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WHO collaborative study to assess the suitability of a WHO International Reference Panel for Ebola virus VP40 antigen

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** See Appendix 1*

NOTE:
This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by **12 October 2016** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technologies, Standards and Norms (TSN). Comments may also be submitted electronically to the Responsible Officer: **Dr C M Nübling** (nueblingc@who.int)

Summary

Although the Ebola virus (EBOV) outbreak in West Africa has ended, WHO's preparedness activities continue with the aim to ensure that all countries are operationally ready to effectively and safely detect, investigate, and report potential Ebola virus disease (EVD) cases, and to mount an effective response to any EVD outbreak. Thus, the need remains for the development of point-of-care (POC) rapid tests for specific and accurate diagnoses of EBOV without requirements for complex lab equipment or highly trained staff. This report describes the work undertaken for the development and evaluation of candidate EBOV recombinant protein preparations to serve as WHO International Reference Reagents (IRR) for EBOV VP40 for monitoring the performance of POC tests for antigen detection and presumptive diagnosis of EVD. Nine freeze-dried samples, derived from recombinant EBOV VP40, GP and/or NP protein and formulated in defibrinated plasma, were tested for EBOV antigens by 8 laboratories from 4 countries in a blinded manner. Assay methods used include POC rapid tests for detection of EBOV VP40, GP and/or NP as well as enzyme linked immunosorbent assays (ELISA) for VP40 and sequential detection of GP and VP40 by western blot analysis. The study assessed the ability of the participating laboratories to correctly identify samples as positive or negative for EBOV antigen(s). Where available, a semi-quantitative assessment of sample reactivity is also presented.

The study showed that some assays were not able to detect certain EBOV antigens. This may be due to low assay sensitivity, the absence of relevant antigen epitopes or conformations and/or differences in epitope recognition by antibodies used in the assays antigen capture or detection. The consequences of using recombinant EBOV antigens derived from bacterial and eukaryotic expression systems are discussed. The freeze-dried candidates containing VP40 only and showing weakly positive/trace reactivity (sample 5) and low but unequivocal reactivity (sample 3) along with the negative control (sample 8) may have utility as a WHO IRR panel for use in monitoring the performance of rapid POC tests for the detection of VP40 provided the potential limitations of the panel are understood.

Initial data from accelerated thermal degradation of the material obtained at 1 month is insufficient for predicting the long-term stability of the candidate IRR and it is recommended that they are shipped at -20°C. Further work is required for developing reference materials for EBOV GP and NP assays.

Introduction

In support of the World Health Organization's ongoing activities related to the 2014-2015 West Africa Ebola virus (EBOV) outbreak, NIBSC has undertaken projects for the standardization and control of assays used in the diagnosis, treatment or prevention of Ebola virus disease (EVD). In 2015, two international collaborative studies were completed establishing WHO International Reference Reagents (IRR) for the calibration and control of anti-EBOV antibody assays for serology [1] and reverse transcription-polymerase chain reaction (RT-PCR) assays for the diagnosis of EVD [2]. This report describes the work undertaken for the development and evaluation of candidate EBOV recombinant protein preparations to serve as IRRs for EBOV VP40 for monitoring the performance of rapid point-of-care (POC) tests for antigen detection and presumptive diagnosis of EVD.

The large-scale diagnostic efforts of the recent EBOV epidemic relied on centralized deployable laboratory units and other designated laboratories to perform RT-PCR assays for the detection of EBOV in order to appropriately direct symptomatic patients to either an Ebola Treatment Centre or other setting. While these laboratories were vitally important for patient management and transmission interruption, it was recognized that they could not address all the diagnostic needs of the EVD outbreak. Diagnosis relied largely on suspect cases arriving at local holding centers where blood was obtained by venipuncture and transported to centralized laboratories for testing by RT-PCR. The challenges of collecting and handling blood under appropriate biosafety containment and the logistics of transporting samples to a remote testing laboratory often prolonged the turnaround time between sample collection and the reporting of results. In the meantime, patients were being held locally for several hours to days until a diagnosis could be made, thus delaying the treatment of EBOV-positive patients and unnecessarily isolating negative patients placing them at risk of exposure to EBOV. A community-based, decentralised approach to diagnosis by RT-PCR was not feasible because the method requires significant laboratory infrastructure and the expertise of laboratory scientists proficient in molecular biology techniques operating under appropriate biosafety containment [reviewed in 3].

To minimize delays in patient triage and care, WHO and partners such as the Foundation for Innovative New Diagnostics (FIND) and other experts recognized the urgent need for POC rapid diagnostic tests that could be used to diagnose EBOV without requirements for complex lab equipment or highly trained staff [4-6]. WHO recommends that, in the absence of RT-PCR, a rapid antigen test that has reasonable sensitivity in patients with high concentrations of EBOV in the blood may have utility as a screening tool for assisting in patient triage and case management in settings without laboratory infrastructure [5].

Through its Emergency Use Assessment and Listing (EUAL) procedure for rapid assessment of EBOV diagnostics for procurement to affected countries, WHO has listed four PCR-based assays and three rapid antigen tests for the detection of EBOV [7]. Two of the listed PCR assays are automated cartridge or pouch-based platforms that integrate RNA extraction and real-time RT-PCR detection. These self-contained PCR platforms are designed to reduce the biohazard risk of sample handling and shorten the turnaround time for results reporting. They can be setup in decentralised laboratories and operated by technicians with minimal training, but still require dedicated instruments housed in laboratories with adequate infrastructure. The three EUAL rapid tests for antigen detection are lateral flow immunoassays designed for use at the point of contact or care and can be performed by health care personnel with minimal training [7].

This report describes the international collaborative study undertaken to assess the suitability of candidate EBOV VP40 protein preparations to serve as WHO IRR for use in monitoring the performance of POC tests for the detection of VP40. Freeze-dried samples, derived from recombinant EBOV VP40, GP and/or NP protein and formulated in defibrinated plasma, were tested for EBOV antigen by each laboratory in a blinded manner. Assay methods used include POC rapid tests for detection of EBOV VP40, GP and/or NP as well as enzyme linked immunosorbent assays (ELISA) for VP40 and sequential detection of GP and VP40 by western blot analysis. Analysis of results was carried out to assess the ability of the participating laboratories to correctly identify samples as positive or negative for EBOV antigen(s). Where available, a semi-quantitative assessment of sample reactivity is also presented.

The aims [8] of this WHO international collaborative study are to

- assess the suitability of candidate preparations to serve as WHO International Reference Reagents for use as a monitor for EBOV antigen assays
- assess the performance of each candidate in a range of EBOV antigen assays performed in different laboratories
- recommend to WHO ECBS for establishment the candidate(s) found to be suitable to serve as WHO International Reference Reagents.

The findings of the collaborative study suggest that the ability of some assay methods to detect EBOV antigens may be dependent on assay sensitivity, the presence of relevant antigen epitopes or conformations and/or differences in epitope recognition by antibodies used in the assays antigen capture or detection. The freeze-dried candidates containing VP40 only and showing weakly positive/trace reactivity (sample 5) and low but unequivocal reactivity (sample 3) along with the negative control (sample 8) may have utility as a WHO IRR panel for use in monitoring the performance of rapid POC tests for the detection of VP40 provided the potential limitations of the panel are understood. Initial data from accelerated thermal degradation of the material obtained at 1 month is insufficient for predicting the long-term stability of the candidate IRR and it is recommended that they are shipped at -20°C.

Materials and Methods

EBOV Ag source materials

In all cases, EBOV antigen preparations issued by NIBSC contain non-infectious recombinant proteins. The study samples are based on the following source materials:

- **VP40 produced at NIBSC**
  The protein is full length, native, VP40 based on H. sapiens-wt/GIN/2014/Makona-Kissidougou-C15 (EBOV/Mak-C15) (GenBank KJ660346) and expressed in *Escherichia coli.*

- **VP40, GP and NP provided by SunYoung Jeong, SD Biosensor Inc., Republic of Korea.**
  These are full length viral protein VP40 (1 – 326 aa), soluble glycoprotein GP (15-364 aa), partial nucleoprotein NP (247-493aa). Each protein was produced in *E. coli* using sequences for isolate EBOV/H. sapiens-wt/SLE/2014/Makona-G3838 (EBOV/MakG3838) (GenBank KM233105) and supplied to NIBSC in carbonate buffer.

- **Virus-like particles (VLP) produced at NIBSC**
  EBOV VLPs were produced by co-transfection of HEK293T cells with plasmids codon optimised for the expression of EBOV VP40, GP and NP. The VLPs were purified on a 20% sucrose cushion in phosphate buffer. Sequences are derived from EBOV/Mak-C15.

Study Samples

Preparation, filling and freeze-drying of study samples

Manufacturers of WHO EUAL assays provided kits to NIBSC for assessment of the source materials and formulation and validation of the study samples. These are the SD Q Line Ebola Zaire Ag Assay (SD Biosensor Inc., Korea), OraQuick Ebola Test (Orasure Technologies, USA) and ReEBOV Antigen Rapid Test (Corgenix Inc, USA). Assays were performed according to the manufacturers' instructions. An in-house EBOV VP40 ELISA was also used to assess the study samples. For the ELISA, rabbit anti-EBOV VLP antibody (IBT Bioservices) was used to capture VP40 antigen present in samples, which was detected by a mouse anti- EBOV VP40 antibody (Fitzgerald Industries International). The secondary antibody was anti-mouse IgG conjugated to HRP (Jackson Immunoresearch).

The study samples were formulated by dilution in a pool of thrombinized and declotted plasma samples screened negative for HBsAg, anti-HIV and anti-HCV. The pooled defibrinated plasma was also shown to be negative for VP40, GP and NP in the donated POC and in-house EBOV Ag assays. Formulated samples were dispensed in 120µL aliquots into 1.0 mL 2D barcoded plastic screw-top tubes (Fluidx, UK) and freeze-dried using a Virtis Genesis 25EL freeze drier following standard operating procedures. Robust intact freeze-dried cakes were formed upon freeze-drying (Figure 1). The study samples were stored at -20°C or below until shipped on dry ice to participants under NIBSC dispatch reference CS571.

Table 1 lists the coded collaborative study samples. Three candidate IRR panel members were prepared. Samples 3 and 5 comprise of VP40 produced at NIBSC and formulated to give unequivocal reactivity (sample 3) or weak/trace reactivity (sample 5) in pre-study validation assays. Sample 8, consisting of thrombinized plasma, was produced as a negative control. Additional study samples included freeze-dried preparations of VP40, obtained from SD Biosensor Inc. and formulated at NIBSC to give trace, unequivocal or strongly positive results; as well as preparations of VP40, GP and NP combined in solution (SD Biosensor Inc.) or in the form of VLPs (NIBSC). The terms "low", "medium" and "high" used in Table 1 are descriptors indicating the qualitative reactivity of a given sample. Samples with the same descriptor cannot be assumed to have equivalent amounts of a given antigen.

Study protocol

The final version of the study protocol is given in Appendix 2. Briefly, 3 sets plus 1 spare set of 9 coded study samples were provided to the participants. Participants were requested to assay the samples using the method(s) in routine use in their laboratory for the detection of EBOV antigens. Laboratories were permitted to use multiple methods to test the study materials provided that the study design was followed for each method. An excel spread sheet was provided to the participants for returning sample results and describing the assay method(s) used.

Design of study

Participants were requested to:

- perform 3 independent assays preferably on separate days.
- Use a freshly opened and reconstituted study sample for each assay. Detailed directions for reconstituting the study samples were provided in the instructions-for-use.
- For each independent assay, test each reconstituted sample undiluted according to assay procedure.
- include all study samples in each assay run.
- for each independent assay, indicate whether each sample tested is considered positive or negative according to assay criteria.

Participants

Eight laboratories from 4 countries completed the study. The participants were from PR China (1), Republic of Korea (1), UK (1), and USA (5). All laboratories are referred to by code number allocated at random and not representing the order of listing in Appendix 1. Participating organisations include manufacturers of assay kits, government research, medical counter-measure and regulatory organisations.

Assay methods

Codes for the quantitative and qualitative assays used by participants in the collaborative study are summarised in Table 2. The assay methods fall into 2 general categories: *i*) rapid POC tests for procurement under EUAL or under development and *ii*) enzyme immunoassays for research use only (RUO). Laboratories using POC tests listed the types of samples that could be tested according to the package insert. These included capillary or venipuncture whole blood (1 method); whole blood, serum and plasma (2 methods); capillary whole blood, EDTA-whole blood and EDTA-plasma (1 method).

Stability study

The candidate EBOV VP40 samples have been placed at -20˚C, +4˚C, 37˚C, 45 ˚C and 56 ˚C and will be retrieved at specific time points for assessment. To date, accelerated degradation data has been obtained for the 1-month time point. The degradation samples were assayed concurrently in the in-house ELISA for VP40. The stability of the candidate material was assessed using the Arrhenius model for accelerated degradation studies with potencies expressed relative to the samples stored at -20˚C [9,10].
EBOV VP40 samples stored at +4˚C, 37˚C, 45 ˚C for 3 months were assayed for residual moisture by coulometric Karl Fischer titration (Mitsubishi CA100, A1-Environsciences Ltd., Luton, UK).

Results and data analysis

Study sample formulations

The recombinant EBOV proteins used in the sample formulations are based on either EBOV/Mak-C15 or EBOV/MakG3838 sequences (see Materials and Methods). Except for EBOV/MakG3838 GP protein (aa 15-364) and NP protein (aa 247-493), the recombinant proteins are full-length. A BLAST search of the reference sequences indicated that the translated sequence for EBOV/MakG3838 has 100 % identity for full-length VP40, 100% identity for GP (aa 15-364) and 99% identity for NP (aa 247-493) with respect to sequences encoded by EBOV/Mak-C15 (Appendix 3).

Formulation of the freeze-dried samples

VP40 obtained from two sources (both derived from bacteria) was used to formulate VP40 only-containing samples. In preliminary work, serial dilutions of the starting materials were tested to estimate the target reactivities of antigen. The dilutions that gave trace to weakly positive results were selected for formulating and freeze-drying samples 4 and 5 (Table 1). The dilutions giving VP40 amounts 4-fold higher than the weakly positive samples were selected for preparing low but unequivocally positive samples 2 and 3. Strongly positive samples 1 and 6 were formulated at 8-fold higher amounts of VP40 with respect to samples 4 and 5. Two freeze-dried multiplexed preparations containing VP40, GP and NP obtained from two sources were also prepared (sample 7 and 9). Validation assays indicated that further optimization of the formulations was needed before their potential use as IRRs. For example, the VP40:GP:NP ratios have not been determined for the multiplexed samples. Nevertheless, these preparations were included as additional study samples. A diluent-only freeze-dried preparation was also prepared to serve as a negative control (sample 8). In order to minimize the risk of handling in high-containment situations, all study samples were produced in plastic 1.0 mL screw-top tubes (Figure 1) instead of glass ampoules or vials. The qualitative reactivities of EBOV VP40 antigens in the final freeze-dried preparations were confirmed in validation assays before dispatch and assessment in the collaborative study.

Collaborative study data received

Eight laboratories returned data sets for 9 assay methods (Table 2). Laboratory 8 returned results from 2 assay methods designated as 8a and 8b. Five laboratories (1, 4, 6, 7, 8a) returned data for POC assays for the detection of VP40 only (3 data sets), NP only (1 data set), VP40, GP and NP simultaneously (1 data set). Three laboratories (2, 3, 8b) reported results for EIA RUO assays for the detection of VP40 only (3 data sets). Laboratory 5 indicated that they tried different tests but only obtained consistent results with western blot analysis for the presence of GP and VP40 (1 data set).

Scoring study samples as positive or negative for Ebola virus antigens

The scoring of the samples as positive or negative in assays for the detection of VP40 only is shown in Table 3. Table 4 lists the results of the assay targeting NP only and Table 5 lists the results for the assay targeting VP40, GP and NP combined. Table 6 lists the results of the western blot analysis for VP40 and GP. Assay results shown to be unexpected with respect to validation or consensus results are highlighted in grey (Tables 3-5).

VP40 assays

With the exception of method 8a, VP40 was not detected in the negative control (sample 8), by any of the assay methods. Laboratory 8a scored the negative control as negative in two assays and trace-positive in two assays, giving an overall interpretation for sample 8 as indeterminate. As expected, all of the laboratories listed in Table 3 scored as positive, samples 1, 2, 3, 6, 7, and 9, which have medium to high reactivities for VP40. Samples 4 and 5, which were formulated to give a weakly positive signal for VP40, where also scored as positive by most laboratories; however, laboratory 2 scored sample 4 as negative and laboratory 8a scored sample 5 as indeterminate trace-positive in 2 out 4 assay runs (highlighted in grey in Table 3). Laboratory 8a commented that, according to the package insert, results for samples 5 and 8 would be considered as indeterminate and, in the clinical setting, an additional patient sample would be requested for re-testing and /or RT-PCR confirmation. Laboratory 8a indicated that they cannot rule out the occurrence of interference by the thrombinized and declotted plasma used in the references preparations.

NP assay
Using an assay method targeting NP only, laboratory 6 returned expected negative scores for negative sample 8 as well as for the sample formulations containing no NP (samples 1- 6). Laboratory 6 returned a strongly positive result for sample 7 which contains VP40/GP/NP derived from eukaryotic cells (Table 4). Laboratory 6 scored sample 9, a formulation of VP40/GP/NP derived from bacteria and containing an NP fragment, as negative (highlighted in grey in Table 4).

VP40-GP-NP assay
Table 5 lists the sample results for the assay method for the simultaneous detection of VP40, GP and NP. As expected, laboratory 4 scored the negative control (sample 8) as negative for VP40, GP and NP, and those samples containing only VP40 (samples 1-6) as negative for GP and NP. Laboratory 4 also scored samples 1-4 and 6 as positive for VP40. The more challenging sample 5 was scored negative in one out of three assay runs. For the samples containing VP40, GP and NP combined, sample 8 (bacterial source) was scored positive for all three antigens while sample 7 (eukaryotic source) was scored positive for VP40 and NP but, unexpectedly, negative for GP (highlighted in grey in Table 5).

VP40-GP assay
Table 6 and Figure 2 show the sample results for the sequential detection of VP40 and GP by western blot analysis. As expected, laboratory 5 scored the negative control (sample 8) as negative for VP40 and GP. Of the two samples containing GP (samples 7 and 9), laboratory 5 detected GP in sample 7, which contains full-length GP expressed in eukaryotic cell culture, but not in sample 9, which contains amino acids 15-364 of GP expressed in bacteria as a soluble protein. Similarly, of the eight samples containing VP40 (samples 1-7 and 9), laboratory 5 detected VP40 in sample 7, which is the only sample containing VP40 produced in eukaryotic cells.

Stability assessment of candidate VP40 antigen preparation

An accelerated degradation study was performed in order to predict the long-term stability of the candidate NIBSC EBOV VP40 preparations when stored at the recommended temperature of -20˚C. The potency of samples 3, 5 and 6 stored at the elevated temperatures for 1 month were expressed relative to the material stored at –20ºC (Table 7). As these initial results are based on limited data it was only possible to fit the data for sample 3 to the Arrhenius model (Table 8). The other data sets do not show a sufficiently consistent pattern of loss to allow the data to fit the Arrhenius model. As such, no predicted loss of potency over time can be made at this point. From this initial result it is recommend that the materials should be shipped at -20°C until further data has been gathered.

As a parameter that is used to assess the seal integrity of sample containers, the residual moisture content of freeze-dried materials can serve as an indicator of product stability over time. Table 9 shows the percent residual moisture contents for sample 8 (negative) and sample 3 [Candidate WHO IRR NIBSC VP40 (medium)] stored for 3 months at 4°C, 37°C and 45°C. Although the data is limited, it is clear that the moistures are all high compared to typical moistures seen for vials and ampoule formats [8]. Although the samples have high moisture contents, it appears that the storage temperature does not make an impact on the determined moisture over the time period.

Discussion

Real-time RT-PCR performed on blood samples is presently the standard method for the diagnosis of EVD in outbreak settings [5]. To run a RT-PCR assay, clinical laboratory

personnel with specialist expertise are required to operate dedicated equipment which must be housed in a facility with significant infrastructure. In resource-poor settings, this places RT-PCR out of reach of local front-line holding centers. In the recent EVD outbreak, biosafety and logistical challenges of collecting, handling and transporting samples to centralized testing centers often resulted in prolonged delays to diagnosis [reviewed in 3]. WHO recommends that nucleic acid testing using technologies such as PCR should be the method of choice for diagnosing EVD, however, a rapid antigen test that has reasonable sensitivity in patients with high concentrations of Ebola virus in the blood may have utility in settings without laboratory infrastructure if the benefits and limitations of the test are understood and appropriately managed [5, 7]. To give a fast presumptive diagnosis to patients suspected of having EVD, POC tests for detecting EBOV antigen may allow for a more rapid, although less sensitive and less specific, alternative to RT-PCR. Given the serious implications of false positive or false negative results, POC tests are intended for use as screening tools only and their results must be confirmed by RT-PCR according to established testing algorithms. To date three POC antigen tests, all based on the detection of EBOV VP40, have been listed by WHO under the EUAL procedure for emergency procurement [7].

In March 2016, the WHO Director-General terminated the Public Health Emergency of International Concern (PHEIC) regarding the EVD outbreak in West Africa [11]. Consequently, WHO no longer accepts new applications for EVD diagnostics through its EUAL mechanism; however, all applications submitted prior to this announcement will continue to be assessed by WHO under the EUAL procedure. WHO continues to encourage manufacturers of EVD diagnostics to apply for full prequalification [12].

Although the EBOV outbreak in West Africa has ended, WHO's preparedness activities continue with the aim to ensure that all countries are operationally ready to effectively and safely detect, investigate, and report potential EVD cases, and to mount an effective response to any EVD outbreak [11]. Thus, the need remains for the development of POC rapid tests for specific and accurate diagnoses of EBOV without requirements for complex lab equipment or highly trained staff [4-6].

WHO international collaborative studies have been conducted previously to establish WHO biological reference materials for use in the standardization and control of EBOV antibody and nucleic acid assays [1,2]. In this study, a range of assays has been used to assess the suitability of freeze-dried candidate 1st WHO IRR panel members for use in monitoring the performance of POC assays for the detection of VP40.

The candidate 1st WHO IRR panel for VP40 consists of 2 preparations of VP40 that have been diluted to give reactivities that approach the limit of detection of the VP40 assays reported in this study. The more concentrated panel member (sample 3) was shown to have low but unequivocal reactivity for VP40 across POC assays and it is expected that sample 3 should be positive in every assay run. Sample 5, which contains ~4-fold lower amounts of VP40 than sample 3, was shown to be more challenging in the assays with most reporting qualitatively lower positive signals than sample 3. The exceptions for sample 3 were assays by laboratories 4 and 8b, which returned negative results. The inability to detect VP40 in sample 3 by laboratory 8b may be a consequence of the assay procedure which involves a pre-dilution step lowering the amount of VP40 to below the detection limit of the assays. The WHO IRR panel also includes a negative control sample containing no EBOV proteins and comprises the defibrinated plasma diluent used in the formulation of the study materials (Tables 3 and 5).

Additional samples were included in the study and containing varying amounts of different EBOV antigens from different sources. The performance of the VP40 only-containing samples derived from SD Biosensor Inc. (e.g. samples 2 and 4) was comparable to that of the VP40 samples derived from NIBSC in most assays (Tables 3 and 5). Neither sample, however was detected by western blot (Table 6 and Figure 1).

Although not optimized for use as IRRs, the samples containing VP40, GP and NP as VLP (sample 7) or combined in solution (sample 9) were included in the study to assess how they performed across different assays. Both samples were positive or strongly positive in POC and ELISA assays for VP40 (Tables 3 and5), but, neither sample was found suitable in assays for assessing reactivity across assays for GP or NP i.e. sample 9 was strongly positive for NP by laboratory method 4, but negative for NP by method 6; and sample 7 was negative for GP by laboratory 4. The western blot method was only able to detect VP40 and GP derived from the eukaryotic expression system. The differences in reactivities for these two preparations could be due to differences in antigen preparations (e.g. epitopes missing from partial sequences or due to aggregation); how the antigens were expressed (bacterial or eukaryotic) or in assay methods (e.g. differences in epitope recognition by antibodies used in antigen capture or detection). All VP40 preparations were produced in bacteria except for sample 7, which contains virus-like particles produce in eukaryotic cells with oligomeric GP and VP40 which are assumed to be in their native biologically-relevant conformations associated with lipids. Interestingly, all assays except the western blot test (laboratory 5) detected VP40 produced in bacteria. The lack of detection of the VP40 produced in bacteria may not be due to the sensitivity of the Western blot test but may rather be due to posttranslational modifications and/or the conformation of VP40 produced in bacteria.

Instead of glass ampoules or vials, which are normally used for WHO International Standards, the EBOV Ag samples were freeze-dried in plastic screw-top microtubes (Figure 1). This novel approach was taken in order to fill small volumes and also to avoid using glass which could pose an injury risk to laboratory personnel operating in high containment situations. The performance of the plastic tubes in terms of product stability has not been extensively studied. To date, samples from the 1 month time-point have been assessed for accelerated degradation and additional data is required before the long-term stability of the candidates can be determined. Also, the candidate VP40 preparations have higher residual moistures than observed generally for plasma filled in ampoules or vials and so their stability may be impaired on long-term storage. 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 [8]. Thus, further optimisation of the freeze-drying conditions may be necessary before materials can be supplied in the plastic microtubes with the moisture levels that are comparable to typical freeze-dried WHO biological reference materials.

In conclusion, the candidate 1st WHO IRR panel for VP40 consists of 2 preparations of VP40 with reactivities that approach the limit of detection of the VP40 assays reported in this study. Sample 3 is expected to score unequivocally positive in VP40 Ag assays and, when used alongside the negative control (sample 8), may have utility in monitoring operator or kit performance. Sample 5, with a 4-fold lower amount of VP40 than sample 3, has been shown to be indeterminate in some VP40 assays. Sample 5 may be used by assay developers to aid in the assessment of an assay's limit of detection. The 1st WHO IRR panel must not be used as a substitute for the controls provided by the kit manufacture, but instead may be used as an

adjunct to the kit controls. Further work is required for developing reference materials for EBOV GP and NP assays. Additional data is required to predict the long-term stability of the freeze-dried material.

Proposal

It is proposed that samples 3, 5 and 8 are established as the 1st WHO reference panel for EBOV VP40 for use by laboratories in the qualitative assessment of the performance of assays kits for the detection of VP40 provided that the potential limitations of the materials are made clear to the end-user. The panel is not suitable for assessing assays for the detection of EBOV antigens other than VP40 and it should not be used as a substitute for assay kit controls. **Further development and stability assessments are needed before EBOV antigen preparations can be made available to serve as WHO IRRs for use as part of the WHO prequalification procedure POC tests.**

Approximately 500 panels are available for distribution.

Comments from participants

SD Bioscience provided a correction to the EBOV reference sequence quoted for their donated antigens (EBOV/MakG3838 instead of EBOV Mayinga). This information has been corrected throughout the report.

Laboratory 5 specifically commented that "…the results raise concerns about the VP40 antigen produced in bacteria as a standard to qualify POC diagnostic tests. Furthermore, VP40 mainly exists associated to membranes in the infected cells and virus particles that may require the use of detergents in the POC tests. Membrane associated VP40 such as in the VLPs provides an antigen in its natural conformation that will need to be extracted for detection constituting a better antigen for the qualification of POC tests".
We have added these concerns to the report and amended the proposal to address this issue (shown in **bold**).

Acknowledgements
We gratefully acknowledge the important contributions of the collaborative study participants and the donors of the POC assay kits used in developing the test materials. We would also like to thank NIBSC Standards Production and Development for the production and distribution of the candidate materials. We also thank David Wood, Micha Nuebling and Robyn Meurant of the WHO for their support, guidance and advice.

References

1. Wilkinson DE, Page M, Almond N, Anderson R, Berry N, et al. (2015) WHO collaborative study to assess the suitability of an interim standard for antibodies to Ebola virus.
2. Wilkinson DE, Page M, Almond N, Anderson R, Ashall J, et al. (2015) WHO collaborative study to assess the suitability of interim standards for Ebola virus NAT assays.
3. Broadhurst MJ, Brooks TJ, Pollock NR (2016) Diagnosis of Ebola Virus Disease: Past, Present, and Future. Clin Microbiol Rev 29: 773-793.
4. FIND (2014) Joint WHO/FIND Meeting on Diagnostics and Ebola Control, Geneva, Switzerland, 12 December 2014.
5. WHO (2015) Selection and use of Ebola in vitro diagnostic assays. Emergency guidance. Available at: http://www.who.int/csr/resources/publications/ebola/ivd-assays/en/
6. WHO (2014) Target Product Profile for Zaïre ebolavirus rapid, simple test to be used in the control of the Ebola outbreak in West Africa. Available at: http://www.who.int/medicines/publications/target-product-profile.pdf?ua=1
7. WHO (2016) Public Reports: WHO list of IVDs for Ebola virus disease accepted for procurement through the EUAL Procedure for IVDs. Available at http://www.who.int/medicines/ebola-treatment/emp_ebola_diagnostics/en/
8. WHO (2006) Recommendations for the preparation, characterization and establishment of international and other biological reference standards. WHO Technical Report Series, No. 932. Available at: http://www.who.int/biologicals/reference_preparations/establishment/en/
9. Kirkwood TBL (1977) Predicting the Stability of Biological Standards and Products. Biometrics 33: 736-742.
10. Kirkwood TBL, Tydeman MS (1984) Design and analysis of accelerated degradation tests for the stability of biological standards II. A flexible computer program for data analysis. Journal of Biological Standardization 12: 207-214.
11. WHO (2016) Ebola Situation Report - 30 March 2016. Available at: http://apps.who.int/ebola/current-situation/ebola-situation-report-30-march-2016
12. WHO (2016) Ebola EUAL Status after PHEIC Termination. Available at: http://www.who.int/diagnostics_laboratory/procurement/purchasing/en/

Tables

Table 1. Collaborative study samples

Sample Code	Description#	Reactivity in preliminary assays	Presentation
1	SD Bioscience VP40 (high)	strongly positive	120 µL formulation freeze-dried in 1mL plastic tubes
2	SD Bioscience VP 40 (medium)	unequivocal positive	
3	Candidate WHO IRR NIBSC VP40 (medium)	unequivocal positive	
4	SD Bioscience VP40 (low)	weakly/trace positive	
5	Candidate WHO IRR NIBSC VP40 (low)	weakly/trace positive	
6	NIBSC VP40 (high)	strongly positive	
7	NIBSC VLPs (VP40-GP-NP) (unknown relative amounts*)	unequivocal positive for VP40 weakly positive for GP by ELISA or negative for GP by POC assay strongly positive for NP by POC assay	
8	Candidate WHO IRR pooled de-fibrinated plasma	negative	
9	SD Bioscience antigens (VP40-GP-NP) (medium for each antigen*)	unequivocal positive for VP40, GP and NP by POC assay	

The amounts of antigen in the study samples have not been quantitatively determined. High, medium, and low indicate the qualitative amounts of antigen as assessed during formulation of the study samples.
*The ratios of antigens have not been determined.

Table 2. Assay methods

Target EBOV antigen	Assay	No. of data sets
VP40	POC EUAL	3
NP	POC	1
VP40, GP, NP	POC EUAL	1
VP40	Luminex-based RUO	1
VP40	ELISA RUO	2 in-house
VP40, GP	western blot RUO	1

Abbreviations: (POC) point-of-care; EUAL = WHO emergency use assessment and listing; IP = Immunoprecipitation; RUO = research use only

Table 3. Samples scored as positive or negative for Ebola in VP40 antigen detection assays

VP40		Sample#								
Lab Code	Readout details	1 VP40 (high) SD Biosensor	2 VP40 (medium) SD Biosensor	3 VP40 (medium) NIBSC	4 VP40 (low) SD Biosensor	5 VP40 (low) NIBSC	6 VP40 (high) NIBSC	7 VP40-GP-NP VLPs (unknown) NIBSC	8 Negative	9 VP40-GP-NP (medium) SD Biosensor
1	n=3	PPP	PPP	PPP	PPP	PPP	PPP	PPP	NNN	PPP
2	n=3	PPP	PPP	PPP	NNN	PPP	PPP	PPP	NNN	PPP
	mean semi-quant	+	+	+	-	+	++	+++	-	+
3	n=3	PPP	PPP	PPP	PPP	PPP	PPP	PPP	NNN	PPP
	mean readout	1.414	0.339	1.212	0.150	0.363	2.216	2.131	0.013	0.311
7	n=3	PPP	PPP	PPP	PPP	PPP	PPP	PPP	NNN	PPP
	mean readout <12=Neg	297	196	67	107	21	165	194	6	217
8a	n=4	PPPP	PPPP	PPPP	PPPP	TrPPTr	PPPP	PPPP	NNTrTr	PPPP
	median visual score	5	3	1	2	1Tr	3	5	0Tr	3
8b	n=4 1/10 pre-dilution	PPPP	PPPP	PPPP	PPPP	NNNN	PPPP	PPPP	NNNN	PPPP
	mean readout cut-off = 0.055	0.380	0.145	0.086	0.078	0.052	0.225	1.868	0.045	0.345

Abbreviations: n = number of independent assays reported. P= positive; N = negative; Tr = trace reactivity.
Notes: # High, medium, and low indicate the qualitative amounts antigen present as assessed during formulation of the study samples.
Results highlighted in grey are unexpected with respect to consensus or pre-study validation results (See Discussion).

Table 4. Samples scored as positive or negative for Ebola NP antigen detection assays.

NP		Sample#								
Lab Code	Readout details	1 VP40 (high) SD Biosensor	2 VP40 (medium) SD Biosensor	3 VP40 (medium) NIBSC	4 VP40 (low) SD Biosensor	5 VP40 (low) NIBSC	6 VP40 (high) NIBSC	7 VP40-GP-NP VLPs (unknown) NIBSC	8 Negative	9 VP40-GP-NP (medium) SD Biosensor
6	n=3	NNN	NNN	NNN	NNN	NNN	NNN	PPP	NNN	NNN
	overall score	-	-	-	-	-	-	strongly +	-	-

Abbreviations: n = number of independent assays reported. P= positive; N = negative.
Notes: # High, medium, and low indicate the qualitative amounts antigen present as assessed during formulation of the study samples.
Results highlighted in grey are unexpected with respect to pre-study validation results (See Discussion).

Table 5. Samples scored as positive or negative for Ebola VP40-GP-NP antigen detection assays.

VP40/GP/NP		Sample#								
Lab Code	Target Antigen & Readout details	1 VP40 (high) SD Biosensor	2 VP40 (medium) SD Biosensor	3 VP40 (medium) NIBSC	4 VP40 (low) SD Biosensor	5 VP40 (low) NIBSC	6 VP40 (high) NIBSC	7 VP40-GP-NP VLPs (unknown) NIBSC	8 Negative	9 VP40-GP-NP (medium) SD Biosensor
4 n=3	VP40 overall score	PPP	PPP	PPP	PPP	ppN	PPP	PPP	NNN	PPP
		++	+	+	+	+/-	++	++	-	+
	GP overall score	NNN	NNN	NNN	NNN	NNN	NNN	NNN	NNN	PPP
		-	-	-	-	-	-	-	-	++
	NP overall score	NNN	NNN	NNN	NNN	NNN	NNN	PPP	NNN	PPP
		-	-	-	-	-	-	+++	-	+++

Abbreviations: n = number of independent assays reported. P= positive; p = weakly positive; N = negative.
High, medium, and low indicate the qualitative amounts antigen present as assessed during formulation of the study samples.
Results highlighted in grey are unexpected with respect to pre-study validation results (See Discussion).

Table 6. Samples scored as positive or negative for Ebola VP40 and GP by sequential western analysis.

VP40/GP		Sample#								
Lab Code	Target Antigen & Readout details	1 VP40 (high) SD Biosensor	2 VP40 (medium) SD Biosensor	3 VP40 (medium) NIBSC	4 VP40 (low) SD Biosensor	5 VP40 (low) NIBSC	6 VP40 (high) NIBSC	7 VP40-GP-NP VLPs (unknown) NIBSC	8 Negative	9 VP40-GP-NP (medium) SD Biosensor
5 n=3	VP40	NNN	NNN	NNN	NNN	NNN	NNN	PPP	NNN	NNN
	GP	NNN	NNN	NNN	NNN	NNN	NNN	PPP	NNN	NNN

Abbreviations: n = number of independent assays reported. P= positive; N = negative.
High, medium, and low indicate the qualitative amounts antigen present as assessed during formulation of the study samples.
Results highlighted in grey are unexpected with respect to pre-study validation results (See Discussion).

Table 7. Thermal degradation assessment of VP40 antigen preparations stored for 1 month at the indicated temperatures. Potencies are expressed relative to the material stored at -20ºC (assigned a unitage of 1 for the purpose of this assessment).

Sample Code	Description#	-20°C	4°C	37°C	45°C	56°C
6	NIBSC VP40 (high)	1	0.94	0.84	0.61	0.40
3	Candidate WHO IRR NIBSC VP40 (medium)	1	0.85	0.87	0.51	0.26
5	Candidate WHO IRR NIBSC VP40 (low)	1	0.71	0.60	0.15	0.14

High, medium, and low indicate the qualitative amounts of antigen present as assessed during formulation of the study samples.

Table 8. The % loss per month for sample 3 [Candidate WHO IRR NIBSC VP40 (medium)] was predicted using the Arrhenius model.

Temperature (°C)	% Loss per Month	95% Upper Confidence Limit (% Loss)
-20	0.059	0.323
4	1.169	4.128
20	6.346	15.071
37	28.463	38.631

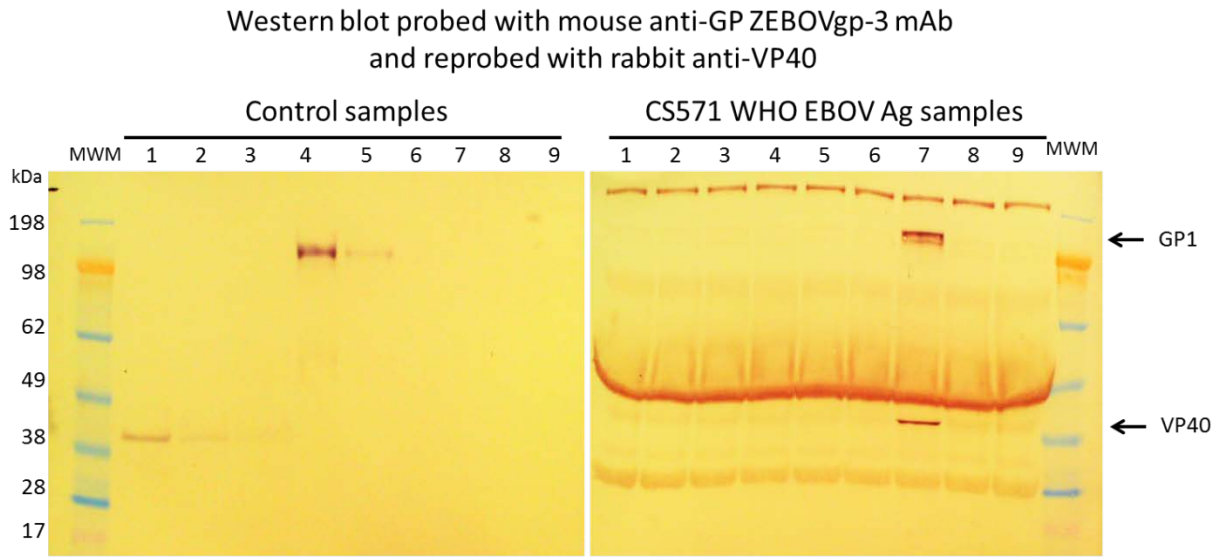
Table 9. Percent residual moisture content of sample 8 (negative) and sample 3 [Candidate WHO IRR NIBSC VP40 (medium)] stored for 3 months at the indicated temperatures.

Temperature	Sample	Moisture (%)	Individual determinations
4°C	8	3.93	3.91, 3.95
	3	12.73	12.07, 13.38
37°C	8	4.04	4.09, 3.98
	3	6.79	4.78, 8.80
45°C	8	2.86	2.77, 2.94
	3	9.39	9.01, 9.77

Figures

Figure 1. Candidate WHO IRR NIBSC VP40 (sample 3) 120 μL freeze-dried in novel 1mL plastic screw top tubes.

Figure 2. Samples tested by laboratory 5 for Ebola GP and VP40 by sequential western analysis.



Control Samples
1: 20 ng purified ZEBOV VP40 expressed in bacteria
2: 10 ng purified ZEBOV VP40 expressed in bacteria
3: 5 ng purified ZEBOV VP40 expressed in bacteria
4: 200,000 TCID50 rVSV-ZEBOVgp
5: 100,000 TCID50 rVSV-ZEBOVgp
6: 200,000 TCID50 wt VSV
7: 100,000 TCID50 wt VSV
8: Blank
9: Blank

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

Name	**Laboratory**	**Country**
Thomas Wang	InTec Products, Inc	PR China
SunYoung Jeong and Dong-hyuk Kim	SD Biosensor, Inc.	Republic of Korea
Giada Mattiuzzo and Sophie Myhill	National Institute for Biological Standards and Control (NIBSC)	UK
Javan Esfandiari, Louise Sigismondi, Katie Ann Paden and Dhammika Gunasekera	Chembio Diagnostics Inc.	USA
Matthew L. Boisen	Corgenix Inc	USA
Gerardo Kaplan and Michael Ibrahim	Office of Blood Research and Review, Center for Biologics Evaluation and Research (CBER), U.S. Food and Drug Administration (FDA)	USA
Graham Yearwood, Tiffany Miller and Nicholas Statile	OraSure Technolgies	USA
Mark Poli and Keersten Ricks	US Army Medical Research Institute of Infectious Diseases	USA

Appendix 2

Collaborative Study Protocol

Protocol for the WHO collaborative study to assess the suitability of an International Reference Reagent for Ebola virus antigen

Background

In support of the World Health Organization's ongoing activities related to the recent Ebola virus (EBOV) outbreak in West Africa, NIBSC is undertaking a project to develop an International Reference Reagent for EBOV Viral Protein antigen for use in monitoring the performance of diagnostic assays.

Aims

The aims of this WHO international collaborative study are to

- assess the suitability of candidate preparations to serve as WHO International Reference Reagents for use as a monitor for EBOV antigen assays
- assess the performance of each candidate in a range of typical assays performed in different laboratories
- recommend to WHO ECBS for establishment the candidate(s) found to be suitable to serve as WHO International Reference Reagents.

Study Samples

In all cases, EBOV antigen preparations issued by NIBSC contain non-infectious recombinant proteins. Any study samples or other materials of human origin will have undergone screening for HBsAg, anti-HIV and HCV RNA.

The Study Samples are based on the following source materials.

NP, GP and VP40 provided by SunYoung Jeong, SD Biosensor Inc., S. Korea:

These are soluble glycoprotein GP (15-364 aa), partial nucleoprotein NP (247-493aa) and full length viral protein VP40 (1 – 326 aa); all the proteins were produced in *E.coli* using EBOV Mayinga sequences and supplied to NIBSC in carbonate buffer.

Virus-like particles (VLP) produced at NIBSC:

EBOV VLPs are produced by co-transfection of three plasmids (pCMV3-codon optimised VP40, pCMV3-codon optimised NP, pCAGGS-codon optimised GP) into HEK293T cells using polyethylenimine. Sequences are derived from EBOV strain *H. sapiens*-wt/GIB/2014/Kissidougou-C15. VLPs are purified on a 20% sucrose cushion in phosphate buffer.

VP40 produced at NIBSC:

The protein is full length, native, VP40 based on 2014 Kissidougou-C15 and expressed in *E. coli*.

Coded study samples

The source materials have been formulated in thrombinized and declotted plasma. The study samples are provided to the participant coded and blinded. Nine samples are provided for assay. The coded samples may include both negative and positive samples.

The study samples have been freeze-dried. The directions for reconstitution of the freeze-dried materials are given in the instructions for use included with the study samples. Laboratories will receive at least 4 sets of study samples which will allow 3 independent assays by one method plus 1 spare. Laboratories with more than one assay method will receive additional sample sets per method (subject to availability).

We aim to dispatch the study samples in April 2016.

The freeze-dried study samples should be stored at -20°C or below. The study samples shall not be administered to humans.

Assay Methods

For testing the Study Samples, participants are requested to use the method(s) in routine use in their laboratory for the detection of EBOV antigens. Laboratories may use multiple methods to test the study materials provided that the study design is followed for each method.

Design of study

Participants are requested to:

- perform 3 independent assays preferably on separate days.
- Use a freshly opened and reconstituted Study Sample for each assay. To prepare the samples for assay, bring the freeze-dried samples to ambient temperature and reconstitute samples as described in the Instructions for Use. Samples should be used within 10 minutes to 1 hour after reconstitution.
- For each independent assay, test each reconstituted sample neat according to assay procedure.
- include all study samples in each assay run
- for each independent assay, indicate whether each sample tested is considered positive or negative according to assay criteria.

Results analysis

Participants are requested to return their results to NIBSC within 2 weeks of receipt of the study materials. An excel spread sheet is provided so that all essential information can be recorded including details of assay methodology (Figure 1). If multiple assay methods are undertaken, a separate worksheet for each method should be completed. The final version of the reporting spread sheet 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. Analysis of the study will assess the reactivities of the study samples in the different assay methods.

Figure 1. Example Excel reporting sheet.

Results Form: WHO collaborative study to assess the suitability of an reference reagent for EBOV Ag assays

reporting sheet

Participant's name	
Organisation	
Method/Ag	
Kit Manufacturer	

Have you provided the requested information for this method by completing the questionnaire "Ebola WHO CS questionnaire Final 160415"?

If not, please complete the questionnaire which can be obtained from Dianna Wilkinson dianna.wilkinson@nibsc.org

Reconstitute samples as described in the Instructions for Use. Test each sample neat according to your assay procedure.

		Record the reactivity of each sample (e.g. +, -)									Comments
		Sample									
		1	2	3	4	5	6	7	8	9	
Assay 1 date											
kit Lot#											
Date kit opened											
Expiry date											
Assay 2 date											
kit Lot#											
Date kit opened											
Expiry date											
Assay 3 date											
kit Lot#											
Date kit opened											
Expiry date											

Return results to
Dr. Dianna Wilkinson
Study Organizer
Division of Virology
National Institute for Biological Standards and Control (NIBSC)
Blanche Lane, South Mimms, Potters Bar, Hertfordshire, EN6 3QG UK

Prior to submission of study findings to WHO, a draft study report will be sent to participants for comment. The report will include results analysis, proposed conclusions and recommendations on the selection and use of the most appropriate EBOV antigen preparation(s) to serve as the WHO International Reference(s). The finalised report will then be submitted to the WHO ECBS who will decide on the suitability of the preparation to serve as International Reference Reagent for EBOV antigen.

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.
- in order to address the need for Ebola reference materials, participants are permitted to use the study samples for purposes that fall outside the collaborative study. To better inform the subsequent implementation of any interim standard, participants are encouraged to provide any information gained to the study organizer about their use of the study materials. THE CANDIDATE MATERIALS ARE PROVIDED TO PARTICIPANTS “AS IS”, WITHOUT ANY REPRESENTATION OR WARRANTY OF SATISFACTORY QUALITY OR FITNESS FOR A PARTICULAR PURPOSE.
- 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.
- the study materials are described in the study protocol. Participants accept responsibility for the safe handling and disposal of the materials provided.

- (For non-UK recipients) I confirm that these materials may be legally imported without delay in their delivery. For guidelines: **http://www.nibsc.org/asset.ashx?assetid=a83f80e8-4be1-45cf-92e2-a636a149ed9a** Note: In all cases, EBOV antigens issued by NIBSC are non-infectious recombinant proteins. Any study samples or other materials of human origin will have undergone screening for blood-borne viruses.

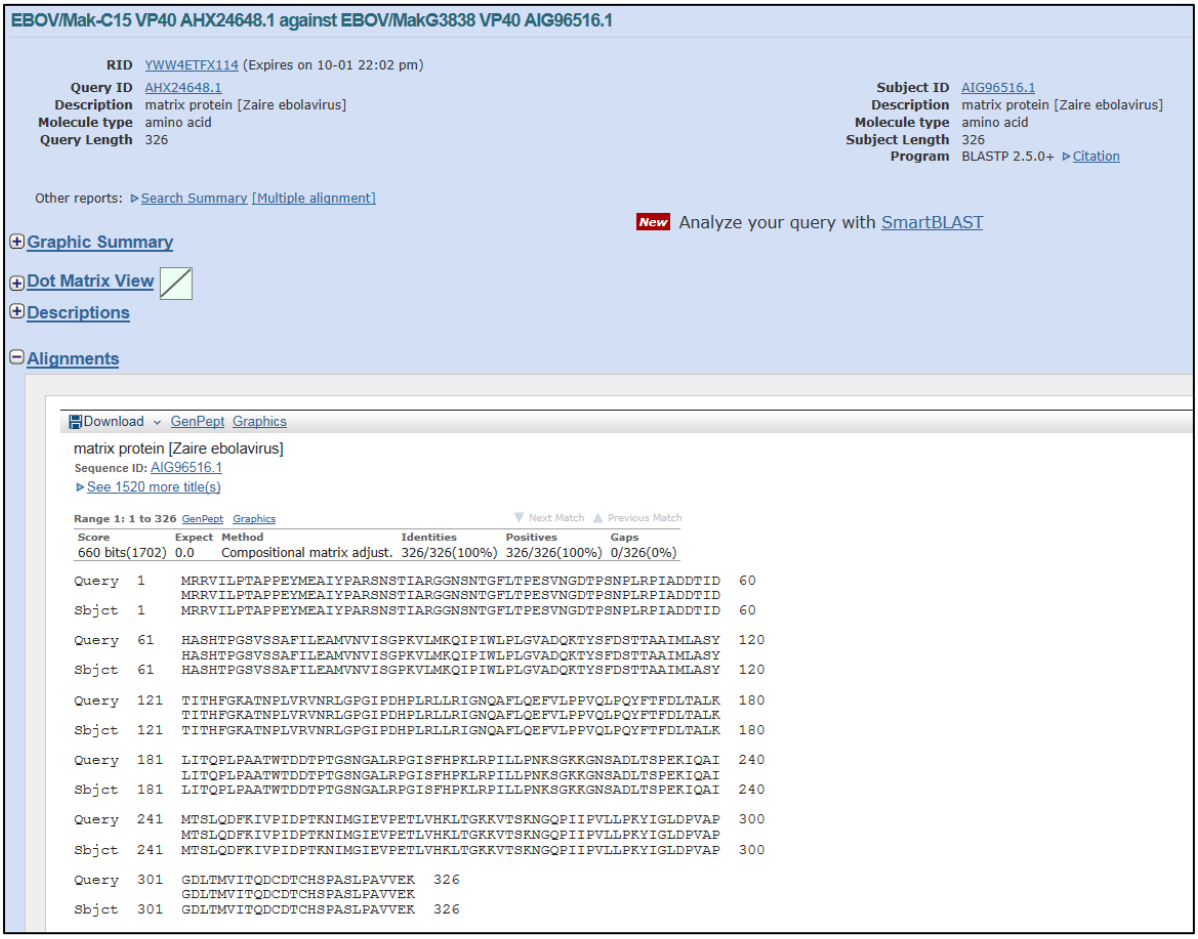
The deadline for completed results spread sheets is 2 weeks from receipt of study materials. All completed results spread sheets should be returned electronically to:

Dr Dianna Wilkinson
Principal Scientist
Viral Vaccines Section
Division of Virology
National Institute for Biological Standards and Control
Blanche Lane
South Mimms
Hertfordshire
EN6 3QG
UK

Tel. +44(0)1707 641314
dianna.wilkinson@nibsc.org

Appendix 3

BLAST alignment of EBOV/Mak-C1 translated antigen sequences against EBOV/MakG3838



EBOV/Mak-C15 VP40 AHX24648.1 against EBOV/MakG3838 VP40 AIG96516.1

RID YWW4ETFX114 (Expires on 10-01 22:02 pm)

Query ID	AHX24648.1	Subject ID	AIG96516.1
Description	matrix protein [Zaire ebolavirus]	Description	matrix protein [Zaire ebolavirus]
Molecule type	amino acid	Molecule type	amino acid
Query Length	326	Subject Length	326
		Program	BLASTP 2.5.0+ ▹Citation

Other reports: ▹Search Summary [Multiple alignment]

New Analyze your query with SmartBLAST

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⊕Descriptions

⊖Alignments

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matrix protein [Zaire ebolavirus]

Sequence ID: AIG96516.1

▹See 1520 more title(s)

Range 1: 1 to 326 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
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Query  121  TITHFGKATNPLVRVNRLGPGIPDHPLRLLRIGNQAFLQEFVLPPVQLPQYFTFDLTALK  180
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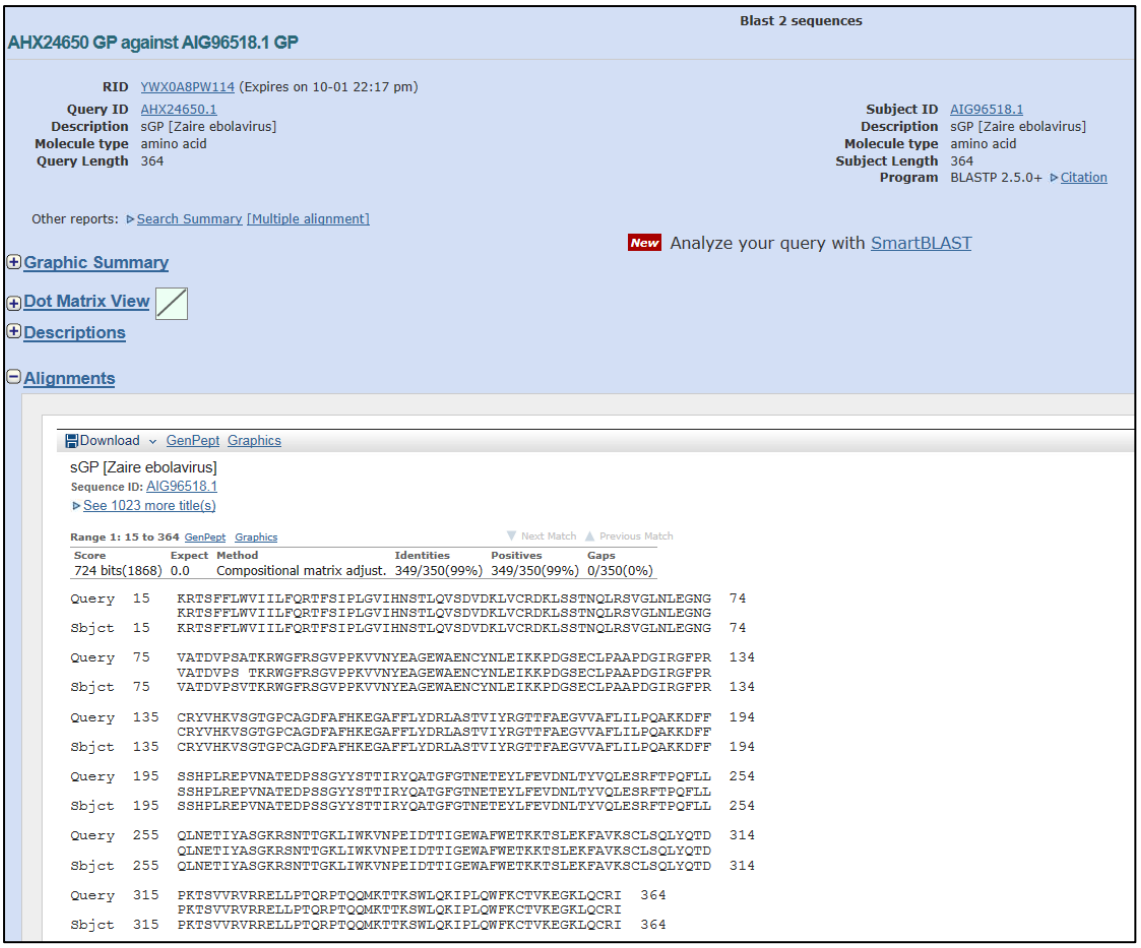
Appendix 3
continued



Blast 2 sequences

AHX24650 GP against AIG96518.1 GP

RID YWX0A8PW114 (Expires on 10-01 22:17 pm)

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Molecule type	amino acid	Molecule type	amino acid
Query Length	364	Subject Length	364
		Program	BLASTP 2.5.0+ ▹Citation

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sGP [Zaire ebolavirus]
Sequence ID: AIG96518.1
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Range 1: 15 to 364 GenPept Graphics ▼Next Match ▲Previous Match

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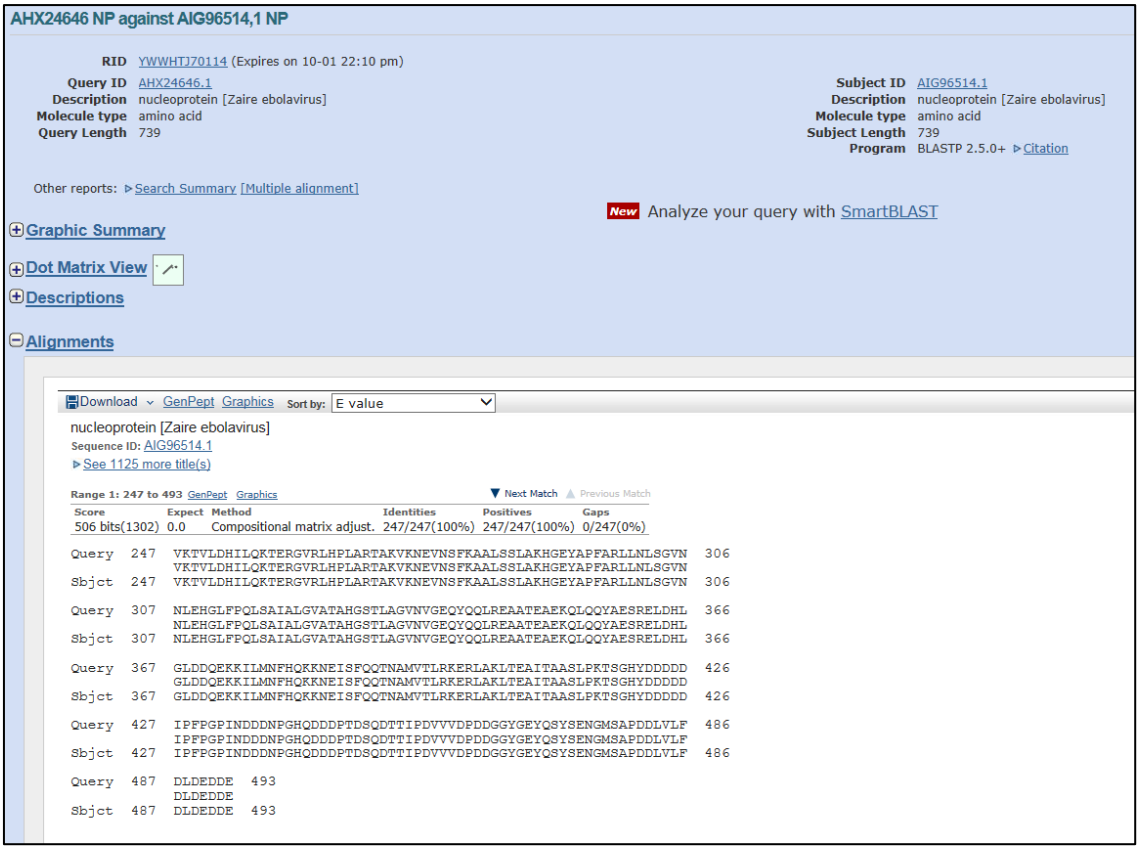

AHX24646 NP against AIG96514,1 NP

RID YWWHTJ70114 (Expires on 10-01 22:10 pm)

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Molecule type	amino acid	Molecule type	amino acid
Query Length	739	Subject Length	739
		Program	BLASTP 2.5.0+ ▹Citation

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nucleoprotein [Zaire ebolavirus]
Sequence ID: AIG96514.1
▹See 1125 more title(s)

Range 1: 247 to 493 GenPept Graphics ▼Next Match ▲Previous Match

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Query  427  IPFPGPINDDDNPGHQDDDPTDSQDTTIPDVVVDPDDGGYGEYQSYSENGMSAPDDLVLF  486
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