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Collaborative Study to Evaluate a Candidate World Health Organization International Standard for Zika Virus for Nucleic Acid Amplification Technique (NAT)-Based Assays

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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 **16 September 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

The aim of the collaborative study was to assess the suitability of a candidate International Standard (IS) for Zika virus (ZIKV) for nucleic acid amplification technique (NAT)-based assays. Potency of the candidate IS and related reference preparations was evaluated using a range of NAT-based assays for ZIKV RNA with the aim of assigning an internationally agreed unitage to the candidate WHO IS.

The candidate IS consisted of an inactivated, lyophilized ZIKV preparation formulated in a stabilizing, neutral solution and intended for dilution using a range of different types of sample matrix. The virus strain used for the preparation of the candidate IS originated from a ZIKV-infected patient from French Polynesia, closely related to ZIKV strains currently circulating in the Asia-Pacific region and central and South America. Further strains from the Asian ZIKV lineage were included in the study as well as two preparations derived from African ZIKV isolates. The samples consisted of a mixture of inactivated ZIKV reference materials as well as clinical materials (urine or plasma) from ZIKV-infected patients. In addition, a panel of *in vitro* transcribed RNAs covering partial ZIKV genome sequences were included in the study.

The samples for evaluation were distributed to 24 laboratories from 11 different countries. The samples were assayed on three separate days and the data were collated and analysed at the Paul-Ehrlich-Institut (PEI). Data were returned by 21 of the participating laboratories, in total 37 sets of data were analysed; 19 from quantitative assays and 18 from qualitative assays. The assays used consisted of a mixture of in-house developed and commercial assays (currently available or in development). The results showed that all samples were detected consistently by the majority of participants. The candidate standard is very stable under recommended storage conditions, i.e. at or below -20ºC, and is therefore suitable for long term use. On-going real-time and accelerated stability studies of the candidate IS are in progress.

It is proposed that the heat-inactivated and lyophilized preparation with cell culture-derived French Polynesian ZIKV strain be established as the 1st IS for ZIKV RNA with an assigned unitage of 50,000,000 International Units per ml.

Introduction

Zika virus (ZIKV) was first identified in 1947 and circulated in Africa between the 1960s and 1980s, emerging in 2007 in Yap Island, Micronesia (Duffy *et al*., 2009). In 2013/2014 ZIKV appeared in Tahiti, French Polynesia (Cao-Lormeau *et al*., 2014), spreading to New Caledonia and Cook Islands in 2014 (Musso *et al*., 2014), and subsequently causing the on-going outbreak in Brazil (Zanluca *et al*., 2015; Campos *et al*., 2015) with wider dissemination of the virus in Central and South America and continued circulation in Pacific region. In other parts of the world, for example North America and Europe, there are reports of ZIKV infection in travellers returning from affected areas. ZIKV is commonly transmitted by *Aedes aegypti* and *Aedes albopictus*, the same vectors that transmit Chikungunya and Dengue viruses.
Infection with ZIKV is usually asymptomatic or results in usually mild disease typically presenting with fever, headache, malaise, conjunctivitis and skin rash (Musso and Gubler, 2016), however, complications with ZIKV infection include microcephaly and brain abnormalities in the fetus (Rasmussen *et al*. 2016; Driggers *et al*., 2016) as well as other neurological conditions in adults such as Guillain-Barré syndrome (Cao-Lormeau *et al*., 2016). ZIKV, like other arboviruses, has the potential to be transmitted by transfusion of blood and blood products

(Cunha *et al*., 2016; Barjas-Castro *et al*., in press). Sexual transmission of ZIKV is being increasingly reported (Musso *et al*., 2015; Deckard *et al*., 2016).
There are no specific therapies or vaccines for ZIKV. Suspected clinical cases of ZIKV infection can only be confirmed by detection of virus RNA in plasma, or other bodily fluids such as urine (Anonymous, 2016) and saliva using nucleic acid amplification techniques (NAT). False positive results may occur with serological testing due to cross-reactivity with other flaviviruses such as Dengue virus; testing algorithms for discrimination are restricted to specialist laboratories. Currently, there is no standardization of NAT-based assays for the detection of ZIKV RNA. In early 2016, the World Health Organization (WHO) asked the Paul-Ehrlich-Institut (PEI), a WHO collaborating centre for quality assurance of blood products and *in vitro* diagnostic devices, to develop an International Standard (IS) for ZIKV RNA for use in NAT assays.

Study materials

Candidate International Standard – 11468/16

The ZIKV strain PF13/251013-18, selected for the preparation of the candidate IS, was isolated in 2013 from the serum of a French Polynesian patient (Aubry *et al*., 2016; Trösemeier *et al*., 2016). For preparation of the candidate IS, ZIKV was propagated in Vero E6 cells using Dulbecco's modified Eagle medium, without serum. Supernatants were harvested 8 to 10 days post-infection, clarified by low speed centrifugation and stored at -80°C prior to final formulation and lyophilization.

Heat inactivation of the stock virus was performed by initially filtering through a 0.45 µm filter and allowing 30 ml aliquots of the stock to equilibrate to 56°C in a water bath and then incubating for a period of 1 hour. The titre of the bulk virus stock before heat-inactivation was 7.07 $\log_{10}$ $TCID_{50}$/ml. The heat inactivated ZIKV stock was tested for infectious virus by large volume plating/bulk titration (28.8 ml). The $\log_{10}$ reduction factor for the heat treatment was $\geq 7.89 \pm 0.31$. The candidate IS was dispensed in 0.5 ml aliquots per vial for lyophilization (see below), and the calculated residual infectious virus load per vial, after heat-inactivation and dilution of the bulk preparation, is ≤ -1.98 $\log_{10}$ $TCID_{50}$.

For the lyophilization the inactivated virus stock was diluted 1:7.2 in a solution containing hydroxyectoin (Bitop AG, Witten, Germany) at a final concentration of 0.6 M. The filling and lyophilization was performed by an ISO 13485:2003 accredited Swiss company and processing took place between the 4th and 7th of April, 2016. For processing, 0.5 ml volumes were dispensed into 4 ml screw-cap glass vials. After completion of the freeze-drying procedure, the vacuum was broken by the introduction of nitrogen gas and the vials sealed. The vials were further secured with screw caps prior to storage at -20°C. A total of 4092 vials were prepared. The coefficient of variation (% CV) of the filled vials was 0.69% (n=27). Residual moisture was determined by Karl Fischer analysis and was 1.35% (n=14). Testing of samples of 11468/16 post-lyophilization revealed that there was an approximate drop in ZIKV RNA titre of 0.7-0.8 $\log_{10}$ copies/ml compared to the bulk.

Because the candidate IS was to be assigned a unitage with respect to the ZIKV RNA content, homogeneity of the filling/freeze-drying was assessed using real time-PCR. Extraction of RNA was performed using 200 µL of the sample using the QIAamp MinElute Virus Spin Kit (Qiagen GmbH, Hilden, Germany). Elution of the viral nucleic acid was performed using 70 µL of elution buffer, and 5 µL of the eluate was used for the RT-PCR. ZIKV RNA was detected using reverse-transcription real time PCR. Amplification reactions were performed using the

LightMix® Modular Zika Virus kit (TIB Molbiol GmbH, Berlin, Germany) and the LightMix® Modular Equine Arteritis Virus (EAV) RNA extraction control. Detection of ZIKV and EAV RNA was performed using the LightCycler 480 (Roche Applied Science GmbH, Mannheim, Germany) in accordance with the manufacturer's instructions. The assay is able to detect 10 copies of ZIKV RNA per reaction. A standard curve was prepared using an *in vitro* ZIKV RNA transcript (GenExpress Gesellschaft für Proteindesign mbH, Berlin, Germany). The crossing threshold or cycle threshold (C_T) values were determined for 30 vials of the candidate IS; the mean C_T value was 20.09, with a CV of 1.96%, indicating that the filling was of acceptable homogeneity.

Vials of the candidate WHO IS are held at the Paul-Ehrlich-Institut, Paul-Ehrlich-Straße 51-59, D-63225 Langen, Germany. The vials are kept at -20°C with continuous temperature monitoring.

All manufacturing records are held by PEI and are available on request by the WHO Expert Committee on Biological Standardization.

Inactivated reference materials

11469/16

This preparation was provided by ZBS 1 - Highly Pathogenic Viruses, Robert Koch Institute (RKI), Germany. Zika virus strain Uganda MR766 (Dick *et al*. 1952) was kindly provided by Dr. Maria João Alves (CEVDI/INSA National Institute of Health Dr. Ricardo Jorge, Portugal) via the European Network for Diagnostics of "Imported" Viral Diseases – (ENIVD). For thisreference material, the virus has been propagated in cell culture at the RKI, inactivated by heat-treatment and gamma irradiation, and diluted in a proprietary matrix and lyophilized. The standard is stored at +4°C. Details are given in the following link: https://www.rki.de/DE/Content/Institut/OrgEinheiten/ZBS/ZBS1/Zika_PCR_standard.pdf?__blob=publicationFile

11470/16 and 11471/16

These preparations were provided by the Center for Biologics Evaluation and Research/U.S. Food and Drug Administration (CBER/FDA). The ZIKV strains were isolated in Cambodia (11470/16) and Puerto Rico (11471/16) in 2010 and 2015, respectively. The isolates were propagated in cell culture and heat-inactivated, diluted in pooled human plasma and stored frozen at ≤ -60°C.

11474/16

This was prepared by the PEI using the same inactivated virus preparation and dilution as the candidiate IS, however, this preparation was diluted in pooled human plasma and lyophilized. Vials of 11474/16 were stored frozen at -20°C

114579/16

This was prepared by the National Institute for Biological Standards and Control (NIBSC), United Kingdom. This preparation (NIBSC code 16/110) has been produced using the MP 1751 ZIKV strain from Uganda (Haddow *et al*., 1964) as supplied by Public Health England Culture Collection. Details are given in the following link: https://www.phe-culturecollections.org.uk/products/viruses/detail.jsp?refId=1308258v&collection=ncpv. The virus was propagated in cell culture, heat-inactivated and diluted in pooled human plasma prior to lyophilization. Vials of 11579/16 were stored frozen at -20°C.

Clinical materials

11467/16
This material was a pool of two ZIKV-positive urine samples from Europe, one patient developed ZIKV infection after travelling to South America and the other was obtained from a patient who became infected after sexual intercourse with a partner who had visited South America. The pooled material was stored frozen at ≤ -60ºC.

11472/16
This viraemic plasma sample came from a Brazilian patient infected with ZIKV. Plasma has been stored as liquid/frozen material at ≤ -60ºC after ~1:50 dilution in pooled human plasma which tested negative for ZIKV RNA at the PEI.

11473/16
This viraemic plasma sample came from a Brazilian blood donor infected with ZIKV (Cunha *et al*., 2016; Barjas-Castro *et al*., 2016). Plasma has been stored as liquid/frozen material at ≤ -60ºC after ~1:10 dilution in pooled human plasma which tested negative for ZIKV RNA at the PEI.

In vitro transcribed RNAs

11475/16-11478/16

Two sets of *in vitro* transcribed RNAs (IVTs) were included in the study and these were prepared by the University of Bonn, Germany. The IVTs have been described elsewhere: http://www.who.int/bulletin/online_first/16-175950.pdf, futher information is provided in Appendix 2. For use in the study, the IVTs were diluted in 10 mM Tris pH 8.0 containing carrier nucleic acid and provided at 2 different concentrations i.e. 10,000 copies/µl and 1,000 copies/µl.

The phylogenetic relationship of the ZIKV strains included in the collaborative study are shown in Figure 1. Sequences were not available for all strains included in the study.

Collaborative Study

The collaborative participants are listed in Appendix 1; 24 laboratories from 11 different countries agreed to participate in the study. For the purposes of data analysis, each laboratory has been referred to by a code number allocated at random and not representing the order of listing in Appendix 1.

All collaborative study materials were shipped to participating laboratories on dry ice and participants requested to store the materials at or below -60°C until use. Participants received samples representing both African and Asian ZIKV lineages and samples represented a mixture of inactivated reference materials as well as a small number of clinical samples and *in vitro* RNA transcripts.

The samples included in the panel are described above and listed in Table 1. The approximate titres of the study materials were provided to the participants for guidance (Table 2). Sufficient materials were provided for three separate assay runs. Additional vials were provided for laboratories using larger extraction volumes. In the case of sample 11467/16 (pooled ZIKV-positive human urine), because of limited sample volume, this sample was only distributed to

those laboratories that confirmed that they tested this type of sample routinely. Laboratory 13 only received inactivated samples and the IVTs.

Participants were asked to test the panel using their routine assay for ZIKV RNA, testing the panel of samples in three separate assay runs, using fresh vials of each sample for each run. Where laboratories performed quantitative tests, they were requested to report results in copies/ml, suggested dilutions to test for each sample were proposed in the study protocol (Table 2). In the case of qualitative assays, participants were requested to assay each sample by a series of one $\log_{10}$ dilution steps, to obtain an initial estimate of an end-point. For the two subsequent assays, they were requested to assay half-$\log_{10}$ dilutions around the end-point determined in their first assay. If, in the second assay, all dilutions were positive, or all negative, then the dilution series were to be adjusted accordingly for the final assay run. Participants were asked to note if replicate extractions and replicate amplification/detection steps were performed. Results were reported as either positive i.e. ZIKV RNA detected or negative. Electronic data sheets and a method reporting form were provided so that all relevant information (e.g. C_T values for the respective dilutions where real time PCR methods were used or signal to cut-off (S/Co) values - e.g. for transcription-mediated assays) could be reported.

For the preparation of dilutions, participants were requested to use their usual diluent representing the matrix of the normal test specimens e.g. ZIKV negative plasma. It was recommended to dilute sample 11468/16 in plasma, urine, or other types of sample matrix that might be used for ZIKV diagnostic testing. It was suggested to dilute sample 11469/16 using cell culture medium.

Several lyophilized preparations were evaluated in the study and these were reconstituted before use by participants using molecular grade, nuclease-free water. Samples 11468/16 and 11474/16 were reconstituted in 0.5 ml of water; samples 11469/16 and 11579/16 was reconstituted in 0.1 and 1.0 ml of water, respectively. After addition of water, it was recommended that the samples be left for a minimum of 20 minutes with occasional agitation before use. All other samples were provided as liquid/frozen materials.

Concerning the IVTs, participants were requested to test the IVTs by addition of 2 µl of RNA directly to the amplification/detection reaction. Because these IVTs do not cover the entire ZIKV genome, participants were asked to review the sequences (Appendix 2) to determine if they included the target region of their assay(s). In the case of quantitative assays participants were requested to report results as copies/ml. For the qualitative assays, results for testing of the IVTs were to be reported as C_T values, or as positive (RNA detected) or negative (RNA not detected). Because the IVTs were not intended to undergo extraction, it was obvious that fully automated systems might not be suitable for evaluation of these samples.

Statistical Methods

Quantitative Assays

Quantitative assays were performed over a range of specific dilutions expected to fall in the linear range of the majority of assays (Table 2).
Evaluation of quantitative results includes the complete dilution range used by the participants, as with correction of the dilution factor the data show comparable results between dilutions. Estimates (expressed in $\log_{10}$ NAT-detectable units/ml) for each laboratory, assay and matrix were derived by the arithmetic mean from up to 3 runs and 3 replicates.

Qualitative Assays

For qualitative data analysis, results from all assays were pooled to give the number of positives out of the total number tested at each dilution. If it is assumed that a single 'detectable unit' will give a positive result, and that the probability of a positive result follows a Poisson distribution, the EC63 (the dilution at which 63% of the samples are expected to be positive) was chosen as the end-point. For each laboratory and sample, these end-points were estimated by means of probit analysis. For assays where the change from complete negative to complete positive results occurred in two or fewer dilution steps for all samples, the Spearman-Kaerber method was applied for EC63 estimation. The calculated end-point was used to give estimates expressed in $\log_{10}$ NAT-detectable units/ml after correcting for the equivalent volume of the test sample.

Combination of quantitative and qualitative data

Quantitative and qualitative data were combined by means of mixed linear models to calculate estimates per sample and laboratory, per sample and assay type (qualitative, quantitative), per sample and matrix (plasma, medium, saliva, serum, urine or direct testing for the IVTs), and overall estimates for each sample. Distribution of participants, matrix, and assay types were graphically presented in histogram form.

For assignment of the potency to the candidate IS, data from 70 assays were combined using a mixed linear model with random factors *laboratory*, *assay type* (qualitative/quantitative), and *matrix*. Data from all 21 laboratories was included with 17 participants providing data for more than one assay. Most assays were quantitative (41 assays, 29 qualitative assays) and tested in plasma (35 assays; urine: 23, saliva: 9, serum: 2, and medium: 1 assay). No assays had to be excluded.

Relative potencies

Potencies of all samples, for the quantitative assays, were estimated relative to the candidate IS 11468/16 using parallel line analysis of log transformed data. In the case of the qualitative assays, the relative potencies were determined using parallel line analysis of probit transformed data.

C_T values

For assays reporting C_T values, these were evaluated for both qualitative and quantitative methods (relative to the candidate IS 11468/16) using a parallel line model for each laboratory combined for all evaluable (i.e. valid) assay runs. Relative potencies from C_T values were shown as forest plots to allow for a comparison of participants, assay types, and, where applicable, matrix.

Parallel line and probit analysis were performed according to methods as described in chapter 5.3, "Statistical analysis of results of biological assays and tests", of the European Pharmacopoeia (8th Edition, Strasbourg, France Council of Europe; 2015). The statistical analysis was performed with SAS®/STAT software, version 9.4, SAS System for Windows, and CombiStats, version 5.0, EDQM, Council of Europe (www.combistats.eu).

Results

Data received

Data were received from a total of 21 of the 24 participating laboratories. In total, 37 sets of data were returned; 19 from quantitative assays and 18 from qualitative assays. Some laboratories reported results for more than one type of assay. Where a laboratory performed more than one assay method, the results from the different methods were analysed independently, as if from separate laboratories, and coded, for example, laboratory 16a and laboratory 16b.

The types of methods used by the participants are listed in Table 3. Assays included in the study targeted several different regions of the ZIKV genome – these included the propeptide, envelope, NS1, NS2A, NS2B, NS5 as well as the 3' UTR. The assays included in-house developed assays, ones based on scientific publications as well as commercial assays (including ones still in development). The vast majority of assays were based on real-time PCR. Different types of extraction method were used (Table 3). The types of matrix evaluated for the dilution of sample 11468/16 are indicated in Table 3 (these do not necessarily reflect the types of matrix validated for the respective tests).

The urine sample (11467/16) was distributed to 12 laboratories, of these 7 returned results that could be analysed.

Laboratory 2a used the ZIKV reference material provided by the RKI as a calibrator in their quantitative assay. Laboratory 5 used the European Virus Archive reference material as calibrator for three quantitative assays (5a-5c). Other laboratories performing quantitative assays either used IVTs or ZIKV with quantified genomic titres for quantitation.

Laboratory 10 did not test sample 11469/16.

Laboratory 15 performed three different types of assay (quantitative), in the case of 15c, results were reported only for the panel of IVTs.

Laboratory 16 used 2 different extraction kits, one for runs 1 and 2 (same extract) and a different kit for the third run.

The different ZIKV strains from both the African and Asian lineages were well detected by the assays used in the study. In some cases, differences in the efficiency of detection were observed for some of the panel members.

Quantitative Assay Results – Mean Estimates

The individual laboratory mean estimates for the panel of samples, are shown in Tables 4-7. These data are based on quantitative assays with values reported in copies/ml or genome equivalents/ml (accounted for dilution). The laboratory mean absolute estimates of NAT-detectable units/ml ($\log_{10}$) are shown in histogram form in Figures 2-8. Data are presented in the tables as NAT-detectable units per ml. The majority of laboratories had estimates that were within approximately 2.5 $\log_{10}$ NAT-detectable units for sample 11468/16, the candidate IS. In the case of 11468/16, this was diluted in several different types of matrix – mainly plasma and urine although other types of matrix including saliva, serum and cell culture medium were used – the reported potencies are shown in Figure 3. As can be seen, there were no major differences in

the reported potencies of 11468/16 when diluted in different types of diluent. In the case of saliva, the potencies of 11468/16 seem to be slightly higher; however, data were only reported from 2 laboratories. Variability between different quantitative assays is in part due to different types of calibrator as well as assay design. For example, laboratories 5 and 11, for the majority of the biological samples, reported consistently higher potencies than others. This is likely to be due to the individual calibrators, since several of the assays employed by these laboratories were used by other participants reporting lower potencies.

Qualitative Assay Results – Mean Estimates

The individual laboratory mean estimates, based on the results of the qualitative assays, for the panel of samples are shown in Tables 4, 5 and 7. The laboratory mean absolute estimates of NAT-detectable units/ml ($\log_{10}$) are shown in histogram form in Figures 2-8, the data from the qualitative assays is shown underlined in the respective boxes in each histogram. The majority of laboratories had estimates that were within approximately 2.0-2.5 $\log_{10}$ NAT-detectable units for sample 11468/16, the candidate IS. A similar spread of data is seen for 11468/16 as per the quantitative assays for the different types of matrix. Variability between the qualitative data reflects the different sensitivities of the assays. This observation is not unexpected and is in line with previous studies.

IVTs

Data returned by participants for the set of IVTs is summarized in Table 8. Quantitative values in copies/ml or genome equivalents/ml were reported for the quantitative assays. In the case of the qualitative assays, participants reported either C_T values or scored the samples as either positive or negative. The data for the quantitative assays is shown in histogram form in Figures 7-8. In some cases, the IVTs did not contain the target region and were not tested; in others, fully automated systems were employed and it was not possible to add the IVTs to the amplification/detection reaction.

Determination of Overall Laboratory Means – Combined Qualitative and Quantitative Results

The overall mean values for the candidate IS and the other samples for the qualitative and quantitative assays are shown in Table 9 and the precision of estimates between laboratories (95% confidence interval). The combined overall mean values for both the qualitative and quantitative tests are shown in Table 10 together with the standard deviations and the range of estimates. The overall means fit well with the values expected for the panel of samples (Table 2).

Qualitative assays gave consistently lower mean estimates for the panel of biological samples than for the quantitative assays, probably as a result of lower sensitivity of the qualitative assays. The highest standard deviations were observed with the IVTs, although the data sets were more limited and may be a reflection of the lower number of results for this set of samples.

Relative Potencies

On the basis of the combined data from both qualitative and quantitative assays, the candidate WHO IS was determined to have a potency of 7.70 $\log_{10}$ units/mL (95% CI 7.04-8.35). The potencies of the panel of samples were calculated relative to sample 11468/16 – the candidiate IS, taking the value of sample 11468/16 as 7.70 $\log_{10}$ units/mL. The relative potencies for the

quantitative and qualitative assays are shown in Figures 10-15. For samples 11467/16, 11469/16-11474/16 and 11579/16, the biological samples, it can be observed that in each case there is a marked reduction in assay variability when data are expressed against the common standard. However, for the IVTs, expression of the data relative to 11468/16 (quantitative assays) results in no significant improvement of agreement between assays.
Expression of the IVT mixture 11478/16 against 11476/16, the universal IVT calibrator – taking the value of the latter as 5.57 $\log_{10}$ units/ml (Table 10), and using data from methods that tested both types of transcript, there was an improvement in agreement. However, expression of 11468/16, the candidate WHO IS, against 11476/16 did not result in any marked reduction in assay variation between laboratories.
Precision of relative potency estimates is shown by forest plots (Figure 9) for the different labs and assays where C_T values have been reported. In this case, the values have been expressed relative to the 11468/16 diluted in plasma. For some laboratories 95% confidence intervals are far wider than for others, in some cases due to higher intra-laboratory variability, but more often simply due to a lower amount of data available for evaluation. In the plots, the qualitative assays are indicated by a star on the horizontal axis and the confidence intervals are indicated by the vertical bar for each laboratory/assay. Not unexpectedly, the plots for sample 11474/16 relative to 11468/16 are most consistent, reflecting the fact that the same virus strain was used to prepare both samples.

Stability Studies

Stability of the candidate IS is under continuous assessment, through both real-time and accelerated thermal degradation stability studies. Vials of the candidate panel samples have been stored at -20°C (the recommended storage temperature). For the accelerated thermal degradation, vials were incubated at +37°C and +45°C for 1 week and 8 weeks. After incubation at the respective temperatures, the contents of the vials were reconstituted in 0.5 ml of nuclease free water and analysed by real-time PCR as described above.

Results of Stability Studies

Accelerated thermal degradation studies have been performed by incubation of vials of 11468/16 at higher temperature i.e. +37° and +45C° and compared to vials stored at -20°C, the normal storage temperature. Duplicate vials were evaluated for each temperature/time point and PCR was performed in duplicate. There was no evidence for a reduction in ZIKV RNA after 8 weeks incubation at +45C° (Table 11). After reconstitution of 11468/16 and storage at -80°C, no loss of titre was observed after three freeze-thaw cycles. Accelerated and real-time stability studies are on-going.

Conclusions

In this study, a wide range of quantitative and qualitative assay formats were used to evaluate the candidate ZIKV RNA IS and other ZIKV preparations. Approximately half of the assays were developed in-house with the rest being available commercially or in development by kit manufacturers. The panel of samples, both Asian and African lineages included in the study, were well detected by the participating laboratories, with some differences in efficiency of detection of some of the samples. Although evaluation of additional strains and careful review of primer and probe design is necessary to ensure that assays are able to detect all expected virus variants (Corman *et al*., 2016).

Despite several laboratories reporting quantitative data as copies/ml or genome equivalents per ml, there was a wide variation in reported values of the respective ZIKV samples and IVTs in the study. Test result reporting was based on RNA transcripts or virus preparations to prepare standard curves for quantitative assays. However, for the biological samples in the panel, agreement was improved by expression of potencies relative to 11468/16, the candidate IS. This finding suggests that a common reference material can help in harmonizing results between different assays and between different laboratories.

The relative potency data provide some evidence for commutability of the candidate standard for evaluation of ZIKV from infected persons, because there was an improvement in agreement of results for samples 11472/16 and 11473/16 – the clinical plasma samples, as well as the marked improvement seen for sample 11467/16 – the pool of two ZIKV-positive clinical urine samples. From the relative potency data it was shown that there was an improvement in agreement between a range of inactivated reference preparations prepared from several different strains of ZIKV, and improvement in agreement was observed not just between Asian lineages of the virus, but, also between older African isolates that have been extensively passaged in the laboratory. There was no improvement of agreement with the IVTs when expressed relative to the candidiate IS. There was agreement however, between the IVTs. However, these (bio)-synthetic materials may standardize the amplification/detection part of NAT, not the extraction, which is a critically important step.

The data from the study also provide an evaluation of the mean estimates for several secondary reference materials included in the study and these include materials from the RKI (11469/16), CBER/FDA (11470/16 and 11471/16) and NIBSC (11579/16).

Real-time stability studies have indicated that the candidate IS is stable under normal conditions of storage, i.e., at -20ºC or below for 3 months and therefore suitable for long term use as well as at elevated temperatures i.e. after 8 weeks incubation at 45°C there was no reduction in ZIKV RNA which would support shipment at ambient temperature. Stability studies for the candidate IS are on-going. The draft “Instructions for Use” for the candidate IS, include details for storage and reconstitution of the material (Appendix 3); each vial contains the dried residue of 0.5 ml of inactivated ZIKV to be reconstituted in 0.5 ml of water.

Recommendations

Based upon the results of the collaborative study, it is proposed that the heat-inactivated ZIKV strain, code number 11468/16, should be established as the 1st International Standard for ZIKV RNA with a unitage of 50,000,000 IU/ml. The custodian laboratory is the Paul-Ehrlich-Institut.

Responses from participants

After circulation of the draft report for comment, replies were received from twenty-one participants; all were in agreement with the conclusions of the report. The majority of the comments were editorial in nature and the report has been amended accordingly. In some cases statistical methods have been clarified. One participant suggested removing the sections related to IVT panels. IVT panel and testing result may not be relevant and the IVT panels may not be appropriate as universal control and reference material. Another commented that the concentration of the candidate WHO IS seems to be quite high.

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Table 1 Collaborative study materials

Code number Presentation	Description of preparation	Origin Strain Accession number
11467/16 Liquid/frozen	Human urine containing ZIKV; pool of 2 ZIKV clinical samples	Europe 2016
11468/16 Lyophilized	Cell culture derived virus; heat inactivated	French Polynesia, 2015 Strain: PF13/251013-18 GenBank: KX369547
11469/16 Lyophilized	Cell culture derived virus; heat inactivated and gamma irradiated	Uganda, 1947 Strain: MR766 RKI* and EVAg
11470/16 Liquid/frozen	Heat inactivated cell culture derived virus diluted in pooled human plasma	Cambodia, 2010 Strain: FSS13025 GenBank JN860885
11471/16 Liquid/frozen	Heat inactivated cell culture derived virus diluted in pooled human plasma	Puerto Rico, 2015 Strain: PRVABC59 GenBank: KU501215
11472/16 Liquid/frozen	Viraemic plasma diluted in pooled human plasma	Brazil, 2016
11473/16 Liquid/frozen	Viraemic plasma diluted in pooled human plasma	Brazil, 2016 GenBank: KU321639
11474/16 Lyophilized	Heat inactivated cell culture derived virus diluted in pooled human plasma	French Polynesia, 2013 Strain: PF13/251013-18 GenBank: KX369547
11579/16 Lyophilized	Heat inactivated cell culture derived virus diluted in pooled human plasma	Uganda, 1962 Strain: MP 1751 PHE Virus Collection**#
11475/16 Liquid/frozen	IVT RNA - Universal Control	EVAg
11476/16 Liquid/frozen	IVT RNA - Universal Control	EVAg
11477/16 Liquid/frozen	IVT RNA Mixture	EVAg
11478/16 Liquid/frozen	IVT RNA Mixture	EVAg

*http://www.rki.de/DE/Content/Institut/OrgEinheiten/ZBS/ZBS1/Zika_PCR_standard.pdf?__blob=publicationFile (This material is also distributed by the European Virus Archive - EVAg).
**Virus obtained from the Public Health England Cultures Collection - https://www.phe-culturecollections.org.uk/products/viruses/detail.jsp?refId=1308258v&collection=ncpv
Sequencing of this virus strain is available as part of the ZiBRA project - https://raw.githubusercontent.com/zibraproject/zibraproject.github.io/master/data/Zika_MP1751_NCPV_consensus.fasta
N.B. Resequencing of PF13/251013-18 (11468/16 and 11474/16) has been performed as part of the ZiBRA project, details: http://zibraproject.github.io/data/

Table 2 Collaborative study materials – nominal concentrations

Code number	Nominal concentration	Suggested dilutions for analysis
11467/16	2-3 $\log_{10}$ copies/ml	Neat
11468/16	7.5-8.5 $\log_{10}$ copies/ml	10^{-3} to 10^{-5}
11469/16	6.0-7.5 $\log_{10}$ copies/ml	10^{-2} to 10^{-4}
11470/16	5-6.5 $\log_{10}$ copies/ml	10^{-1} to 10^{-3}
11471/16	5-6.5 $\log_{10}$ copies/ml	10^{-1} to 10^{-3}
11472/16	3.5-5.0 $\log_{10}$ copies/ml	Neat - 10^{-1}
11473/16	3.5-5.0 $\log_{10}$ copies/ml	Neat - 10^{-1}
11474/16	7.5-8.5 $\log_{10}$ copies/ml	10^{-3} to 10^{-5}
11579/16	6.0-7.5 $\log_{10}$ copies/ml	10^{-2} to 10^{-4}
11475/16	4 $\log_{10}$ copies/µl	2 µl added directly to reaction
11476/16	3 $\log_{10}$ copies/µl	2 µl added directly to reaction
11477/16	4 $\log_{10}$ copies/µl	2 µl added directly to reaction
11478/16	3 $\log_{10}$ copies/µl	2 µl added directly to reaction

Table 3 Assay protocols used by participants

Lab. code	Assay type*	Extraction method	NAT method	Matrix	IC (reference material)	Reference
1	Qual.	cobas® 6800/8800 (Roche)	Real-time RT-PCR (cobas® Zika, Roche Molecular Systems)	Plasma	Y	
2a	Quant.	NucleoSpin® RNA Virus isolation kit (Macherey- Nagel)	Real-time RT-PCR (Zika virus Single check kit Genekam Biotechnology AG)	Plasma	N (ENIVD ZIKV reference material)	
2b	Qual.	NucleoSpin® RNA Virus isolation kit (Macherey- Nagel)	Real-time PCR (Zika virus Single check kit Genekam Biotechnology AG)	Plasma Urine	N	
3a	Qual.	Utility channel cobas® 6800 (Roche)	Real-time RT-PCR (TaqMan)	Plasma Urine	Y	
3b	Qual.	Utility channel cobas® 6800 (Roche)	Real-time RT-PCR (TaqMan)	Plasma Urine	Y	
4	Quant.	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (RealStar® Zika Virus RT-PCR Kit 1.0, altona Diagnostics)	Plasma Serum Urine	Y (IVT)	
5a	Quant.	MagNA Pure Total Nucleic acid Kit (Roche)	Real-time RT-PCR (TaqMan)	Plasma Urine Saliva	Y (EVAg ZIKV reference material)	Lanciotti *et al*., 2008 (envelope)
5b	Quant.	MagNA Pure Total Nucleic acid Kit (Roche)	Real-time RT-PCR (TaqMan)	Plasma Urine Saliva	Y (EVAg ZIKV reference material)	Corman *et al*., 2016 (NS1)
5c	Quant.	MagNA Pure Total Nucleic acid Kit (Roche)	Real-time RT-PCR (RealStar® Zika Virus RT-PCR Kit 1.0, altona Diagnostics)	Plasma Urine Saliva	Y (EVAg ZIKV reference material)	
6	Qual.	MagNA Pure 96 Viral NA Large Volume Kit (Roche)	Real-time RT-PCR (LightMix® Modular Zika Virus TIB MOLBIOL)	Plasma	Y	
7	Qual.	Exiprep™16 Dx Viral RNA kit (Bionner)	Real-time RT-PCR (Multiplex PCR - ZIKV, DENV, CHIKV - Bioneer)	Plasma Urine	Y	
8	Qual.	AmpliPrep TNAI kit (Roche)	Real-time RT-PCR (TaqMan)	Plasma Urine	N	Lanciotti *et al*., 2008 (envelope)
9a	Quant.	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (LightMix® MDx all Flavi TIB MOLBIOL)	Plasma	Y (IVT)	
9b	Quant.	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (LightMix® MDx Multiplex Zika Virus, Dengue Virus, Chikungunya virus TIB MOLBIOL)	Plasma	Y (IVT)	

9c	Quant.	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (LightMix® Modular Zika Virus TIB MOLBIOL)	Plasma Urine Saliva	Y (IVT)	
9d	Quant.	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (TaqMan)	Plasma Urine	Y (IVT)	Lanciotti *et al*., 2008 (envelope)
10	Qual.	VERSANT Sample Preparation 1.0 kit reagents (Siemens)	Real-time RT-PCR (VERSANT® Zika RNA 1.0 Assay (kPCR) Kit Siemens)	Plasma Serum Urine	Y	
11a	Quant.	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (TaqMan)	Plasma	N (ZIKV stock – quantified in copies/ml)	Lanciotti *et al*., 2008 (envelope)
11b	Quant.	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (TaqMan)	Plasma	N (ZIKV stock – quantified in copies/ml)	Lanciotti *et al*., 2008 (envelope)
12a	Qual.	MagNA Pure 96 Viral NA Small Volume Kit (Roche)	Real-time RT-PCR (TaqMan)	Plasma Urine	N	Corman *et al*., 2016 (NS1)
12b	Qual.	MagNA Pure 96 Viral NA Small Volume Kit (Roche)	Real-time RT-PCR (TaqMan)	Plasma Urine	N	Corman *et al*., 2016 (envelope)
12c	Qual.	MagNA Pure 96 Viral NA Small Volume Kit (Roche)	Real-time RT-PCR (FTD Zika virus. FastTrack Diagnostics)	Plasma Urine	Y	
12d	Quant.	MagNA Pure 96 Viral NA Small Volume Kit (Roche)	Real-time RT-PCR (TaqMan)	Plasma Urine	N (IVT)	Corman *et al*., 2016 (NS1)
12e	Quant.	MagNA Pure 96 Viral NA Small Volume Kit (Roche)	Real-time RT-PCR (TaqMan)	Plasma Urine	N (IVT)	Corman *et al*., 2016 (envelope)
13	Qual.	QIAamp Viral RNA Mini Kit (Qiagen)	Real Time RT-PCR (Zika Virus (ZIKA) Kit Liferiver)	Plasma Urine	Y	
14a	Qual.	QIAamp MinElute Virus Spin Kit (Qiagen)	Real-time RT-PCR (TaqMan)	Plasma	N	Lanciotti *et al*., 2008 (envelope)
14b	Qual.	QIAamp MinElute Virus Spin Kit (Qiagen)	Real-time RT-PCR (RealStar® Zika Virus RT-PCR Kit 1.0, altona Diagnostics)	Plasma	Y	
15a	Quant.	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (TaqMan)	Serum Urine	N (IVT)	
15b	Quant.	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (RealStar® Zika Virus RT-PCR Kit 1.0, altona Diagnostics)	Serum Urine	Y	
15c	Qual.	QIAamp Viral	Real-time RT-PCR	IVTs		Faye *et al*.,

		RNA Mini Kit (Qiagen)	(TaqMan)	only		2013 (NS5)
16	Quant.	RNeasy mini Kit (Qiagen) - run 1 / 2 QIAamp Viral RNA Mini Kit (Qiagen) – run 3	Real-time RT-PCR (TaqMan)	Plasma Urine	N (IVT)	Lanciotti *et a*l., 2008 (envelope)
17	Qual.	Automated proprietary magnetic target capture method – Procleix Panther System	Transcription mediated amplification/hybridization protection assay (Procleix ZIKV Assay, Hologic Inc.)	Plasma	Y	
18	Quant.	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (RealStar® Zika Virus RT-PCR Kit 1.0, altona Diagnostics)	Plasma Urine	Y (IVT)	
19a	Quant.	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (TaqMan)	Plasma	N (IVT)	Lanciotti *et al*., 2008
19b	Qual.	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (TaqMan)	Plasma	N	Lanciotti *et al*., 2008
20	Qual.	NucliSENS® easyMag® (bioMérieux)	Real-time RT-PCR (TaqMan)	Plasma Urine	N	Faye *et al*., 2013 (NS5)
21	Qual.	NucliSENS® easyMag® (bioMérieux)	Reverse transcription PCR xMAP® MultiFLEX™ Mosquito-borne Panel (ZIKV, DENV)	Plasma Serum Urine	Y	

*Assay type:Qualitative (Qual.) and quantitative (Quant.) assays
Laboratories were requested to test sample 11469/16 by dilution in cell culture medium. In the case of laboratory 15 serum was used as a dilution matrix, this was foetal bovine serum
Laboratory 3a and 3b used the same extraction platform, but performed PCR using 2 different regions of the ZIKV genome; laboratory 11a and 11 b used the same extraction and amplification/detection assays, the difference between 11a and 11b was realted to the volume of extacted RNA tested

Table 4 Laboratory mean estimates from quantitative and qualitative assays (log_{10} NAT-detectable units/ml) – samples 11467 (urine) and 11468/16 (diluted in urine)

Sample Assay type	Laboratory code	NAT detectable units/ml (log_{10})
11467/16 Quantitative assays	5a	3.91
	5b	4.48
	5c	4.23
	9c	2.44
	9d	2.74
	12d	3.38
	12e	2.45
	15a	1.89
	18	1.95
11467/16 Qualitative assays	20	1.74
	21	1.70
11468/16 Quantitative assays	4	8.52
	5a	9.56
	5b	9.62
	5c	9.57
	9a	7.69
	9b	8.43
	9c	8.42
	9d	8.62
	12d	8.62
	12e	7.89
	15a	5.91
	16	7.09
	18	7.72
11468/16 Qualitative assays	3a	6.52
	3b	6.43
	7	7.65
	8	7.13
	12a	7.59
	12b	7.26
	12c	6.73
	13	7.45
	20	6.47
	21	7.70

Table 5 Laboratory mean estimates from quantitative and qualitative assays (log_{10} NAT-detectable units/ml) – samples 11468/16 and 11469/16 diluted in medium/plasma

Sample Assay type	Laboratory Code	NAT detectable units/ml (log_{10})
11468/16 Quantitative assays (medium)	18	7.68
11469/16 Quantitative assays (medium)	2a	6.32
	4	7.89
	5a	8.89
	5b	9.05
	5c	8.92
	9a	7.36
	9b	7.95
	9c	7.95
	9d	7.56
	11a	6.97
	11b	6.89
	12d	7.50
	12e	6.86
	15a	6.32
	16	5.46
	18	6.87
	19a	5.54
11469/16 Qualitative assays (medium)	1	7.00
	2ba	4.86
	3	6.52
	3b	6.91
	6	7.26
	7	6.95
	8	6.61
	10	Not tested
	12a	5.85
	12b	6.52
	12c	5.73
	13	6.92
	17	7.61
	19b	6.73
	20	5.36
	21	4.89
11469/16 Qualitative assays (plasma)	14a	6.79
	14b	7.33

Table 6 Laboratory mean estimates from quantitative assays (log_{10} NAT-detectable units/ml) for sample 11468/16 diluted in saliva

Laboratory code	NAT detectable units/ml (log_{10})
5a	9.69
5b	8.91
5c	9.66
9Sb	8.22
9Sc	8.16
9Sd	8.53
9b	7.99
9c	7.77
9d	8.11

Laboratory 9 performed additional testing (9Sb, 9Sc, 9Sd) using saliva as a diluent for the respective assays.

Table 7 Laboratory mean estimates from quantitative and qualitative assays (log_{10} NAT-detectable units/ml) – samples 11468/16, 11470/16-11474/16 and 11579/16 diluted in plasma and serum

Lab. code	Assay type	Sample						
		11468/16	11470/16	11471/16	11472/16	11473/16	11474/16	11579/16
2a	Quantitative assays	7.48	5.40	5.76	4.73	4.73	8.17	6.42
4 (plasma)		8.00	5.87	6.51	4.97	4.82	8.31	7.41
4 (serum)		8.29						
5a		9.64	7.25	7.70	6.18	5.99	9.83	8.67
5b		9.66	7.26	7.84	6.35	6.23	9.81	8.67
5c		9.51	7.27	7.74	6.18	6.01	9.60	8.70
9a		7.68	5.85	6.26	4.90	4.72	7.58	6.85
9b		8.57	6.09	6.60	5.02	4.81	8.60	7.73
9c		8.53	6.05	6.55	5.01	4.86	8.54	7.61
9d		8.56	6.21	6.62	5.10	4.93	8.61	7.01
11a		9.71	7.60	7.71	6.57	6.28	10.05	8.71
11b		9.73	7.46	7.90	6.64	6.29	10.12	8.46
12d		8.57	6.39	6.85	5.37	5.31	8.72	7.81
12e		7.95	5.70	6.15	4.79	5.04	7.96	7.23
15a		7.51	5.14	5.67	4.09	3.44	7.57	6.51
16		7.17	4.13	4.61	3.22	3.45	6.43	5.70
18		7.61	5.05	5.62	3.80	3.36	7.47	6.75
19a		7.62	5.22	5.76	3.89	4.09	7.53	6.00
1	Qualitative assays	7.16	5.40	5.79	4.57	4.79	8.01	5.37
2b		4.65	4.32	4.81	2.99	2.75	6.07	5.37
3a		6.52	5.02	5.52	4.02	3.52	7.02	6.19
3b		6.86	5.49	5.69	3.89	3.64	6.91	6.16
6		7.83	5.58	5.69	4.32	4.15	8.98	6.83
7		7.66	5.88	6.36	4.90	5.00	7.88	6.66
8		8.21	5.03	5.81	4.21	3.92	8.36	6.71
10		8.02	5.76	6.22	4.68	5.13	8.35	7.25
12a		7.59	5.03	5.54	4.54	4.85	6.73	6.49
12b		6.80	4.63	5.27	4.42	4.27	7.27	6.51
12c		6.33	4.73	5.34	4.33	4.34	7.54	6.12
13		8.57	5.67	6.26			8.26	6.77
14a		7.91	5.91	5.81	4.58	4.58	7.37	6.42
14b		7.51	6.03	6.19	4.75	4.75	8.00	6.64
17		8.22	5.79	6.73	5.12	5.00	8.51	7.26
19b		8.38	5.44	6.06	4.19	4.92	7.73	6.00
20		8.87	4.90	5.41	4.42	4.47	6.99	6.27
21 (plasma)		7.49	5.11	6.15	4.70	4.70	7.49	6.28
21 (serum)		7.70						

Laboratory 13 did not receive samples 11472/16 and 11473/16 for testing. For sample 11579/16, laboratory 16, the value is based on a single determination as all other dilutions fell below the limit of detection.

Table 8 Data returned for direct analysis of the panel of IVTs

IVT Preparation	Zika virus Universal calibrator RNA 1.0		Zika virus *In Vitro* RNA Mix	
Sample code	11475/16	11476/16	11477/16	11478/16
Nominal. conc. (cps/ml)	5.0E+06	5.0E+05	5.0E+06	5.0E+05
Qualitative assays Laboratory code				
1	n.r.	n.r.	n.r.	n.r.
2b*	C_T 21.5	C_T 23.7	C_T 24.0	C_T 23.4
3a	C_T 28.1	C_T 31.5	C_T 27.2	C_T 30.6
3b	n.r.	n.r.	n.r.	n.r.
6	n.r.	n.r.	C_T 29.1	C_T 31.4
7	C_T 27.5	C_T 31.2	C_T 30.3	C_T 33.2
8	C_T 25.7	C_T 28.3	C_T 27.9	C_T 30.1
10	n.r.	n.r.	n.r.	n.r.
12a	Pos.	Pos.	Pos.	Pos.
12b	Pos.	Pos.	Pos.	Pos.
12c	Pos.	Pos.	Pos.	Pos.
13	C_T 25.3	C_T 29.0	C_T 27.4	C_T 31.0
14a	C_T 21.6	C_T 23.9	C_T 22.9	C_T 25.2
14b	C_T 22.6	C_T 24.8	n.r.	n.r.
17	n.r.	n.r.	Pos.**	n.r.
19b	Pos.	Pos.	Pos.	Pos.
20	C_T 28.9	C_T 32.0	C_T 28.2	C_T 31.41
21	n.d.	n.d.	Pos.	Pos.
Quantitative assay Laboratory code				
2a	C_T 21.5	C_T 23.7	C_T 24.0	C_T 23.4
4	1.2E+07 cps/ml	8.6E+05 cps/ml	n.r.	n.r.
5a	6.5E+07 geq/ml	7.2E+06 geq/ml	1.2E+07 geq/ml	9.9E+05 geq/ml
5b	2.6E+07 geq/ml	3.0E+06 geq/ml	3.6E+07 geq/ml	2.5+05 geq/ml
5c	2.8E+07 geq/ml	1.6E+06 geq/ml	n.d.	n.d.
9a	n.d.	n.d.	n.d.	n.d.
9b	n.d.	n.d.	3.6E+06 cps/ml	3.8E+05 cps/ml
9c	n.d.	n.d.	7.0E+06 cps/ml	6.7E+05 cps/ml
9d	2.2E+07 cps/ml	1.9E+06 cps/ml	5.4E+06 cps/ml	4.3E+05 cps/ml
11a	7.5E+06 cps/ml	9.6E+05 cps/ml	2.1E+06 cps/ml	2.7E+05 cps/ml
11b	5.9E+06 cps/ml	9.3E+05 cps/ml	1.5E+06 cps/ml	1.8E+05 cps/ml
12d	3.5E+06 cps/ml	2.8E+05 cps/ml	4.2E+06 cps/ml	3.8E+05 cps/ml
12e	4.0E+06 cps/ml	4.5E+05 cps/ml	7.8E+05 cps/ml	7.8E+04 cps/ml
15a	n.r.	n.r.	n.r.	n.r.
15b	C_T 26.2	C_T 29.5	n.r.	n.r.
15c	C_T 28.4	C_T 32.5	C_T 28.5	C_T 32.7
16	1.7E+07 cps/ml	1.5E+06 cps/ml	5.6E+06 cps/ml	4.1E+05 cps/ml
18	n.r.	n.r.	n.r.	n.r.
19a	4.8E+05 cps/ml	5.6E+04 cps/ml	1.1E+05 cps/ml	1.2E+04 cps/ml

n.r. Not reported; n.d. Not detected

Lab 2a/2b Average of 3 C_Ts reported for both assays; Lab 3a Average of 3 C_Ts; Lab 4 Average of 9 values; Lab 5 Average of 3 values; Lab 6 Average of 6 C_Ts; Lab 7 Average of 9 C_Ts; Lab 8 Average of 9 C_Ts; Lab 9 Average of 6 values (9b), 6 values (9c), 4 values (9d); Lab 11 Average of 3 values (11a), 3 values (11b); Lab 12 Average of 3 values (12d), 3 values (12e); Lab 13 Average of 3 C_Ts; Lab 14 Average of 3 C_Ts (14a), ; Lab 15 Average of 9 C_Ts; only C_T values were reported (15b and 15c); Lab 16 Average of 6 values; Lab 17 Samples was diluted in buffer and subjected to automated extraction/amplification and found positive at a dilution of 1:100,000 for 5/5 replicates. 2/5 replicates were positive at the 10,000,000 dilution; Lab 19 Average of 9 C_Ts (19a); Lab 20 Average of 5 C_Ts

Table 9 Overall mean estimates for the panel of samples – qualitative and quantitative assays

Sample	Assay type	Units/ml log_{10}	95% CI		Units/ml	95% CI	
11467/16	Qual.	1.72	1.41	2.03	5.25E+01	2.59E+01	1.06E+02
11467/16	Quant.	2.75	1.56	3.93	5.56E+02	3.62E+01	8.55E+03
11468/16	Qual.	7.41	6.38	8.44	2.57E+07	2.38E+06	2.77E+08
11468/16	Quant.	8.08	7.22	8.93	1.19E+08	1.68E+07	8.51E+08
11469/16	Qual.	6.46	5.93	7.00	2.92E+06	8.58E+05	9.91E+06
11469/16	Quant.	6.93	6.15	7.70	8.49E+06	1.42E+06	5.07E+07
11470/16	Qual.	5.35	5.07	5.62	2.23E+05	1.18E+05	4.21E+05
11470/16	Quant.	5.78	5.04	6.52	6.02E+05	1.10E+05	3.30E+06
11471/16	Qual.	5.87	5.59	6.15	7.45E+05	3.92E+05	1.42E+06
11471/16	Quant.	6.26	5.55	6.97	1.83E+06	3.57E+05	9.37E+06
11472/16	Qual.	4.40	4.08	4.71	2.49E+04	1.20E+04	5.16E+04
11472/16	Quant.	4.77	4.00	5.54	5.87E+04	1.00E+04	3.43E+05
11473/16	Qual.	4.43	4.02	4.83	2.67E+04	1.05E+04	6.81E+04
11473/16	Quant.	4.63	3.88	5.38	4.26E+04	7.55E+03	2.40E+05
11474/16	Qual.	7.73	7.30	8.17	5.40E+07	1.99E+07	1.47E+08
11474/16	Quant.	8.23	7.45	9.01	1.69E+08	2.79E+07	1.02E+09
11475/16	Quant.	6.57	5.67	7.48	3.74E+06	4.66E+05	3.00E+07
11476/16	Quant.	5.57	4.68	6.45	3.68E+05	4.84E+04	2.79E+06
11477/16	Quant.	6.10	5.13	7.07	1.26E+06	1.36E+05	1.17E+07
11478/16	Quant.	5.08	4.15	6.00	1.19E+05	1.42E+04	1.00E+06
11579/16	Qual.	6.42	6.09	6.75	2.61E+06	1.22E+06	5.58E+06
11579/16	Quant.	7.11	6.39	7.84	1.29E+07	2.43E+06	6.88E+07

95% CI – 95% confidence interval
SD – standard deviation
Copies or genome equivalents: accounted for dilution; quantal values: estimated EC63

Table 10 Overall mean estimates for the panel of samples – combined data from qualitative and quantitative assays

Sample	N	Units/ml log_{10}	95% CI		SD	Min.	Max.	Units/ml	95% CI	
11467/16	11	2.46	1.59	3.33	0.92	1.70	4.48	2.92E+02	3.93E+01	2.16E+03
11468/16	70	7.70	7.04	8.35	1.04	4.65	9.73	4.97E+07	1.09E+07	2.26E+08
11469/16	34	6.69	6.02	7.36	1.00	4.86	9.05	4.89E+06	1.05E+06	2.28E+07
11470/16	35	5.53	5.03	6.04	0.61	4.13	7.60	3.41E+05	1.07E+05	1.09E+06
11471/16	35	6.05	5.55	6.54	0.57	4.61	7.90	1.12E+06	3.56E+05	3.50E+06
11472/16	34	4.56	4.02	5.10	0.66	2.99	6.64	3.63E+04	1.05E+04	1.25E+05
11473/16	34	4.52	3.96	5.08	0.72	2.75	6.29	3.30E+04	9.06E+03	1.20E+05
11474/16	35	7.95	7.34	8.55	0.90	6.07	10.12	8.86E+07	2.19E+07	3.58E+08
11475/16	12	6.57	5.67	7.48	1.18	4.30	7.81	3.74E+06	4.66E+05	3.00E+07
11476/16	12	5.57	4.68	6.45	1.12	3.30	6.86	3.68E+05	4.84E+04	2.79E+06
11477/16	12	6.10	5.13	7.07	1.12	4.30	7.56	1.26E+06	1.36E+05	1.17E+07
11478/16	12	5.08	4.15	6.00	1.02	3.30	6.39	1.19E+05	1.42E+04	1.00E+06
11579/16	35	6.74	6.16	7.31	0.74	5.37	8.71	5.49E+06	1.46E+06	2.06E+07

95% CI – 95% confidence interval
SD – standard deviation
Copies or genome equivalents: accounted for dilution; quantative values: estimated EC63
N – number of datasets

Table 11 Stability of 11468/16 at 1 week and 8 weeks

Temperature	**Log_{10} copies/ml**	
	1 week	8 weeks
- 20°C	8.03	8.10
+ 37°C	N.D.	8.06
+ 45°C	8.03	8.10

Figure legends

Figure 1
Maximum likelihood phylogeny of ZIKV strains evaluated in the study. The tree was estimated from published complete and partial (>80%) coding region sequences. Taxa are labeled with accession number, sampling location, and sampling date. The name of the sequence generated in this study is in bold. Numbers next to phylogenetic nodes denote bootstrap percentages (100 replicates). The strain MP1571 is available at the following link:
https://raw.githubusercontent.com/zibraproject/zibraproject.github.io/master/data/Zika_MP1751_NCPV_consensus.fasta
In the case of MR766, the sequence of sample 11469/16, was found to be identical to accession number KU955594 (bases 51-10,615). KU955594 was used in the construction of the phylogenetic tree. Arrows indicate the strains included in the study. Sequences are not available for samples 11467/16 and 11472/16.

Figure 2-8
Histograms showing results for quantitative and qualitative assays conducted by 21 laboratories for the detection of ZIKV RNA. Number of laboratories is indicated on the vertical axis. Laboratory code numbers are indicated in the respective boxes. The colour of the boxes corresponds to the sample/dilutional matrix i.e. red boxes – plasma, yellow – urine, green – serum, blue – saliva, purple – medium. Underlined – qualitative data, other data is based on quantitative assays.

Figure 9 Forest plots
C_T values, potencies in NAT detectable units, relative to 11468/16 plasma sample, when assigned a value of 7.70 $\log_{10}$ NAT-detectable units/ml, (estimates depicted as "+" with 95% confidence intervals as vertical lines; horizontal reference line: overall sample estimate estimated from quantitative and qualitative data. Matrix: D=directly tested (IVTs), M=medium, P=plasma, A=saliva, S=serum, U=urine). The star denotes data from qualitative assays.

Figure 10-16
Histograms showing potencies of the study samples relative to 11468/16, the candidate WHO IS for ZIKV RNA, when assigned a value of 7.70 $\log_{10}$ NAT detectable units/ml. Number of laboratories is indicated on the vertical axis. Laboratory code numbers are indicated in the respective boxes. The colour of the boxes corresponds to the sample/dilutional matrix i.e. red boxes – plasma, yellow – urine, green – serum, blue – saliva, purple – medium. Underlined – qualitative data, other data based on quantitative assays.

Figure 17
Histograms showing potencies of the study samples 11478/16 and 11468/16 relative to 11476/16, the Universal ZIKV calibrator RNA, based on quantitative assays. Number of laboratories is indicated on the vertical axis. Laboratory code numbers are indicated in the respective boxes. The colour of the boxes corresponds to the sample/dilutional matrix i.e. red boxes – plasma, yellow – urine, green – serum, blue – saliva.

Figure 1 Phylogenetic analysis of the ZIKV strains evaluated in the study

Figure 2 Laboratory mean estimates for ZIKV-positive urine pool - sample 11467/16

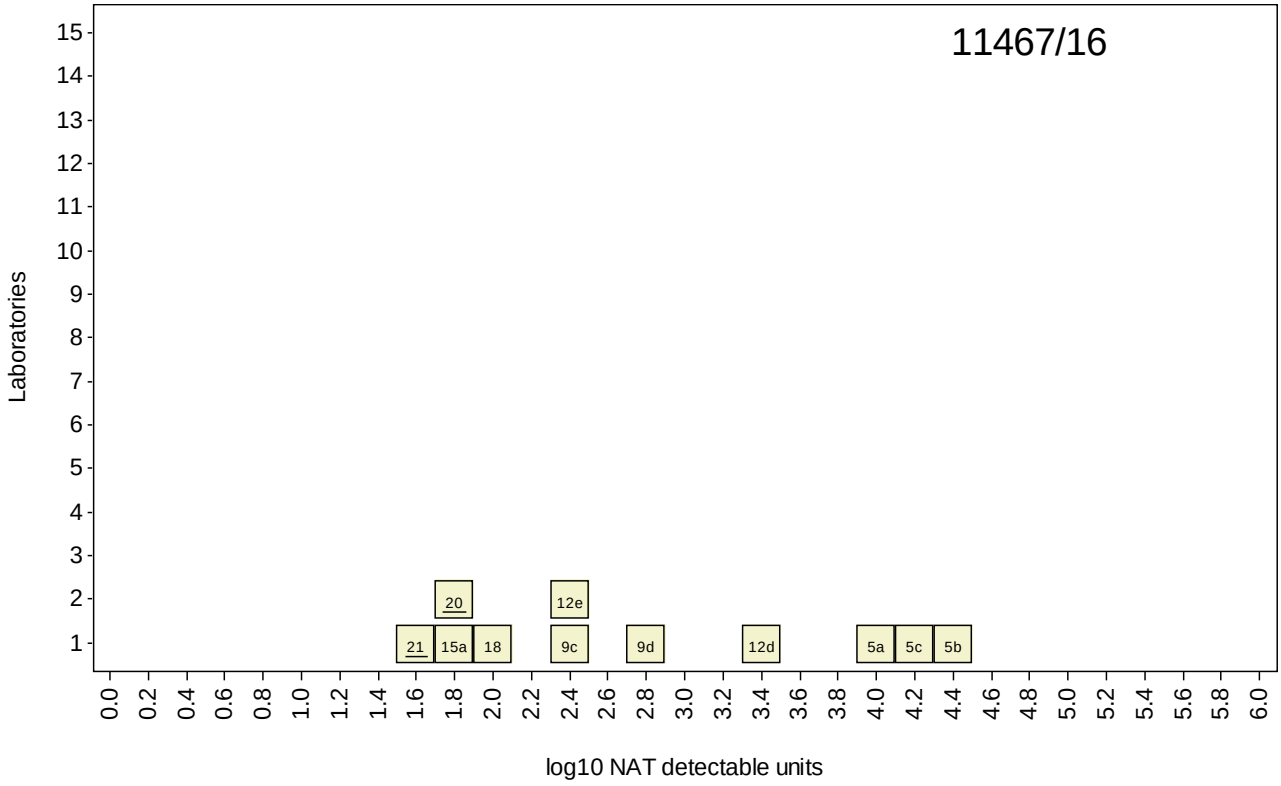


Figure 3 Laboratory mean estimates for inactivated, lyophilized Asian ZIKV strain PF13/251013-18 - samples 11468/16 and 11474/16

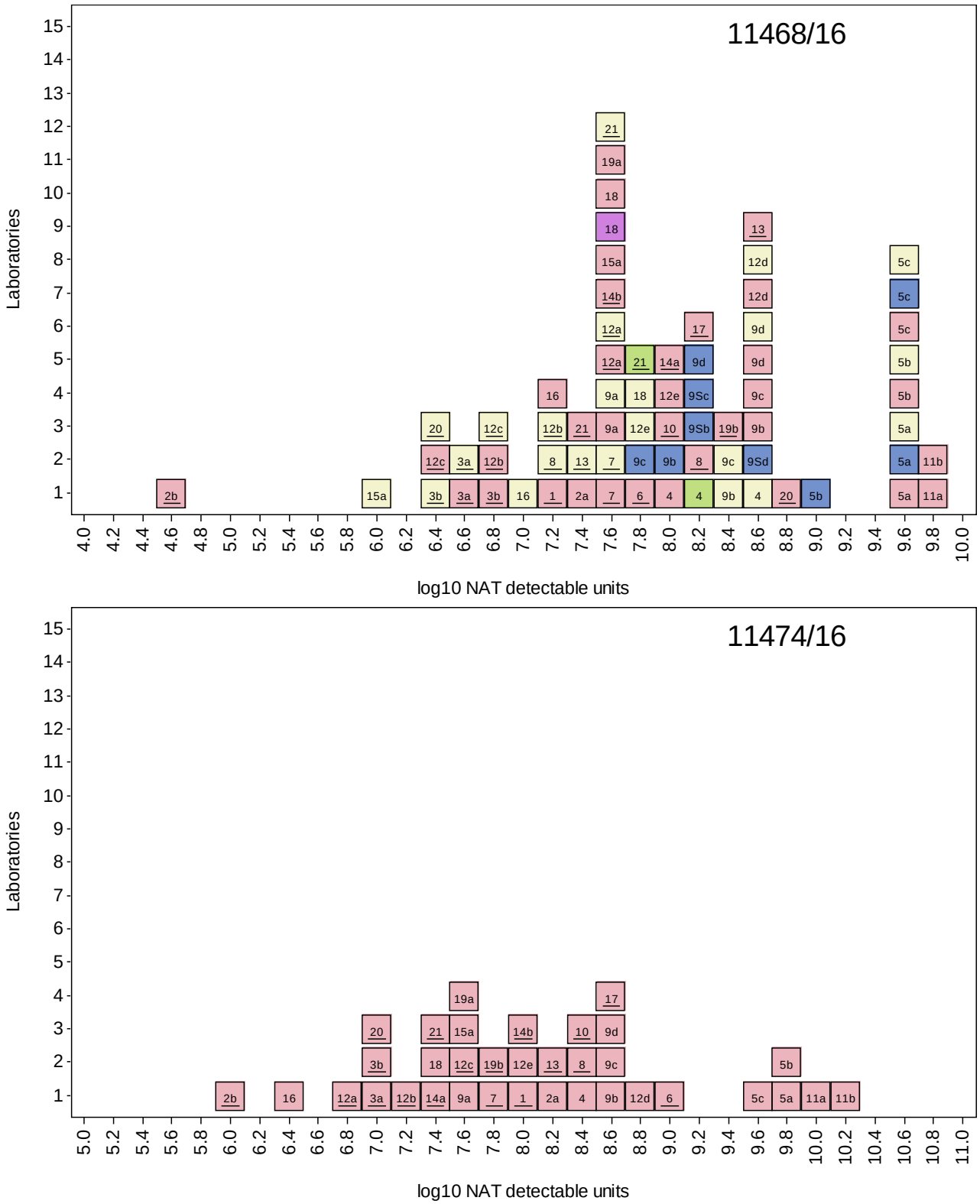


Figure 4 Laboratory mean estimates for inactivated, lyophilized African ZIKV strains MR776 and MP 1751 – samples 11469/16 and 11579/16, respectively

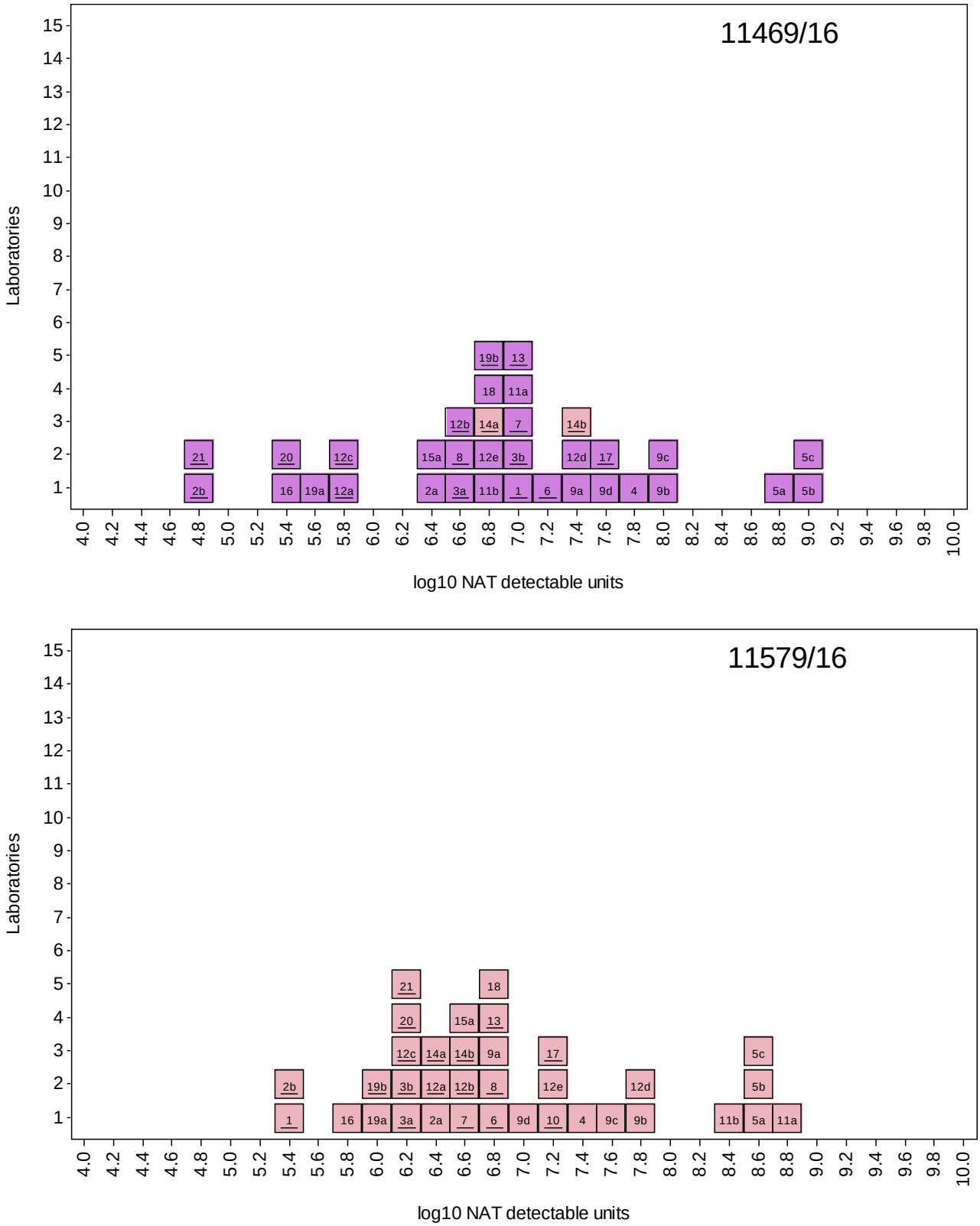
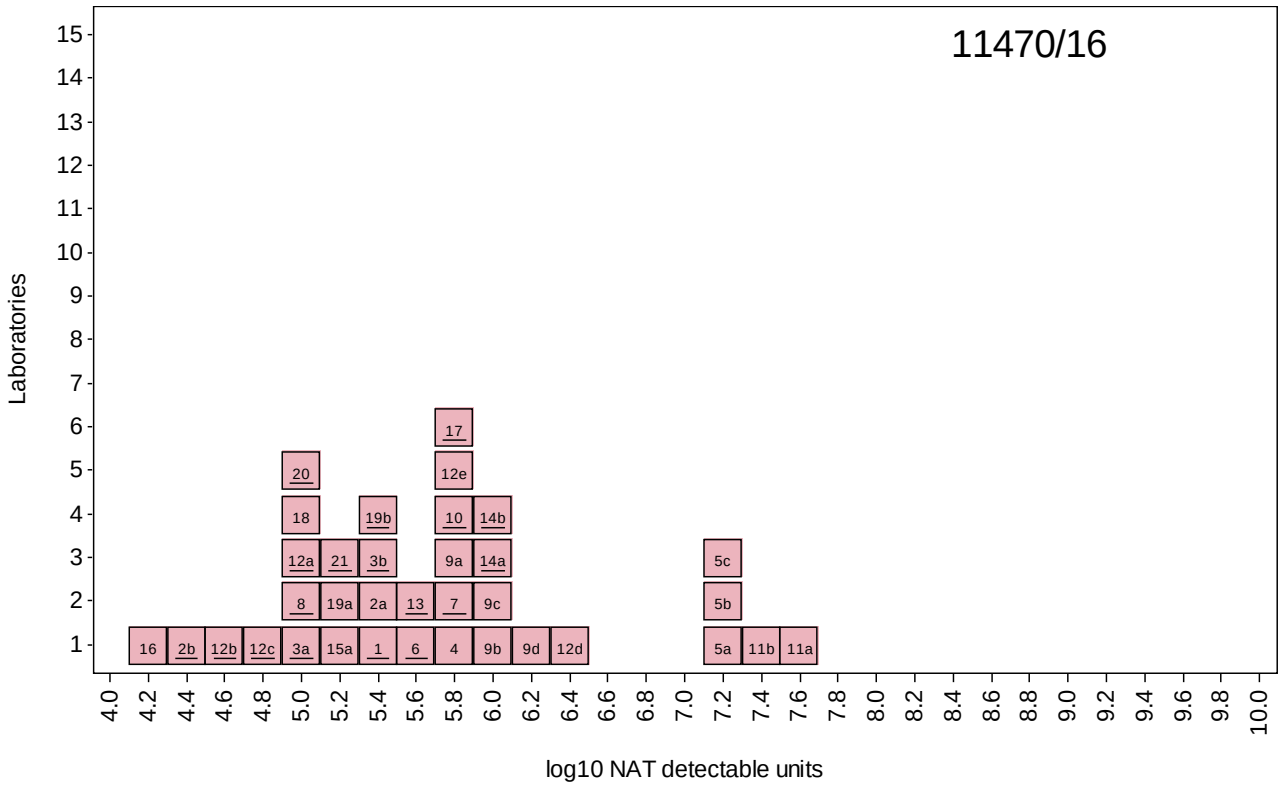


Figure 5 Laboratory mean estimates for inactivated liquid/frozen Asian ZIKV strains FSS13025 and PRVABC59 - samples 11470/16 and 11471/16, respectively



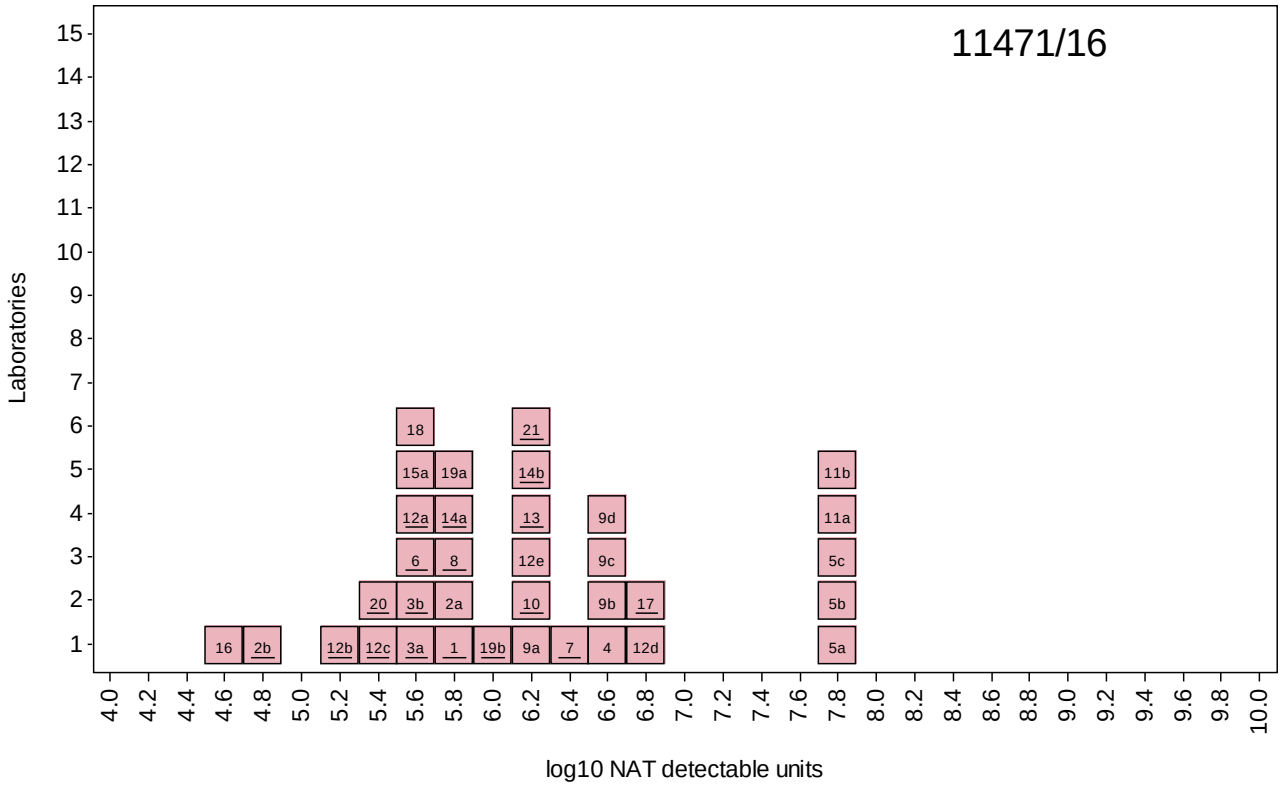


Figure 6 Laboratory mean estimates for Brazilian ZIKV-positive plasma samples 11472/16 and 11473/16

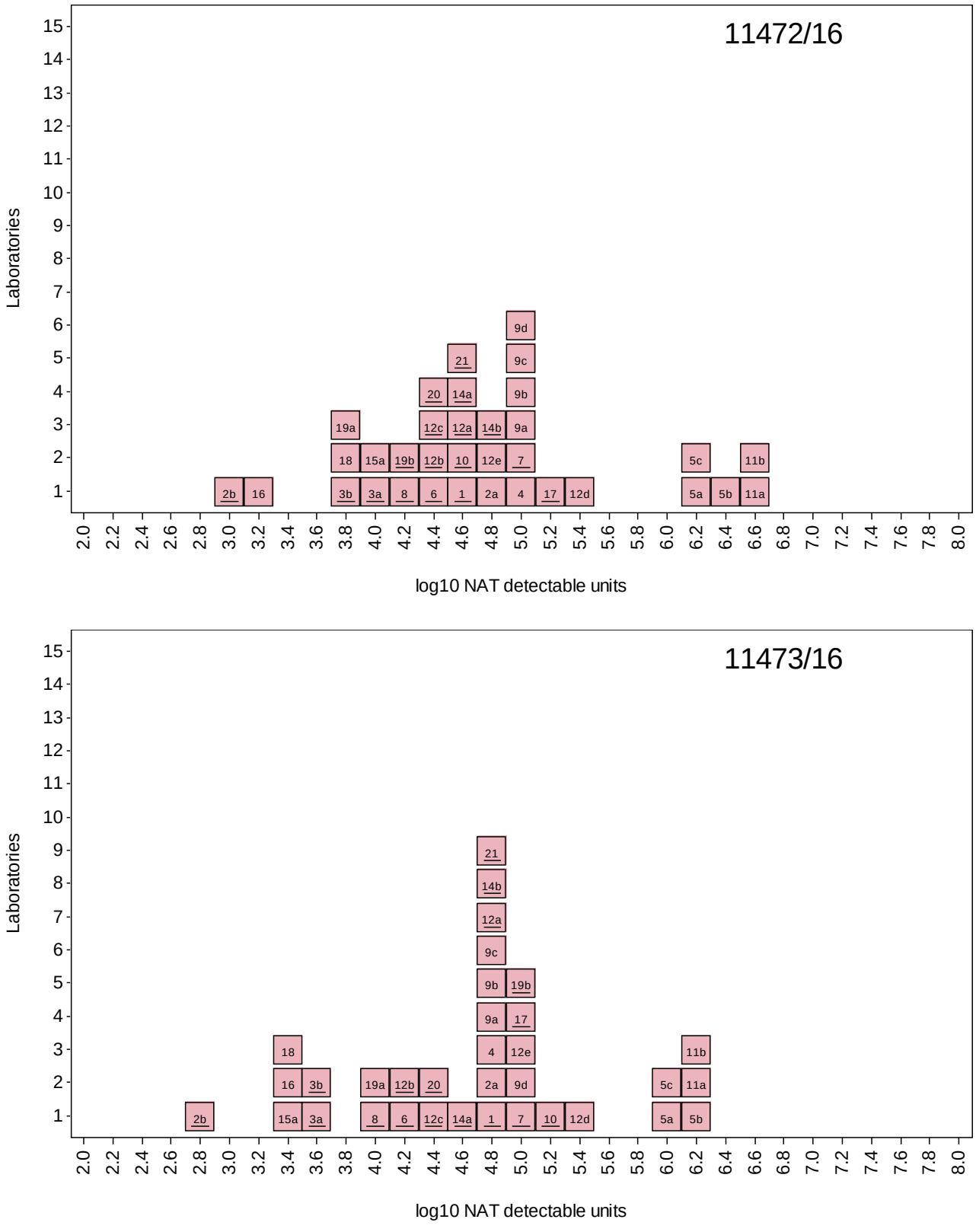
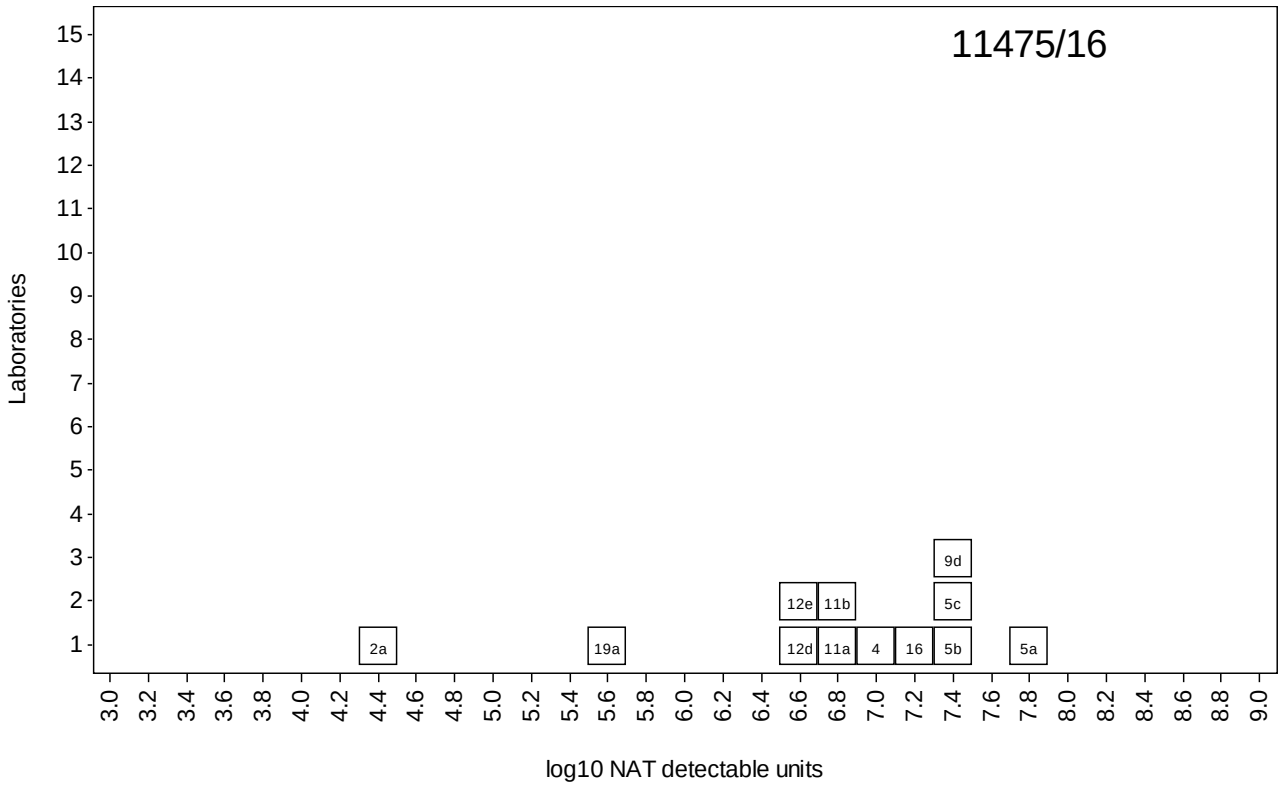


Figure 7 Laboratory mean estimates for ZIKV Universal calibrator RNA – samples 11475/16 and 11476/16



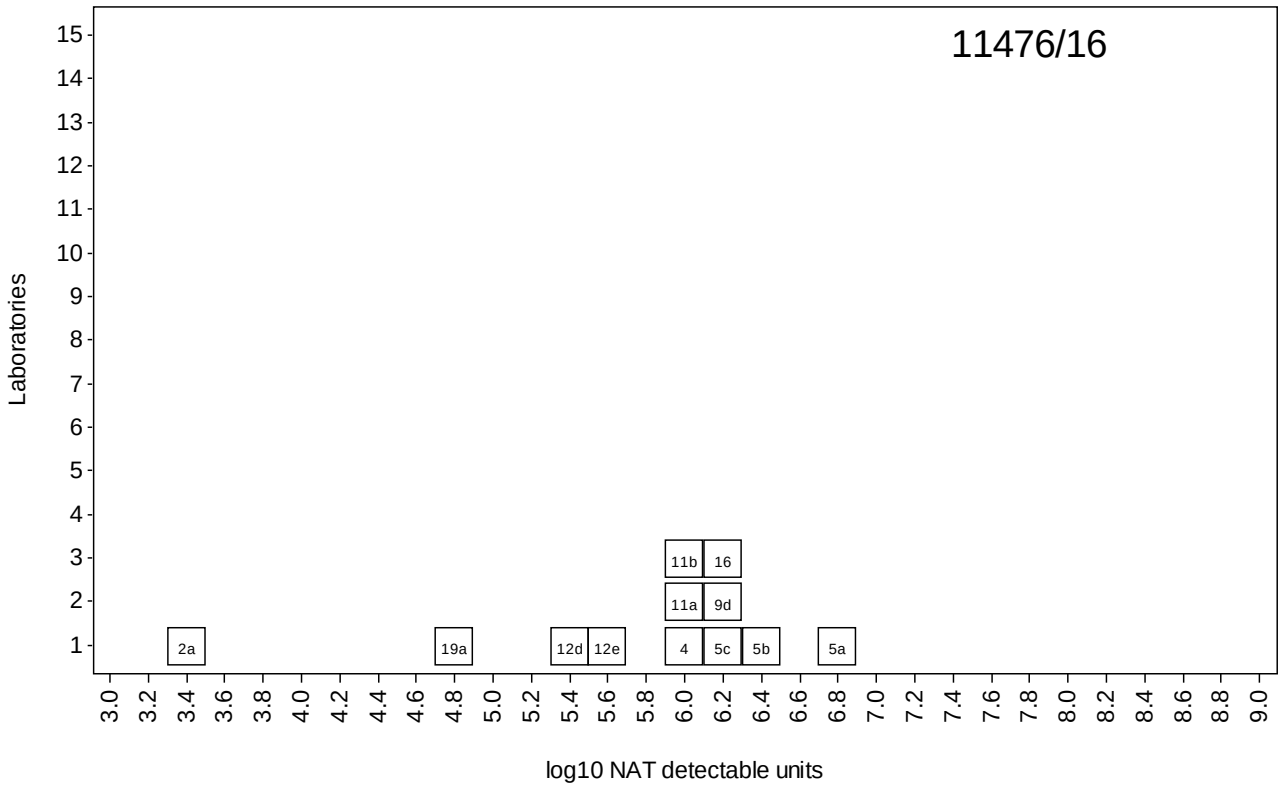


Figure 8 Laboratory mean estimates for ZIKV *in vitro* RNA Mix – samples 11477/16 and 11478/16

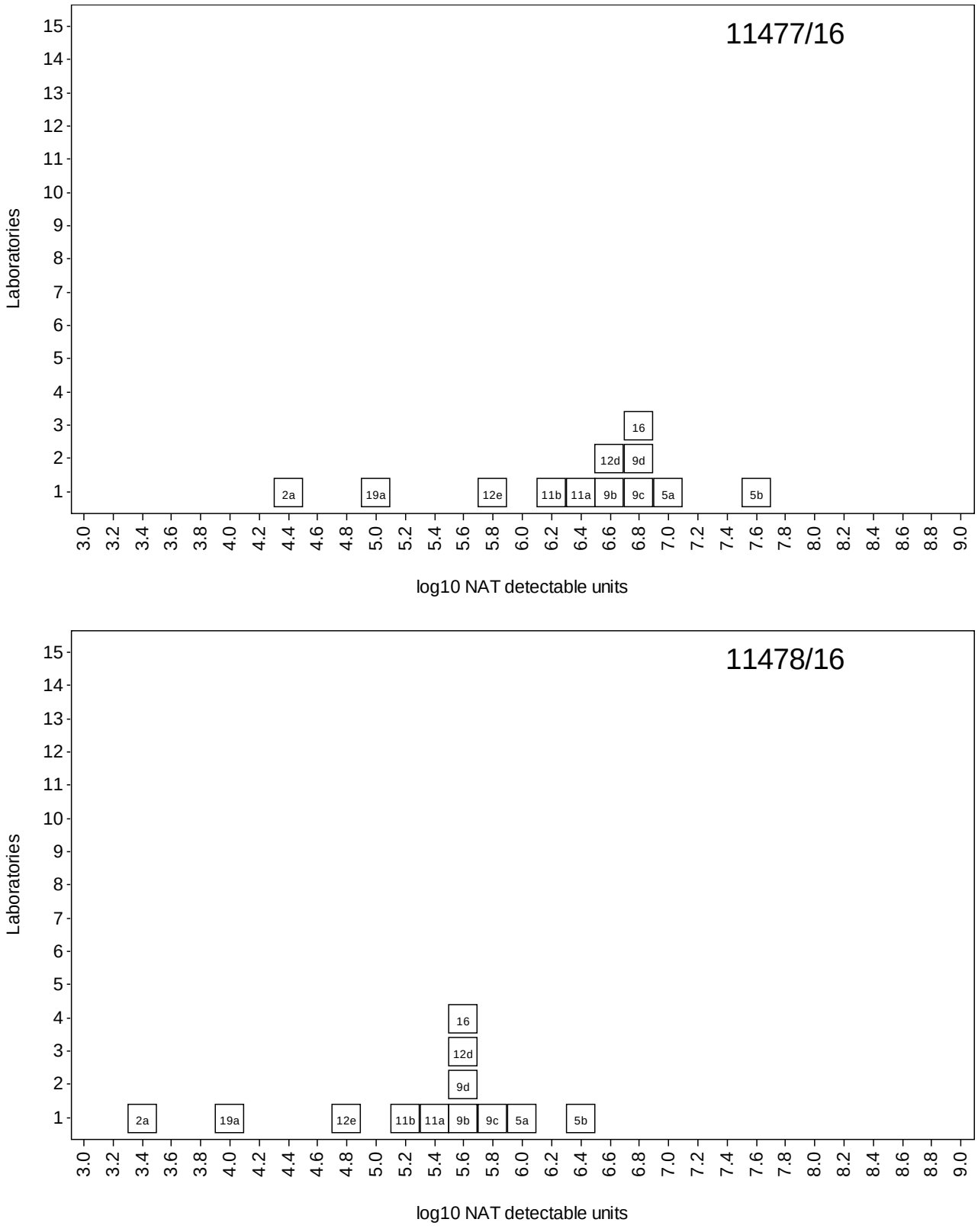
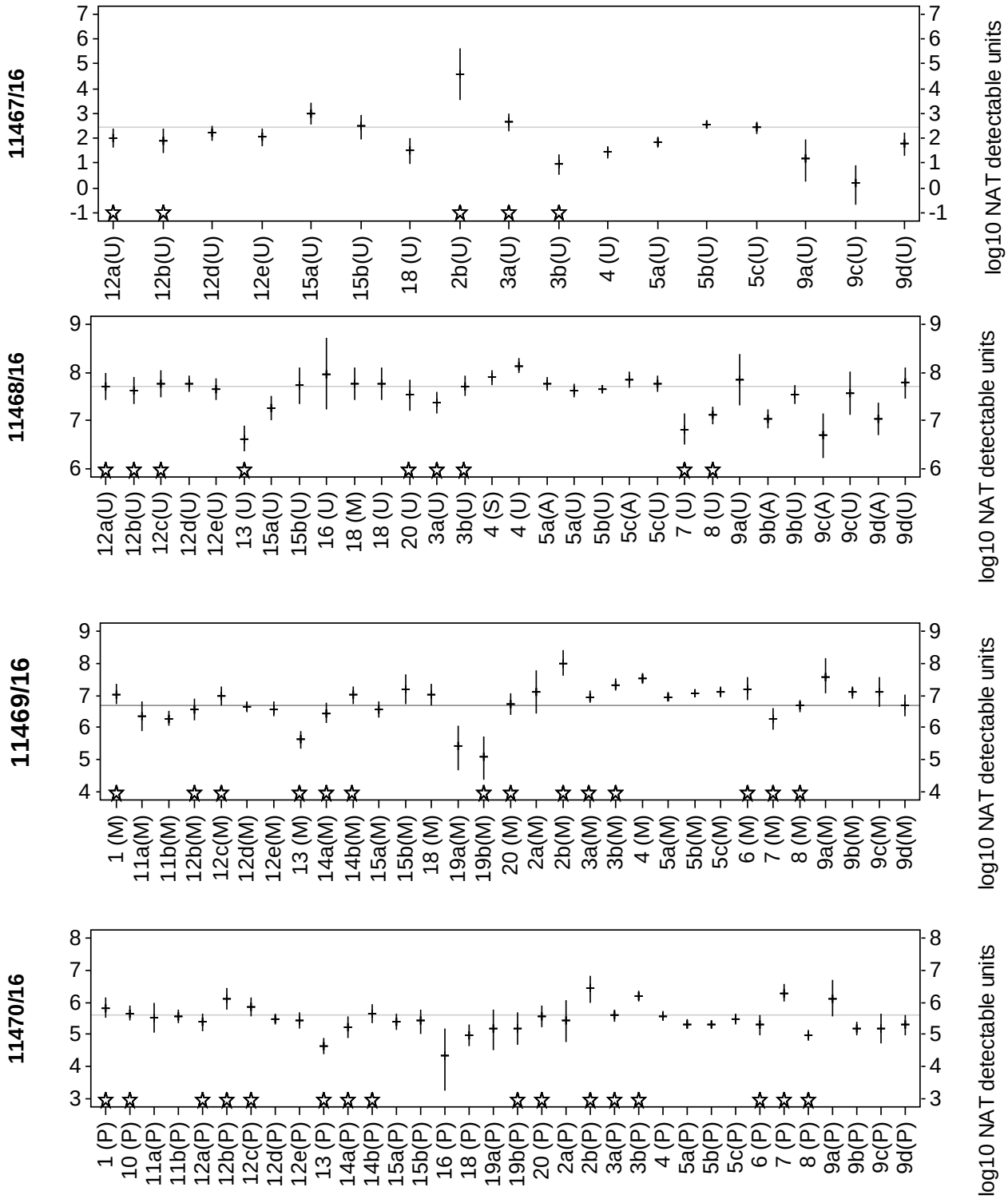
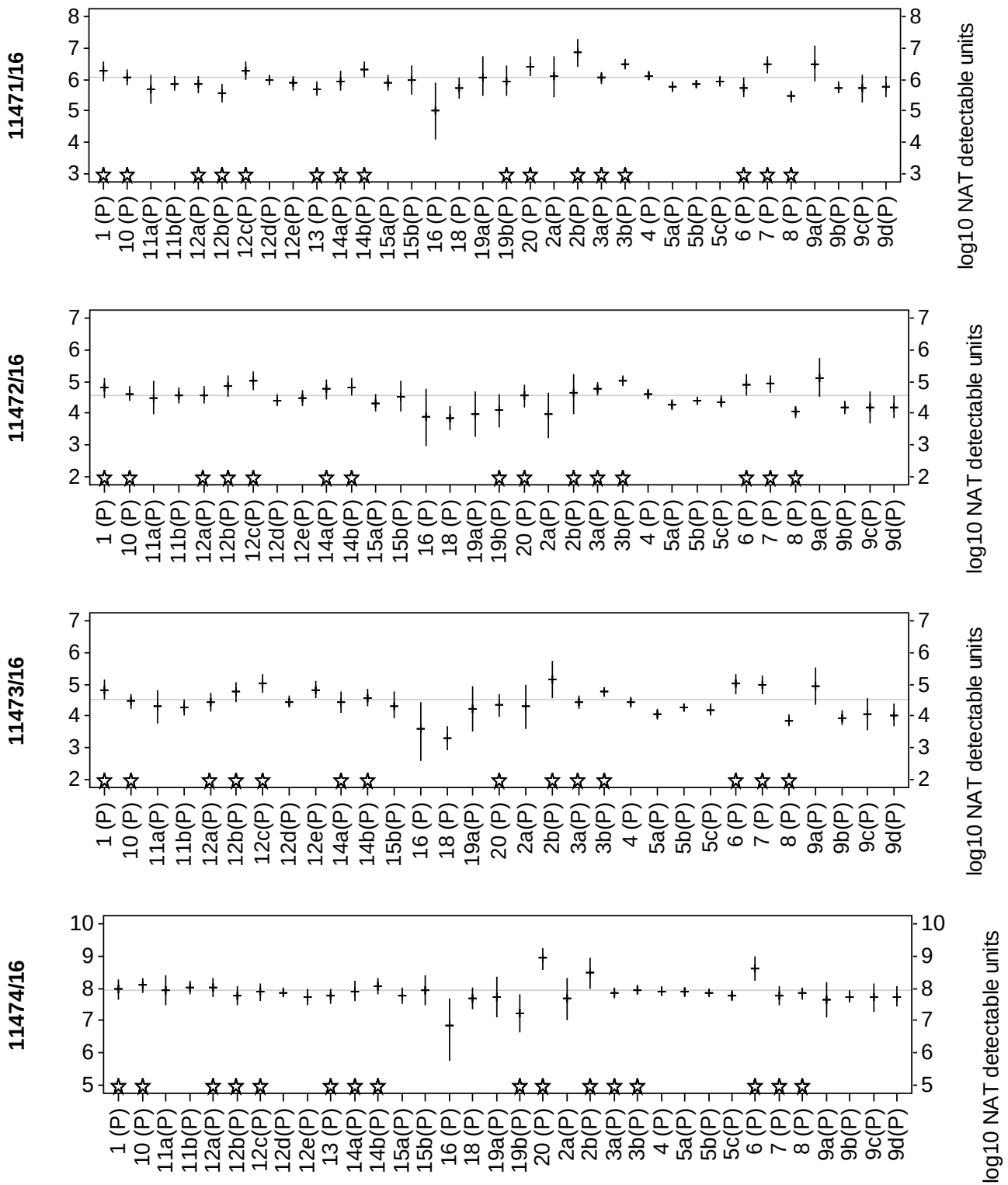
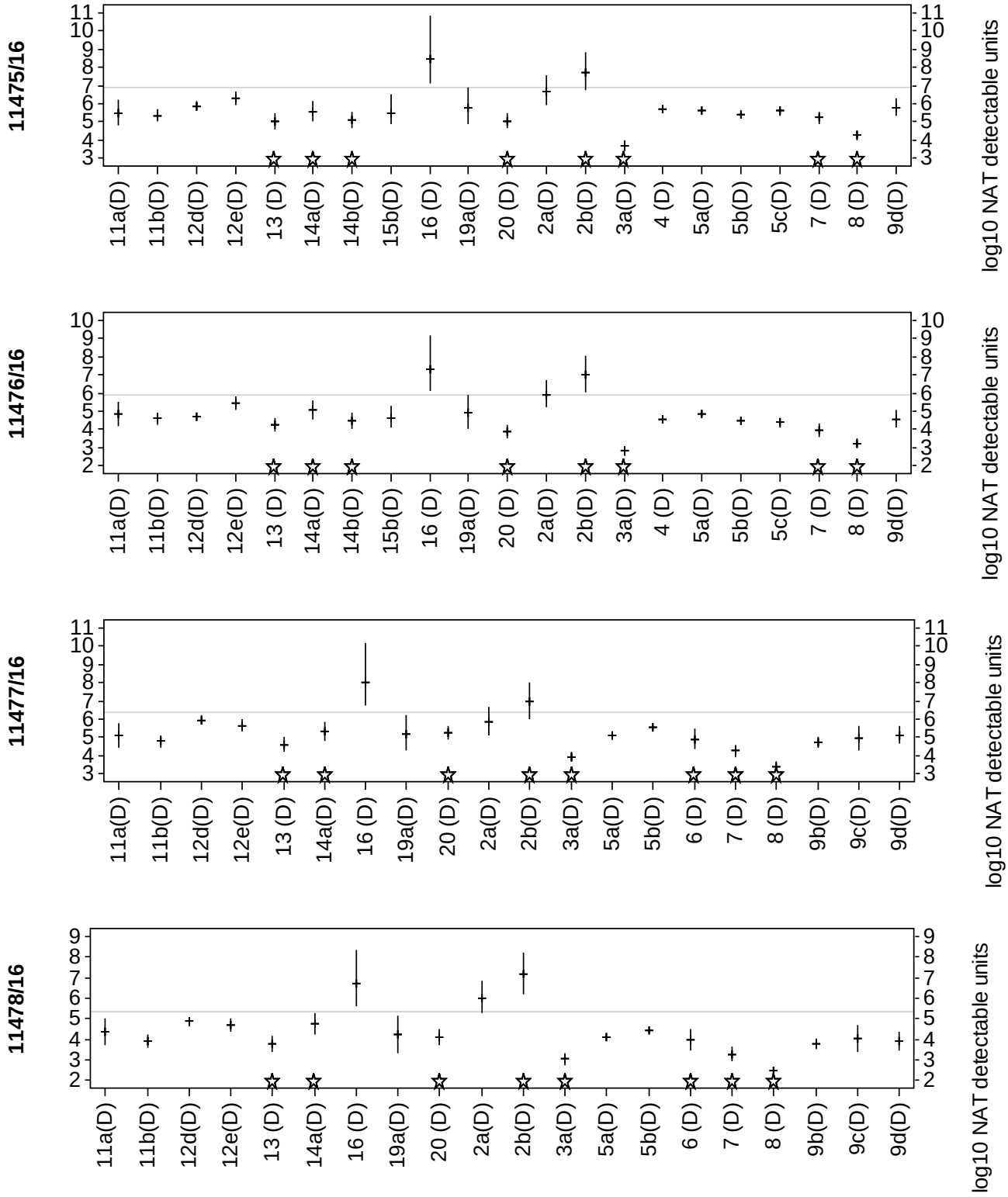


Figure 9 Forest plots




11471/16
11472/16
11473/16
11474/16
log10 NAT detectable units
1 (P)
10 (P)
11a(P)
11b(P)
12a(P)
12b(P)
12c(P)
12d(P)
12e(P)
13 (P)
14a(P)
14b(P)
15a(P)
15b(P)
16 (P)
18 (P)
19a(P)
19b(P)
20 (P)
2a(P)
2b(P)
3a(P)
3b(P)
4 (P)
5a(P)
5b(P)
5c(P)
6 (P)
7 (P)
8 (P)
9a(P)
9b(P)
9c(P)
9d(P)


11475/16
11476/16
11477/16
11478/16
log10 NAT detectable units
11a(D)
11b(D)
12d(D)
12e(D)
13 (D)
14a(D)
14b(D)
15b(D)
16 (D)
19a(D)
20 (D)
2a(D)
2b(D)
3a(D)
4 (D)
5a(D)
5b(D)
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6 (D)
7 (D)
8 (D)
9b(D)
9c(D)
9d(D)

11579/16

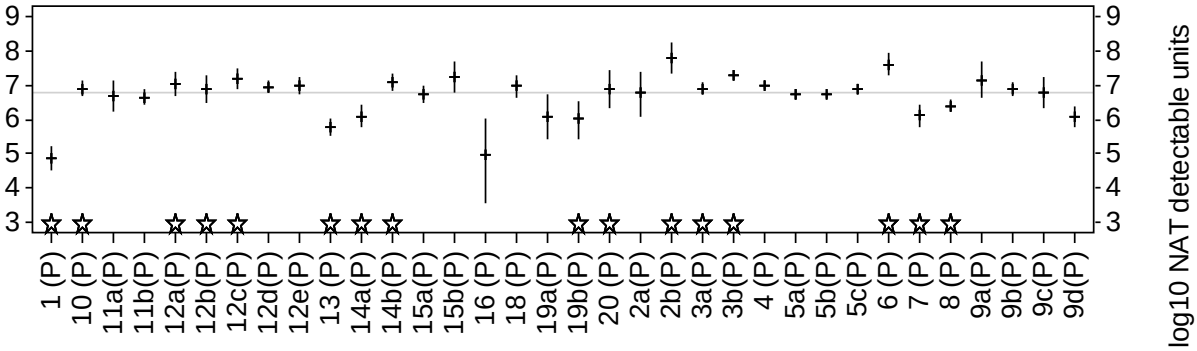

9
8
7
6
5
4
3
1 (P)
10 (P)
11a(P)
11b(P)
12a(P)
12b(P)
12c(P)
12d(P)
12e(P)
13 (P)
14a(P)
14b(P)
15a(P)
15b(P)
16 (P)
18 (P)
19a(P)
19b(P)
20 (P)
2a(P)
2b(P)
3a(P)
3b(P)
4 (P)
5a(P)
5b(P)
5c(P)
6 (P)
7 (P)
8 (P)
9a(P)
9b(P)
9c(P)
9d(P)
log10 NAT detectable units

Figure 10 Potency of 11467/16 relative to 11468/16

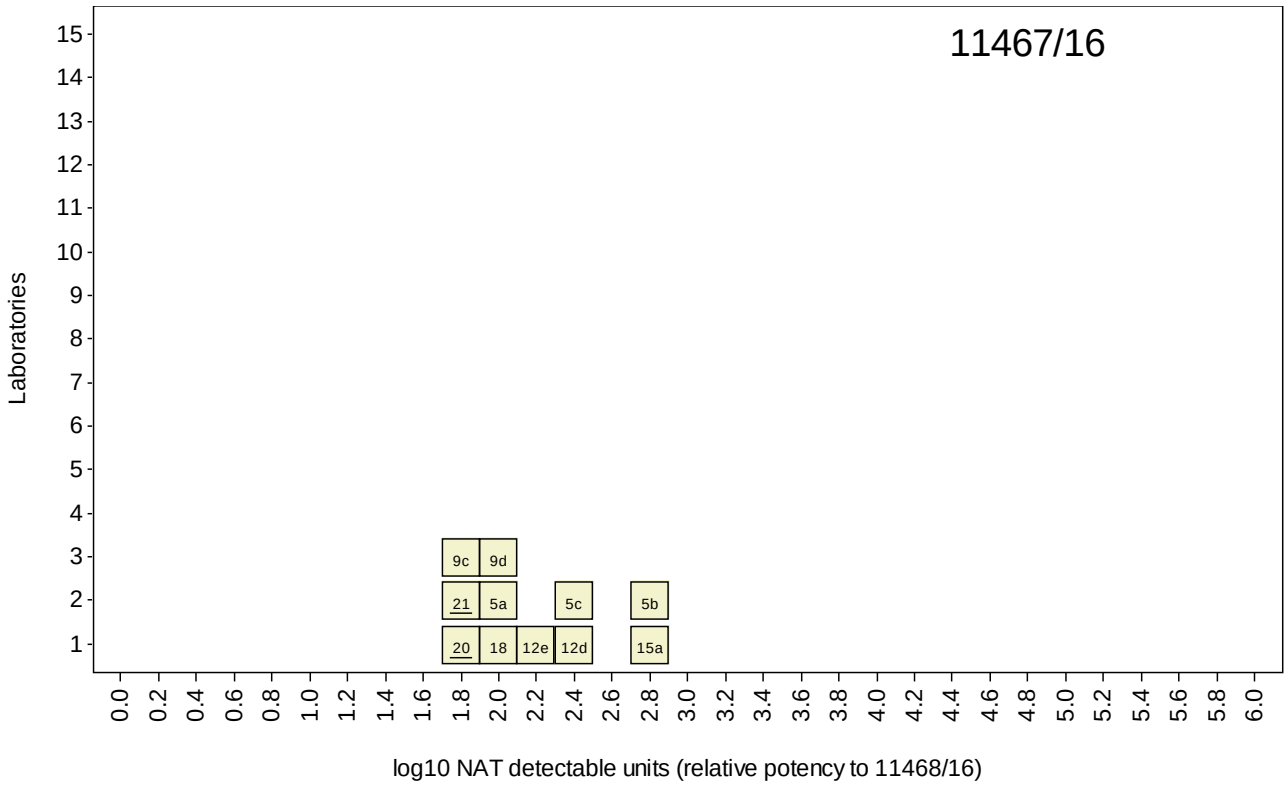


Figure 11 Potency of 11474/16 relative to 11468/16

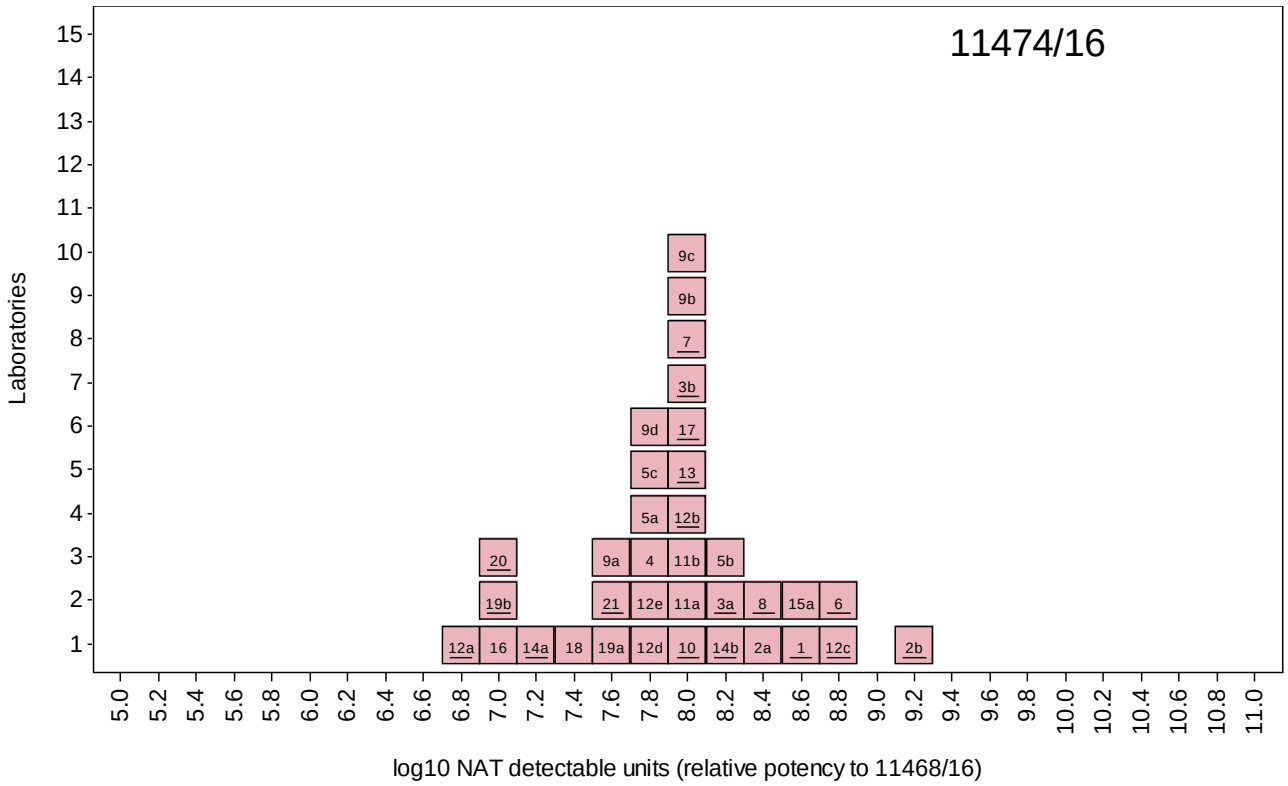
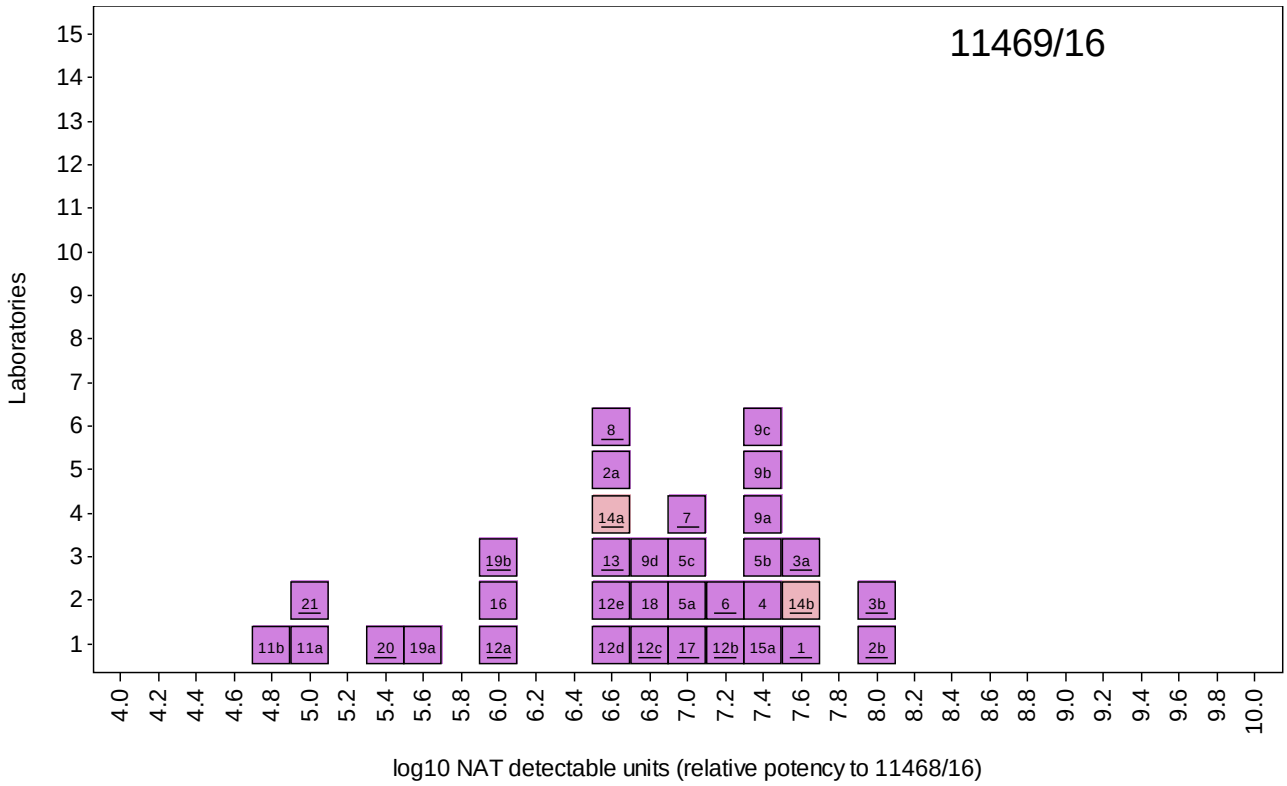


Figure 12 Potencies of 11469/16 and 11579/16, the African strains, relative to 11468/16



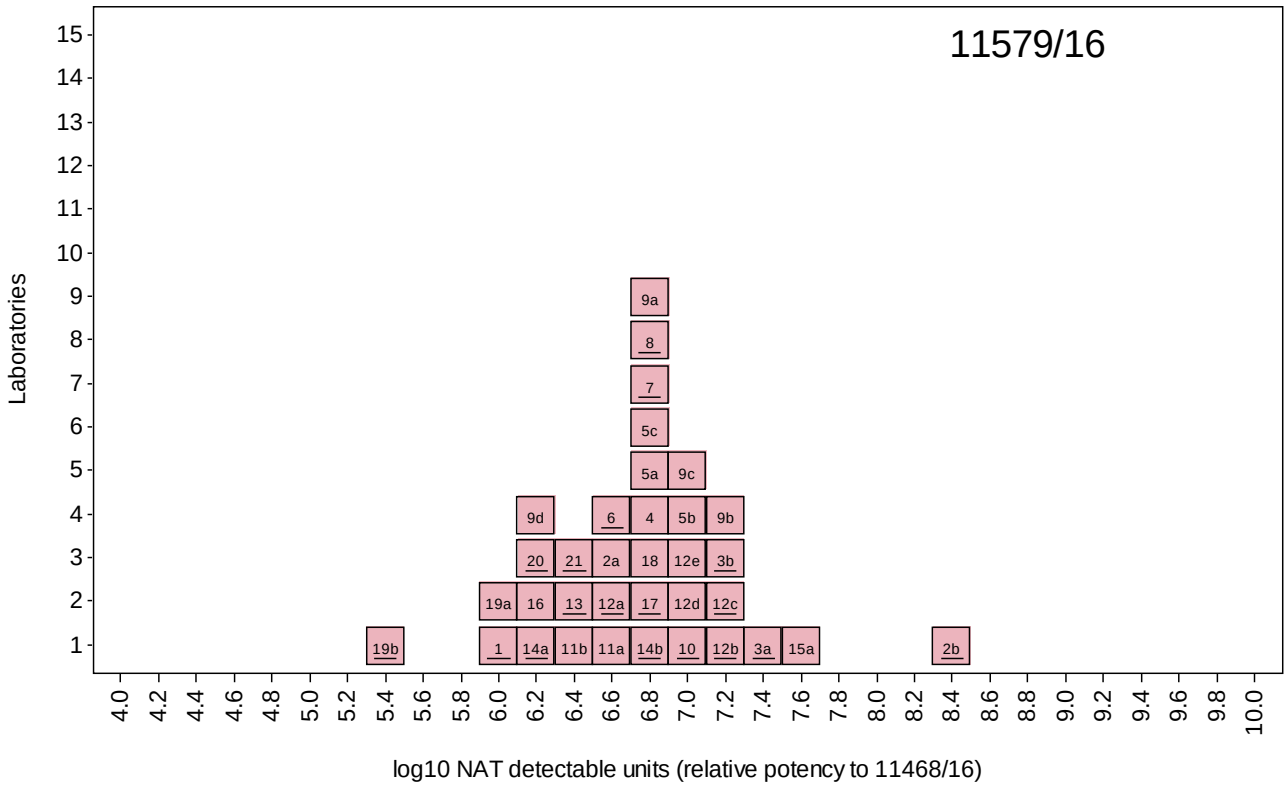


Figure 13 Potencies of 11470/16 and 11471/16 relative to 11468/16

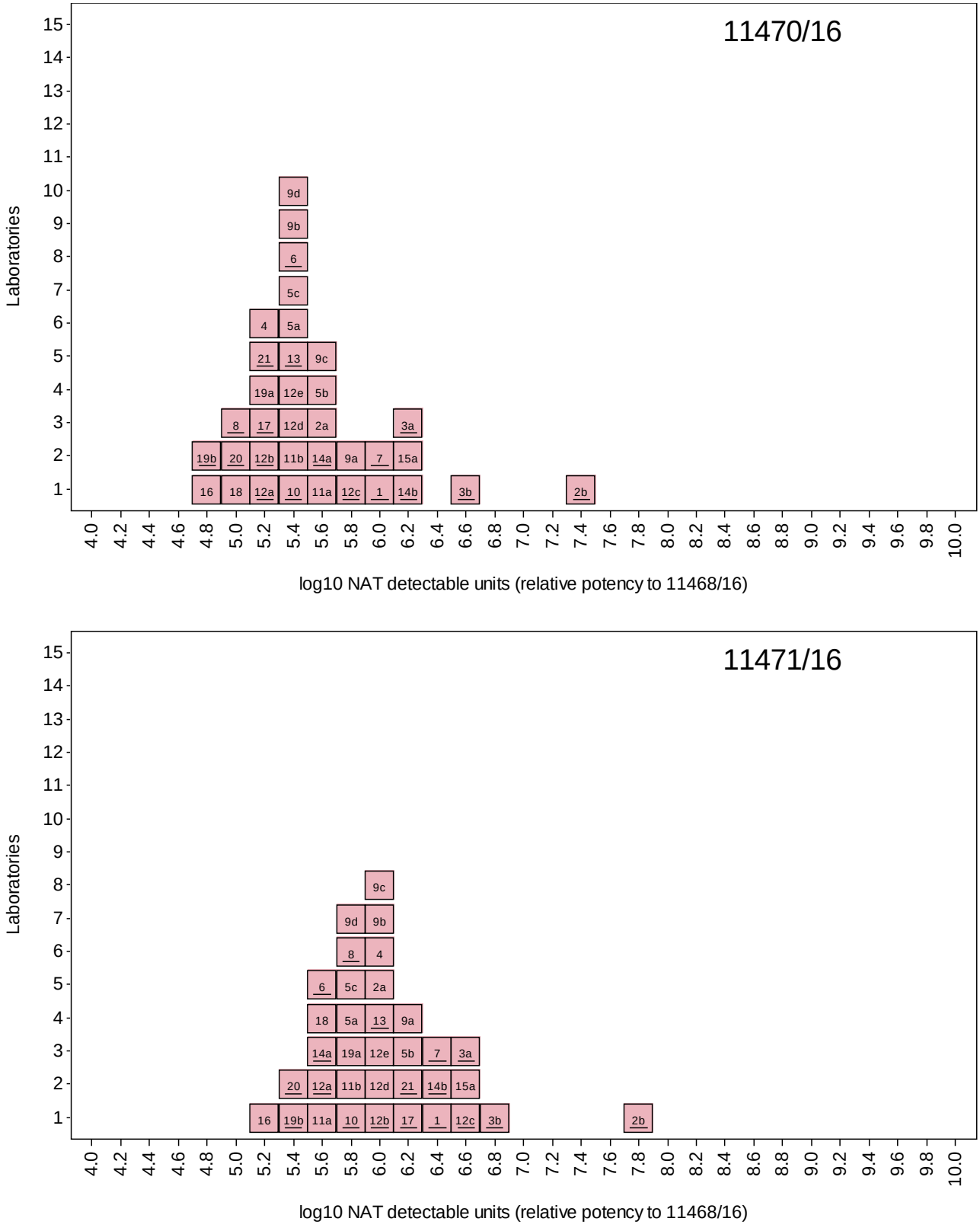
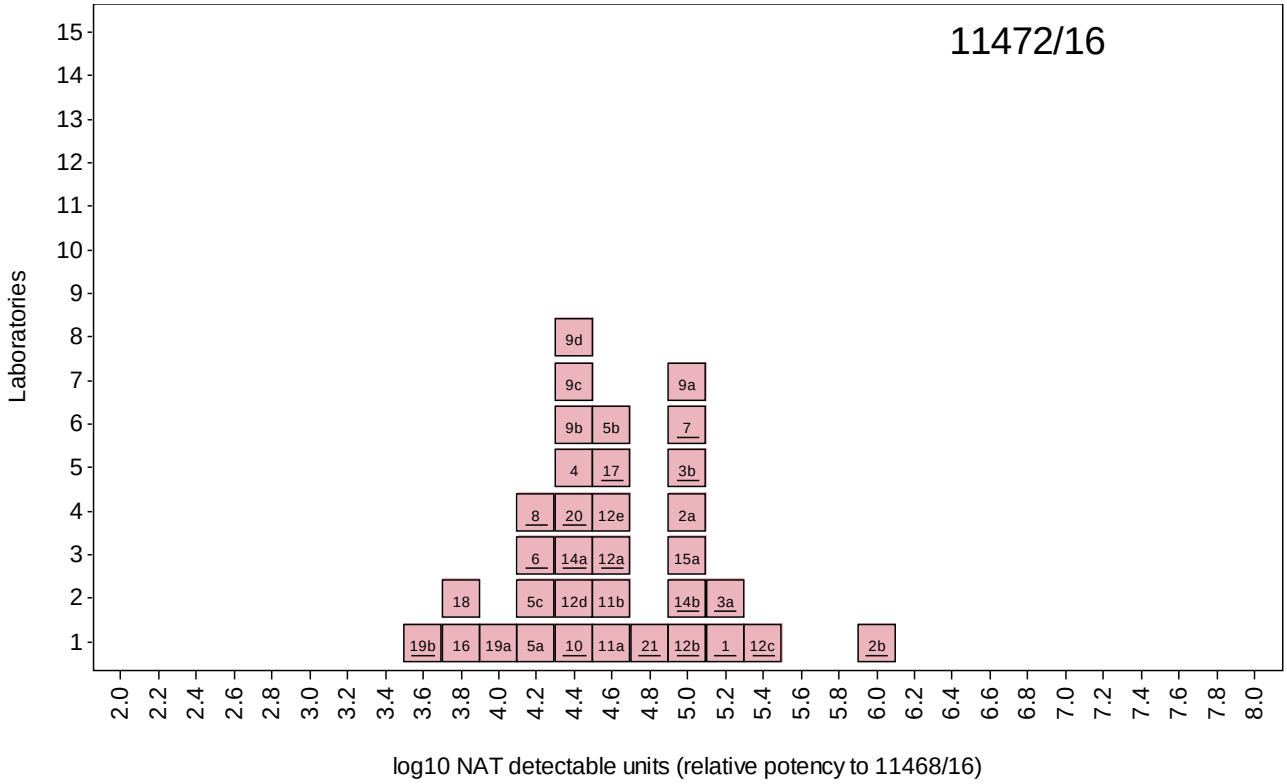


Figure 14 Potencies of 11472/16 and 11473/16 relative to 11468/16



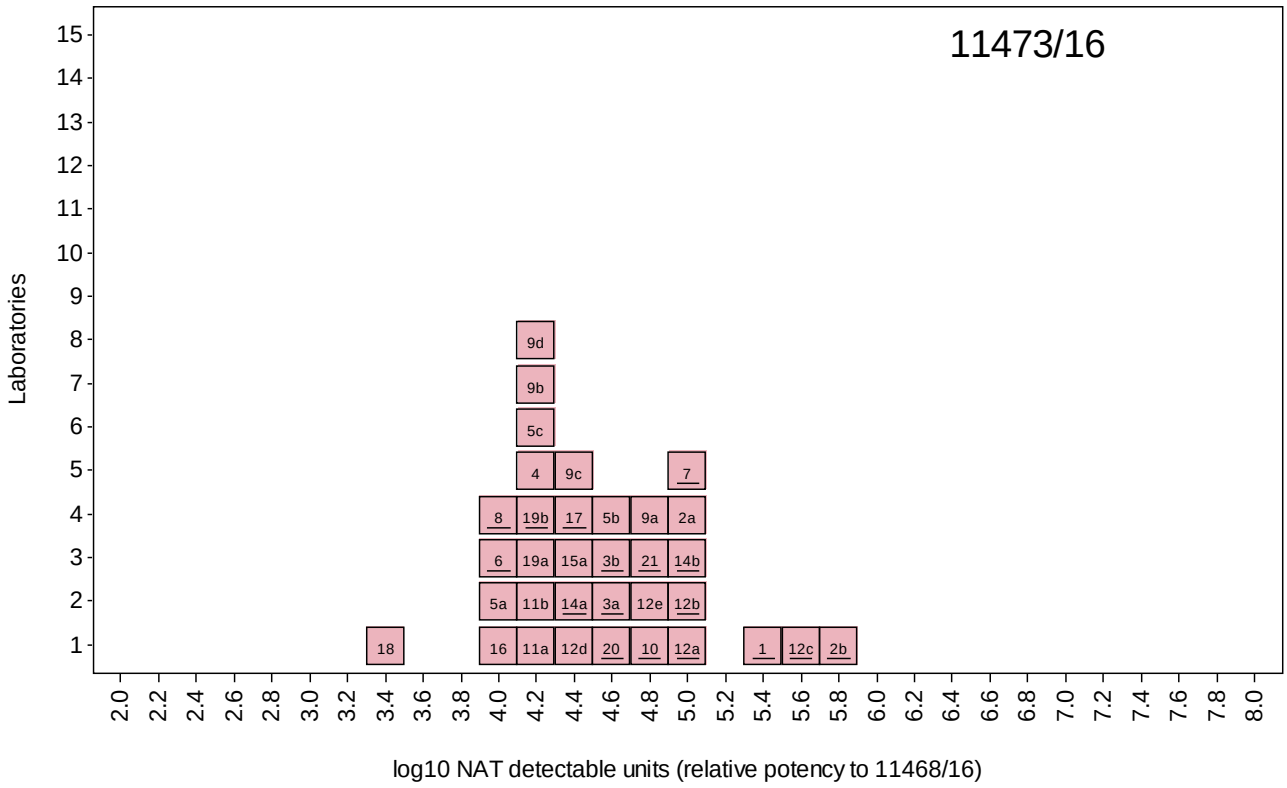


Figure 15 Potencies of 11475/16 and 11476/16 relative to 11468/16

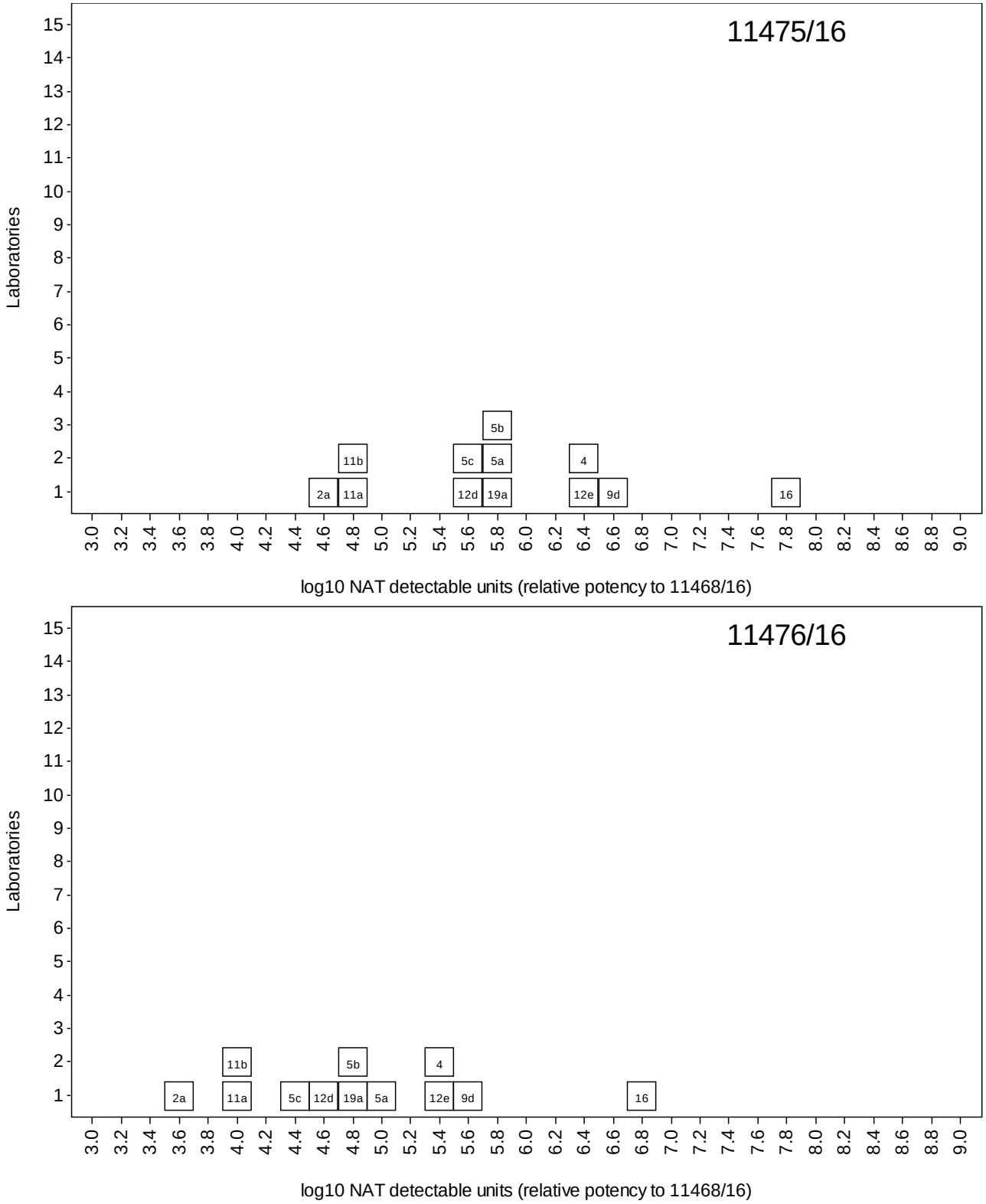


Figure 16 Potencies of 11477/16 and 11478/16 relative to 11468/16

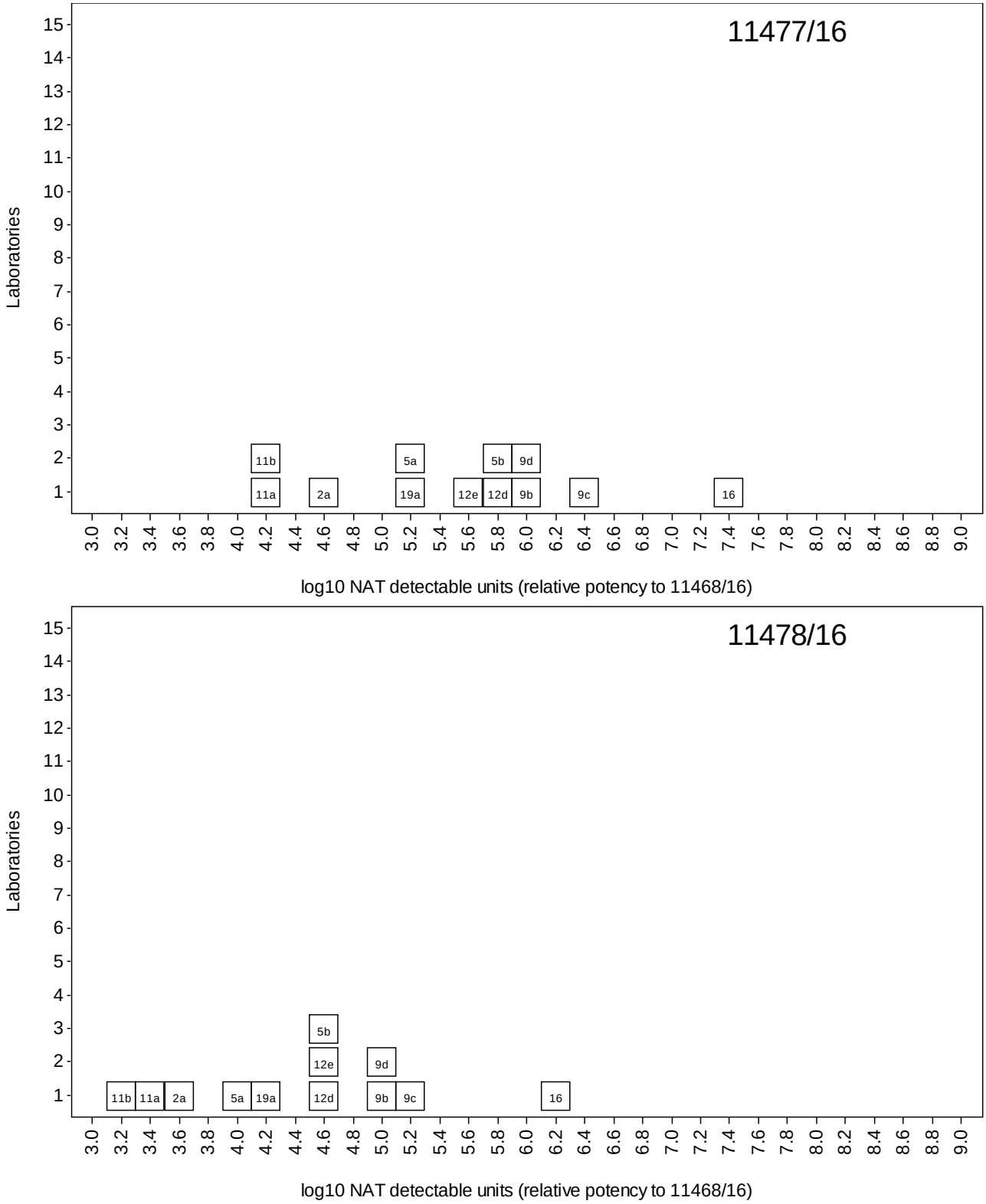
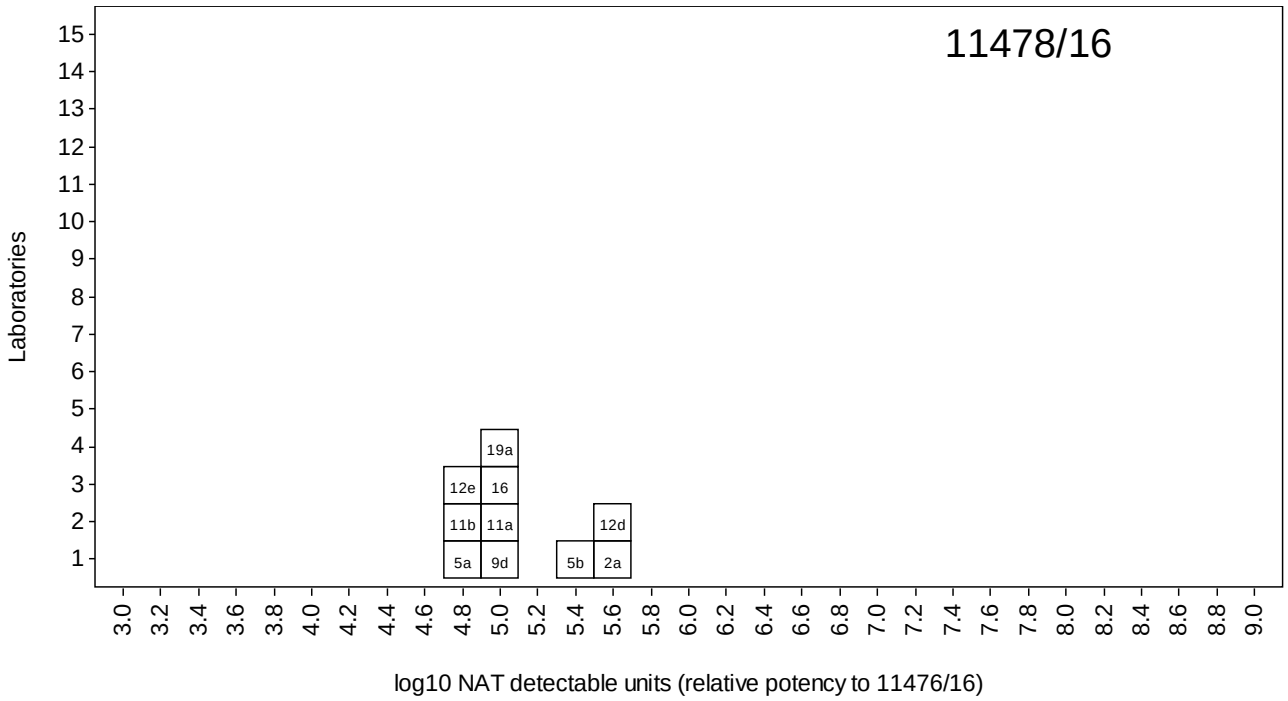
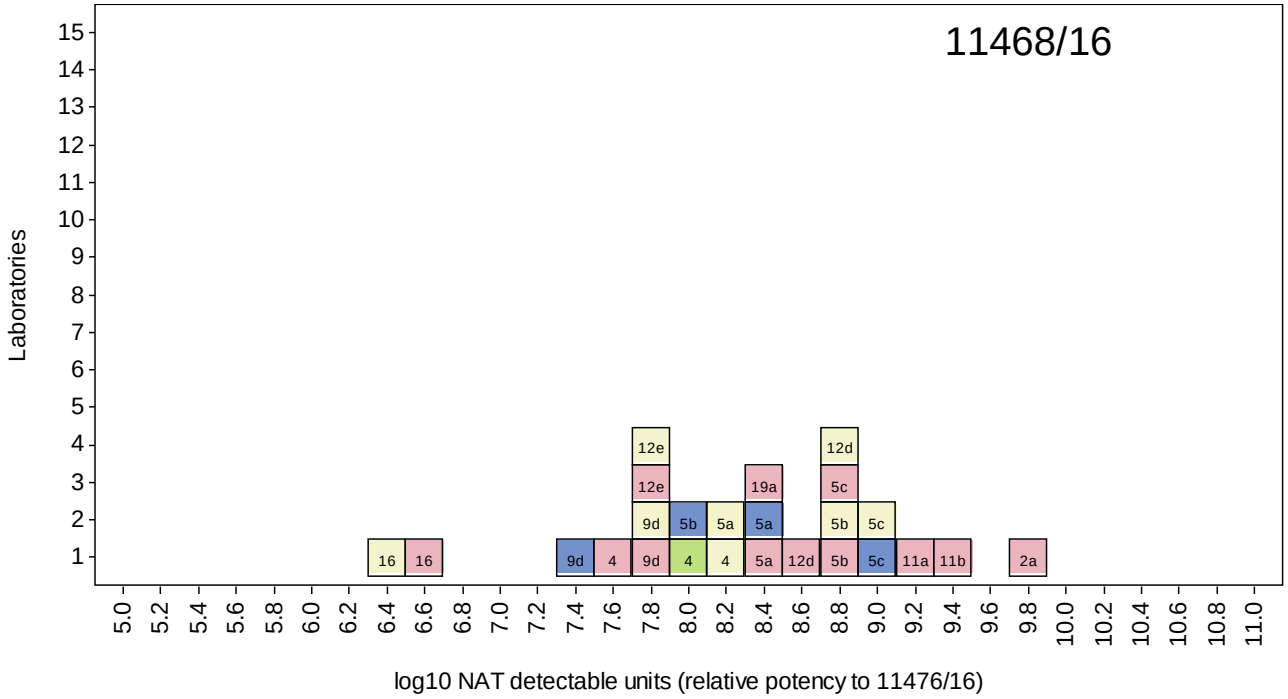


Figure 17 Potencies of 11478/16 and 11468/16 relative to 11476/16





Appendix 1

List of participants, alphabetically according to organization

Scientist(s)	Affiliation
Stephan Ölschläger/Hussein El Halas	altona Diagnostics GmbH Hamburg, Germany
Jonas Schmidt-Chanasit	Bernhard-Nocht-Institut für Tropenmedizin Hamburg, Germany
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Sonia Bakkour/Michael Busch	Blood Systems Research Institute San Francisco, United States of America
Maria Rios/Caren Chancey/Rafaelle Fares/Evgeniya Volkova/Andriyan Grinev	Center for Biologics Evaluation and Research/U.S. Food and Drug Administration, Silver Spring, USA
Ann Powers/Nisha Duggal	Centers for Disease Control and Prevention Fort Collins, United States of America
Maria João Alves/Líbia Zé-Zé	Instituto Nacional de Saúde Doutor Ricardo Jorge Águas de Moura, Portugal
Chantal Reusken	Erasmus MC Rotterdam, the Netherlands
Ana Bispo de Filippis/Patrícia Alvarez Baptista/Lia Laura Lewis Ximenez de Souza Rodrigues	Fundação Oswaldo Cruz Rio de Janeiro, Brazil
Gudrun Baersch/Sudhir Bhatia	Genekam Biotechnology AG Duisburg, Germany
José Eduardo Levi	Fundação Pró-Sangue/Hemocentro de São Paulo São Paulo, Brazil
Kui Gao/Jeffrey Linnen	Hologic Inc. San Diego, United States of America
Victor M. Corman, Jan Felix Drexler	Institut für Virologie/German Centre for Infection Research (DZIF – partner sites Bonn-Cologne) Bonn, Germany
Jean-Jacques de Pina/Didier Musso	Institut Louis Malardé Papeete, Tahiti, French Polynesia
Giulio Pisani/Matteo Simeoni/Sara Fabi	Istituto Superiore di Sanità Rome, Italy
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Livia Schrick/Andreas Nitsche/Heinzfried Ellerbrok	Robert Koch Institute Berlin, Germany
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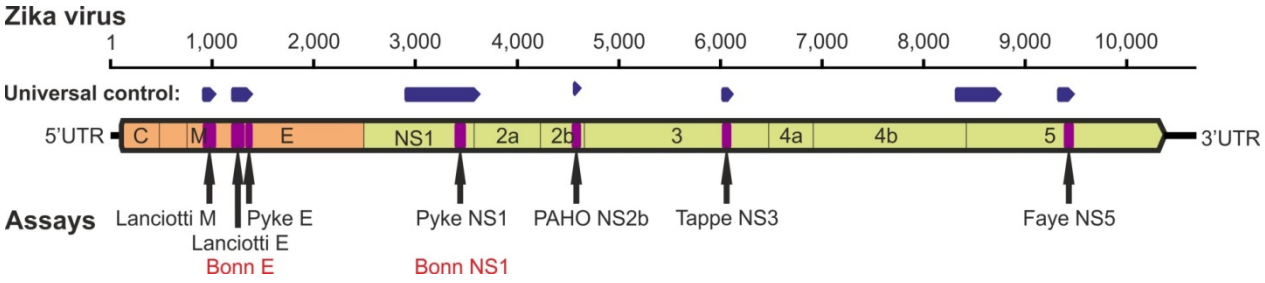
Marco Koppelman	Sanquin Blood Supply Foundation Amsterdam, the Netherlands
Walter Zhang	Shanghai ZJ Bio-Tech Co., Ltd. (Liferiver) Shanghai, China
Cynthia Wagner	Siemens Healthcare Diagnostics Berkeley, United States of America
Marco Kaiser	TIB MOLBIOL Syntheselabor GmbH Berlin, Germany

Appendix 2

Certificate of Analysis - Universal calibrator RNA 1.0 Sequence (EVAg/Institute of Virology, Bonn) 11475/16 and 11476/16



5'-
GGGAGAGCUUUUGGGAAGCUCAACGAGCCAAAAAGUCAUAUACUUGGUCAUGAUACUGCUGAUUGCCCCGGCAUACAGCA
UCAGGUGCAUAGGAGUCAGCAAUAGGGACUUUGUGGAAGGUAUGUCAGGUGGGACUUGGGUUGAUAUUGUCUUGGAACAU
GGAGGUUGUGUCACCGUAAUGGCACAGGACAAACCGCAUGGCGGAGGUAAGAUCCUACUGCUAUGAGGCAUCAAUAUCAG
ACAUGGCUUCGGACAGCCGCUGCCCAACACAAGGUGAAGCCUACCUUGACAAGCAAUCAGACACUCAAUAUGUCUGCAAA
AGAACGUUAGUGGACAGAGGCUGGGGAAAUGGAUGUGGACUUUUUGGCAAAGGGAGUCUGGUGACAUGCGCUAAGUUUGC
AUGCUCCAAGAAAAUGACCGGGAAGAGCAUCCAGCCAGAGAAUCUGGAGUACCGGAUAAUGCUGUCAGUUCACUCAAGGU
UAGAGAAGAUUAUUCAUUAGAGUGUGAUCCAGCCGUUAUUGGAACAGCUGUUAAGGGAAAGGAGGCUGUACACAGUGAUC
UAGGCUACUGGAUUGAGAGUGAGAAGAAUGACACAUGGAGGCUGAAGAGGGCCCAUCUGAUCGAGAUGAAAACAUGUGAA
UGGCCAAAGUCCCACACAUUGUGGACAGAUGGAAUAGAAGAGAGUGAUCUGAUCAUACCCAAGUCUUUAGCUGGGCCACU
CAGCCAUCACAAUACCAGAGAGGGCUACAGGACCCAAAUGAAAGGGCCAUGGCACAGUGAAGAGCUUGAAAUUCGGUUUG
AGGAAUGCCCAGGCACUAAGGUCCACGUGGAGGAAACAUGUGGAACAAGAGGACCAUCUCUGAGAUCAACCACUGCAAGC
GGAAGGGUGAUCGAGGAAUGGUGCUGCAGGGAGUGCACAAUGCCCCCACUGUCGUUCCGGGCUAAAGAUGGCUGUUGGUA
UGGAAUGGAGAUAAGGCCCAGGAAAGAACCAGAAAGCAACUUAGUAAGGUCAAUGGUGACUGCAGGAUCAACUGAUCACA
UGGAUCACUUCUCCCUUGGAGUGCUUGUGAUUCUGCUCAUGGUGCAGGAAGGGCUGAAGAAGAGAAUGACCACAAAGAUC
AUCCUGUGGCAUGAACCCAAUAGCCAUACCCUUUGCAGCUGGAGCGUGGUACGUAUACGUGAAGACUGGAAAAAGGAGUG
GUGCUCUAUGGGAUCAAACCUGGAGAUGAGUAUCUGUAUGGAGGUGGGUGCGCAGAGACUGACGAAGACCAUGCACACUG
GCUUGAAGCAAGAAUGCUCCUUGACAAUAUUUACCUCCAAGAUGGCCUCAUAGCCUCGCUCUCUACACAUGAGAUGUACU
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AGGAGGCCAGUGAAAUAUGAGGAGGAUGUGAAUCUCGGCUCUGGCACGCGGGCUGUGGUAAGCUGCGCUGAAGCUCCCAA
CAUGAAGAUCAUUGGUAACCGCAUUGAAAGGAUCCGCAGUGAGCACGCGGAAACGUGGUUCUUUGACGAGAACCACCCAU
AUAGGACAUGGGCUUACCAUGGAAGCUAUGAGGCCCCCACACAAGGGUCAGCGUCCUCUCUAAUAAACGGGGUUGUCAGG
CUCCUGUCAAAACCCUGGGAUGUGGUGACUGGAGUCACAGGAAUAGCCAUGACCGACACCACACCGUAUGGUCAGCAAAG
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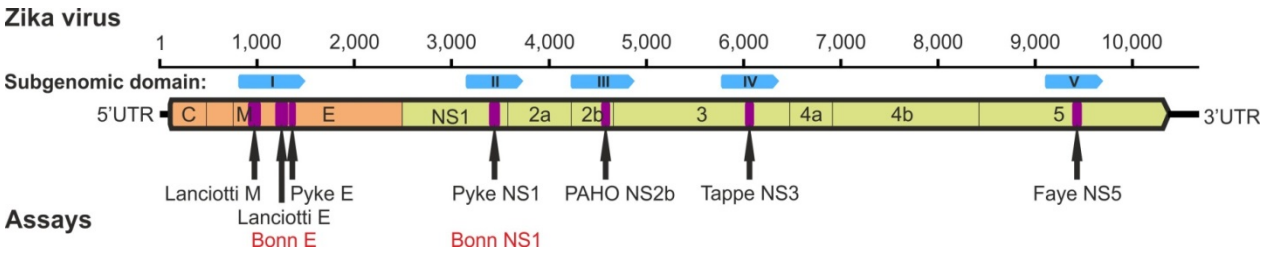
-3'

Certificate of Analysis - Universal calibrator RNA 1.0 Sequence (EVAg/Institute of Virology, Bonn) 11477/16 and 11478/16 – IVT I



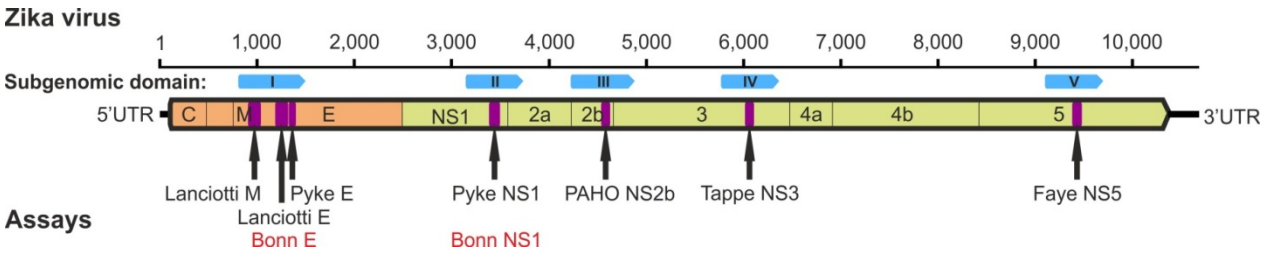
Zika virus IVT I

A specific RT-PCR was performed on KJ776791. The product was cloned into a vector, sequenced and *in vitro* transcribed. *In vitro* transcript: Vector Sequence: Position 1-36, 727-816 Insert Sequence: Position 37-726

5'-
GGGCGAATTGAATTTAGCGGCCGCGAATTCGCCCTTGGAATCAAGAGAATACACAAAGCACTTGATTAGAGTCGAAAATT
GGATATTCAGGAACCCTGGTTTCGCTTTAGCAGCAGCTGCCATCGCGTGGCTTTTGGGAAGCTCAACGAGCCAAAAAGTC
ATATACTTGGTCATGATACTGCTGATTGCCCCGGCATACAGCATCAGGTGCATAGGAGTCAGCAATAGGGACTTTGTGGA
AGGTATGTCAGGTGGGACTTGGGTTGATGTTGTCTTGGAACATGGAGGTTGTGTCACCGTAATGGCACAGGACAAACCGA
CTGTCGACATAGAGCTGGTTACAACAACAGTCAGCAACATGGCGGAGGTAAGATCCTACTGCTATGAGGCATCAATATCA
GACATGGCTTCGGACAGCCGCTGCCCAACACAAGGTGAAGCCTACCTTGACAAGCAATCAGACACTCAATATGTCTGCAA
AAGAACGTTAGTGGACAGAGGCTGGGGAAATGGATGTGGACTTTTTGGCAAAGGGAGCCTGGTGACATGCGCTAAGTTTG
CATGCTCCAAGAAAATGACCGGGAAGAGCATCCAGCCAGAGAATCTGGAGTACCGGATAATGTTGTCAGTTCATGGCTCC
CAGCACAGTGGGATGATCGTTAATGACACAGGACATGAAACTGATGAGAATAGAGCGAAGGTTGAGATAACGCCCAATTC
ACCAAGAAGGGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCGTAATCATGG
TCATAGCTGTTTCCTG

-3'

Certificate of Analysis - Universal calibrator RNA 1.0 Sequence (EVAg/Institute of Virology, Bonn) 11477/16 and 11478/16 – IVT II



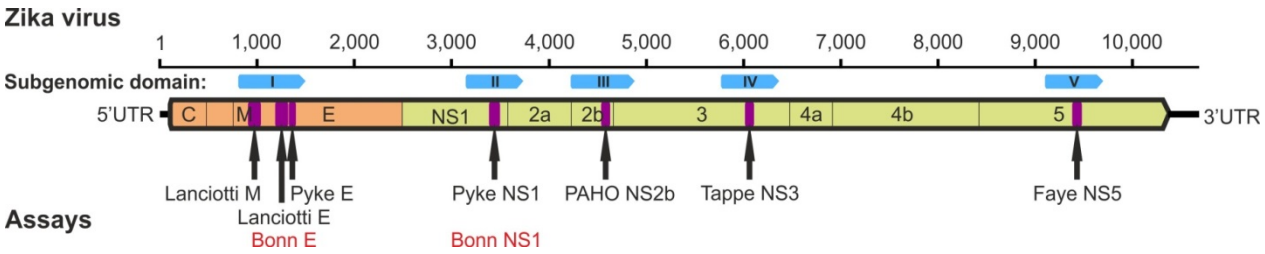
Zika virus IVT II

A specific genome region was amplified by PCR, cloned into a vector and *in vitro* transcribed. *In vitro* transcript: Vector Sequence: 1-36, 632-721 Insert Sequence: 37-631

5'-
GGGCGAATTGAATTTAGCGGCCGCGAATTCGCCCTTGATGAAAACATGTGAATGGCCAAAGTCCCACACATTGTGGACAG
ATGGAATAGAAGAGAGTGATCTGATCATACCCAAGTCTTTAGCTGGGCCGCTCAGCCATCACAATACCAGAGAGGGCTAC
AGGACCCAAATGAAAGGGCCATGGCACAGTGAAGAGCTTGAAATTCGGTTTGAGGAATGCCCAGGCACTAAGGTCCACGT
GGAGGAAACATGTGGAACAAGAGGACCATCTCTGAGATCAACCACTGCAAGCGGAAGGGTGATCGAGGAATGGTGCTGCA
GGGAGTGCACAATGCCCCCACTGTCGTTCTGGGCTAAAGATGGCTGTTGGTATGGAATGGAGATAAGGCCCAGGAAAGAA
CCAGAAAGCAACTTAGTAAGGTCAATGGTGACTGCAGGATCAACTGATCACATGGATCACTTCTCCCTTGGAGTGCTTGT
GATTCTGCTCATGGTGCAGGAAGGGCTGAAGAAGAGAATGACCACAAAGATCATCATAAGCACATCAATGGCAGTGCTGG
TAGCTATGATCCTGGGAGGATTTTCAATGAGTGACCTGGCTAAGCTTGCAATTTTGATGGGTGCCACCTTCAAGGGCGAA
TTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCT
G

-3'

Certificate of Analysis - Universal calibrator RNA 1.0 Sequence (EVAg/Institute of Virology, Bonn) 11477/16 and 11478/16 – IVT III



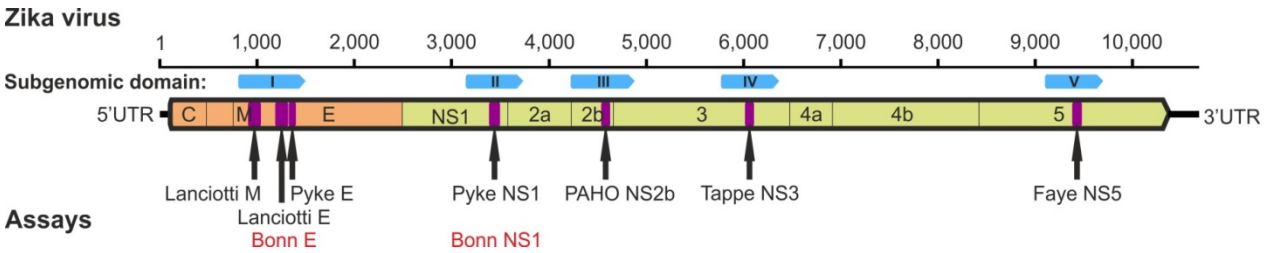
Zika virus IVT III

A specific RT-PCR was performed on KJ776791. The product was cloned into a vector, sequenced and *in vitro* transcribed. *In vitro* transcript: Vector Sequence: 1-36, 674-763 Insert Sequence: 37-673

5'-
GGGCGAATTGAATTTAGCGGCCGCGAATTCGCCCTTCACAGCTGTTGGCCTGATATGTGCATTGGCTGGAGGGTTCGCCA
AGGCAGATATAGAGATGGCTGGGCCCATGGCCGCGGTCGGTCTGCTAATTGTCAGTCACGTGGTCTCAGGAAAGAGTGTG
GACATGTACATTGAAAGAGCAGGTGACATCACATGGGAAAAAGATGCGGAAGTCACTGGAAACAGTCCCCGGCTCGACGT
GGCGCTAGATGAGAGTGGTGATTTCTCCCTGGTGGAGGATGACGGTCCCCCCATGAGAGAGATCATACTCAAGGTGGTCC
TGATGACCATCTGTGGCATGAACCCAATAGCCATACCCTTTGCAGCTGGAGCGTGGTACGTATACGTGAAGACTGGAAAA
AGGAGTGGTGCTCTATGGGATGTGCCTGCTCCCAAGGAAGTAAAAAAGGGGGAGACCACAGATGGAGTGTACAGAGTAAT
GACTCGTAGACTGCTAGGTTCAACACAAGTTGGAGTGGGAGTTATGCAAGAGGGGGTCTTTCACACTATGTGGCACGTCA
CAAAAGGATCCGCGCTGAGAAGCGGTGAAGGGAGACTTGATCCATACTGGGGAGATGTCAAGCAGGATCTGGTGTCATAC
TGTGGTCCATGGAAGCTAGATGCCGCCTGGGACAAGGGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGG
TTAATTCTGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTG

-3'

Certificate of Analysis - Universal calibrator RNA 1.0 Sequence (EVAg/Institute of Virology, Bonn) 11477/16 and 11478/16 – IVT IV



Zika virus IVT IV

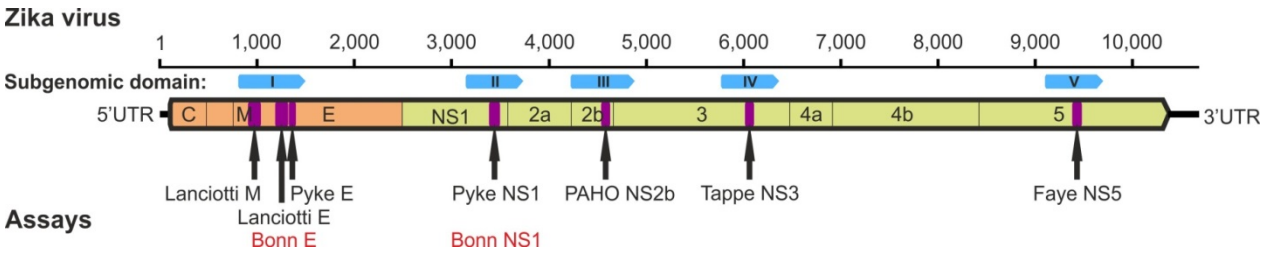
A specific RT-PCR was performed on KJ776791. The product was cloned into a vector, sequenced and *in vitro* transcribed. *In vitro* transcript: Vector Sequence: 1-36, 638-727 Insert Sequence: 37-637

5'-

GGGCGAATTGAATTTAGCGGCCGCGAATTCGCCCTTCAGCAGAAAGACTTTTGAGACAGAGTTCCAGAAAACAAAACATC
AAGAGTGGGACTTTGTCGTGACAACTGACATTTCAGAGATGGGCGCCAACTTTAAAGCTGACCGTGTCATAGATTCCAGG
AGATGCCTAAAGCCGGTCATACTTGATGGCGAGAGAGTCATTCTGGCTGGACCCATGCCTGTCACACATGCCAGCGCTGC
CCAGAGGAGGGGGCGCATAGGCAGGAATCCCAATAAACCTGGAGATGAGTATCTGTATGGAGGTGGGTGCGCAGAGACTG
ACGAAGACCATGCACACTGGCTTGAAGCAAGAATGCTCCTTGACAATATTTACCTCCAAGATGGCCTCATAGCCTCGCTC
TATCGACCTGAGGCCGACAAAGTAGCAGCCATTGAGGGAGAGTTCAAGCTTAGGACGGAGCAAAGGAAGACCTTTGTGGA
ACTCATGAAAAGAGGAGATCTTCCTGTTTGGCTGGCCTATCAGGTTGCATCTGCCGGAATAACCTACACAGATAGAAGAT
GGTGCTTTGATGGCACGACCAACAACACCATAATGGAAGACAGTGTGCCGGCAGAGGTGTGGACCAGACACGGAGAGAAG
GGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCGTAATCATGGTCATAGCTG
TTTCCTG

-3'

Certificate of Analysis - Universal calibrator RNA 1.0 Sequence (EVAg/Institute of Virology, Bonn) 11477/16 and 11478/16 – IVT V



Zika virus IVT V

A specific RT-PCR was performed on KJ776791. The product was cloned into a vector, sequenced and *in vitro* transcribed. *In vitro* transcript: Vector Sequence: 1-36, 634-723 Insert Sequence: 37-633

5'-

GGGCGAATTGAATTTAGCGGCCGCGAATTCGCCCTTGTGGCTAGGGGCTAGATTTCTAGAGCTCGAAGCCCTTGGATTCT
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CTAGAAGAGATGAGTCGCATACCAGGAGGAAGGATGTATGCAGATGACACTGCTGGCTGGGACACCCGCATCAGCAGGTT
TGATCTGGAGAATGAAGCTCTAATCACCAACCAAATGGAGAAAGGGCACAGGGCCTTGGCATTGGCCATAATCAAGTACA
CATACCAAAACAAAGTGGTAAAGGTCCTTAGACCAGCTGAAAAAGGGAAAACAGTTATGGACATTATTTCGAGACAAGAC
CAAAGGGGGAGCGGACAAGTTGTCACTTACGCTCTTAACACATTTACCAACCTAGTGGTGCAACTCATTCGGAATATGGA
GGCTGAGGAAGTTCTAGAGATGCAAGACTTGTGGCTGCTGCGGAGGTCAGAGAAGGTGACCAACTGGTTGCAGAGCAACG
GATGGGATAGGCTCAAACGAATGGCAGTCAGTGGAGATGATTGCGTTGTGAAGCCAATTGATGATAGGTTTGCAAGGGCG
AATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCGTAATCATGGTCATAGCTGTTTC
CTG

-3'

Appendix 3

Draft Instructions for Use for the Interim Zika Virus Reference Material for NAT-based Assays

Paul-Ehrlich-Institut
Bundesinstitut für Impfstoffe und biomedizinische Arzneimittel
Federal Institute for Vaccines and Biomedicines

A WHO Collaborating Centre
for Quality Assurance of Blood Products and
in vitro Diagnostic Devices

Candidate World Health Organization International Standard for Zika virus RNA for Nucleic Acid Amplification Techniques (NAT)-Based Assays

PEI code 11468/16

(Version 1.0, July 2016)

1. INTENDED USE

The candidate World Health Organization (WHO) International Standard for Zika virus (ZIKV) RNA is intended to be used in the standardization of nucleic acid amplification technique (NAT)-based assays for ZIKV. The establishment of an international standard is urgently needed in the standardization, harmonization and quality control of the NAT tests and patient management. The standard has been prepared using an Asian strain of ZIKV (PF13/251013-18), isolated from a patient serum in French Polynesia in 2013. The material has been lyophilized in 0.5 ml aliquots and stored at -20°C. The material has been evaluated in an international collaborative study involving 24 laboratories performing a wide range of ZIKV NAT assays. Further details of the collaborative study are available in the report (1).

2. UNITAGE

This reagent has been assigned a unitage of 50,000,000 NAT-detectable Units/ml.

3. CONTENTS

Each vial contains 0.5 ml of lyophilized plasma containing non-infectious ZIKV. The virus has been diluted in a solution of hydroxyectoin (0.6 M) and lyophilized. The material is intended for dilution in a diluent appropriate for the assay matrix being tested e.g. plasma, urine etc.

4. CAUTION

THIS PREPARATION IS NOT FOR ADMINISTRATION TO HUMANS.

The preparation contains heat inactivated, non-infectious ZIKV.

As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures probably will include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

5. USE OF MATERIAL

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

The material is supplied lyophilized and should be stored at or below-20°C. Each vial should be reconstituted in 0.5 ml of sterile nuclease-free water. The product should be reconstituted just prior to use. The product should be used for the calibration of secondary reference preparations for ZIKV RNA. The If not all the material is used immediately, laboratories may aliquot the remaining material into suitable volumes which should be stored at or below -70°C.

6. STABILITY

As the stability studies with accelerated conditions indicate high stability of the lyophilized reference material under the recommended storage conditions (at or below -20°C), there is no expire date assigned to the international standard. This approach complies with the recommendations for the preparation, characterization and establishment of international and other biological reference standards (2). The reference material is held at the Paul-Ehrlich-Institut (PEI) within assured, temperature-controlled storage facilities. During its life cycle the stability is monitored at regular intervals. The international standard remains valid with the assigned potency and status until withdrawn or amended.

Reference materials should be stored on receipt as indicated on the label. Once, diluted or aliquoted, users should determine the stability of the material according to their own method of preparation, storage and use.

Users who have data supporting any deterioration in the characteristics of any reference preparation are encouraged to contact PEI.

7. REFERENCES

The accession number for the ZIKV virus strain is KX369547 (3).

1. Baylis SA, Hanschmann KMO, Schnierle BS, Trösemeier JH, J. Blümel J. Collaborative Study to Evaluate a Candidate World Health Organization International Standard for Zika Virus for Nucleic Acid Amplification Technique (NAT)-Based Assays WHO/BS/2016.2286.

2. World Health Organization. Recommendations for the preparation, characterization and establishment of international and other biological reference standards (revised 2004). WHO Technical Report Series 2006. 932, 73-131.

3. Trösemeier J-H, Musso D, Blümel J, Thézé J, Pybus OG, Baylis SA. Genome Sequence of a Candidate World Health Organization Reference Strain for Zika Virus for Nucleic Acid Testing. Genome Announcements, in press.

1. ACKNOWLEDGEMENTS

We are grateful to the Didier Musso at the Institut Louis Malardé for supplying the virus strain used to develop the international standard and to all study participants.

2. FURTHER INFORMATION

Further information for this material can be obtained as follows: whoccivd@pei.de or
WHO Biological Reference Preparations:
http://www.who.int/biologicals/en/

3. 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 whoccivd@pei.de

4. CITATION

In any circumstance where the recipient publishes a reference to PEI materials, it is important that the correct name of the

Paul-Ehrlich-Institut
Paul-Ehrlich-Str. 51-59
63225 Langen, Germany

Email: whoccivd@pei.de
Web: http://www.pei.de

preparation, the PEI code number, the name and the address of PEI are cited correctly.

5. MATERIAL SAFETY SHEET

Physical properties (at room temperature)		
Physical appearance		Lyophilized powder
Fire hazard		None
Chemical properties		
Stable	Yes	Corrosive: No
Hygroscopic	No	Oxidising: No
Flammable	No	Irritant: No
Other (specify) ZIKV	Contains inactivated, non-infectious	
Handling:	See caution, section 4	
Toxicological properties		
Effects of inhalation:	Not established - avoid	
Effects of ingestion:	Not established - avoid	
Effects of skin absorption:	Not established - avoid	
Suggested First Aid		
Inhalation	Seek medical advice	
Ingestion	Seek medical advice	
Contact with eyes	Wash thoroughly with water. Seek medical advice	
Contact with skin	Wash thoroughly with water. Seek medical advice	
Action on Spillage and Method of Disposal		
Spillage of vial 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.		

6. LIABILITY AND LOSS

Information provided by the Institute is given after the exercise of all reasonable care and skill in its compilation, preparation and issue, but it is provided without liability to the Recipient in its application and use.

It is the responsibility of the Recipient to determine the appropriateness of the materials supplied by the Institute to the Recipient ("the Goods") for the proposed application and ensure that it has the necessary technical skills to determine that they are appropriate. Results obtained from the Goods are likely to be dependent on conditions of use by the Recipient and the variability of materials beyond the control of the Institute.

All warranties are excluded to the fullest extent permitted by law, including without limitation that the Goods are free from infectious agents or that the supply of Goods will not infringe any rights of any third party.

The Institute shall not be liable to the Recipient for any economic loss whether direct or indirect, which arise in connection with this agreement.

The total liability of the Institute in connection with this agreement, whether for negligence or breach of agreement or otherwise, shall in no event exceed 120% of any price paid or payable by the Recipient for the supply of the Goods.

If any of the Goods supplied by the Institute should prove not to meet their specification when stored and used correctly (and provided that the Recipient has returned the Goods to the Institute together with written notification of such alleged defect within seven days of the time when the Recipient discovers or ought to have discovered the defect), the Institute shall either replace the Goods or, at its sole option, refund the handling charge provided that performance of either one of the above options shall constitute an entire discharge of the Institute's liability under this Condition.