

Table 21. Rabies vaccine potencies for sample C expressed relative to the 6th IS (6.6 IU/mL in 0.5 mL) in SRD.

Lab Code	Assay	LCL	Potency (IU/mL)	UCL	Intra-laboratory %GCV	LCL	Laboratory GM Potency (IU/mL)	UCL
1	1	7.047	7.632	8.337				
1	2	7.330	7.871	8.511	4.7%	7.177	7.380	7.589
1	3	6.907	7.193	7.510				
3c	1	7.136	7.716	8.857				
3c	2	7.631	7.954	8.396	8.3%	6.982	7.442	7.933
3c	3	6.554	6.838	7.164				
7c	1	5.712	6.221	6.763				
7c	2	5.908	6.407	6.941	3.3%	5.989	6.224	6.468
7c	3	5.477	6.004	6.561				
8	1							
8	2	ns	ns	ns	n/a	~	n/a	~
8	3							
13a	1	5.586	6.397	7.310				
13a	2	5.555	6.321	7.175	6.9%	5.712	6.093	6.501
13a	3	5.004	5.669	6.374				
13b	1	5.743	6.517	7.391				
13b	2	5.982	6.741	7.604	7.2%	6.082	6.533	7.018
13b	3	4.575	5.893	7.306				

Abbreviations
LCL= Lower 95% confidence limit
UCL= Upper 95% confidence limit
GCV=Geometric coefficient of variation
GM = Geometric Mean
ns = No sample received for testing
n/a = Not applicable

		LCL		UCL
N=5	overall GM Potency (IU/mL)	5.970	6.711	7.543
Sample C	Overall %GCV		9.9%	

Table 22. Rabies vaccine potencies expressed relative to the 6th IS by in vitro ABT assay performed and reported by Laboratory 5b.

Sample	Assay	Potency (IU/mL)	Laboratory GM Potency
A	1	7.7	
A	2	6.1	6.7
A	3	6.5	
B	1	6.9	
B	2	6.3	6.6
B	3	6.7	
C	1	17.7	
C	2	16.1	16
C	3	14.4	

Table 23. Glycoprotein antigen content of samples of 16/204 measured in ELISA by RL1. Ampoules of 16/204 were stored at various temperatures for ~ 13 months. Antigen content is expressed as a percentage of the -70°C sample

Temperature (ºC)	**Assay**	**Percentage Glycoprotein antigen content of sample stored at -70°C**	**Geometric Mean (%)**
-70	1	100.0	
	2	100.0	100.0
	3	100.0	
-20	1	95.8	
	2	95.4	96.2
	3	97.3	
4	1	98.4	
	2	95.0	97.5
	3	99.0	
20	1	94.9	
	2	95.9	96.4
	3	98.4	
37	1	89.9	
	2	90.1	89.9
	3	89.8	

Table 24. Summary of overall geometric mean potencies (IU/mL) of the candidate 7th IS (16/204) in NIH, ELISA and SRD tests. To account for possible outliers and anomalous results, Huber's robust means for the three assay methods are also shown.

Assay	NIH	ELISA	SRD
Overall GM Potency (IU/mL)	8.79	4.94	5.77
Overall %GCV	51%	10.5%	8.2%
95% Confidence Interval	7.21-10.72	4.65-5.24	5.49-6.07
Huber's Robust Mean (IU/mL)	8.86	4.89	5.84

Appendix 1
Collaborative study participants
(In alphabetical order by country)

Name	Laboratory	Country
Alejandro Parola, Analia De Nichilo	Fundación Pablo Cassará (Pablo Cassara Foundation)	Argentina
Maria Luisa Brero, Silvina Gil, Javier Espeche, Silvana Deluchi, Carlos Atzori	Servicio Vacunas Bacterianas Centro Nacional de Control de Calidad de Biológicos (CNCCB) Administración Nacional de Laboratorios e Institutos de Salud. (ANLIS) "Dr Carlos G Malbrán"	Argentina
Cynthia M. Allen, Gayle Pulle	Viral Vaccines Division Center for Biologics Evaluation (CBE) Biologics and Genetic Therapies Directorate (BGTD) Health Canada	Canada
Stéphane MAISONNEUVE	Agence Nationale de Sécurité du Médicament et des produits de santé (ANSM)	France
Marie-Paule Furnion, Delphine Painblanc; Nicolas Bardy, Audrey Toinon	SANOFI PASTEUR	France
Kristina Scheerer, Andre Noll, Heidi Scholz	GSK Vaccines GmbH	Germany
Beate Krämer Constanze Göpfert	Paul-Ehrlich-Institut (PEI) Federal Institute for Vaccines and Biomedicines	Germany
Sai D. Prasad, Dipankar Das, Gopal Singh;	Bharat Biotech International Limited, Hyderabad	India
Sunil Gairola	Serum Institute of India PVt. Ltd.	India
Guillermo Vega Rodriguez	Commission for Analytical Control and Coverage Extension (CCAYAC)-COFEPRIS	Mexico
Nadezhda Alekseeva	Krasnoyarsk branch of the Federal State Budgetary Institution 'Information and Methodological Centre for Expertise, Stocktaking and Analysis of Circulation of Medical Products' of the Federal Service on Surveillance in Healthcare	Russian Federation
Srđan Stankov, Dusan Lalosevic	Department for Microbiology, Pasteur Institute Novi Sad	Serbia
Derek Litthauer, Yolandi Roodt, Quinton Meyer	National Control Laboratory of South Africa University of the Free State	South Africa
Supaporn Phumiamorn, Koraphong Pinyosukhee	Institute of Biological Products, Department of Medical Sciences Ministry of Public Health	Thailand
Judith Prince	National Institute for Biological Standardisation (NIBSC)	UK
Alethea Fry	Center for Veterinary Biologics Virology Policy, Evaluation, and Licensing USDA/APHIS/VS/CVB	USA

Appendix 2

Collaborative Study Protocol

Final 13/04/17

Protocol for the WHO collaborative study to calibrate a candidate standard for the 7th International Standard for Rabies Vaccine

Background

Quality control testing for vaccines is mandatory for batch release to ensure safety and potency of each batch. One of the most important tests for rabies vaccines is the potency test which gives an indication of the consistency of production of vaccine batches with respect to batches which have previously been shown to be efficacious in clinical trials. The currently required potency test for rabies vaccine for human use is the National Institutes of Health (NIH) mouse protection test which is described in detail in Laboratory Techniques in Rabies [1]. This assay is also used to test product stability for the purpose of establishing shelf-life.

The native form of the surface glycoprotein of the rabies virus included in rabies vaccines is responsible for the production of protective virus-neutralizing antibodies. The quantification of glycoprotein in rabies vaccines by e.g. single radial immunodiffusion (SRD) or ELISA is used routinely during manufacture at different process stages and in particular at the final bulk stage, allowing definition of the final antigen content per dose of vaccine.

The WHO's Expert Committee on Biological Standardization (ECBS) establishes reference standards for biological substances used in the prevention, treatment or diagnosis of human disease. WHO International Standards are recognized as the highest-order of references for biological substances and are assigned potencies in International Units (IU). Their primary purpose is to calibrate secondary references used in routine laboratory assays in terms of the IU thereby providing a uniform results-reporting system and traceability of measurements independent of the method used. International Standards are prepared in accordance with published WHO recommendations [2].

Rabies vaccines for human and veterinary use are produced in many countries and the minimum potency requirements are expressed in IU. The WHO International Standard for rabies vaccine (inactivated) is used by vaccine manufacturers and control testing laboratories to calibrate secondary references for use in the standardisation of the NIH test and assays for glycoprotein content. The International Standard is also used by laboratories for the standardisation of potency assays for veterinary rabies vaccines e.g. [3].

The stock of the current International Standard (6th IS NIBSC code 07/162) is near depletion and a candidate replacement has been prepared using bulk vaccine manufactured by the same process as the 6th International Standard. The proposal to assess and establish the candidate replacement as the 7th IS for rabies vaccine was endorsed by ECBS in October, 2016.

Laboratories with established assays for rabies vaccine are being recruited to participate in a WHO International Collaborative study to assess the suitability of the candidate (NIBSC 16/204) to serve as the 7th IS for Rabies vaccine.

Study Aims

The aims of this WHO international collaborative study [2] are to assess candidate NIBSC 16/204 against the 6th International Standard in

- The NIH mouse protection assay

- in vitro assays of rabies vaccines for glycoprotein content such as SRD and ELISA
- mouse immunisation and challenge assays
- mouse immunisation and serology assays

The study will be conducted in 2 phases with phase 1 involving the assessment and calibration of the candidate in assays of rabies vaccines in the NIH mouse protection assay. Phase 2 will involve the assessment of the candidate in mouse immunisation and challenge/serology assays. Participants of both study phases will be given the opportunity to assess the candidate in their in vitro assays (e.g. ELISA, SRD).

Materials

Candidate 7th IS for Rabies vaccine (NIBSC code 16/204)

The bulk vaccine material provided to NIBSC for the preparation of the candidate IS is derived from a beta-propiolactone-inactivated Pitman-Moore (PM) strain of virus grown in Vero cells. Human albumin was added as a stabiliser and the batch used in the production of the rabies bulk was produced from a plasma pool which had been tested and found negative for HBsAg, HCV RNA by NAT and anti-HIV 1+2.

6th IS for Rabies vaccine (NIBSC code 07/162), the 5th IS for Rabies Vaccine (NIBSC code RAV) and coded samples

The current IS [4] 07/162 will be used to calibrate the candidate 7th IS. An additional 1-2 vaccines may also supplied for inclusion in each assay. Some laboratories performing the NIH assay may also receive for testing the 5th IS [5] which is available in limiting amounts. Study samples will be provided blinded. Instructions for preparation and use of the study samples will be provided.

Assay Methods

Phase 1

NIH Mouse Protection assay

Participants are requested to assay the study samples in the NIH mouse protection test [1] as established in their laboratory.

in vitro assays for glycoprotein content

Participants are also requested to assay the study materials in any in vitro assays which are performed routinely in their laboratory e.g. SRD or ELISA. As some of the vaccine samples contain human albumin as stabiliser, it has been NIBSC's experience that the SRD gels will have to be soaked in PBS for 24 -48 h to wash out unbound protein.

Phase 2

Mouse Immunisation and Challenge or Mouse Immunisation and Serology

A few participants have agreed to assay the study samples in the mouse immunisation challenge or serology assay as established in their laboratory. Considering the 3Rs approach to animal testing, participants are request not to initiated testing of the samples by mouse serology or single immunisation and challenge until instructed to do so by the study organiser (Dianna Wilkinson).

Design of study

Participants are requested to:

- perform 3 independent assays on different days;
- reconstitute the freeze-dried samples as directed in the instructions for use;
- prepare and test a series of dilutions for the standard and each study sample and **test concurrently**;
- use a freshly opened and reconstituted ampoule for each assay. Sufficient ampoules of the standard and each study sample will be supplied so that a fresh ampoule may be used for each of the two inoculations administered to mice in the NIH test.

Results and data analysis

Participants are requested to return their results to NIBSC within 3 months of receipt of the study materials. If it is not practicable to turn around results within 3 months, please inform Dianna.wilkinson@nibsc.org . Excel spread sheets will be provided so that all essential information can be recorded including details of assay methodology and the raw data obtained from each assay. The use of the reporting spread sheet facilitates the analysis and interpretation of results. The final version of the reporting spread sheets will be e-mailed to each participant following shipment of study materials.

The confidentiality of each laboratory will be ensured with each participant being anonymous to the other laboratories. Assay data will be analysed at NIBSC by an experienced biometrician using standard statistical techniques. Analysis of the study will assess the potencies of each study sample relative to the candidate IS. Intra- and inter-laboratory variability will also be assessed for assay methods.

A draft study report will be sent to participants for comment. The report will include data analysis, proposed conclusions and recommendations to ECBS on the suitability of the candidate to serve as the 7th IS for rabies vaccine.

Participation in the WHO collaborative study is conducted under the following conditions:

- The data obtained in the collaborative study should not be published or cited before the formal establishment of the standard by WHO, without the expressed permission of the NIBSC Study organizer.
- It is normal practice to acknowledge participants as contributors of data rather than co-authors in publications describing the establishment of the standard.
- Individual participant's data will be coded and reported "blind" to other participants during the preparation of the study report, and also in subsequent publications.
- Participants will receive a copy of the report of the study and proposed conclusions and recommendations for comment before it is further distributed.
- Participants accept responsibility for safe handling and disposal of the materials provided.
- Participants agree to abide to local regulations regarding the use of animals in the performance of research.

Deadline for completed results spread sheets is 3 months from receipt of study materials. If it is not practicable to return results within 3 months, please inform Dianna Wilkinson.

All completed results spread sheets should be returned electronically to:

Dr Dianna Wilkinson
Principal Scientist
Viral Vaccines Section

Division of Virology
National Institute for Biological Standards and Control
Blanche Lane
South Mimms
Hertfordshire
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UK

Tel. +44(0)1707 641314

dianna.wilkinson@nibsc.org

References

1. Wilbur, L.A., Aubert, F.A.M., 1996. The NIH test for potency. In: Meslin, F.X., Kaplan, M.M., Koprowski, H. (Eds.), Laboratory Techniques in Rabies. World Health Organization, Geneva, pp. 360–368.
2. WHO, Recommendations for the preparation, characterization and establishment of international and other biological reference standards. WHO Technical Report Series, No. 932., in Expert Committee on Biological Standardization. 2006.
3. Daas, A., L. Bruckner and C. Milne (2015). "EDQM biological reference preparation for rabies vaccine (inactivated) for veterinary use." Pharmeur Bio Sci Notes 2015: 57-72.
4. Ferguson, M., Heath, A. (2008). "Report of the WHO collaborative study to calibrate a candidate replacement for the fifth international standard for rabies vaccine" WHO Technical Report WHO/BS/08.2087. available at: http://apps.who.int/iris/handle/10665/70593
5. Lyng J, Bentzon MW, Ferguson M, Fitzgerald EA. Rabies vaccine standardization: International Collaborative Study for the Characterization of the fifth International Standard for Rabies Vaccine. Biologicals. 1992; 20:301-13.

Appendix 3
Instructions for Use

WHO International Standard
7th International Standard for Rabies vaccine
NIBSC code: 16/204
Instructions for use

1. INTENDED USE

The International Standard (IS) for rabies vaccine is used in the standardisation of rabies vaccines in NIH mouse protection tests and in in vitro assays for glycoprotein content. This material was prepared from a bulk of Vero cell-derived, Pitman Moore strain, produced by the same manufacturing process as the 5th and 6th International Standards.

The candidate 7th IS was calibrated in IU against the 6th IS in a collaborative study in which 16 participants from 12 countries assayed the candidate as blinded duplicates against the 6th International Standard. These samples were assayed by the participants in 30 NIH mouse potency tests, 27 enzyme-linked immunosorbent assays (ELISA) and 18 single radial immunodiffusion (SRD) tests.

2. CAUTION
This preparation is not for administration to humans or animals in the human food chain.

As for 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

The unitage assigned to this material is as follows:

For use in NIH mouse protection tests: 8.9 IU/ampoule i.e. 8.9 IU/ml when reconstituted as directed in 1ml distilled water

For use in ELISA: 2.45 IU per ampoule Pitman Moore rabies virus glycoprotein antigen content i.e. 4.9 IU/ml when the contents are reconstituted as directed in 0.5ml distilled water.

For use in SRD tests: 2.9 IU per ampoule Pitman Moore rabies virus glycoprotein antigen content i.e. 5.8 IU/ml when the contents are reconstituted as directed in 0.5ml distilled water.

4. CONTENTS

Country of origin of biological material: France.
Each ampoule contains the freeze-dried residue of 0.5 ml aliquots of a commercial rabies vaccine bulk containing inactivated Pitman Moore virus grown in Vero cells were filled in DIN ampoules and freeze dried at NIBSC following documented procedures. This fill was 0.5g fill weight with a mean dry weight of 0.0551g. The coefficient of variation (CV) was 0.3478%. Residual moisture measured on 12 samples gave a mean of 0.4 with a CV of 20.5% and oxygen headspace measured in 12 ampoules gave a mean of 0.13% with a CV of 81.37%.

Uncertainty: the proposed unitage does not carry an uncertainty associated with its calibration. The only uncertainty is therefore derived from the variability of the dry fill weight of the ampoule content.

5. STORAGE

The ampoules should be stored at -20° C or below until use.
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.

Tap the ampoule gently to collect the material at the bottom (labelled) end. Ensure that the disposable ampoule safety breaker provided is pushed down on the stem of the ampoule and against the shoulder of the ampoule body. Hold the body of the ampoule in one hand and the disposable ampoule breaker covering the ampoule stem between the thumb and first finger of the other hand. Apply a bending force to open the ampoule at the coloured stress point, primarily using the hand holding the plastic collar.

Care should be taken to avoid cuts and projectile glass fragments that might enter the eyes, for example, by the use of suitable gloves and an eye shield. Take care that no material is lost from the ampoule and no glass falls into the ampoule. Within the ampoule is dry nitrogen gas at slightly less than atmospheric pressure. A new disposable ampoule breaker is provided with each DIN ampoule.

7. USE OF MATERIAL

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

For use in in vivo NIH tests, reconstitute the contents of the ampoule in 1mL distilled water. The resultant solution is 8.9 IU/mL.

For use in ELISA and SRD assays for glycoprotein antigen content, reconstitute the contents of each ampoule in 0.5mL distilled water. The resultant solution is 4.9 IU/mL rabies virus glycoprotein content by ELISA OR 5.8 IU/mL rabies virus glycoprotein content by SRD.

The ampoules should be shaken gently without the formation of foam to ensure that all contents are completely reconstituted. When a reconstitution volume of 0.5mL is used, the ampoule may be left at ambient temperature for 30 minutes to facilitate complete disolution of the contents.

8. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities and they should be stored on receipt as indicated on the label. It is the policy of WHO not to assign an expiry date to their international reference materials. They remain valid with the assigned potency and status until withdrawn or amended.

Stability studies on reconstituted material have not been undertaken. It is anticipated that the contents of individual ampoules will be used on the day reconstitution as the volumes required in both the in vivo potency tests and and in vitro assays of glycoprotein antigen content mean that the contents would be used in a single assay. However, should users wish to store reconstituted material, they should determine the stability of reconstituted material according to their own method of preparation, storage and use. Multiple freeze/thaw cycles should be avoided.

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

9. REFERENCES

WHO Expert Committee on Biological Standardization report WHO/BS/08.2087 available online.
http://apps.who.int/iris/bitstream/10665/70593/1/WHO_BS_08.2087_eng.pdf

10. ACKNOWLEDGEMENTS

We gratefully acknowledge the important contributions of the collaborative study participants. We would also like to thank Paul Chamberlain and Lindsay Stone, Division of Virology at NIBSC, for initial work on the development and validation of the candidate standard and NIBSC Standards Production and Development for the production of NIBSC 16/204 and distribution of the study materials.
We also thank Ivana Knezevic of the WHO and the International Working Group of the European Partnership for Alternatives to Animal Testing (EPAA) for their support, guidance and advice.

11. FURTHER INFORMATION

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 (Add or amend as necessary)
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	Corrosive: No
Stable: Yes	Oxidising: No
Hygroscopic: No	Irritant: No
Flammable: No	Handling: See caution, Section 2
Other (specify): Contains human albumin	
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: 1.0 g
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable. Attached: Not Applicable

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 http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_biolefstandardsrev2004.pdf (revised 2004). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.