

**EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION  
Geneva, 24 to 28 October 2022****Collaborative Study to Evaluate a Candidate World Health Organization  
International Standard for Antibodies to Chikungunya Virus**

Sally A. Baylis<sup>1\*#</sup>, Constanze Yue<sup>1#</sup>, Marcus Panning<sup>2</sup>, Marie-Christin Pauly<sup>2</sup>, Lia Laura Lewis Ximenez de Souza Rodrigues<sup>3</sup>, Helen Faddy<sup>4</sup>, Graham Simmons<sup>5</sup>, Michael Busch<sup>5</sup>, Barbara S. Schnierle<sup>1</sup>, Hanna Roth<sup>1</sup>, Kay-Martin O. Hanschmann<sup>1</sup> and the Collaborative Study Group§

*<sup>1</sup>Paul-Ehrlich-Institut, Paul-Ehrlich-Straße 51-59, D 63225 Langen, Germany;*

*<sup>2</sup>Universitätsklinikum Freiburg, University of Freiburg, 79106 Freiburg, Germany; <sup>3</sup>Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro 21040-900, Brazil; <sup>4</sup>Australian Red Cross Lifeblood, Brisbane QLD 4000, Queensland, Australia; <sup>5</sup>Vitalant Research Institute, San Francisco, CA 94118-4417, USA*

\*Principal contact: [Sally.Baylis@pei.de](mailto:Sally.Baylis@pei.de)

#Contributed equally

§Listed in 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 **23 September 2022** and should be addressed to the attention: World Health Organization, 1211 Geneva 27, Switzerland, attention: Technical Standards and Specifications (TSS). Comments may also be submitted electronically to the Responsible Officer: **Dr Ivana Knezevic** at email: [knezevici@who.int](mailto:knezevici@who.int).

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## Summary

Chikungunya fever is a mosquito-borne disease characterized by fever and severe joint pain frequently resulting in long-term morbidity with outbreaks reported in Africa, Asia, Europe and the Americas. Chikungunya fever is caused by chikungunya virus (CHIKV), an *Alphavirus*. Currently, there are no licensed vaccines available to prevent chikungunya infections. This collaborative study was undertaken with the aim to assess the suitability of a candidate World Health Organization (WHO) International Standard (IS) for CHIKV-specific antibodies; neutralizing antibodies are potentially an important correlate of protection.

The potency of the candidate IS, related reference preparations and clinical samples were evaluated using a range of virus neutralization and immunoassays with the aim of assigning an internationally agreed unitage to the candidate WHO IS. The candidate IS (1502/19) consisted of a lyophilized anti-CHIKV plasma preparation comprising a pool of three donations from a CHIKV-recovered patient. A second preparation (1504/19) was produced from pooled plasma from ten anti-CHIKV-antibody positive blood donations. Both 1502/19 and 1504/19 were included as duplicate samples in the study to evaluate inter- and intra-assay variability. Seven additional samples were included in the study: five anti-CHIKV-antibody positive clinical plasma samples of differing titres were included to investigate commutability of the candidate ISs; two further samples were included to investigate specificity - an anti-dengue virus /anti-Zika virus antibody positive plasma sample as well as a sample consisting of a pool of anti-Ross River virus (RRV)-positive plasma donations. CHIKV and RRV are both members of the Semliki Forest complex of *Alphaviruses* and serological cross-reactivity within the complex has been described.

The collaborative study materials were distributed to 28 laboratories from 13 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 26 of the participating laboratories. The assays used consisted of a mixture of different types of virus neutralization assays (using CHIKV or reporter viruses or replicon particles), binding assays (some commercially available some developed in-house) such as enzyme-linked immunosorbent assays, immunofluorescence tests, microsphere-based assays, blots and haemagglutination-inhibition assays. The binding assays were directed against CHIKV virions, virus-infected cells, virus-like particles and recombinant envelope proteins (E1 alone or in combination with E2). Laboratories performing neutralization assays used their own virus stocks and in-house developed methods.

The results showed that the candidate ISs were detected consistently by all participants. Intra-assay variation was considerably lower than inter-assay variation for 1502/19 and 1504/19. Both candidate ISs were investigated for their ability to harmonize results and assay variability was substantially reduced to a similar extent, when titres from the panel of samples were expressed relative to either 1502/19 or 1504/19. This harmonization also applied to the anti-RRV antibody positive sample where it was detected.

Both candidate ISs are stable under recommended conditions of storage, i.e. at or below -20°C, and are 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 1502/19 be established as the 1<sup>st</sup> IS for anti-CHIKV (immunoglobulin G) neutralizing antibodies with an assigned unitage of 1,000 International Units per ml.

## Introduction

Chikungunya virus (CHIKV) is an arbovirus first identified during an outbreak of dengue-like illness in 1952-53 in Tanzania (Robinson, 1955; Lumsden, 1955). Since then, frequent outbreaks of CHIKV have been reported in Eastern, Southern, Central and West Africa (Zeller *et al.*, 2016). In the late 1950s, CHIKV appeared in Thailand for the first time. In 1964, CHIKV and dengue virus (DENV) type 2 were isolated from a coinfecting patient in Vellore, Southern India (Myers and Carey, 1967). By the 2000s, CHIKV had emerged in La Réunion (Paquet *et al.*, 2006) and other islands in the Indian Ocean as well as in India, South East Asia and later in Oceania. In 2013, CHIKV was identified in Brazil, being introduced more widely in southern and central America and the Caribbean (Cauchemez *et al.*, 2014; Cunha *et al.*, 2020). Sporadic CHIKV outbreaks have occurred in the Mediterranean region with the first introduction identified in Italy in 2007 (Angelini *et al.*, 2007).

CHIKV is an enveloped, positive-sense RNA Alphavirus belonging to the *Togaviridae* family (Chen *et al.*, 2018). The virus is maintained in a sylvatic cycle involving non-human primates and mosquito species present in forest canopies; the urban cycle involves transmission of the virus between humans and *Aedes aegypti* and *Aedes albopictus* mosquito vectors. The emergence of CHIKV in La Réunion was associated with a single amino acid change (Ala→Val) in the E1 envelope protein of the virus (Tsetsarkin *et al.*, 2007). This mutation affected vector specificity resulting in an increase in CHIKV infectivity in *Ae. albopictus* leading to rapid emergence and spread of the virus beyond La Réunion. Further point mutations have been identified that increase infectivity and enhance dissemination (Cunha *et al.*, 2020). There are three main genotypes of CHIKV - East/Central/South African (ECSA), Asian and West African (WA). The ECSA genotype gave rise to the Indian Ocean lineage (IOL) following the outbreak in La Réunion in 2006 (Kariuki Njenga *et al.*, 2008) whilst the Asian genotype gave rise to the Asian/American lineage (AAL) during outbreaks in the Americas in the last decade (Lanciotti and Lambert, 2016; Archila *et al.*, 2022).

Between ~72%-97% of people infected with CHIKV will develop symptoms. Chikungunya fever, the disease caused by CHIKV infection, is characterized by an acute infection with high fever, rash, fatigue, myalgia and polyarthralgia/polyarthrititis. Symptoms of chikungunya fever are similar to those caused by DENV and Zika virus (ZIKV), which co-circulate in most endemic areas, making differential diagnosis challenging. The name chikungunya, in the Tanzanian Makonde dialect, means “bent over in pain” referring to debilitating arthralgia and joint pain in patients. Symptoms can persist in ~30% of patients often lasting several months or even years resulting in significant morbidity (Puntasecca *et al.*, 2021; Doran *et al.*, 2022). In some patients, less frequent clinical manifestations of CHIKV include neurologic, cardiac and ocular symptoms as well as hepatitis and haemorrhage. Neonatal encephalitis and neuro-developmental problems may occur as a result of vertical transmission. CHIKV has been associated with a relatively low mortality, with a case fatality rate (CFR) of ~0.1%. However, more recent surveillance data from 2019, compiled by the World Health Organization (WHO) has identified a significantly higher CFR of 1.8% in certain populations, particularly those with comorbidities and the elderly (Puntasecca *et al.*, 2021). Since there are no antiviral therapies for CHIKV, treatment relies upon management of symptoms.

Because of the significant morbidity and disability-adjusted life years impact associated with long-term rheumatic sequelae following CHIKV infection (Puntasecca *et al.*, 2021), and the increased upper range of the CFR, effective vaccines to prevent infection and disease and counter outbreaks are urgently needed. Several candidate CHIKV vaccines are in the pipeline, which are at different

stages of pre-clinical and clinical development, although none have been licensed yet. The vaccines, include ones based on inactivated viruses, attenuated CHIKV strains, vectored vaccines, nucleic acid-based vaccines as well as virus-like particles and recombinant proteins (Smalley *et al.*, 2016; Goulas *et al.*, 2018). There are several CHIKV genotypes/lineages circulating worldwide, however, there is only a single serotype. With long-lasting cross-protection between different lineages, vaccines are expected to protect against different CHIKV strains (Smalley *et al.*, 2016).

Several lines of evidence have shown that protection against CHIKV is primarily correlated with the induction of neutralizing antibodies (Lum *et al.*, 2013; Yoon *et al.*, 2015; Milligan *et al.*, 2019). The evidence includes passive transfer of IgG antibodies from convalescent patients affording protection in mice; neutralizing monoclonal antibodies directed against the CHIKV E1 and E2 envelope proteins modulate disease in mouse models with some being protective in lethal challenge models in immunocompromised animals; the presence of anti-CHIKV plaque reduction neutralization test (PRNT) titres of > 10 seemed to prevent development of symptoms associated with CHIKV (Yoon *et al.*, 2015); the early development of neutralizing antibodies is associated with protection against arthralgia; natural infection appears to confer lifelong immunity to CHIKV (Kam *et al.*, 2012).

Because of the rapid and transient nature of CHIKV outbreaks and the widespread immunity as a consequence of high infection rates, evaluation of efficacy of candidate vaccines in phase III clinical trials is logistically challenging. Clinical end-points of clinical trials would typically include reduction and/or prevention of symptomatic disease and seroconversion, including the generation of neutralizing antibody responses. An alternative to clinical efficacy being considered by national regulatory authorities is combining data from human clinical trials and animal studies using bridging data with well-controlled assays as possible route for vaccine licensing. However, there is a wide range of assays in use to measure neutralizing antibodies, using different types of virus or antigens with differing operational conditions and reporting methods resulting in substantial variability. This lack of standardization makes comparison of results challenging. The aim of this study was to develop a suitable antibody reference material to be able to compare results of neutralization assays for anti-CHIKV antibodies, including those generated during natural infection as well as those produced in response to different candidate vaccines. It is hoped, that the use of such a reference material or WHO International Standard (IS) will be useful in determining antibody titres that correlate with protection against CHIKV.

Clinical diagnostic testing for anti-CHIKV antibodies also lacks standardization with variability in performance of laboratory developed/in-house and commercially available immunoassays. Reference material for anti-CHIKV antibodies will be useful for serological assay standardization, mainly as controls for assay performance across different platforms and different test formats. More standardization will result in better understanding of CHIKV (sero-)epidemiology.

The Paul-Ehrlich-Institut (PEI), Federal Institute for Vaccines and Biomedicines, as a WHO Collaborating Centre for both the quality assurance of blood products and *in vitro* diagnostic devices and for the standardization and evaluation of vaccines, developed two candidate anti-CHIKV antibody preparations for testing and comparison across assays and laboratories to evaluate their suitability as a WHO IS. This study evaluated the potency of the two proposed candidate materials for a WHO IS for anti-CHIKV antibodies in parallel with other antibody preparations obtained from CHIKV convalescent patients and blood donors, using assays in routine use in the participants' laboratories. The aim was to select the most suitable candidate standard for assay harmonization and agree on an internationally assigned unitage for the candidate standard following statistical analysis of the study data at the PEI.

## Study materials

### Candidate International Standard – 1502/19

The candidate IS (1502/19) was prepared using a pool of three plasma donations from a German patient who contracted chikungunya whilst travelling in Brazil in 2016. The patient was diagnosed with CHIKV infection whilst in Brazil and the diagnosis was reconfirmed upon return to Germany. The patient displayed signs of arthralgia over a period of several months. Plasma was collected ~8-11 months after the onset of symptoms. The plasma was obtained from the voluntary, anonymous donor by plasmapheresis on three separate occasions. The individual plasma donations tested positive for anti-CHIKV antibodies, but were negative for other *Alphavirus* antibodies and anti-DENV and anti-ZIKV antibodies using a mixture of enzyme-linked immunosorbent assays (ELISAs) and viral pseudotyping assays (Henss *et al.*, 2019; Henss *et al.*, 2020).

The plasma samples from the donor were tested by NAT to ensure the absence of Human Immunodeficiency Virus Type 1 (HIV-1) Group M RNA, HIV-1 Group O RNA, Human Immunodeficiency Virus Type 2 (HIV-2) RNA, Hepatitis C Virus (HCV) RNA, and Hepatitis B Virus (HBV) DNA using the cobas TaqScreen MPX Test, v2.0 (Roche Diagnostics GmbH, Mannheim, Germany). No HIV-1/2 RNA, HCV RNA or HBV DNA were detected. In addition, the plasma was tested for the presence of CHIKV RNA, DENV RNA and ZIKV RNA using the ExiPrep™ Dx Viral RNA Kit (Bioneer Corporation, Daejeon, Republic of Korea) on the ExiPrep™ 16 Dx platform incorporating an internal control. The entire eluate was analyzed by PCR; set-up was performed using the ExiSpin™ device (Bioneer Corporation) and amplification/detection reactions using the Exicycler™ 96 Real-Time Quantitative Thermal Block (Bioneer Corporation) using the AccuPower® ZIKV(DENV,CHIKV) Multiplex Real-Time RT-PCR Kit (Bioneer Corporation). The plasma samples were negative for CHIKV RNA, DENV RNA and ZIKV RNA.

This candidate IS was evaluated in the Gesellschaft zur Förderung der Qualitätssicherung in medizinischen Laboratorien (INSTAND) External Quality Assessment (EQA) Scheme Group No. 402 Virus Immunology – Chikungunya Virus in 2017, the report is included in Appendix 2. The candidate IS was designated 402009 in the EQA study. The study investigated qualitative and quantitative analysis of anti-CHIKV IgM and IgG in the EQA panel as well as anti-CHIKV IgG avidity. The EQA study was used to determine how the candidate IS would perform across a wide range of assay types.

For the lyophilization the pooled plasma donations were diluted 1:1 with cell culture grade water. Processing was performed during February 2019. For the processing, 1.0 ml volumes were dispensed into 7 ml amber glass vials. After completion of the freeze-drying procedure, the vacuum was broken by the introduction of nitrogen gas and the vials sealed and capped with Flip Off Tear Off caps. All the operations were performed in a qualified class A clean room (for filling equipment and in front of the freeze dryer). Residual moisture was determined by Karl Fischer analysis. The number of filled vials, coefficient of variation of the filled volume and residual moisture content of the vials is shown in the production summary (Table 1). The filling and lyophilization was performed at the Division of Reference Standards European Directorate for the Quality of Medicines and HealthCare, Strasbourg, France. Vials of the candidate IS are intended for reconstitution in 0.5 ml of cell culture grade water.

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.

### **Candidate International Standard – 1504/19**

A second candidate IS (1504/19) was prepared by pooling ten anti-CHIKV positive Puerto Rican blood donations collected between November 2016 and January 2017. The donors were likely infected during the CHIKV epidemic of 2014 (Sharp *et al.*, 2014; Simmons *et al.*, 2016). All donations were anti-CHIKV IgG positive; two donations were borderline anti-CHIKV IgM positive – the rest were negative. All donations were anti-DENV IgG positive and anti-DENV IgM negative. Three donations were anti-ZIKV IgG positive and one of these was also anti-ZIKV IgM positive. All samples tested negative for blood-borne viruses by NAT as described above. Processing was performed as described above in February 2019. The number of filled vials, coefficient of variation of the filled volume and residual moisture content of the vials is shown in the production summary (Table 1). Vials of the candidate IS are intended for reconstitution in 0.5 ml of cell culture grade water.

Vials of the candidate WHO IS are stored as described above for 1502/19.

### **Clinical materials**

Several clinical materials were included in the study to evaluate, in a limited way, commutability of the candidate ISs; further samples were included to investigate assay specificity. The samples included a mixture of anti-CHIKV-positive plasmas (covering a range of low to high titres), anti-DENV, anti-ZIKV positive plasma as well as anti-Ross River virus (RRV) plasma. The anti-RRV plasma was included in order to control for specificity since RRV, like CHIKV is also an *Alphavirus* and both belong to the Semliki Forest complex where antigenic cross-reactivity is well known.

Plasma samples were obtained from randomly selected Brazilian patients with clinically diagnosed CHIKV infection collected in 2016; CHIKV, ZIKV and DENV infections were diagnosed using virus-specific commercial ELISAs (Euroimmun AG, Lübeck, Germany) and by triplex NAT as described above. Full-length sequence analysis of one virus isolate from this patient cohort (sample P9 in the study, was a pool of equal volumes of plasma collected 87 and 146 days post-symptom onset) provided evidence of infection by a virus strain belonging to the ECSA genotype (S. Baylis, H. Roth, unpublished observations). Samples were collected from Brazilian patients from the Viral Hepatitis Ambulatory/FIOCRUZ/Rio de Janeiro following IRB approval (May 10, 2016 (Fiocruz IRB ID: 0142/01). All human samples used were collected with the written, informed consent of the patients for diagnostic purposes according to ethical regulations in Brazil. All other samples were obtained from voluntary, anonymous blood donors from Puerto Rico and Australia.

The clinical samples, together with the candidate ISs (each provided in duplicate) are shown in Table 2; all samples have been given a code number P1-P11. Samples were dispensed into volumes suitable for the different assays used by each participating laboratory and stored as liquid/frozen materials. Samples were provided in triplicate. One sample, a pool of anti-RRV antibody-positive plasma donations, was lyophilized – donation testing and processing was performed as described above.

## Collaborative Study

Twenty-eight laboratories from 13 different countries volunteered to participate in the study. In total, 26 laboratories returned results and are listed in Appendix 1. Laboratories from 12 different countries returned results: Australia (2), Austria (2), Canada (1), China (1), French Polynesia (1), Germany (7), India (3), Italy (1), Malaysia (1), Sweden (1), Trinidad and Tobago (1), and the United States of America (5). One laboratory from Brazil and a further laboratory from Australia were unable to return results due to the onset of the Coronavirus disease 2019 (COVID-19) pandemic. The participating laboratories included specialist arbovirus laboratories, vaccine manufacturers, clinical virology laboratories, as well as developers of *in vitro* diagnostic devices. 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 -20°C until use. The samples included in the panel are described above and listed in Table 2. Participants were asked to test the panel using their routine assay for anti-CHIKV IgG antibodies, testing the panel of samples in three separate assay runs, using fresh vials of each sample for each run and performing two independent dilution series where possible. The study protocol is outlined in Appendix 3. For the preparation of dilutions, participants were requested to use their usual diluent.

Several lyophilized preparations were evaluated in the study and these were reconstituted before use by participants using cell culture grade water. Samples P1, P3, P5, P6 and P10 which were all lyophilized were reconstituted in 0.5 ml of water. All other samples were provided as liquid/frozen materials.

## Statistical Methods

The evaluation of raw data was performed with CombiStats version 6.1 (European Directorate for the Quality of Medicines and HealthCare/Council of Europe, Strasbourg, France) - using a sigmoid, 4-parametric dose-response model (quantitative data) and a quantal response model (qualitative data - probit-transformed). Both methods are described in detail in the European Pharmacopoeia, chapter 5.3 (Council of Europe, 2021). With both models, the 50% reduction plaque reduction neutralization or neutralization titres (PRNT<sub>50</sub> or NT<sub>50</sub>, respectively) or EC<sub>50</sub> titres (other assays) were estimated (i.e. the dilution/titre at which 50% of the maximum signal could be observed, or, for qualitative data, the cut-off between positive and negative signals).

Further statistical analysis (i.e. estimation of a consensus value for all combined datasets) was performed with SAS®/STAT software, version 9.4, SAS System for Windows (SAS Institute, Inc., Cary, NC, USA). Individual estimates (both PRNT<sub>50</sub>/NT<sub>50</sub> and EC<sub>50</sub> and potencies relative to samples P1 or P5) were combined using a mixed linear model with random factor 'assay type' (neutralization or binding/other assay) and 'participant'. Combined estimates were accompanied with 95% confidence intervals. The relative potencies of the panel of samples were estimated relative to the candidate ISs i.e. samples P1 (1502/19) or P5 (1504/19) each with an assigned potency of 1,000 U/ml.

The coefficient of variation was used to describe the relative variability of the measurements. The influence of relevant factors (as participant, assay type, sample) on the intermediate precision as well as the intra-assay precision (repeatability) was evaluated by means of a mixed linear model (an

analysis of variance, ANOVA, using fixed and random factors) using log transformed EC<sub>50</sub> estimates of the individual results. This method uses (restricted) maximum likelihood estimates, which may lead to a small difference between the estimated variance and the usual variance estimator. The inter-assay precision (intermediate precision) was estimated as sum of the variances of the relevant factors and is described by the coefficient of variation. For the intra-assay precision (repeatability) the residual variance were used. The measurement uncertainty is described as the estimated total variance from the ANOVA (sum of variances from relevant factors plus residual variance), also denoted as coefficient of variation.

## Stability Studies

Stability of the candidate ISs are under continuous assessment, through both real-time and accelerated thermal degradation stability studies. Vials of the two candidates have been stored at -20°C (the recommended storage temperature) as well as baseline samples stored at -80°C. For the accelerated thermal degradation, vials were incubated at +4°C, +20°C, +37°C and +45°C for 2 weeks, 1 month, 3 months, 6 months and 1 year. Following incubation at the respective temperature, the vials were stored at -80°C until analysis. For analysis, the contents of the vials were reconstituted in 0.5 ml of cell culture grade water and tested for anti-CHIKV IgG in triplicate using a commercially available assay (Chikungunya IgG ELISA - EUROIMMUN Medizinische Labordiagnostika AG, Lübeck, Germany). Testing was performed in accordance with the manufacturer's instructions after appropriate dilution. The titres of the samples were expressed relative to the baseline samples stored at -80°C.

## Results

### INSTAND EQA

In the initial evaluation of the candidate IS (1502/19) in the INSTAND EQA, assays used by participating laboratories included in-house and commercial assays - ELISAs, indirect immunofluorescence tests/assays (IIFTs or IFA), immunoblots and virus neutralization. All laboratories participating in the EQA were able to positively detect IgG in the candidate IS, irrespective of method (46 data sets in total). Data were returned from three different commercially available ELISAs, two commercially available IFAs, two separate in-house IFAs, one commercial immunoblot (5 data sets) and one in-house neutralization assay. Further details are available in Appendix 2. A single laboratory determined the avidity of the candidate standard which was found to be high (97%). In the case of IgM analysis (M. Kammel, H. Zeichhardt, personal communication) 16 laboratories reported the candidate standard as positive, 19 negative and 9 borderline. Where quantitative data were returned for anti-CHIKV IgM, titres of the candidate standard were significantly lower than the EQA sample obtained from an acutely infected patient (24 days after onset of disease). Collectively, these data suggest that the candidate IS (1502/19) contains only very low levels of anti-CHIKV IgM.

### Data received – collaborative study

Data were received from a total of 26 of the 28 participating laboratories. In total, 39 datasets were returned of which, 36 datasets had at least one valid, assay that could be further evaluated. Virus neutralization data were determined by 16 laboratories (live virus (n=15) and virus replicon particle-

based (n=1)). Data from ELISA methods were returned by 15 laboratories representing both commercial (n=8) and in-house developed assays (n=7). The commercial ELISAs were produced by three different manufacturers. Further immunoassays include a microsphere-based assay, a haemagglutination inhibition assay as well as an immunoblot. Eight laboratories reported results for IFAs.

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 26A and laboratory 26B.

The types of methods used by the participants are listed in Table 3.

## Neutralization assays

The combined means for the PRNT<sub>50</sub> and NT<sub>50</sub> titres from the different laboratories are shown in Table 4 and in histogram form in Figures 1A-11A (neutralization data shaded blue). The titres were determined at PEI based upon the data provided by the participants. Consistent detection of all positive for anti-CHIKV antibodies was reported by the participating laboratories using neutralization assays; however, it was not possible to analyse data from Laboratory 3B or Laboratory 19 due to inconsistencies in dilutions and an incomplete data set, respectively. In the case of Laboratory 21C, insufficient dilutions were performed in run 1; however it was possible to analyse data from the subsequent two runs. Laboratory 5 performed repeat testing on two separate occasions on a sub-set of samples that had undergone one round of freeze-thawing.

The mean titres were within  $\sim 2 \log_{10}$  range for sample P1 (1502/19) and its replicate P3. A similar range of values ( $\sim 2\text{--}2.5 \log_{10}$  i.e.  $\geq 100$ -fold) for the mean titres was observed for sample P5 (1504/19) and the replicate sample P10. A range of values was observed for the remaining anti-CHIKV antibody positive samples included in the study and is not unexpected and likely due to differing reagents and procedural differences.

Sample P6 which contained anti-RRV antibodies, was detected by Laboratories 3B, 4, 5, 12, 23A and 27; lower titres were reported by Laboratories 6, 9, 19 and 22A (62.5%). All laboratories reported sample P2 as negative with the exception of Laboratory 23A where a very low titre was reported (Table 4).

For all the neutralization assays, laboratories used their own virus stocks and in-house developed methods (Table 3). Six laboratories used virus strains belonging to the Asian genotype, four of which used the 181/clone 25 vaccine strain (Levitt *et al.*, 1986). Six laboratories used CHIKV strains belonging to the IOL, three of which were based on virus from the 2006 outbreak in La Réunion (in one case using a replicon particle assay). One laboratory used an ECSA strain. Three participating laboratories did not disclose the CHIKV genotype/lineage used in the neutralization assays. There did not appear to be any specific trend regarding under/over reporting of neutralization titres. For example, in the case of Laboratories 4 and 14, neutralization titres were highest for the panel of samples, and these two laboratories used Asian and IOL strains in the neutralization assays, respectively. Laboratory 8 reported generally lower titres across the panel of samples and used a more recent IOL isolate from 2017 following a European outbreak. The single laboratory using the ESCV strain for virus neutralization reported titres in line with the majority of other laboratories. No trend was observed between laboratories detecting anti-RRV neutralizing antibodies in sample P6 and the use of a specific CHIKV genotype/lineage.

## Relative Potencies – Neutralization assays

On the basis of the combined data from the neutralization assays, the mean neutralizing titres were expressed relative to samples P1 (1502/19) and P5 (1504/19), the two candidate ISs. In both cases, the assumed potency was 1,000 U/ml for either P1 or P5. The relative potencies are shown in Table 5 (relative to P1 – 1502/19) and Table 6 (relative to P5 – 1504/19). Figures 1B-11B show the relative potency data for all assays (virus neutralization data is shaded blue). It was not possible to determine relative potencies for either Laboratory 3A or Laboratory 19.

Expression of the relative potencies for all the anti-CHIKV antibody positive samples resulted in a reduction in the variation between assays (with the range varying from  $\sim 0.5$ - $1.1 \log_{10}$ ) when potencies were compared to P1 (1502/19); (range  $\sim 0.7$ - $1.2 \log_{10}$  when data determined relative to P5). This harmonization also applied to the anti-RRV antibody positive sample - where it was detected.

Figure 12A shows the relative potency data across the range of samples for the neutralization methods; the upper panel shows the mean potencies and the lower left panel shows the mean potencies relative to P1 and the lower right panel relative to P5 showing significant reduction in variation. The relative potency data provide some evidence for commutability of the candidate ISs for the clinical samples included in the study.

## Binding assays

A wide range of antibody binding assays were evaluated in the study; for the analysis, the following immunoassays have been grouped together: a) ELISAs, assays based in microspheres, immunoblots and haemagglutination inhibition and rapid tests or b) IFAs.

Laboratory 1 used an in-house developed multiplexed microsphere immunoassay able to detect CHIKV in a panel of viruses which included RRV, Barmah Forest virus (BFV), Sindbis virus (SINV) and CHIKV. Laboratory 1 correctly identified that sample P6 contained anti-RRV antibodies as well as correctly identifying the anti-CHIKV antibody samples included in the panel; no reactivity was observed with the negative control sample (P2).

In the case of Laboratory 2A/2B, using a commercial ELISA, data were reported as Sample/Cut-off ratios for samples P1-P11. Samples P2 and P6 – the anti-CHIKV negative samples were both negative across all assay runs. The rest of the samples were diluted 1:100, 1:200, 1:400 and 1:800 and all duplicate samples tested positive; no end-point was reached. Inspection of the Sample/Cut-off (S/Co) ratios identified the following ranking - P9 (S/Co 37.4), P1/P3 (pooled S/Co 36.2), P7 (S/Co 33.9), P5/P10 (pooled S/Co 27.4), P8 (S/Co 23.3), and P4/P11 (S/Co 13.0 for both samples). Comparing this ranking with the mean potencies of the samples determined across all assays (Table 10) the ranking for the highest (P9) and lowest samples titre samples in the panel (P8, then P4 and P6 which are near identical) were the same. However, the ranking of the other samples is slightly different i.e. P1/P3 and P5/P10 ranked equal third based on mean potencies, but ranked second and fourth respectively for the ELISA; sample P7 ranked second with respect to overall mean potency, but third for the ELISA. The reason for the differences might reflect the use of total CHIKV antigen in the assay design.

Laboratory 8D used a commercially available anti-CHIKV IgM/IgG rapid test based on CHIKV virions. The data for this qualitative, rapid test identified all anti-CHIKV positive samples with the

exception of P4 and P11 which had the overall lowest mean potencies and were negative in the rapid test. Samples P2 (negative control) and P6 (anti-RRV positive) both tested negative.

Laboratory 10 tested the panel of samples by haemagglutination inhibition using inactivated CHIKV.

Laboratory 20 used a commercially available immunoblot to detect and differentiate between anti-CHIKV, anti-ZIKV and anti-DENV antibodies. Virus-like particles are the source of the recombinant antigens for CHIKV and, Equad and NS1 antigens are used for both DENV and ZIKV. Of note, this assay was used in the INSTAND EQA, however, it was subsequently revised to improve assay sensitivity and specificity by changes to the antigen composition/presentation for all three viruses. All the anti-CHIKV antibody positive samples included in the panel tested positive as well as the anti-RRV sample (P6). In the case of the negative control (P2), although sample tested positive for anti-ZIKV and anti-DENV antibodies (the antigens included in the assay include ZIKV NS1 and the envelope protein from DENV); sample P2 was non-reactive for anti-CHIKV antibodies.

Laboratory 25 using a commercially available ELISA (supplier C) repeated results for some samples from the second assay run; some results were unexpectedly high. However, other laboratories using the same method did not report such issues.

Laboratories 3C and 8C using IFAs, tested the samples at a single dilution (1:200 and 1:20, respectively), and with the exception of sample P2 (the negative control), all samples tested positive including P6 (anti-RRV antibody-positive).

The mean potencies of the anti-CHIKV-antibody titres are shown in Table 7. Figures 1A-11A illustrate, in histogram form, these mean potencies. The binding assays, where potencies could be determined, have been divided into immunoassays such as ELISAs (n=13), immunoblots (n=1), microsphere based assay (n=1) and haemagglutination inhibition (n=1) and IFAs (n=8). The ELISAs used a mixture of target antigens: E1; E1/E2 heterodimers (alone or in combination with CHIKV capsid protein (CAP)); CHIKV-infected cells or CHIKV virions. Three commercially available ELISAs were used by participants in the study; supplier A (n=2), supplier B (n=2), supplier C (n=4). Where levels of binding antibodies were determined by IFAs, half of the laboratories used in-house developed assays and the other half used a single commercially available assay. All assays, irrespective of design detected the candidate ISs P1 (and replicate P3) and P5 (and replicate P5). With the exception of Laboratory 26B (albeit at low titre), none of the immunoassays identified sample P2 as positive. In the case of sample P6 (the anti-RRV antibody-positive sample), all the IFA methods found this samples positive (lower dilutions) whilst for the remaining immunoassays listed in Table 7 - 8/15 methods (53%) found sample P6 positive, once again the positivity reflects the antigenic cross-reactivity between CHIKV and RRV.

Samples P7 and P9 have the highest overall potencies across assays and samples P4 and P11 the lowest (Table 10). In general for the IFAs, P7 and P9 were ranked as the highest and samples P4 and P11 the lowest. Similar results were observed for the ELISA data using assays from commercial suppliers B and C where E1/E2 are target antigens as well as Laboratory 15 using an E1-based ELISA and Laboratory 10 (haemagglutination inhibition, using inactivated CHIKV), Laboratory 20 (immunoblot – virus-like particles), Laboratory 23B (E1/E2/CAP).

For the three laboratories using CHIKV virion-based ELISAs, Laboratory 26B found a similar ranking of samples P4, P11 (lowest titres) and P7 and P11; however this was not so clear cut in the case of Laboratories 3A and 22B. For the remaining assays/laboratories the ranking of P4; P11 and

P7 and P9 was not so clear cut – one laboratory (21A) used CHIKV-infected cells whilst Laboratory 1 used CHIKV together with RRV, BFV, and SINV attached to microspheres.

## Relative potencies – Binding assays

On the basis of the combined data from the binding assays (immunoassays including the IFAs), the mean titres were expressed relative to samples P1 (1502/19) and P5 (1504/19), the two candidate ISs. In both cases, the assumed potency was 1,000 U/ml for either P1 or P5. The relative potency data are shown in Table 8 (relative to P1 – 1502/19) and Table 9 (relative to P5 – 1504/19). Figures 1B-11B show the relative potency data for all assays (the immunoassay data are shown in orange and the IFA data in green).

Expression of the relative potencies for all the anti-CHIKV antibody positive samples resulted in a reduction in the variation between assays (with the range varying from  $\sim 0.5$ - $1.6 \log_{10}$ ) when potencies were compared to P1 (1502/19); similar reductions were observed for P5 (1504/19). This harmonization also applied to the anti-RRV antibody positive sample where it was detected.

This relative potency data is further shown in Figure 12B across the range of samples for the immunoassays; the upper panel shows the mean potencies and the lower left panel shows the mean potencies relative to P1 and the lower right panel relative to P5 showing significant reduction in variation. The relative potency data provide some evidence for commutability of the candidate ISs for the clinical samples included in the study.

With the IFAs, relative potency analysis improved agreement between laboratories for the panel of samples either using sample P1 (1502/19) or sample P5 (1504/19) (Figures 1B-11B). However, the reduction in variation was not as pronounced for the IFAs compared to either the neutralization assays or the other immunoassays such as ELISAs. Harmonization was best when P3, P5 and P10 were compared to P1 and when P10, P1 and P3 were compared to P5; P1/P3 and P5/P10 were prepared from pooled plasma donations from a single donor with multiple bleeds or a pool from several donations, respectively. The lower level of harmonization of the other samples (single donations) included in the study, may be a consequence of the target antigens in the immunofluorescence assays which is a mixture of proteins present in the CHIKV-infected cells. In contrast, other types of immunoassay e.g. ELISAs may target specific antigens such as E1, E1/E2 or a functional assay such as a virus neutralization where antibodies target neutralizing epitopes present on viral particles. In the case of laboratory 16, it was not possible to perform relative potency analysis.

## Determination of Overall Laboratory Means – Combined Results

The overall mean values (for all types of assay i.e. virus neutralization as well as the other types of immunoassay) for the candidate ISs P1 (1502/19) and P5 (1504/19) and the other samples are shown in Table 10 and the range of estimates between laboratories. The combined overall mean values for both the qualitative and quantitative tests are shown in Table 10 together with the coefficients of variation and the range of estimates. For the data presented in Table 10, it is clear that samples P1 and the replicate sample P3 are very close in value i.e.  $3.42 \log_{10}$  and  $3.45 \log_{10}$ , respectively. In the case of sample P5 and the replicate sample P10, they have an identical potency of  $3.48 \log_{10}$ . The overall relative potencies against sample P1 (1502/19) or P5 (1504/19) are shown in Tables 11 and 12, respectively. Comparing the 95% confidence intervals for the panel of samples in Table 10 with those shown in Tables 11 and 12 (relative potency data), for each sample there is a reduction in the

95% confidence interval when data is expressed relative to either candidate IS i.e. P1 (1502/19) or P5 (1504/19) once again demonstrating the value of using a standard to facilitate harmonization of results.

### Inter- and intra-assay variation

It was possible to compare inter-and intra-assay variability for the replicate candidate IS preparations P1/P3 and P5/P10. The data are shown in Tables 13 and 14 for the replicate candidate IS preparations P1/P3 and P5/P10, respectively. As expected, inter-assay variability (227% for samples P1/P3 and 224% for samples P5/P10) far exceeds intra-assay variability for the replicate samples (38% for samples P1/P3 and 46% for samples P5/P10).

### Results of Stability Studies

Accelerated thermal degradation studies have been performed by incubation of vials of 1502/19 and 1504/19 at higher temperature i.e. +4°C, +20°C, +37° and +45C° and compared to vials stored at -20°C, the normal storage temperature, and the baseline samples stored at -80°C (Tables 15 and 16). There was a slight drop in titres at the higher temperature.

After 1 year, there was no evidence of loss of titre of the candidate ISs when stored at -20°C the normal storage temperature, moreover storage of the vials at +20°C for 1 year did not result in a loss of potency in either preparation. Collectively, these data indicate acceptable stability of the two candidate ISs.

### Conclusions

In this study, a wide range of immunoassays and virus neutralization protocols were used to evaluate the two candidates ISs for anti-CHIKV antibodies. In general, the panel of samples, including both candidate ISs, were well detected by the participating laboratories, however, data demonstrated wide variations in potencies of the candidate ISs and the other study samples which is not unexpected and regularly observed in similar collaborative studies. However, the aim of such studies is to investigate whether candidate ISs can be used to harmonize data from different assays. Indeed, it was found that both candidate ISs (1502/19 - sample P1 and 1504/19 – sample P5) were able to harmonize data between the virus neutralization assays as well as the immunoassays (including the ELISAs) and to a lesser extent the IFAs evaluated in the study. Overall, the two candidate ISs were found to have very similar potencies and both performed equally well with respect to harmonization of results. Furthermore, the relative potency data provide some evidence for commutability of the candidate standard for evaluation of clinical samples included in the study.

In the study, participants were requested to perform testing for anti-CHIKV IgG antibodies. Prior to the collaborative study it was established that the candidate IS - 1502/19 (sample P1 and its replicate P3 in the study) contained only very low levels of anti-CHIKV IgM antibodies by testing at PEI and during the INSTAND EQA, where the material was either tested negative in some assays or was borderline positive in others. For the candidate IS – 1504/19, this again contained non-detectable levels of anti-CHIKV IgM after pooling. Because anti-CHIKV IgM antibodies not just IgG antibodies are able to neutralize CHIKV (Chua *et al.*, 2017), it was felt that the use of an anti-CHIKV IgG antibody-positive, but IgM-negative reference material might be a more

straightforward choice for an IS and this is reflected in the wording in the draft Instructions for Use (Appendix 4).

With the CHIKV neutralization assays, laboratories used their own virus stocks covering a wide range of genotypes/lineages. There was no trend observed with the use of a specific genotype/lineage with respect to neutralization titres. Candidate IS – P1 (1502/19) was prepared from a recovered chikungunya patient who was infected in Brazil in 2016. In the case of the candidate IS - P5 (1504/19) this was prepared from a pool of plasma donations from Puerto Rico in 2016/2017. Unfortunately, the CHIKV genotype/lineage infecting the respective plasma donors was not known. In Brazil, when the German plasma donor became ill with chikungunya, both the ECSA genotype and the AAL lineage was in circulation there (Nunes *et al.*, 2015). Prior to collection of the plasma donations in Puerto Rico (Simmons *et al.*, 2016), the AAL lineage was prevalent (Chiu *et al.*, 2015; López *et al.*, 2019). For the remaining clinical samples included in the study, collected in Rio de Janeiro, full-length sequence analysis of virus isolated during the symptomatic phase for sample P9 revealed that the strain belonged to the ECSA genotype. Nevertheless, despite different CHIKV strains used by the participants, both candidate ISs harmonized data from different assays irrespective of plasma source.

In the regions where CHIKV outbreaks occur, there is frequent co-circulation of DENV and ZIKV. Therefore, a sample positive for anti-DENV and anti-ZIKV, but negative for anti-CHIKV, was included in the study as a negative control. There were very small number of cases where sample P2 was reported as sporadically reactive for anti-CHIKV antibodies and titres were very low, demonstrating, in general very good specificity across the assays used in the study, irrespective of the format.

In addition, in order to investigate cross-reactivity with other *Alphaviruses* a plasma pool of anti-Ross River virus (RRV) was included in the study. The individual plasma samples in the anti-RRV plasma pool had been previously characterized using a range of assays (including ELISAs and viral pseudotyping – Henss *et al.*, 2019) to ensure that they were specific for anti-RRV and that antibodies to other *Alphaviruses*, that might be expected to be found in Australia, were not present. Across all assays, ~62% found sample P6 reactive for anti-CHIKV antibodies. This observation is not unexpected, because RRV, like CHIKV, belongs to the Semliki Forest complex where such cross-reactivity is well known (Henss *et al.* 2020; Nguen *et al.*, 2020). Relative potency analysis of sample P6 (anti-RRV pooled plasma) against either sample P1 or P5 demonstrated that harmonization of the reported potencies. This observation would suggest it might be possible to use P1 or P5 for standardization of anti-RRV assays, however, a separate candidate standard for anti-RRV (sample P6 itself) has been lyophilized and will be evaluated in a separate study to be presented elsewhere.

For the relative potency analysis, both candidate IS anti-CHIKV antibody samples P1 (1502/19) and P5 (1504/19) were assigned arbitrary unitages of 1,000 units/ml given the similar overall mean potencies of the two candidate ISs (Table 10, 3.44 log<sub>10</sub> (mean of P1 and P3) and 3.48 log<sub>10</sub> (mean of P5 and P10). We propose that 1502/19 be established as the 1<sup>st</sup> World Health Organization International Standard for anti-CHIKV antibodies (immunoglobulin G) with an International Unitage (IU) of 1,000 IU/ml for neutralizing activity following reconstitution in 0.5 ml of cell culture grade water. In the case of the second candidate IS – 1504/19, this may be reserved as a replacement batch for 1502/19 in the future or else it could be used as a secondary standard (with unitage inferred by potency data relative to sample P1 (1502/19) in the study (Table 5). The proposal to select 1502/19 as the IS is partly because of the larger number of vials available and the

fact that material does not contain antibodies to other arboviruses i.e. DENV and ZIKV, although, at least for the assays evaluated in this study, the presence of these antibodies did not affect the performance of 1504/19 the other candidate IS. Clearly, the IS will be valuable for harmonization of anti-CHIKV antibody neutralization assays. Although harmonization of assays determining binding antibodies was demonstrated during the study, in this context, the IS should be used rather more cautiously i.e. simply as a control reagent assuring assay performance – with no unitage being defined for this purpose and being applicable for the detection of binding antibodies of defined specificity e.g. anti-CHIKV E1 IgG; anti-CHIKV E1/E2 IgG, the material should not be used to compare between groups of assays with different specificities. It is important to note that neutralizing antibodies and binding antibodies are not necessarily interchangeable. In the future, it may be possible to correlate a defined protective level of neutralizing antibody (defined in IU) with a surrogate marker that could be measured by an alternative type of immunoassay, however, further studies are required moving forward to establish such a relationship. The use of IU in types of assay other than virus neutralization is not currently recommended.

The data from the study also provide an evaluation of the mean estimates for candidate IS – sample P5 (1504/19) which can be used as either a secondary standard or else a potential replacement for the proposed IS (P1- 1502/01). These materials have very similar titres and showed very little difference in their ability to harmonize data (relative potency analysis).

Real-time stability studies have indicated that both candidate ISs are stable under normal conditions of storage, i.e. at -20°C or below for 6 months and therefore suitable for long term use as well as at elevated temperatures, i.e. after 3 months incubation at +20°C there was no significant reduction in anti-CHIKV antibodies (IgG) which would support shipment at ambient temperature. Initial accelerated thermal degradation analysis indicates a reduction in the levels of anti-CHIKV antibodies (IgG) at higher incubation temperatures (e.g. +37°C). Shipment at extreme temperatures should therefore be avoided. On-going studies on the real-time stability under normal storage conditions as well as studies concerning thermal degradation are in progress.

In conclusion, with the high epidemic potential of CHIKV and the difficulties in the rapid establishment of clinical trials during outbreaks, the establishment of an IS is an important step forward in providing a reference material with a traceable common reporting unit to be able to compare results obtained in different laboratories and better define protective levels of antibodies. Better standardization is essential in order to facilitate a better understanding of CHIKV (sero-) epidemiology globally and with the rapid spread of CHIKV in the last two decades, and the similar clinical presentation with viruses such as DENV and ZIKV, accurate diagnostic testing is essential.

## Recommendations

Based upon the results of the collaborative study, it is proposed that the pooled plasma sample from a convalescent chikungunya patient who acquired CHIKV in Brazil, code number 1502/19, should be established as the 1<sup>st</sup> WHO International Standard for anti-chikungunya virus (immunoglobulin G) neutralizing antibodies with a unitage of 1,000 IU/ml. The custodian laboratory is the Paul-Ehrlich-Institut.

Approximately 2,500 vials are available for distribution (containing 0.5 ml of lyophilized plasma residue per vial).

## Responses from participants

After circulation of the draft report for comment, replies were received from twenty-four participants; all were in agreement with the conclusions of the report. The majority of the comments were editorial in nature, including virus strain names and antigens used in assays and the report has been amended accordingly. One participant commented on the decision to select 1502/19 in preference to 1504/19 – this has been clarified in the Conclusions, with the decision being based on the greater number of vials available for 1502/19 and the absence of antibodies in this preparation to other arboviruses (i.e. DENV and ZIKV). The same participant suggested adding more detail of the circulating CHIKV strains likely to have infected the respective plasma donors in the case of 1504/19, this has been addressed with a further citation (Simmons *et al.*, 2014).

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**Table 1.** Production summary for the candidate International Standards 1502/19 and 1504/19

Code	1502/19	1504/19
Name	Anti-CHIKV	Anti-CHIKV
No. vials	3090	2130
Presentation	7 ml glass vials, flip off-tear off seals	7 ml glass vials, flip off-tear off seals
Nominal fill volume*	1 ml	1 ml
Mean fill mass (g)	1.005 (n=34)	1.007 (n=22)
CV of fill weight (%)	0.12 %	0.21 %
Mean residual moisture (%)	1.52% (n=9)	1.69% (n=9)
CV of residual moisture (%)	8.53 %	8.88 %

\*Vial contents to be reconstituted in 0.5 ml of cell culture grade water

CV coefficient of variation

Fill mass was determined at regular intervals throughout the fill

**Table 2.** Collaborative study materials

<b>Study code number (PEI code number)</b>	<b>Presentation</b>	<b>Description of preparation</b>
P1 (1502/19)	Lyophilized	Pool of three plasma donations from a German convalescent patient infected with CHIKV in Brazil
P2	Liquid/frozen	Plasma from an anti-dengue virus antibody-positive, anti-Zika virus antibody-positive Brazilian patient; negative for anti-CHIKV
P3 (1502/19)	Lyophilized	Pool of three plasma donations from a German convalescent patient infected with CHIKV in Brazil
P4	Liquid/frozen	Plasma from an anti-CHIKV antibody-positive Puerto Rican blood donor
P5 (1504/19)	Lyophilized	Pool of ten anti-CHIKV antibody-positive Puerto Rican blood donations
P6	Lyophilized	Pool of five anti-RRV antibody-positive plasma donations; negative for anti-CHIKV
P7	Liquid/frozen	Plasma from an anti-CHIKV antibody-positive Brazilian convalescent patient
P8	Liquid/frozen	Plasma from an anti-CHIKV antibody-positive Puerto Rican blood donor
P9	Liquid/frozen	Plasma from two bleeds from an anti-CHIKV antibody-positive Brazilian convalescent patient
P10 (1504/19)	Lyophilized	Pool of ten anti-CHIKV antibody-positive Puerto Rican blood donations
P11	Liquid/frozen	Plasma from an anti-CHIKV antibody-positive Puerto Rican blood donor

**Table 3.** Collaborative study participants

Lab code	Assay method	Analyte/CHIKV strain	Readout
1	In-house multiplexed microsphere immunoassay (IgM and IgG)	CHIKV, RRV, BFV, SINV	Mean fluorescent intensity
2A	Commercial indirect ELISA (IgG) - supplier A	CHIKV antigen (total)	OD/End-point dilution
2B	Commercial indirect ELISA (IgG) - supplier A	CHIKV antigen (total)	OD
3A	In-house direct ELISA (Ig)	Purified CHIKV 181/clone 25 virions (Asian genotype)	OD/End-point dilution
3B	Virus neutralization assay	CHIKV (Asian genotype)	PRNT <sub>50</sub>
3C	In-house IFA	CHIKV-infected cells (Asian genotype)	Single dilution (pos./neg.)
4	Virus neutralization assay	CHIKV 181/clone 25-Luc (Asian genotype)	NT <sub>50</sub> (RLU)
5	Virus neutralization assay	Attenuated del5nsP3 CHIKV vaccine strain (IOL)	NT <sub>50</sub>
6	Virus neutralization assay	CHIKV-La Réunion (LR) 2006 strain (IOL)	PRNT <sub>80</sub>
7	No data returned		
8A	Virus neutralization assay	CHIKV Lazio-INMI1-2017 strain (IOL)	NT <sub>50</sub>
8B	Commercial IFA (IgM/IgG)	CHIKV-infected cells	End-point dilution (pos./neg.)
8C	Commercial IFA (IgM/IgG)	CHIKV-infected cells	Single dilution (pos./neg.)
8D	Commercial RDT (IgM/IgG)	CHIKV virions	Single dilution (pos./neg.)
9	Virus neutralization assay	LR2006-OPY1 CHIKV strain (IOL)	NT <sub>50</sub>

Lab code	Assay method	Analyte/CHIKV strain	Readout
10	Haemagglutination inhibition	Inactivated CHIKV	End-point dilution
11	Commercial indirect ELISA (IgG) – supplier B	CHIKV E1/E2	OD/End-point dilution
12	Virus neutralization assay	CHIKV 03/06 strain (IOL)	PRNT <sub>50</sub>
13A	Commercial indirect ELISA (IgG) – supplier C	CHIKV E1/E2 heterodimers	Relative units/ml
13B	Neutralization assay - reporter virus	CHIKV nLuc	NT <sub>50</sub>
14	Neutralization assay - replicon particles	Replicon particles based on LR2006-OPY1 CHIKV strain (IOL)	NT <sub>50</sub>
15	In-house indirect ELISA	E1 protein of LR2006-OPY1 CHIKV strain (IOL)	OD/End-point dilution
16	In-house IFA	CHIKV-infected cells	End-point dilution (pos./neg.)
17A	Commercial indirect ELISA (IgG) – supplier C	CHIKV E1/E2 heterodimers	OD/End-point dilution
17B	Commercial IFA (IgM/IgG)	CHIKV-infected cells	End-point dilution (pos./neg.)
18	Virus neutralization assay	CHIKV 27/05/2014-51 strain (Asian genotype)	NT <sub>50</sub>
19	Virus neutralization assay	CHIKV 181/clone 25 vaccine strain (Asian genotype)	PRNT <sub>50</sub>
20	Commercial immunoblot (IgG)	CHIKV (WA genotype) virus-like particles, DENV NS1, DENV Equad, ZIKV NS1 and ZIKV Equad	Band intensity/End-point dilution
21A	In-house capture ELISA (IgG)	CHIKV-infected cells	OD/End-point dilution
21B	Commercial indirect ELISA (IgG) – supplier B	CHIKV E1/E2	OD/End-point dilution

Lab code	Assay method	Analyte/CHIKV strain	Readout
21C	Virus neutralization assay	CHIKV	PRNT <sub>80</sub>
22A	Virus neutralization assay	CHIKV MY/08/065 strain (ECSA genotype)	PRNT <sub>50</sub>
22B	In-house indirect ELISA	CHIKV MY/08/065 strain (ECSA genotype) - virions	OD/End-point dilution
22C	In-house IFA	CHIKV MY/06/37348 strain (Asian genotype) – detergent-treated infected cell supernatant	End-point dilution (pos./neg.)
23A	Virus neutralization assay	CHIKV 181/clone 25 vaccine strain (Asian genotype)	PRNT <sub>50</sub>
23B	In-house indirect ELISA (IgG)	CHIKV 37997 strain (WA genotype)-virus-like particles (E1/E2/capsid)	ELISA units/ml
24A	Commercial indirect ELISA (IgG) – supplier C	CHIKV E1/E2 heterodimers	Relative units/ml
24B	Commercial IFA (IgM/IgG)	CHIKV-infected cells	End-point dilution
25A	Commercial indirect ELISA (IgG) – supplier C	CHIKV E1/E2 heterodimers	Relative units/ml
25B	Commercial IFA (IgM/IgG)	CHIKV-infected cells	End-point dilution (pos./neg.)
26A	Virus neutralization assay	CHIKV 181/clone 25 vaccine strain (Asian genotype)	PRNT <sub>80</sub>
26B	In-house direct ELISA	Purified CHIKV 181/clone 25 virions (Asian genotype)	OD/End-point dilution
27	Virus neutralization assay	CHIKV	PRNT <sub>80</sub>
28	Data not returned		

Chikungunya virus (CHIKV), Ross River virus (RRV), Barmah Forest virus (BFV), Sindbis virus (SINV), DENV dengue virus, ZIKV Zika virus; Plaque reduction neutralization titre 50 or 80 (PRNT<sub>50/80</sub>); neutralization titre 50 (NT<sub>50</sub>); enzyme-linked immunosorbent assay

(ELISA); immunofluorescence assay (IFA); optical density (OD); positive (pos.); negative (neg.); relative light units (RLU). East/Central/South Africa (ECSA); Indian Ocean lineage (IOL); West African (WA); Luciferase (Luc); conserved fusion loop domain of the envelope protein (Equad); non-structural protein 1 (NS1). Virus neutralization assays are highlighted in blue.

**Table 4.** Neutralization titres - combined geometric means PRNT<sub>50</sub>/NT<sub>50</sub> (log<sub>10</sub>), combination of each independent assay run per laboratory

Lab code	Sample										
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11
4	4.22	-	4.19	3.64	4.33	2.49	4.58	4.19	4.80	4.40	3.93
5	3.58	-	3.57	2.77	3.56	1.85	3.73	3.22	3.82	3.51	3.17
6	3.21	-	3.14	2.16	3.10	1.25	3.01	2.90	3.38	-*	3.07
8A	2.00	-	2.36	2.00	2.38	-	2.33	1.80	2.48	1.93	1.98
9	3.41	-	3.40	2.44	3.20	1.49	3.29	3.02	3.48	3.26	2.82
12	3.50	-	3.55	2.61	3.42	1.90	3.53	3.28	3.48	3.50	3.06
13B	2.96	-	3.13	3.20	3.10	-	3.33	3.20	3.40	3.18	2.87
14	4.20	-	4.43	3.62	4.18	-	4.69	4.06	4.70	4.29	3.81
18	2.74	-	2.79	1.70	2.67	-	2.75	2.60	2.84	2.97	2.41
19	-	-	-	1.90	-	1.55	-	-	-	-	2.51
21C	3.17	-	2.95	2.59	3.29	-	3.04	3.18	2.85	3.19	2.93
22A	3.18	-	3.13	2.06	3.07	1.41	2.78	2.92	-*	2.97	2.66
23A	3.31	0.71	3.29	2.72	3.70	2.12	3.54	3.35	3.62	3.53	2.91
26A	2.95	-	3.00	2.54	3.07	-	3.33	2.83	3.26	2.87	2.51
27	3.81	-	3.89	3.12	3.75	1.93	4.16	3.61	4.31	3.75	3.47

\*Virus neutralization reported (analysis not possible); data were excluded from Laboratory 3B.

**Table 5.** Neutralization titres calculated relative to the candidate IS - sample P1 – 1502/19 (1,000 (3 log<sub>10</sub>) U/ml)

Lab code	Sample									
	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11
4	-	2.97	2.42	3.11	1.28	3.36	2.96	3.58	3.18	2.71
5	-	2.98	2.19	2.98	1.28	3.15	2.64	3.24	2.93	2.59
6	-	2.93	1.97	2.90	1.11	2.80	2.69	3.20		2.86
8A	-	3.36	3.00	3.38	-	3.32	2.80	3.47	2.93	2.97
9	-	2.99	2.04	2.82	1.08	2.88	2.61	3.08	2.85	2.42
12	-	3.05	2.11	2.92	1.40	3.03	2.78	3.02	3.00	2.56
13B	-	3.17	3.06	3.15	-	3.38	3.24	3.44	3.22	2.91
14	-	3.23	2.42	2.98	-	3.49	2.86	3.50	3.05	2.61
18	-	3.05	1.92	2.93	-	3.00	2.85	3.10	3.23	2.67
19	-	-	-	-	-	-	-	-	-	-
21C	-	2.78	2.42	3.13	-	2.87	3.05	2.72	3.03	2.76
22A	-	2.95	1.88	2.89	1.23	2.60	2.74		2.79	2.41
23A	-	2.74	2.41	3.17	1.81	3.01	2.82	3.31	3.00	2.59
26A	-	3.05	2.67	3.12	-	3.39	2.97	3.31	2.95	2.65
27	-	3.05	2.29	2.95	1.11	3.30	2.80	3.48	2.95	2.66

**Table 6.** Neutralization titres calculated relative to the candidate IS - sample P5 – 1504/19 (1,000 (3 log<sub>10</sub>) U/ml)

Lab code	Sample									
	P1	P2	P3	P4	P6	P7	P8	P9	P10	P11
4	2.89	-	2.86	2.31	1.17	3.25	2.85	3.47	3.07	2.60
5	3.02	-	3.00	2.21	1.29	3.17	2.66	3.26	2.95	2.61
6	3.10	-	3.03	2.08	-	2.91	2.79	3.31	-	3.02
8A	2.63	-	2.98	2.62	-	2.95	2.42	3.10	2.55	2.60
9	3.18	-	3.22	2.27	1.34	3.22	2.93	3.38	3.33	2.63
12	3.08	-	3.13	2.19	1.47	3.11	2.86	3.00	3.08	2.64
13B	2.85	-	3.03	2.84	-	3.18	3.09	3.07	3.07	2.76
14	2.99	-	3.15	2.32	-	3.53	2.76	3.53	3.04	2.68
18	3.07	-	3.12	2.03	-	3.07	2.92	3.17	3.30	2.74
19	-	-	-	-	-	-	-	-	-	-
21C	2.87	-	2.65	2.30	-	2.75	2.88	2.54	2.90	2.64
22A	3.11	-	3.06	1.99	1.42	2.71	2.85	-	2.90	2.46
23A	2.83	-	2.53	2.12	1.02	2.84	2.65	3.29	2.83	2.32
26A	2.88	-	2.93	2.60	-	3.26	2.89	3.19	2.80	2.57
27	3.05	-	3.29	2.53	1.18	3.55	2.85	3.71	3.00	2.72

**Table 7.** Immunoassays - combined geometric means EC<sub>50</sub> (log<sub>10</sub>), combination of each independent assay run per laboratory

Assay type	Lab code	Sample										
		P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11
HI	10	3.11	-	3.16	2.05	2.96	1.15	3.26	2.35	3.46	2.96	2.35
IA	1	3.55	-	3.61	3.40	3.95	2.55	4.11	3.39	3.90	3.92	3.21
IA	3A	2.76	-	2.74	2.63	3.12	-	3.23	2.80	3.01	3.10	2.62
IA	11	4.03	-	3.98	-	3.81	-	4.06	3.63	4.55	3.80	
IA	13A	3.17	-	3.18	2.52	3.21	-	3.65	2.87	3.63	3.22	2.58
IA	15	3.01	-	3.08	2.66	3.13	1.67	3.41	2.66	3.39	3.13	2.57
IA	17A	3.36	-	3.34	2.76	3.33	1.90	3.65	3.00	3.60	3.22	2.63
IA	20	3.91	-	3.94	3.40	4.05	2.50	4.34	3.52	4.28	4.02	3.27
IA	21A	3.47	-	3.47	3.47	3.47	-	3.80	2.80	3.63	3.47	2.80
IA	21B	5.41	-	5.26	4.62	5.41	-	5.71	5.11	5.86	5.56	4.95
IA	22B	2.84	-	2.83	2.75	3.18	-	3.63	2.32	3.14	2.89	2.26
IA	23B	3.84	-	3.84	3.40	3.81	2.27	4.18	3.47	4.18	3.77	3.23
IA	24A	3.22	-	3.22	2.57	3.19	1.77	3.53	2.91	3.56	3.21	2.66
IA	25A	3.26	-	3.31	2.58	3.30	1.78	3.50	2.92	3.58	3.13	2.62
IA	26B	3.17	2.62	3.11	2.91	3.50	-	3.53	3.21	3.52	3.47	2.82
IFA	8B	3.33	-	3.31	3.11	3.18	1.95	3.41	3.06	3.41	3.26	3.06
IFA	16		-		2.96		2.66		3.56		3.56	3.86
IFA	17B	3.80	-	3.85	3.35	3.85	2.15	4.10	3.40	4.20	3.90	3.40
IFA	22C	2.85	-	2.85	2.85	2.85	1.65		2.93			2.51
IFA	24B	3.68	-	3.78	3.38	3.88		4.03	2.45	3.93	3.48	2.45
IFA	25B	4.50	-	4.50	4.17	4.50	3.17	5.50	4.67	5.50	4.50	4.33

(Enzyme) immunoassay/immunoassay (IA); haemagglutination inhibition assay (HI); indirect immunofluorescence assay (IFA). It was noted, that Laboratory 3A used the sample P2 as a negative control for the ELISA assays. Laboratories 16, 22C and 24B detected all anti-CHIKV antibody

positive samples; however, no end-point was met for some of the samples or some results were equivocal for some dilutions.

**Table 8.** Immunoassay titres calculated relative to the candidate IS - sample P1 – 1502/19 (1,000 (3 log<sub>10</sub>) U/ml)

Assay type	Lab code	Sample									
		P2	P3	P4	P5	P6	P7	P8	P9	P10	P11
HI	10	-	3.05	1.94	2.85	1.04	3.15	2.25	3.35	2.85	2.25
IA	1	-	3.06	2.85	3.41	1.98	3.56	2.84	3.35	3.37	2.66
IA	3A	-	2.98	2.88	3.37	-	3.47	3.05	3.25	3.34	2.86
IA	11	-	2.95		2.78	-	3.03	2.61	3.36	2.78	
IA	13A	-	3.01	2.35	3.04	-	3.49	2.71	3.46	3.05	2.42
IA	15	-	3.07	2.65	3.12	1.66	3.40	2.66	3.38	3.13	2.56
IA	17A	-	2.97	2.40	2.96	1.52	3.29	2.63	3.24	2.85	2.27
IA	20	-	3.03	2.48	3.14	1.49	3.43	2.61	3.37	3.10	2.35
IA	21A	-	3.00	3.00	3.00	-	3.33	2.33	3.17	3.00	2.33
IA	21B	-	2.85	2.21	3.00	-	3.30	2.70	3.45	3.15	2.55
IA	22B	-	3.00	2.91	3.34	-	3.79	2.49	3.32	3.05	2.43
IA	23B	-	3.00	2.56	3.07	1.43	3.44	2.63	3.44	3.03	2.39
IA	24A	-	3.00	2.35	2.97	1.58	3.31	2.69	3.34	2.99	2.45
IA	25A	-	3.05	2.32	3.03	1.52	3.23	2.64	3.31	3.01	2.35
IA	26B	2.43	2.94	2.74	3.33		3.36	3.05	3.37	3.29	2.65
IFA	8B	-	3.00	2.85	2.85	1.57	3.15	2.77	3.15	3.00	2.77
IFA	16	-									
IFA	17B	-	3.05	2.55	3.05	1.32	3.30	2.60	3.40	3.10	2.60
IFA	22C	-	3.00	3.00	3.00	1.80		3.24			3.00
IFA	24B	-	3.10	2.55	3.20		3.35	1.62	3.25	2.80	1.62
IFA	25B	-	3.00	1.66	3.00	1.66	4.00	3.17	4.00	3.00	2.83

(Enzyme) immunoassay/immunoassay (IA); haemagglutination inhibition assay (HI); indirect immunofluorescence assay (IFA)

**Table 9.** Immunoassay titres calculated relative to the candidate IS - sample P5 – 1504/19 (1,000 (3 log<sub>10</sub>) U/ml)

Assay Type	Lab	Sample									
		P1	P2	P3	P4	P6	P7	P8	P9	P10	P11
HI	10	3.15	-	3.20	2.10	1.20	3.30	2.40	3.50	3.00	2.40
IA	1	2.59	-	2.66	2.45	1.58	3.16	2.43	2.95	2.97	2.26
IA	3A	2.63		2.62	2.51		3.10	2.68	2.88	2.98	2.49
IA	11	3.22	-	3.17	-	-	3.25	2.83	3.69	2.99	-
IA	13A	2.96	-	2.97	2.30	-	3.44	2.66	3.42	3.01	2.37
IA	15	2.88	-	2.95	2.53	1.54	3.28	2.54	3.26	3.01	2.45
IA	17A	3.04	-	3.01	2.43	1.55	3.32	2.67	3.27	2.89	2.30
IA	20	2.61	-	2.90	2.35	1.40	3.33	2.52	3.25	3.01	2.22
IA	21A	3.00	-	3.00	3.00	-	3.33	2.33	3.17	3.0	2.33
IA	21B	3.00	-	2.85	2.21	-	3.30	2.70	3.45	3.15	2.55
IA	22B	2.66	-	2.65	2.57	-	3.45	2.15	2.99	2.71	2.09
IA	23B	2.93	-	2.94	2.46	1.34	3.37	2.53	3.36	2.96	2.29
IA	24A	3.03	-	3.03	2.38	1.59	3.34	2.72	3.38	3.02	2.45
IA	25A	2.97	-	3.02	2.28	1.49	3.20	2.61	3.28	2.98	2.32
IA	26B	2.67	2.06	2.61	2.41	-	3.03	2.73	3.05	2.91	2.32
IFA	8B	3.15	-	3.15	3.00	1.72	3.45	3.08	3.45	3.30	2.92
IFA	16	-	-	-	-	-	-	-	-	-	-
IFA	17B	2.95	-	3.00	2.50	1.26	3.25	2.55	3.35	3.15	2.55
IFA	22C	3.00	-	3.00	3.00	1.80	-	3.08	-	-	2.65
IFA	24B	2.80	-	2.90	2.25	-	3.15	1.32	3.05	2.60	1.32
IFA	25B	2.93	-	2.93	2.67	1.72	3.97	3.01	3.97	3.00	2.83

(Enzyme) immunoassay/immunoassay (IA); haemagglutination inhibition assay (HI); indirect immunofluorescence assay (IFA)

**Table 10.** Overall combined means for samples P1-P11

Sample	N <sub>1</sub>	N <sub>2</sub>	Mean (log <sub>10</sub> )	95% CI	
P1	34	97	3.42	2.98	3.87
P2	2	3	1.66		
P3	34	95	3.45	3.02	3.87
P4	35	93	2.94	2.09	3.76
P5	34	96	3.48	3.06	3.90
P6	22	56	1.98	1.37	2.59
P7	33	90	3.78	2.85	4.71
P8	35	95	3.18	2.71	3.64
P9	32	79	3.78	3.17	4.39
P10	33	90	3.48	3.02	3.94
P11	35	93	2.98	2.54	3.42

N<sub>1</sub> – number of participants with results; N<sub>2</sub> = number of assays overall participants; Mean (log<sub>10</sub>) – consensus mean estimated across assays; 95% CI - 95% confidence interval for mean estimate

**Table 11.** Overall potencies relative to candidate IS - sample P1 (1502/19) with an assumed unit age of 1,000 ( $3 \log_{10}$ ) U/ml.

Sample	N <sub>1</sub>	N <sub>2</sub>	Mean (log <sub>10</sub> )	95% CI	
P2	2	3	1.30		
P3	34	94	3.02	2.94	3.09
P4	33	87	2.51	2.06	2.96
P5	34	94	3.06	2.93	3.18
P6	20	50	1.43	1.10	1.77
P7	33	90	3.30	2.84	3.77
P8	34	90	2.74	2.46	3.01
P9	32	79	3.33	3.17	3.49
P10	32	87	3.03	2.91	3.15
P11	33	88	2.57	2.28	2.87

N<sub>1</sub> – number of participants with results; N<sub>2</sub> = total number of results analyzed per sample; Mean (log<sub>10</sub>) – consensus mean estimated across assays; 95% CI - 95% confidence interval for mean estimate

**Table 12.** Overall potencies relative to candidate IS - sample P5 (1504/19) with an assumed unitage of 1,000 ( $3 \log_{10}$ ) U/ml.

Sample	N <sub>1</sub>	N <sub>2</sub>	Mean ( $\log_{10}$ )	95% CI	
P1	34	92	2.93	2.80	3.07
P2	2	3	1.03		
P3	34	90	2.96	2.80	3.12
P4	33	84	2.46	1.97	2.96
P6	19	47	1.45	1.00	1.90
P7	33	86	3.26	2.84	3.69
P8	34	89	2.68	2.33	3.03
P9	32	74	3.27	3.07	3.47
P10	32	84	2.97	2.85	3.10
P11	33	85	2.52	2.10	2.95

N<sub>1</sub> – number of participants with results; N<sub>2</sub> = total number of results analyzed per sample; Mean ( $\log_{10}$ ) – consensus mean estimated across assays; 95% CI - 95% confidence interval for mean estimate

**Table 13.** Analysis of variance - inter-assay variability and intra-assay variability for P1 and P3

Factor	CV
Sample (P1 or P3)	0.7%
Participant	150%
Assay type*	95%
Inter-assay variability	227%
Intra-assay variability	38%
Measurement uncertainty (overall variability)	246%

CV - coefficient of variation; \*Factor - assay type was not estimable

**Table 14.** Analysis of variance - inter-assay variability and intra-assay variability for P5 and P10

Factor	CV
Sample (P5 or P10)	5%
Participant	154%
Assay type*	88%
Inter-assay variability	224%
Intra-assay variability	46%
Measurement uncertainty (overall variability)	251%

CV - coefficient of variation; \*Factor - assay type was not estimable

**Table 15. Stability of candidate IS sample P1 (1502/19)**

<b>Incubation temperature</b>	<b>Incubation time</b>				
	<b>2 weeks</b>	<b>4 weeks</b>	<b>3 months</b>	<b>6 months</b>	<b>12 months</b>
-20°C	1.07	1.02	0.98	1.00	0.97
+4°C	0.99	1.02	0.98	1.02	0.95
+20°C	1.02	0.99	0.95	0.97	0.93
+37°C	0.98	0.87	0.63	N.T.	N.T.
+45°C	0.83	0.70	N.T.	N.T.	N.T.

N.T. Not tested – heat-treated lyophilized residue was insoluble. Potency expressed relative to -80°C baseline samples of 1502/19.

**Table 16. Stability of candidate IS sample P5 (1504/19)**

<b>Incubation temperature</b>	<b>Incubation time</b>				
	<b>2 weeks</b>	<b>4 weeks</b>	<b>3 months</b>	<b>6 months</b>	<b>12 months</b>
-20°C	1.07	0.97	0.97	0.98	1.02
+4°C	1.02	0.82	1.03	1.00	1.03
+20°C	0.97	1.00	0.97	0.93	0.95
+37°C	0.86	0.89	0.64	0.47	N.T.
+45°C	0.73	0.63	N.T.	N.T.	N.T.

N.T. Not tested – heat-treated lyophilized residue was insoluble. Potency expressed relative to -80°C baseline samples of 1504/19.

**Figure 1-11.** Histograms showing mean potencies for samples P1-P11 and potencies relative to candidate International Standards (sample P1 (1502/19) and sample P5 (1504/19))

Panels' 1A-11A show the mean PRNT<sub>50</sub>/NT<sub>50</sub> or EC<sub>50</sub> for each laboratory for each sample (P1-P11) as log<sub>10</sub> dilution (orange background immunoassays (ELISAs, microsphere assays, immunoblots, haemagglutination inhibition); light green background – indirect immunofluorescence assays; light blue background - virus neutralization assays).

Panels' 1B-11B (left-hand side) show the mean potency (log<sub>10</sub> U/ml, relative to P1 with assumed potency of 1000 U/ml) for each laboratory for each sample (P2-P11). Panels 1B-11B (right-hand side) show the mean potency (log<sub>10</sub> U/ml, relative to P5 with assumed potency of 1000 U/ml) for each laboratory for each sample (P1-P4 and P6-P11).

**Figure 12.** Box and whisker plots showing data harmonization by reporting of potencies of samples relative to the candidate International Standards P1 (1502/19) or P5 (1504/19)

Figure 12A: neutralization assays; Figure 12B, immunoassays including (ELISAs, microsphere and haemagglutination inhibition assays); Figure 12C immunofluorescence assays.

Top panel - mean potencies for each sample for each participating laboratory (where data could be analysed; lower left panel - mean potencies (log<sub>10</sub> U/ml, relative to sample P1 (with assumed potency of 1,000 U/ml) for each laboratory for each sample (P2-P11). Lower right panel - mean potencies (log<sub>10</sub> U/ml) relative to sample P5 (with assumed potency of 1,000 U/ml) for each laboratory for each sample (P1-P4 and P6-P11).

Boxes indicate interquartile range; horizontal lines within each box indicate median; whiskers indicate the ranges from 5% to 95% percentiles.

**Figure 1A Geometric mean potencies of sample P1**

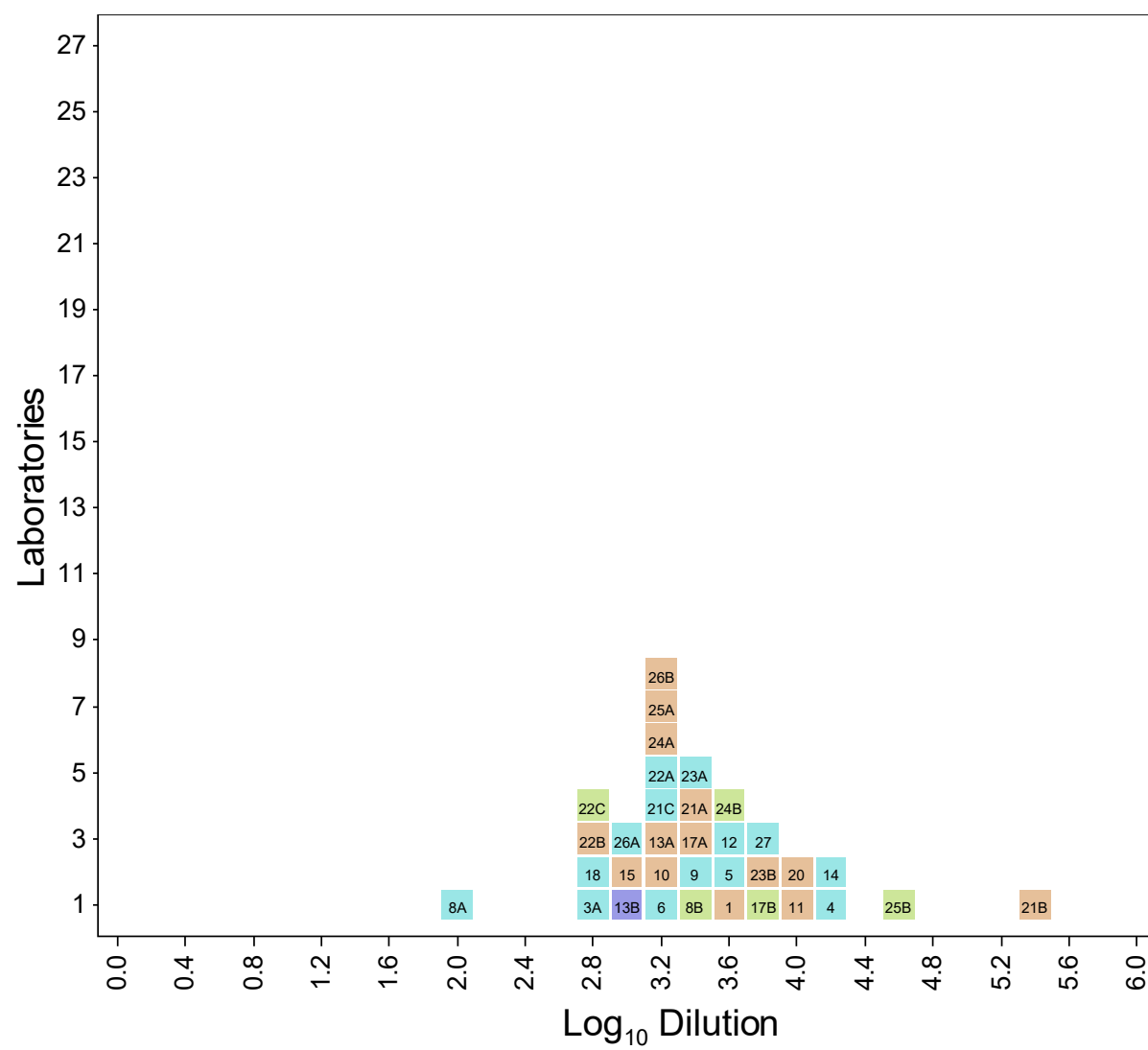


Figure 1B Relative potencies for sample P1

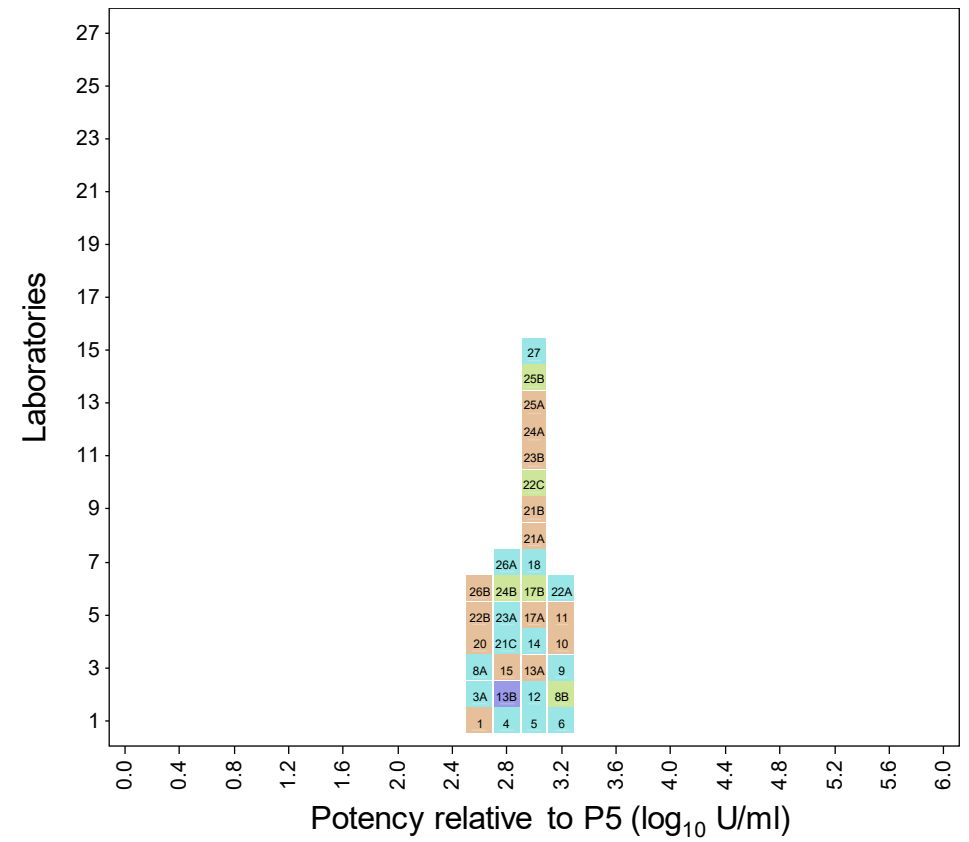


Figure 2A Geometric mean potencies of sample P2

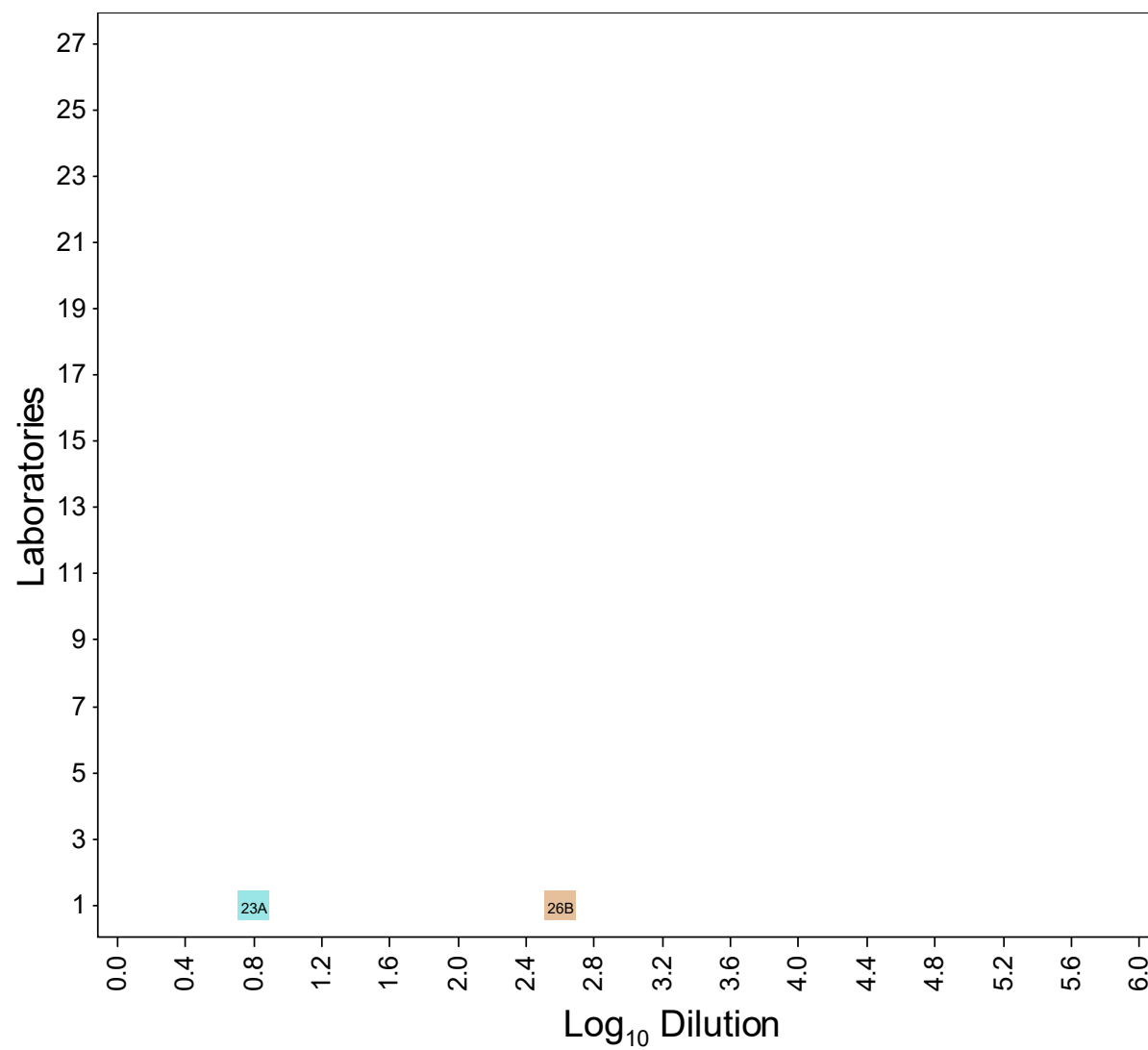


Figure 2B Relative potencies for sample P2

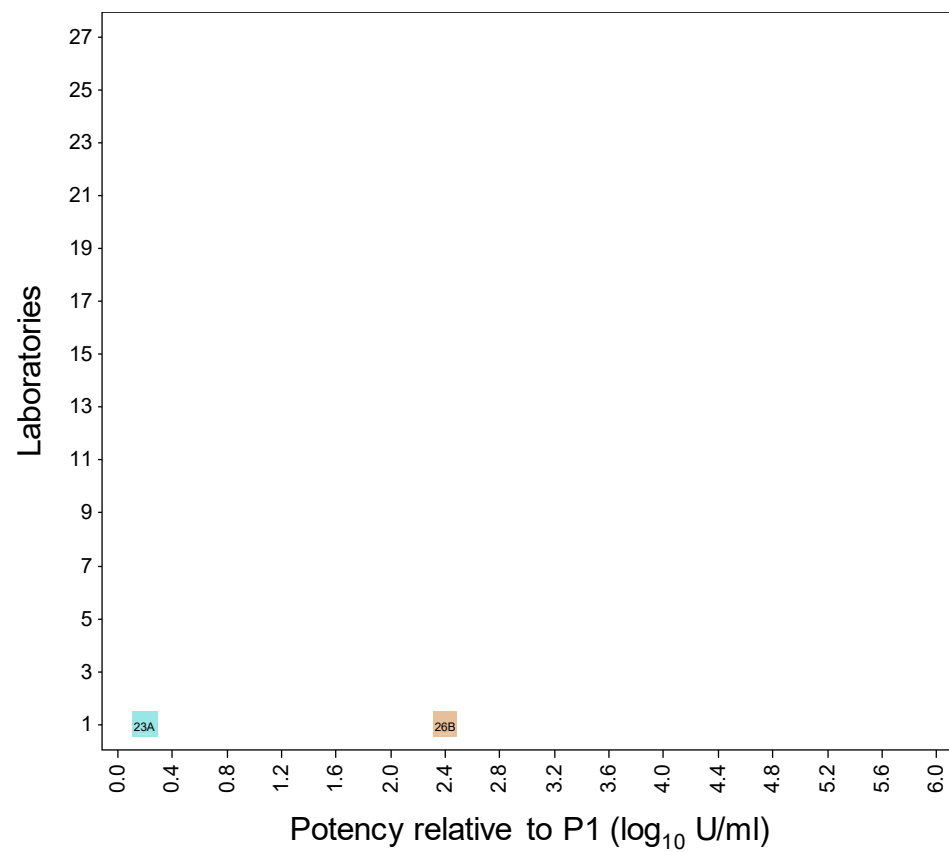


Figure 3A Geometric mean potencies of sample P3

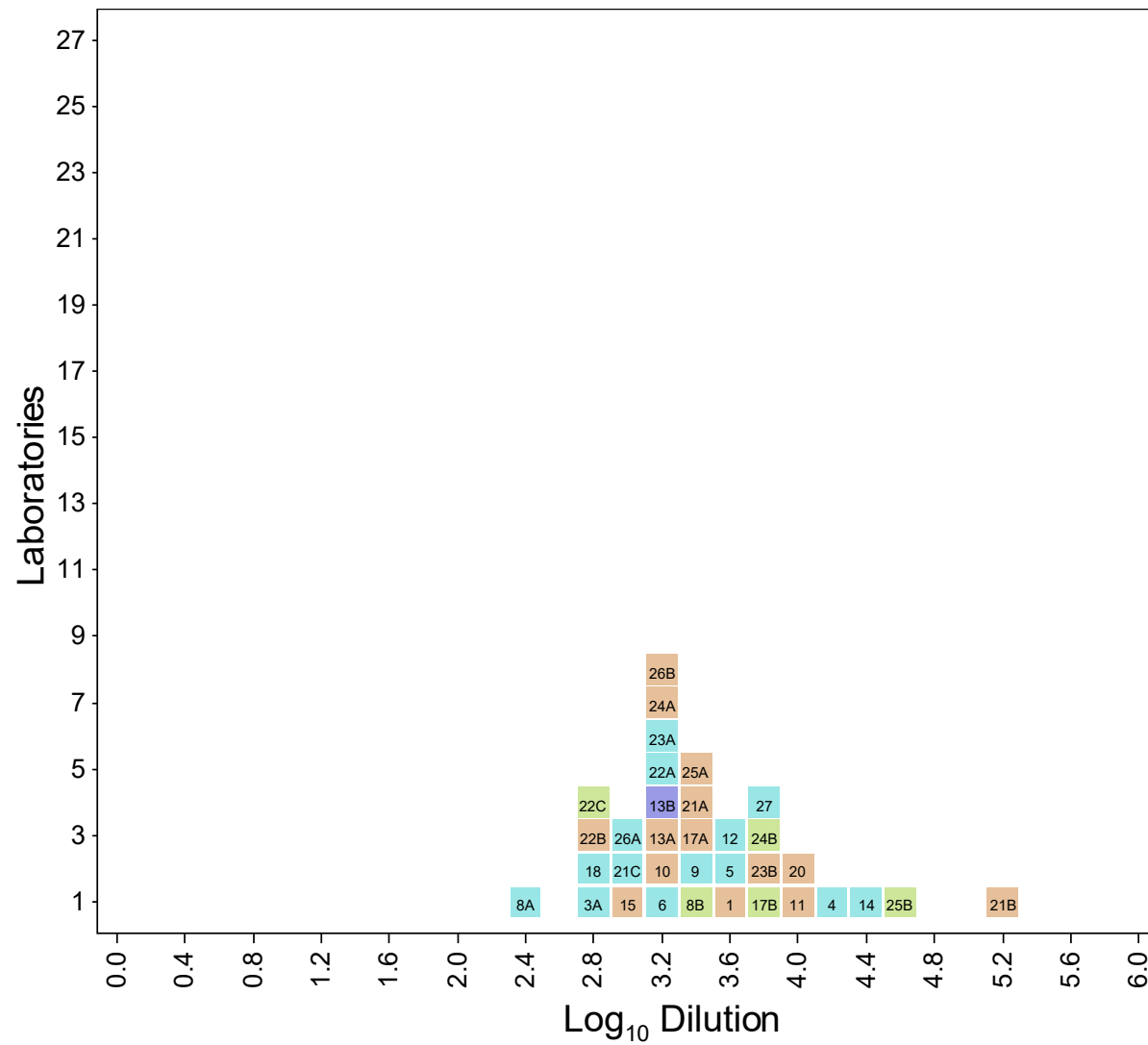


Figure 3B Relative potencies for sample P3

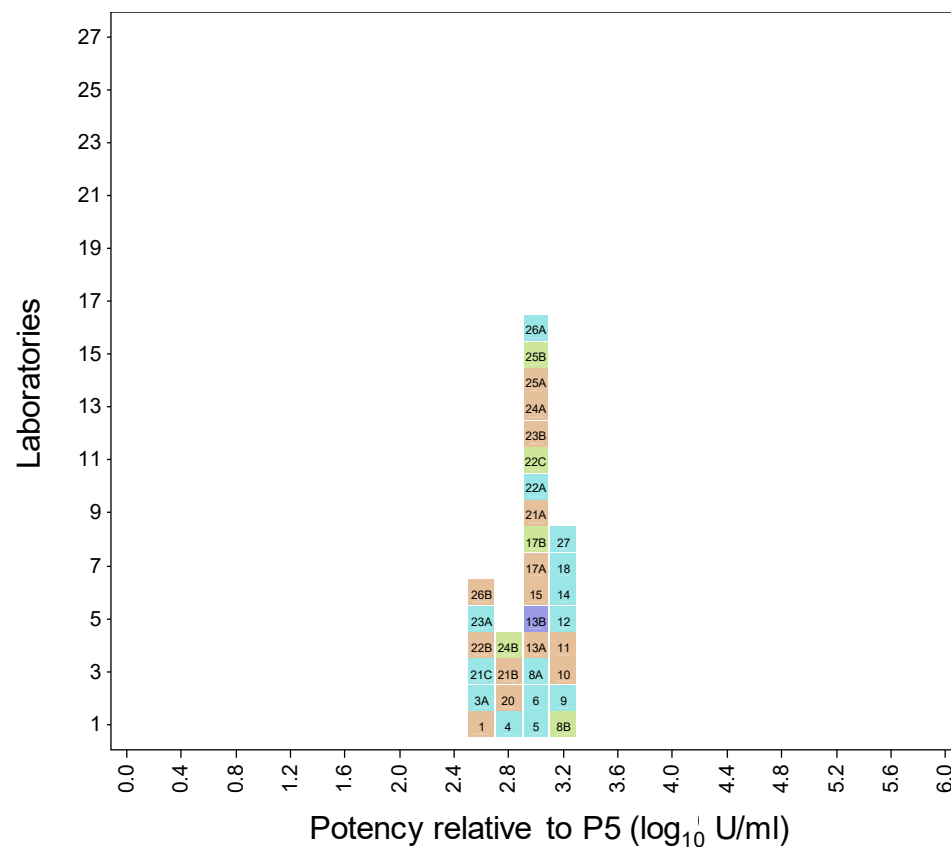
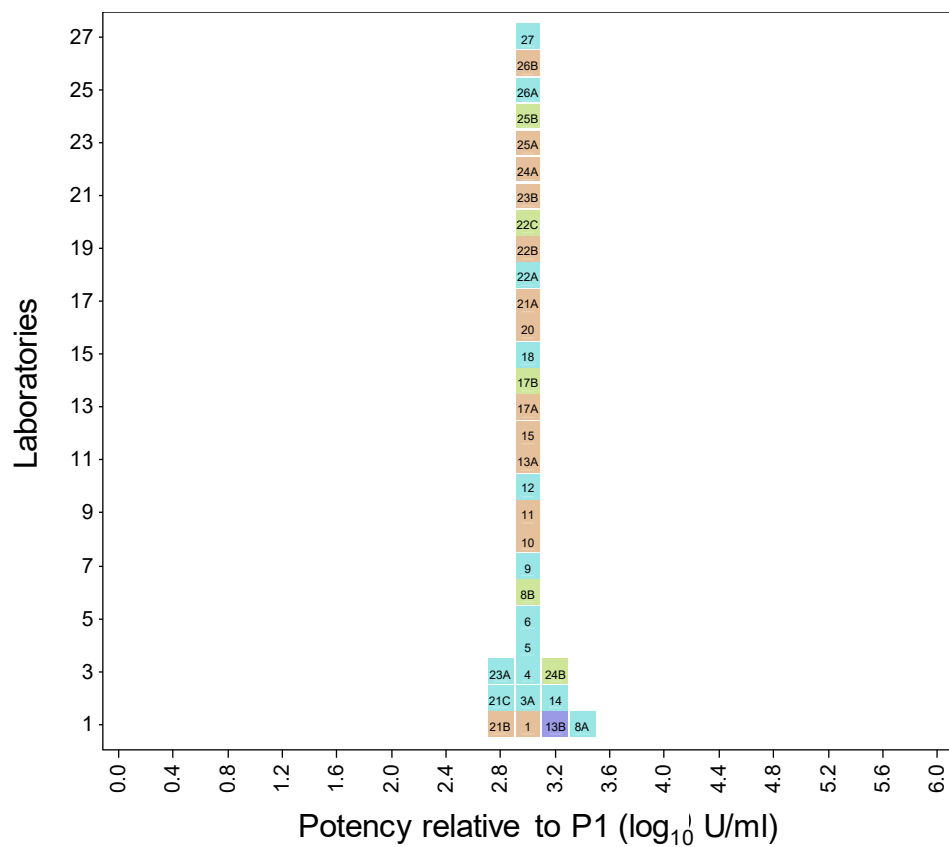


Figure 4A Geometric mean potencies of sample P4

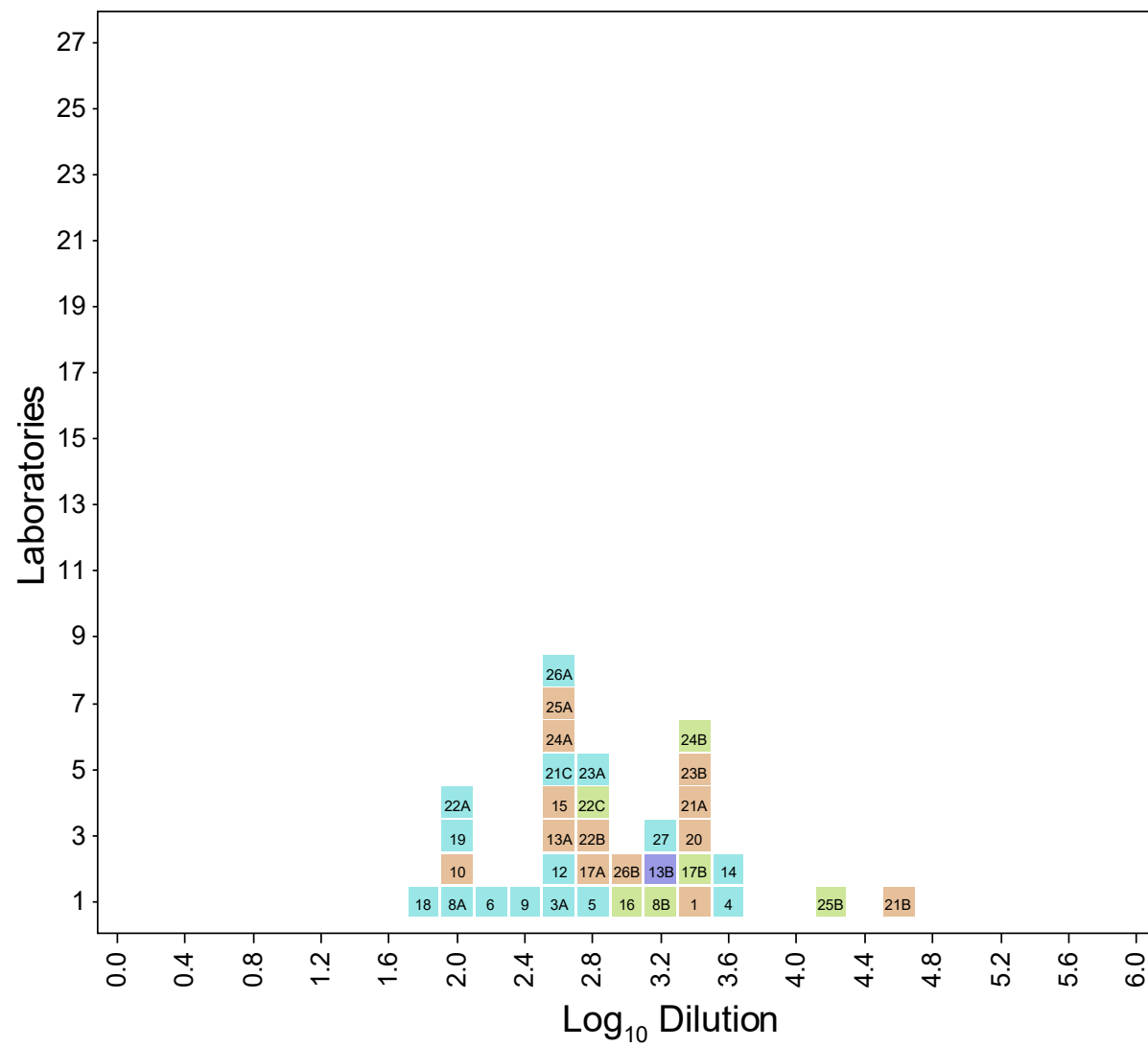


Figure 4B Relative potencies for sample P4

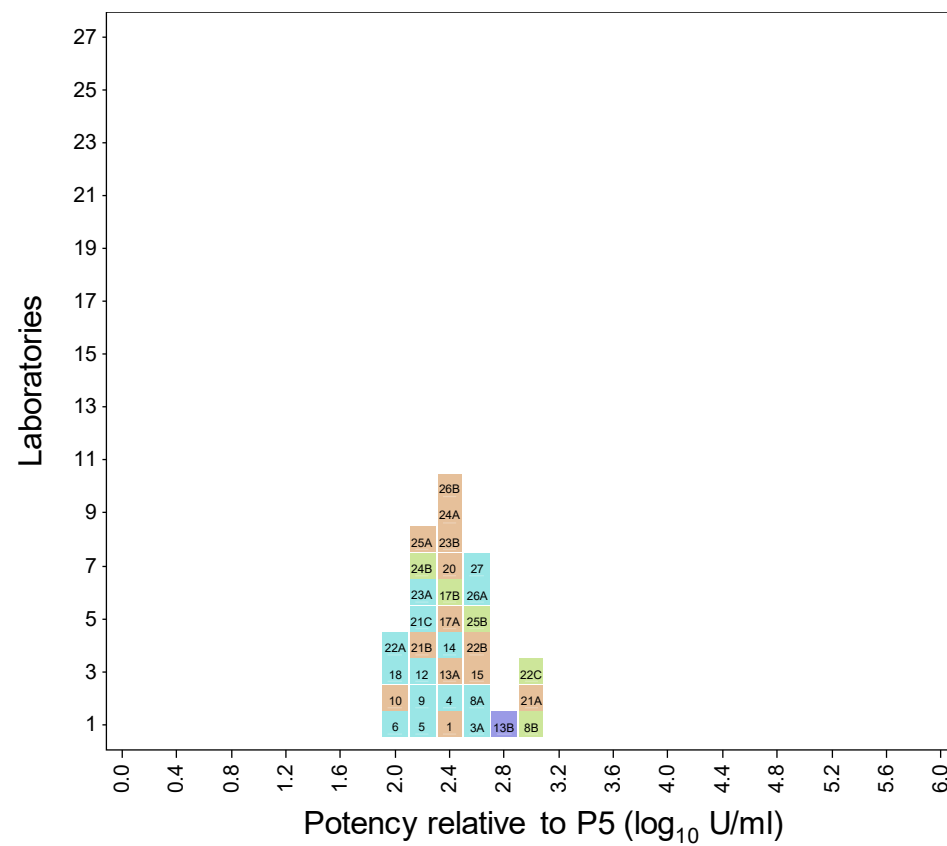
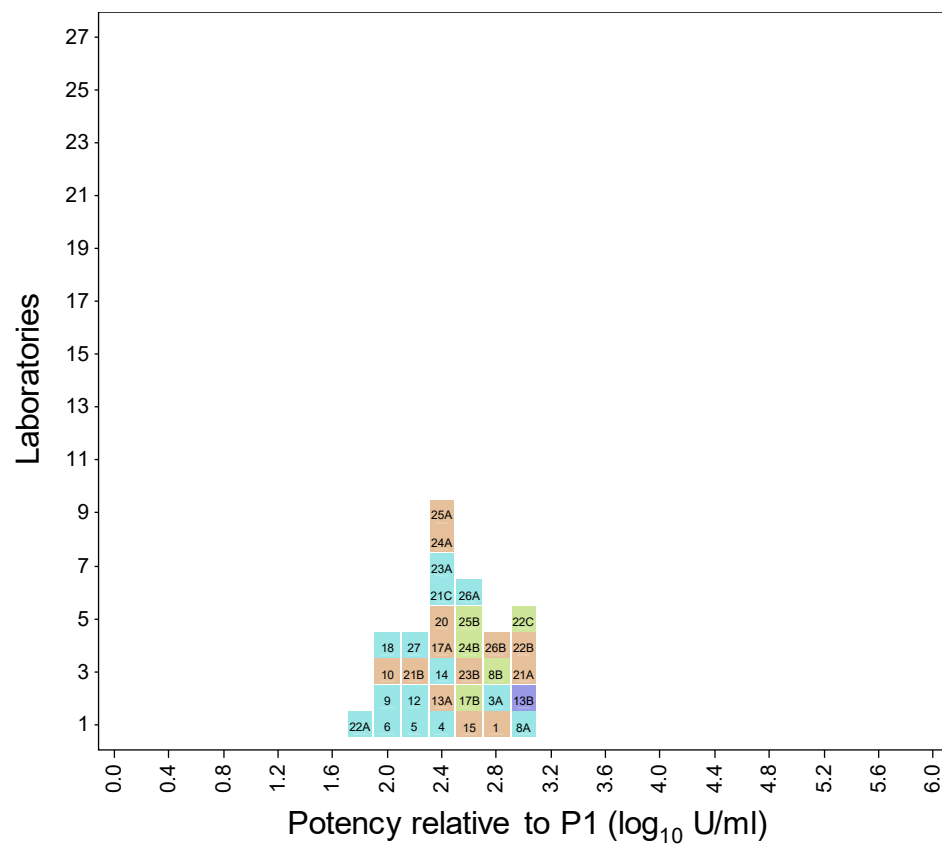


Figure 5A Geometric mean potencies of sample P5

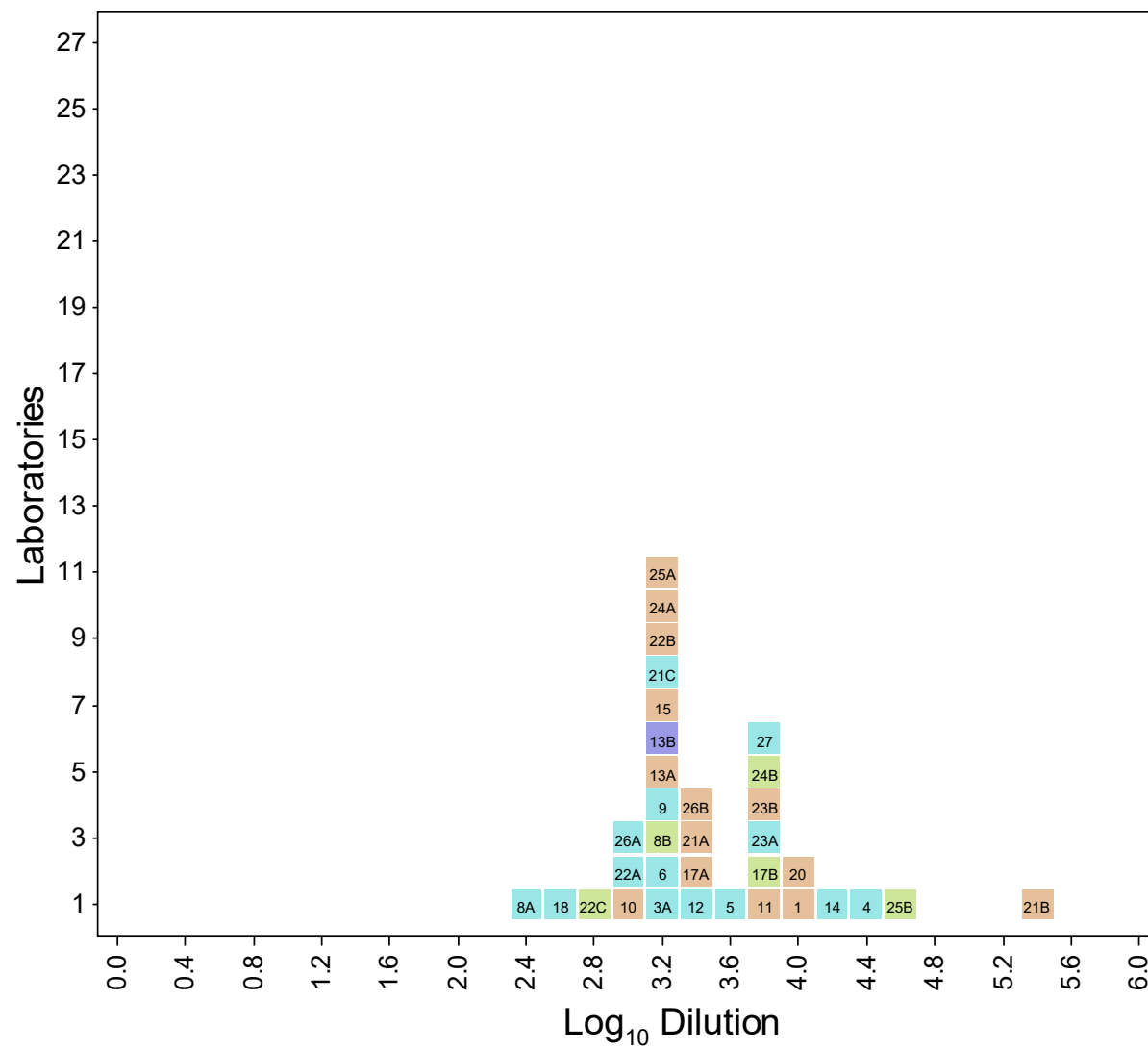


Figure 5B Relative potencies for sample P5

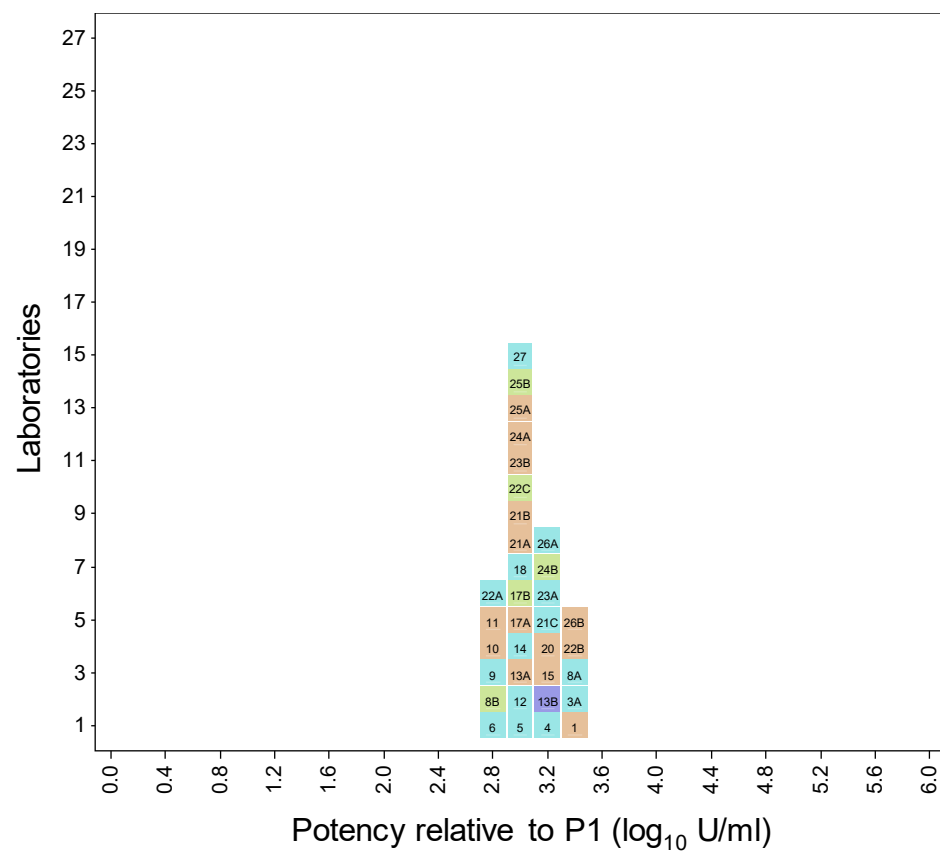


Figure 6A Geometric mean potencies of sample P6

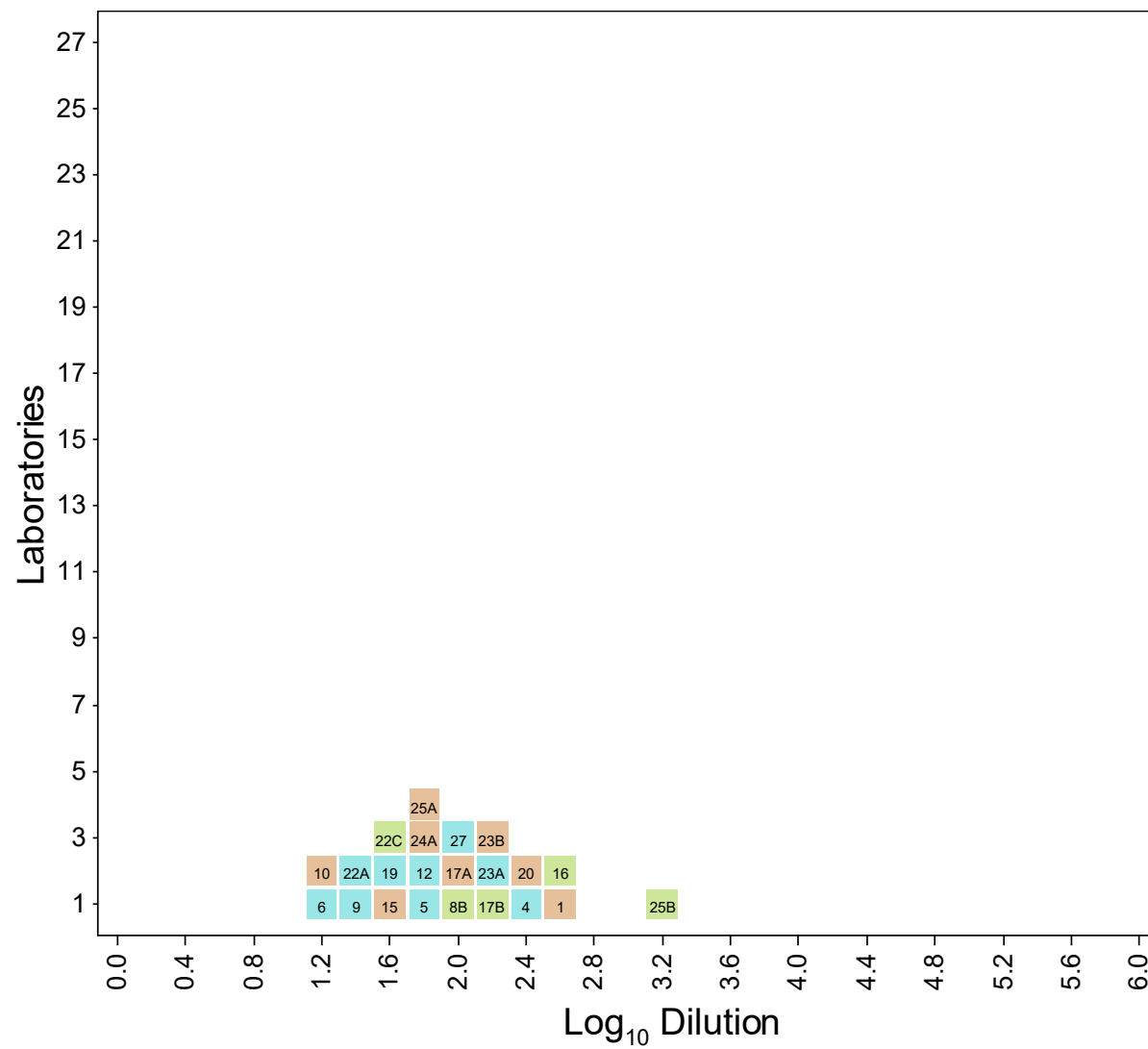


Figure B Relative potencies for sample P6

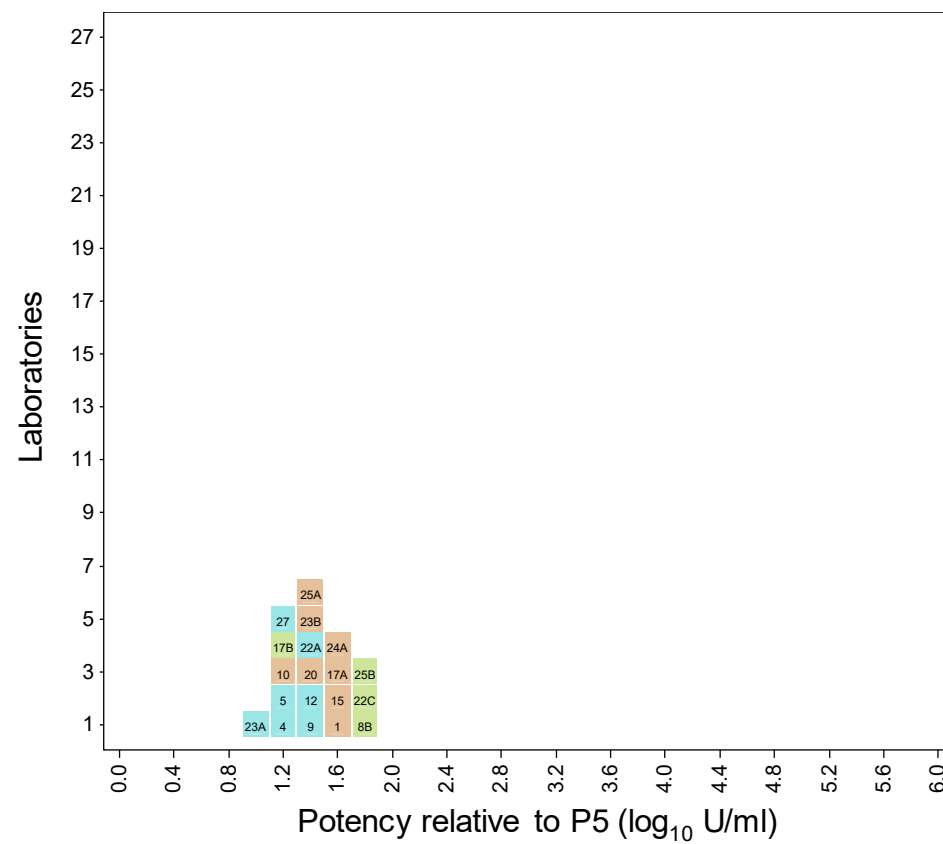
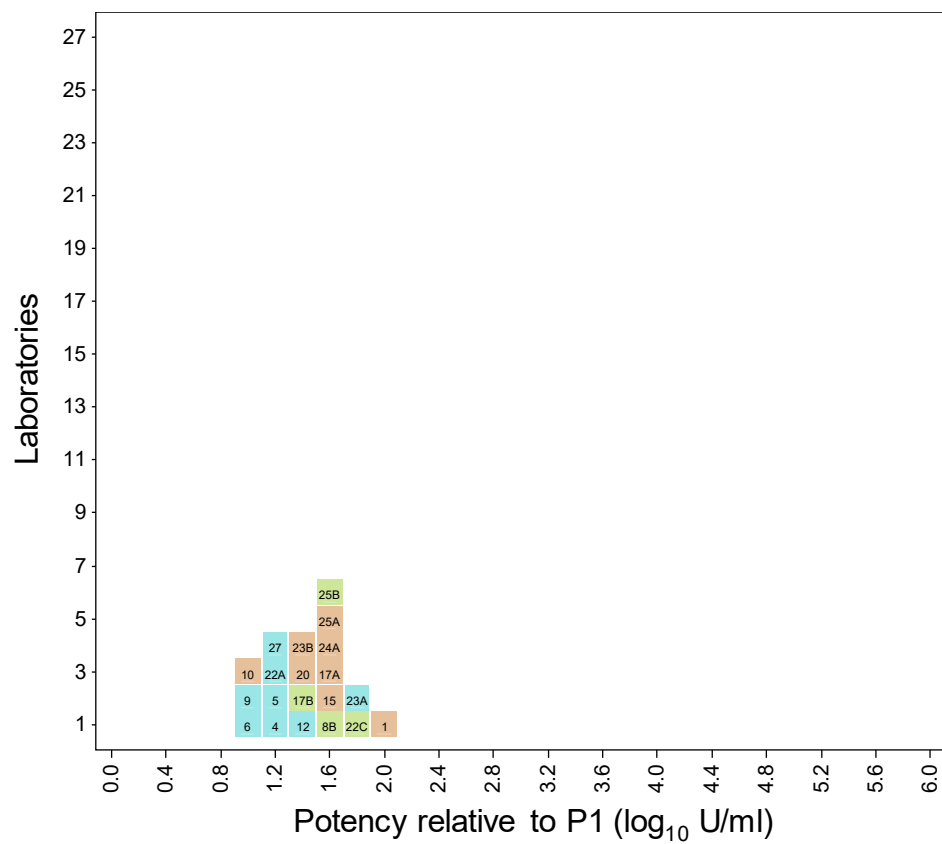


Figure 7A Geometric mean potencies of sample P7

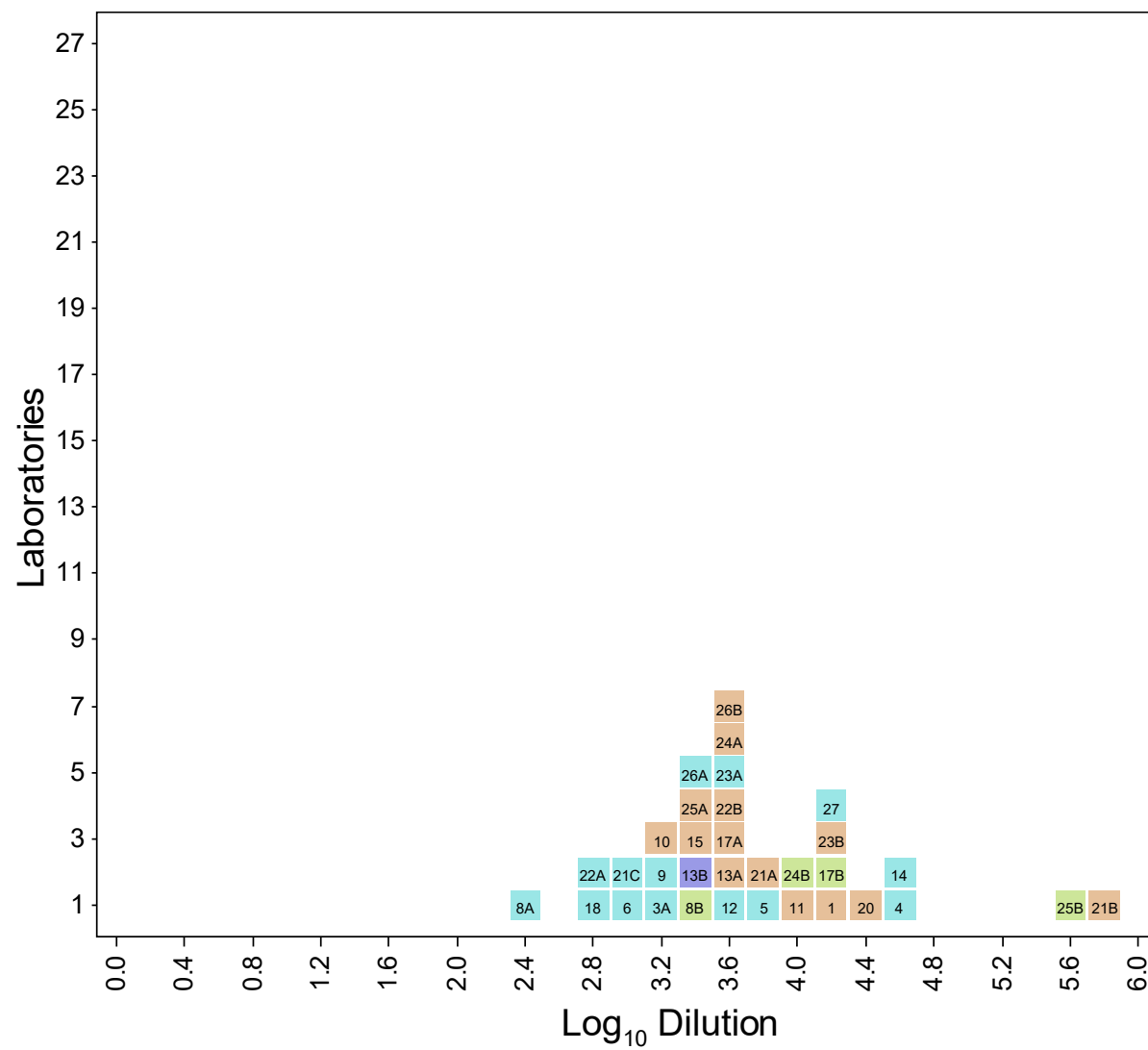


Figure 7B Relative potencies for sample P7

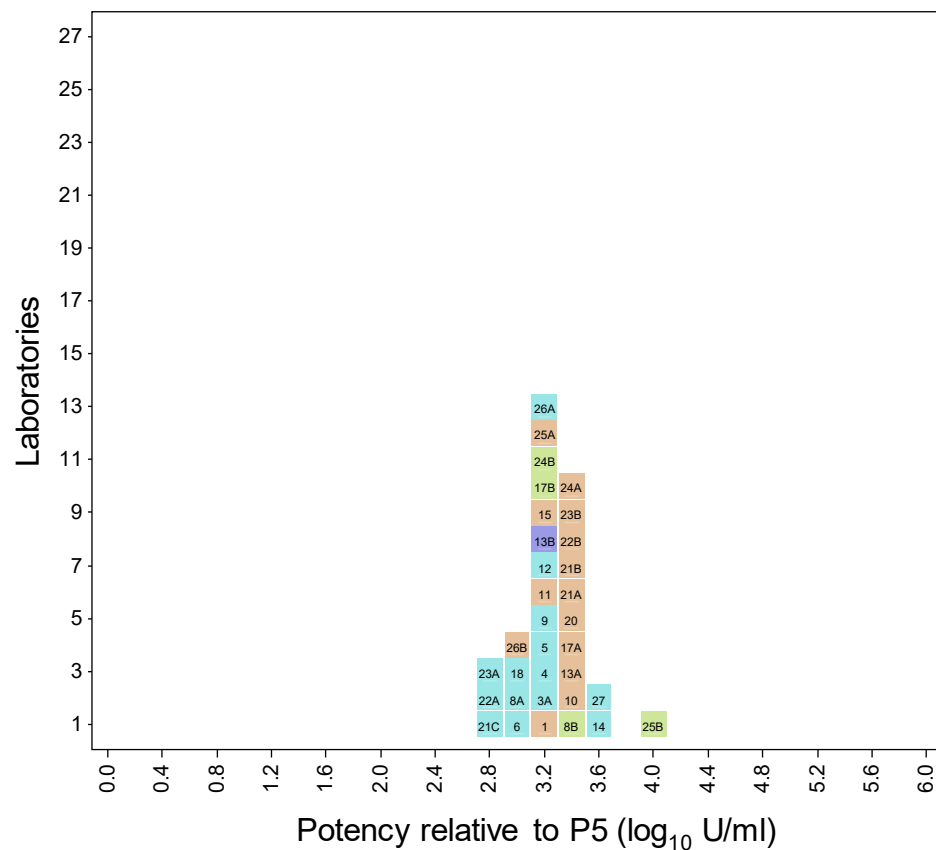
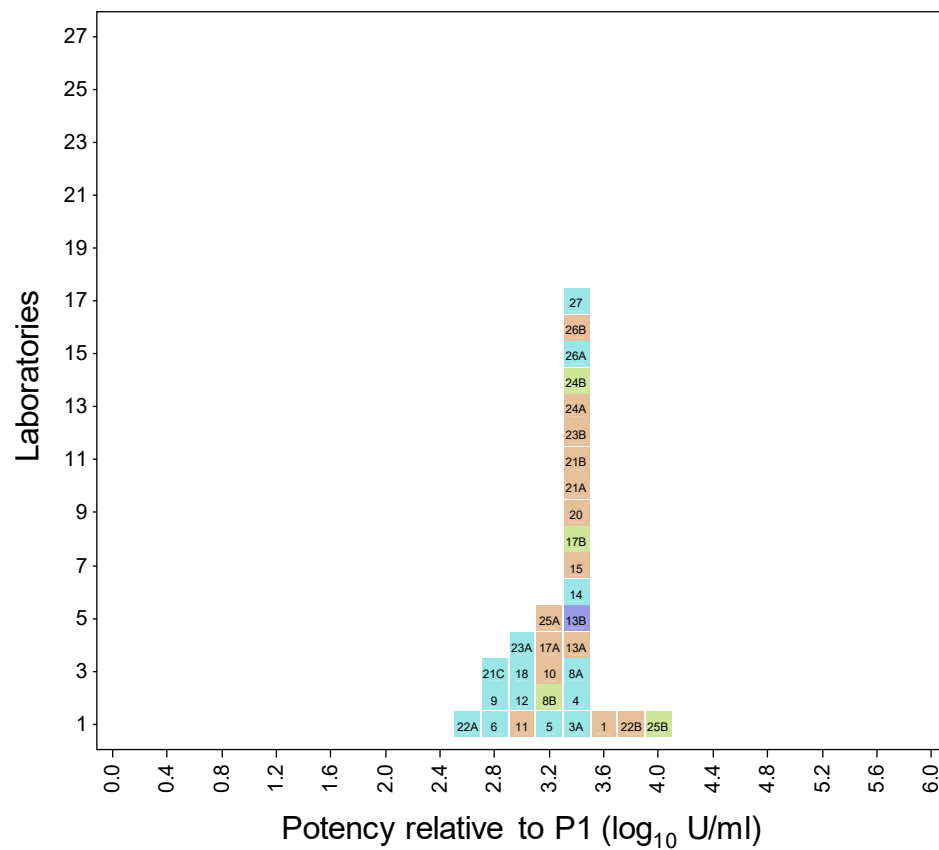


Figure 8A Geometric mean potencies of sample P8

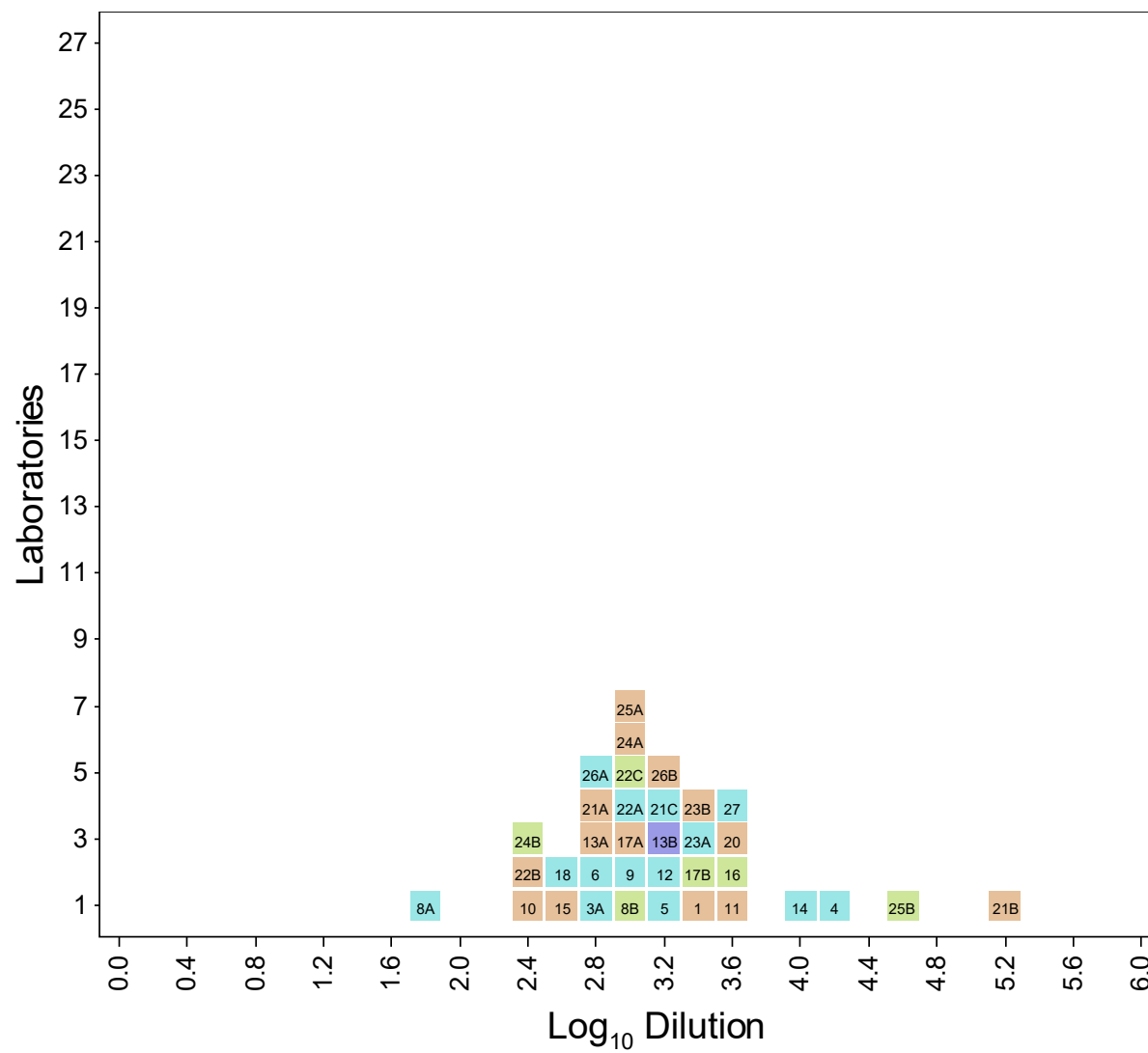


Figure 8B Relative potencies for sample P8

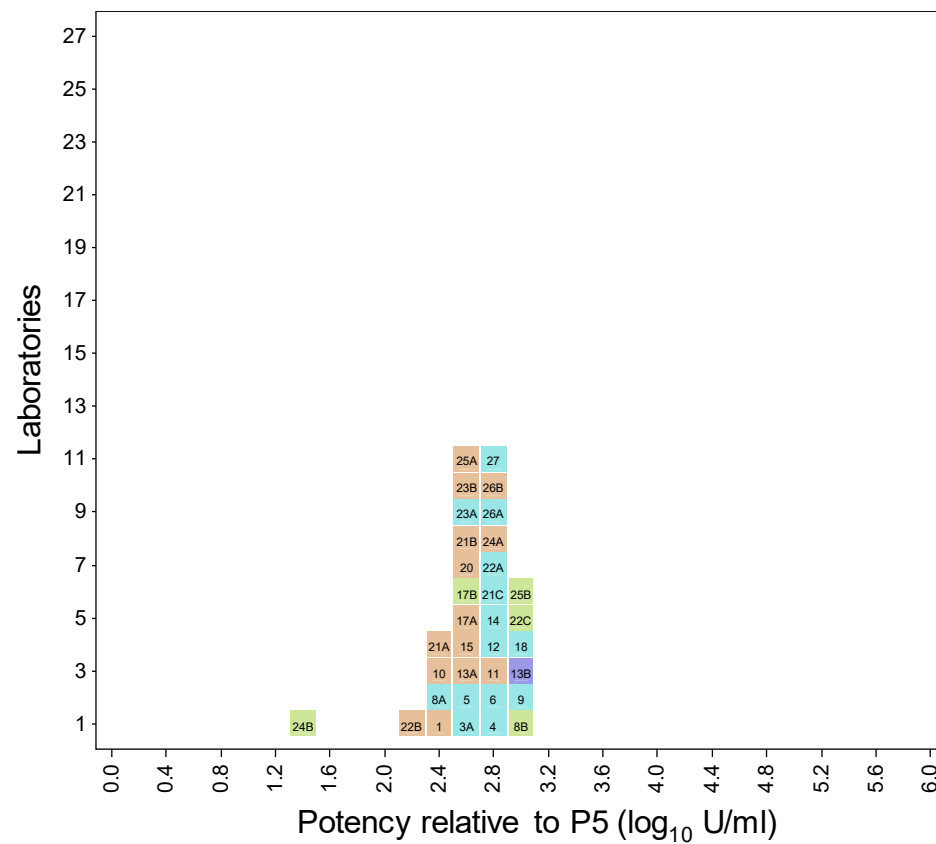
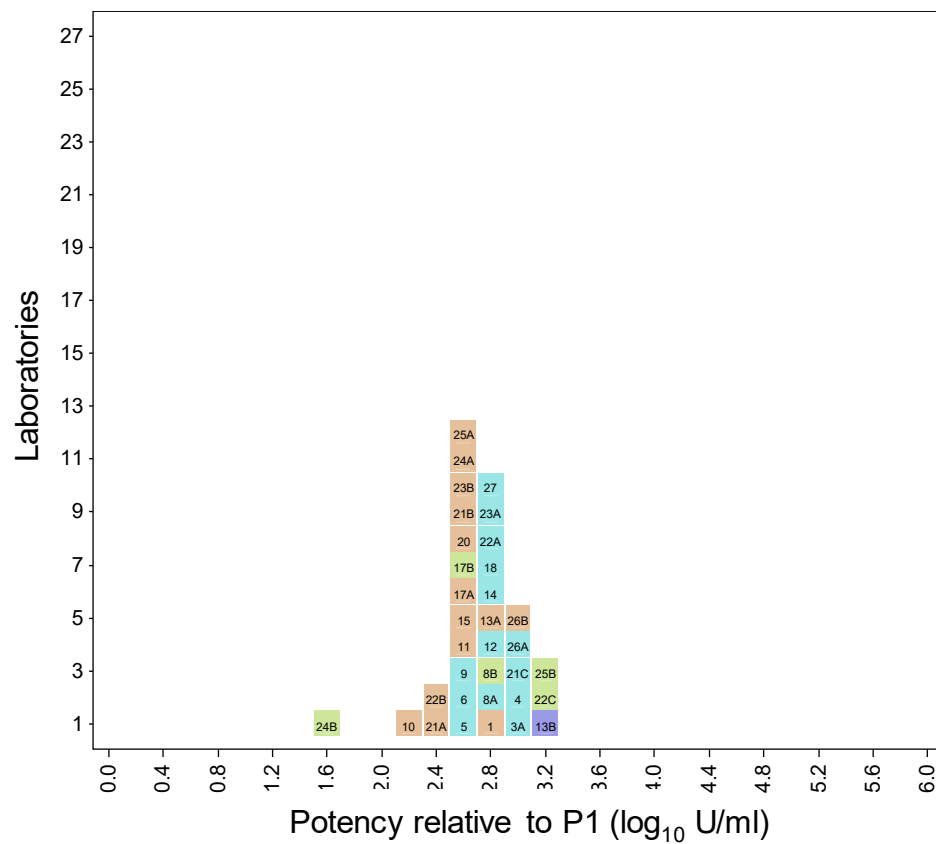


Figure 9A Geometric mean potencies of sample P9

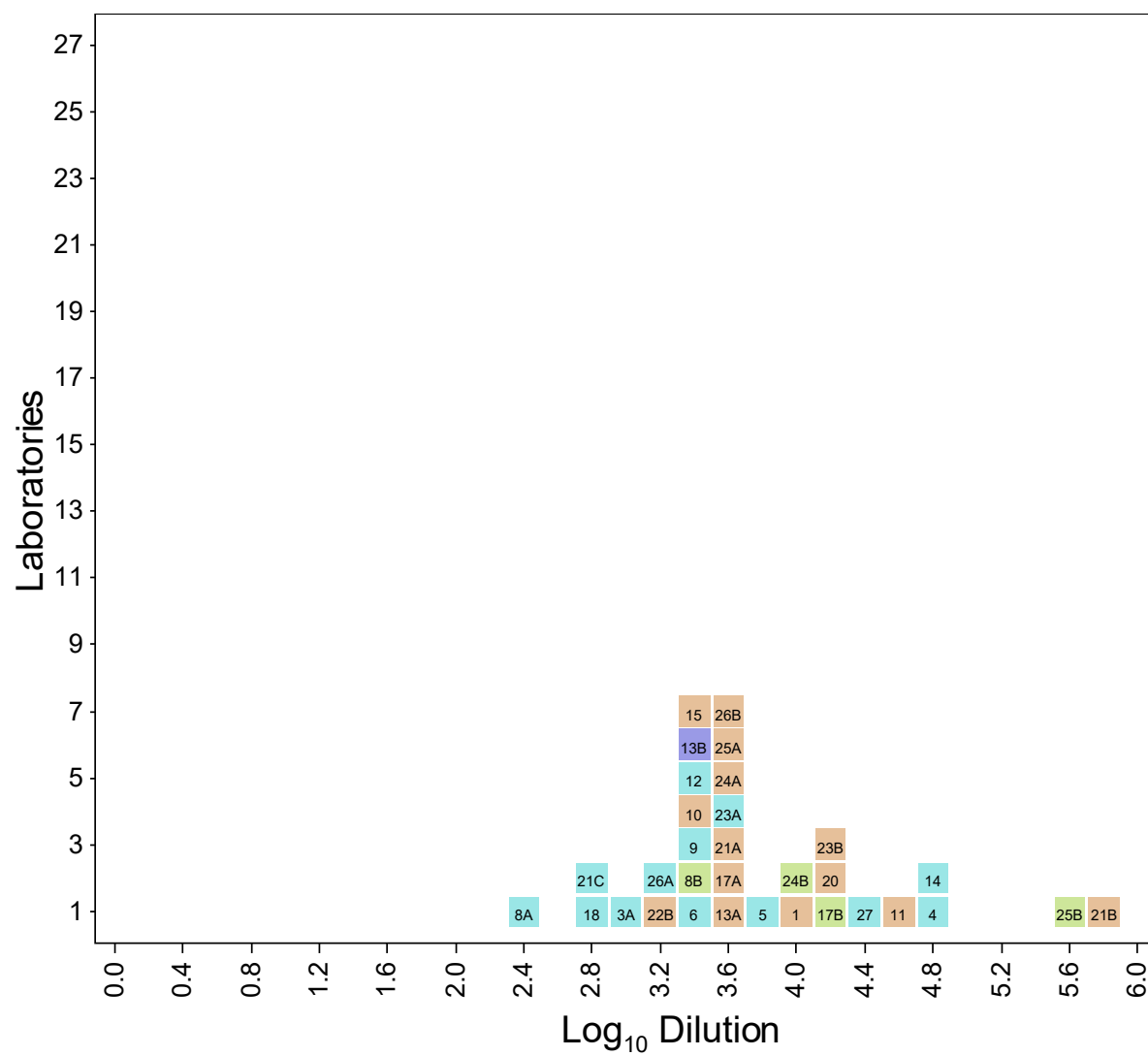


Figure 9B Relative potencies for sample P9

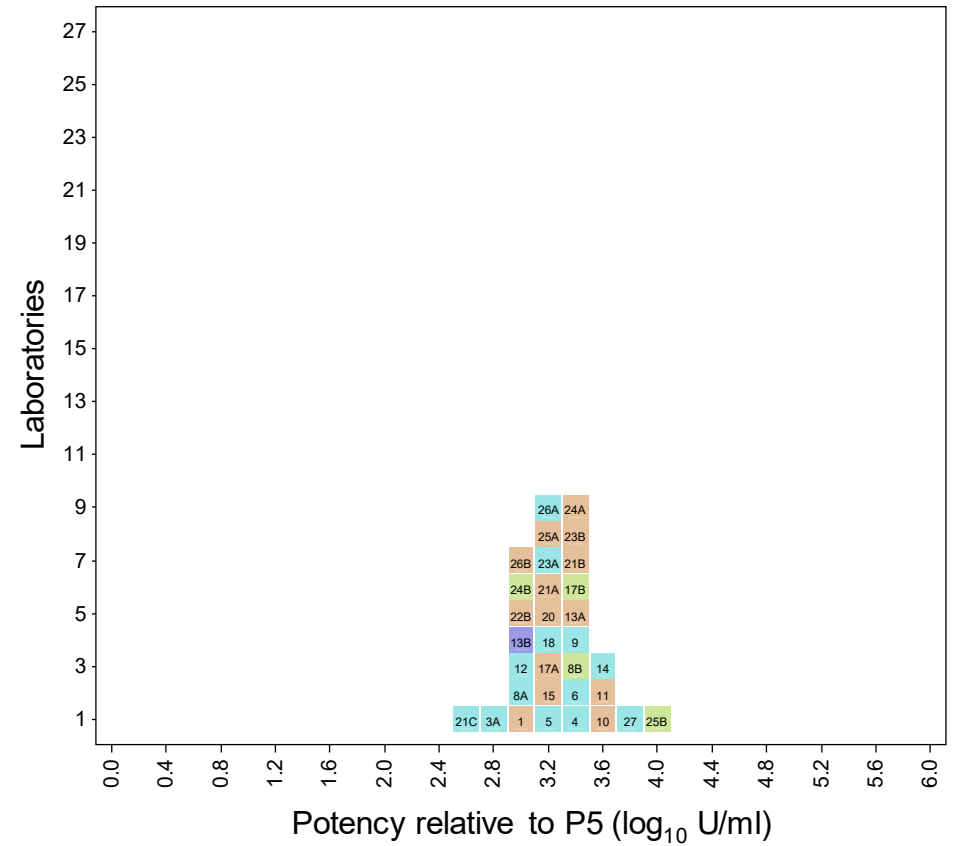
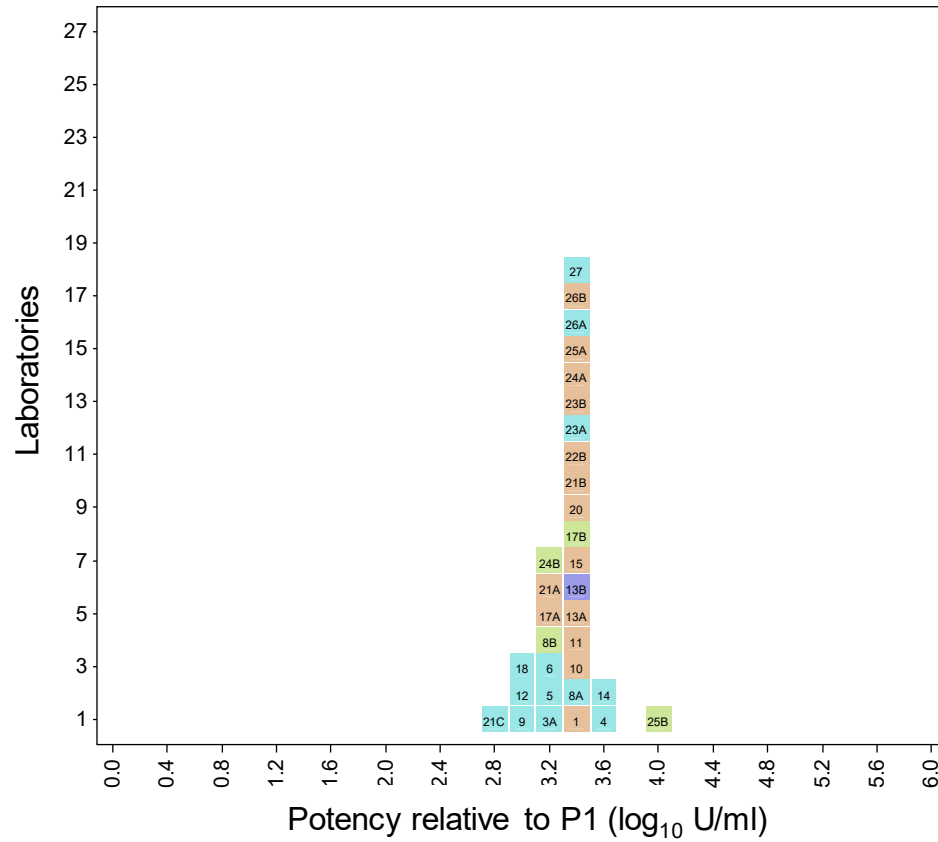


Figure 10A Geometric mean potencies of sample P10

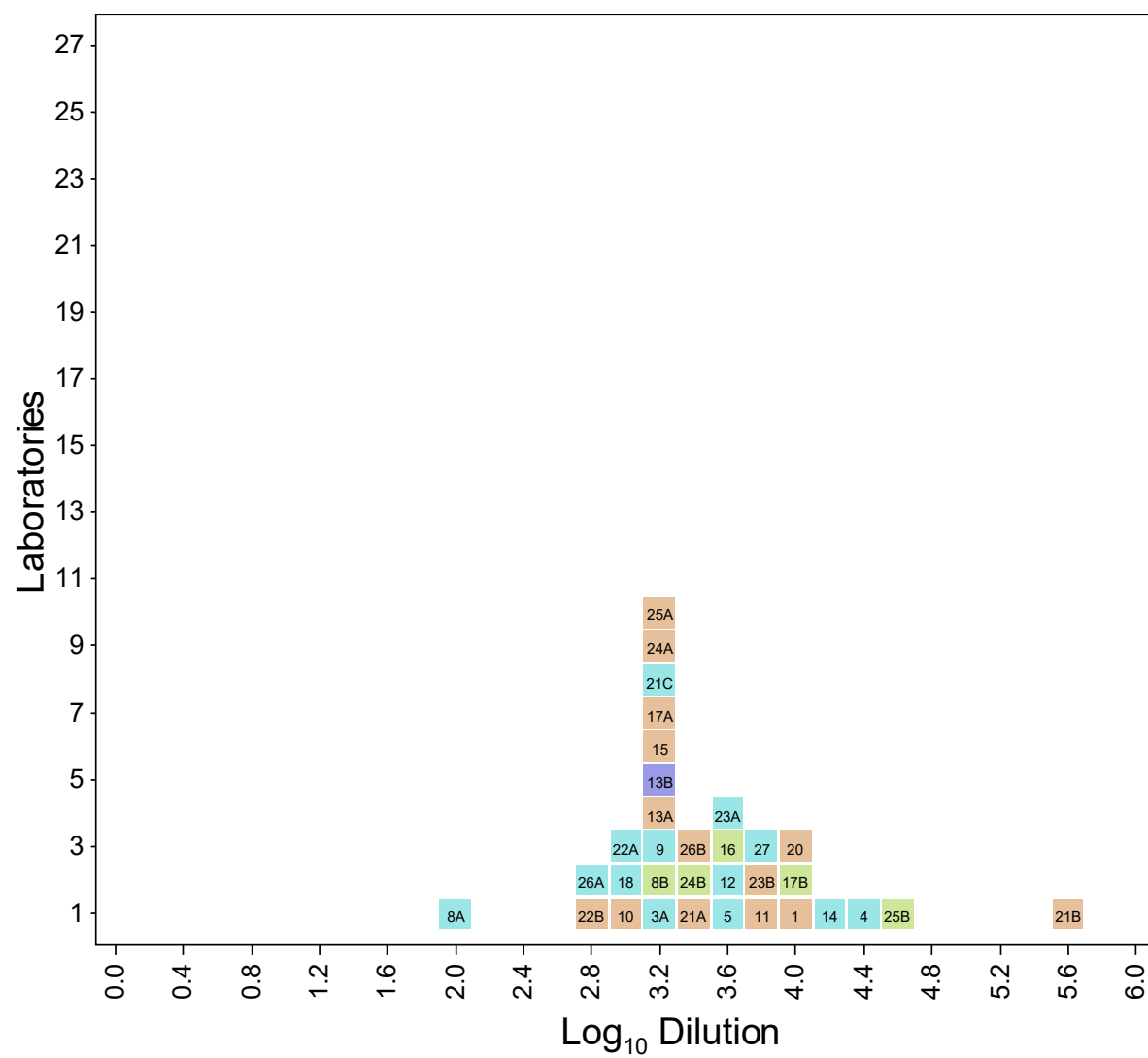


Figure 10B Relative potencies for sample P10

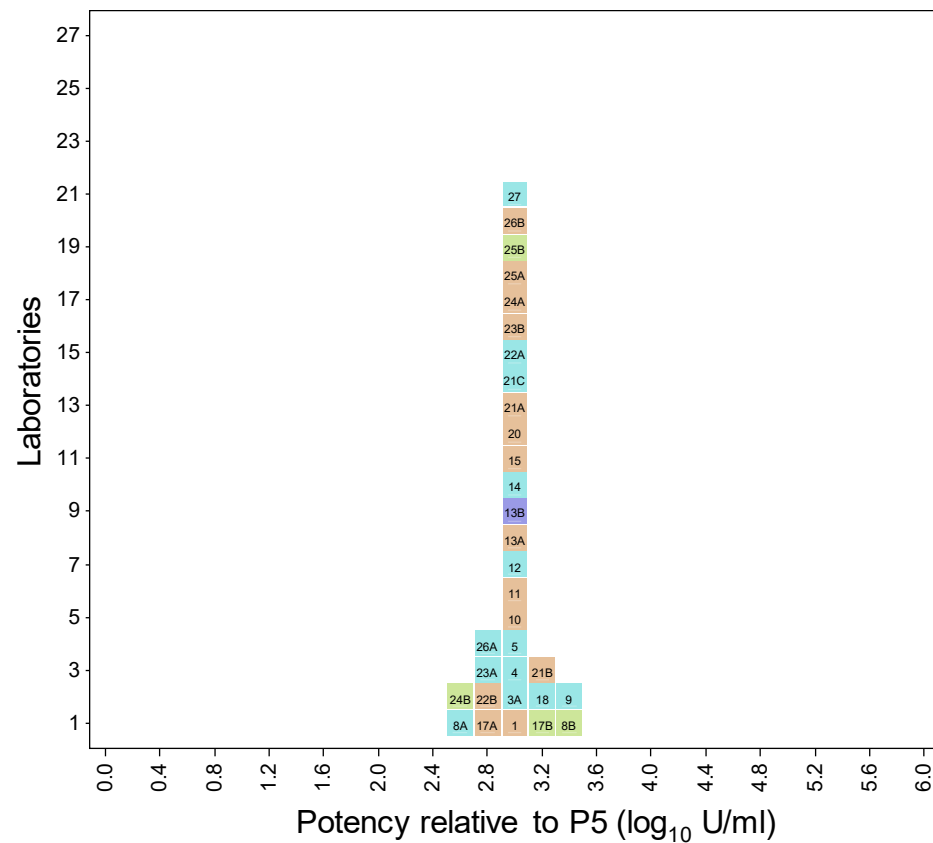
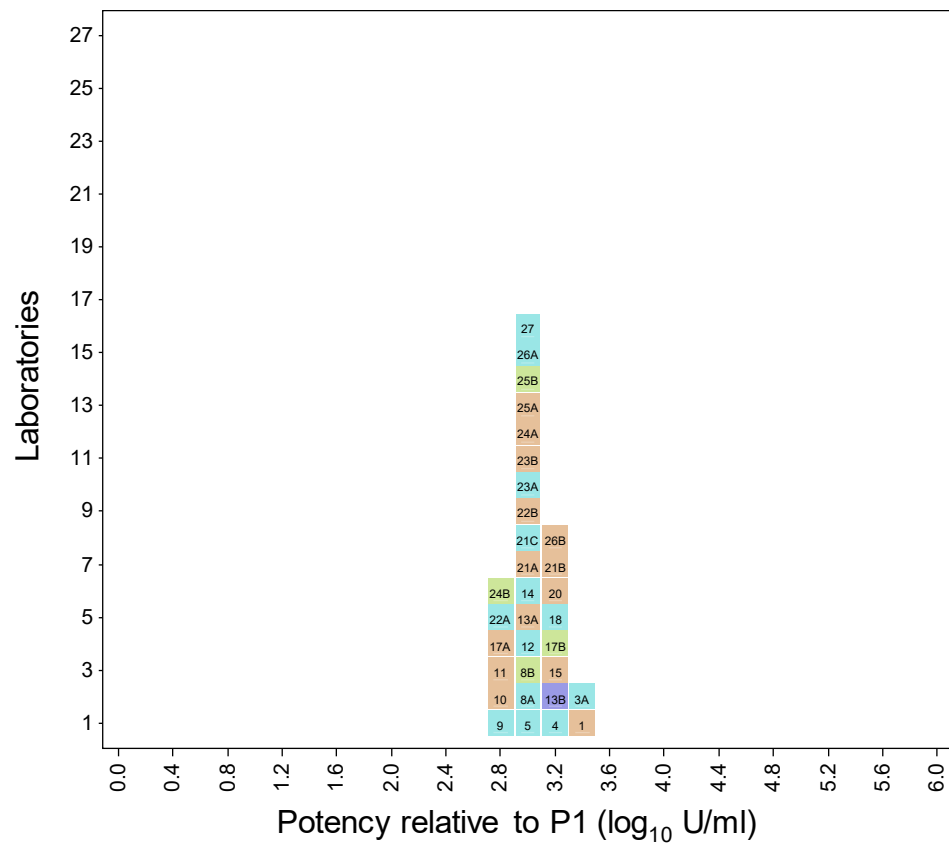


Figure 11A Geometric mean potencies of sample P11

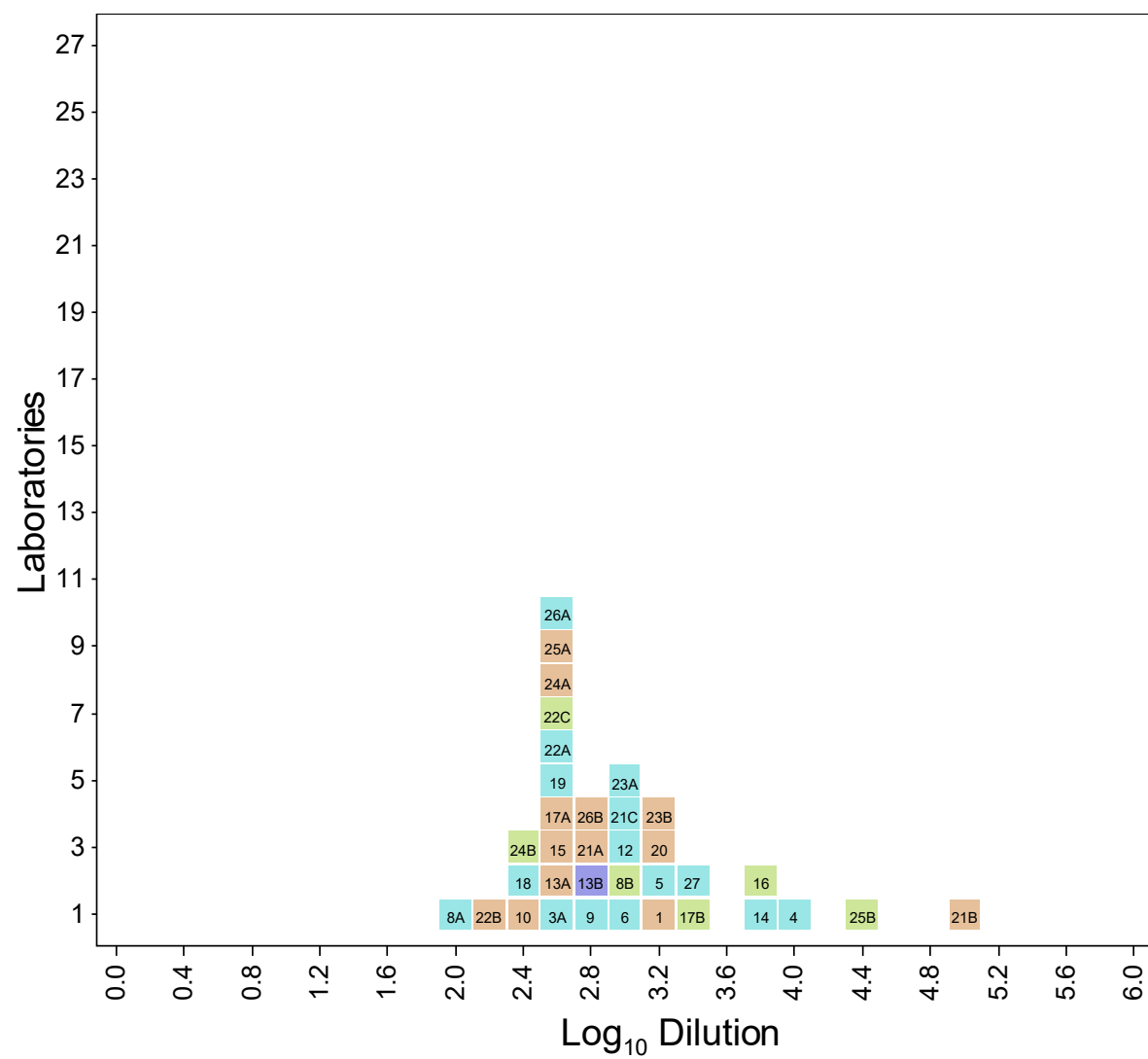


Figure 11B Relative potencies for sample P11

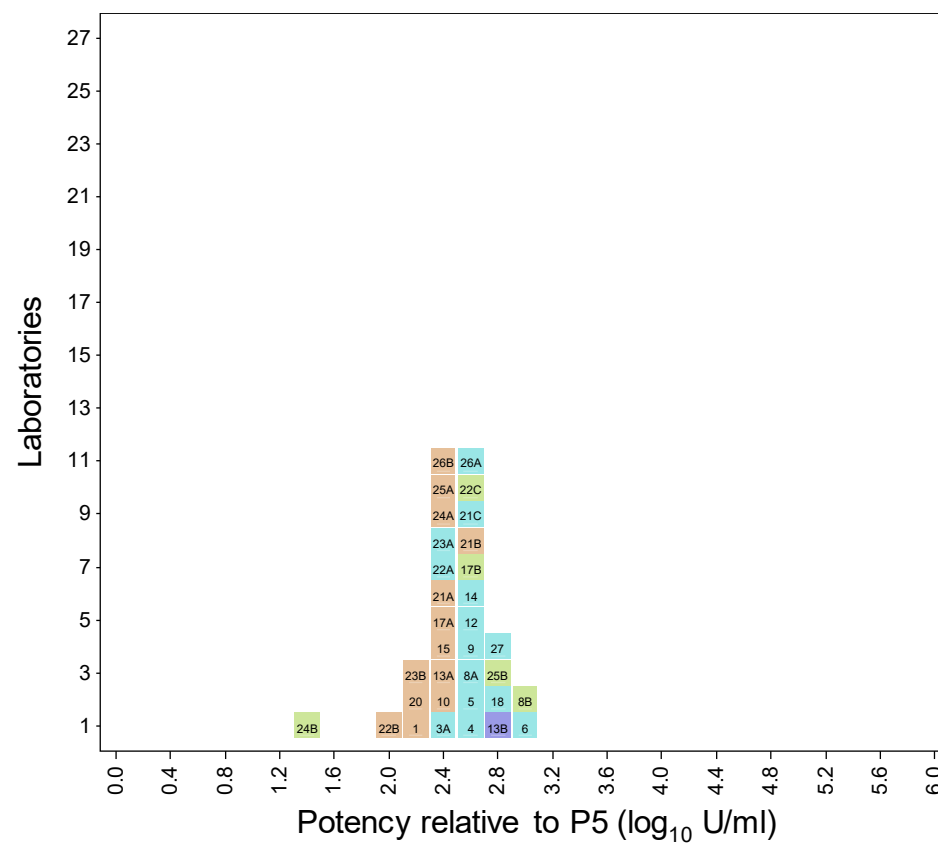
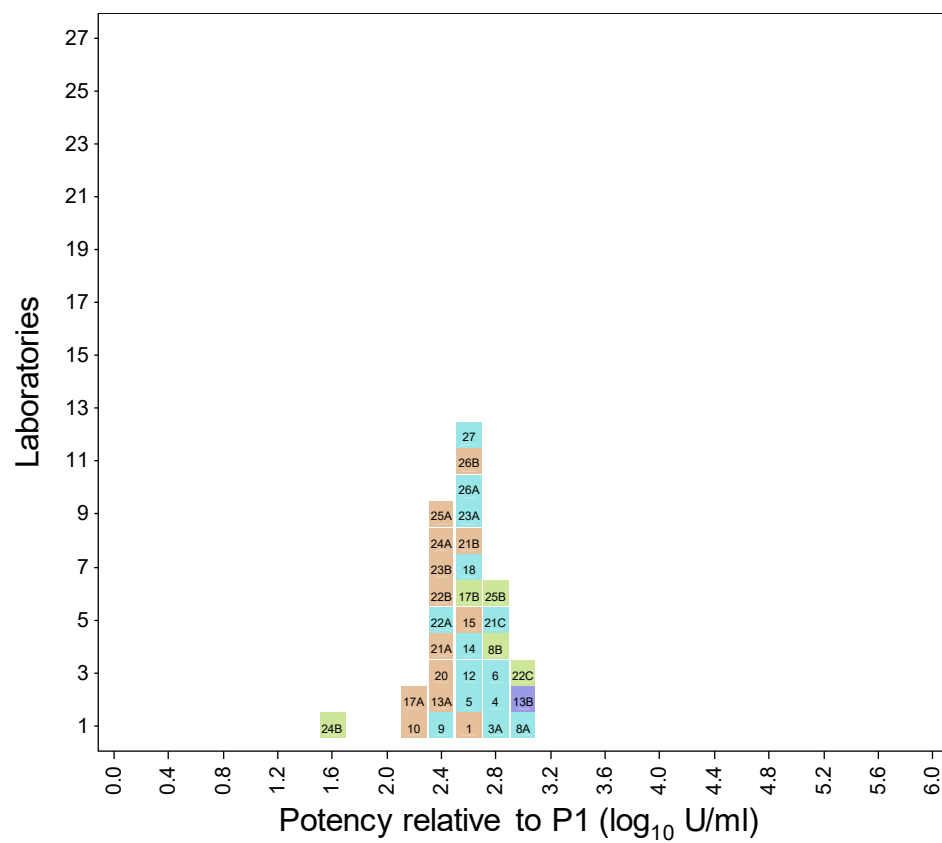
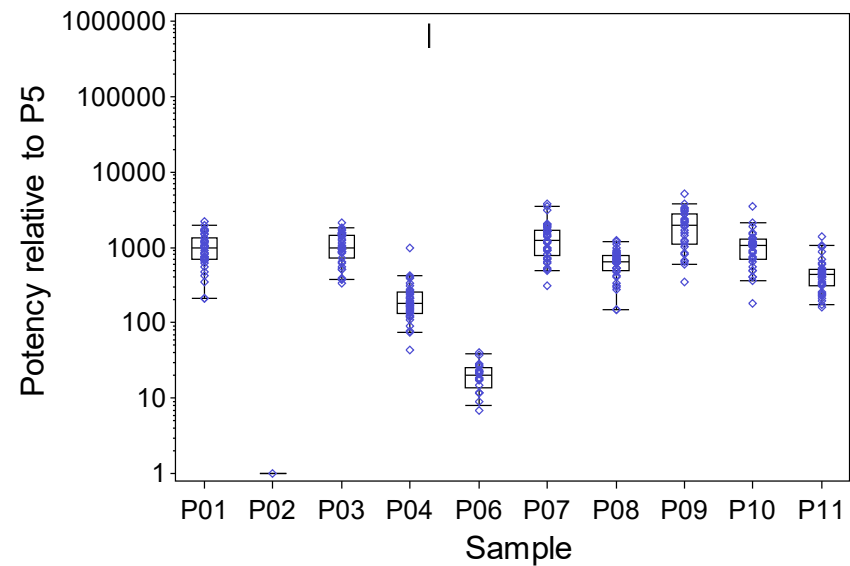
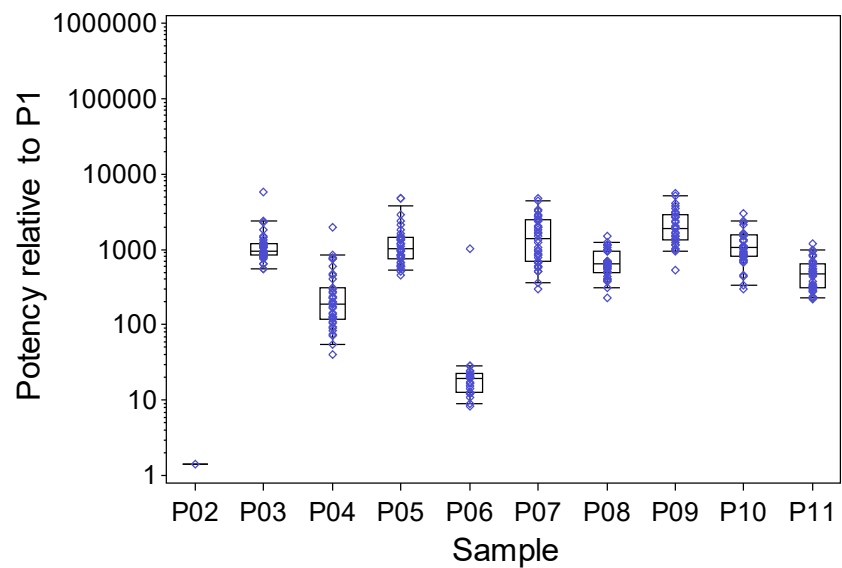
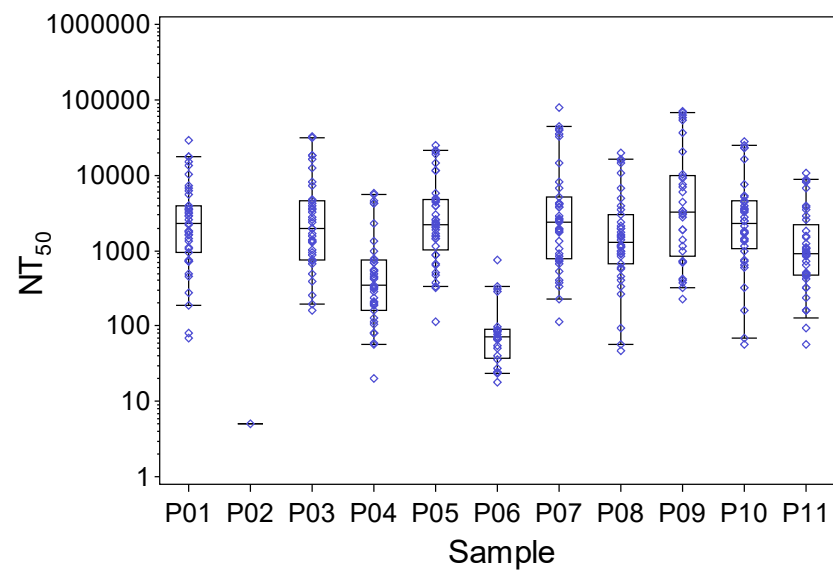


Figure 12A – Virus neutralization assays



**Figure 12B Binding assays (ELISAs, microsphere, immunoblots, haemagglutination inhibition)**

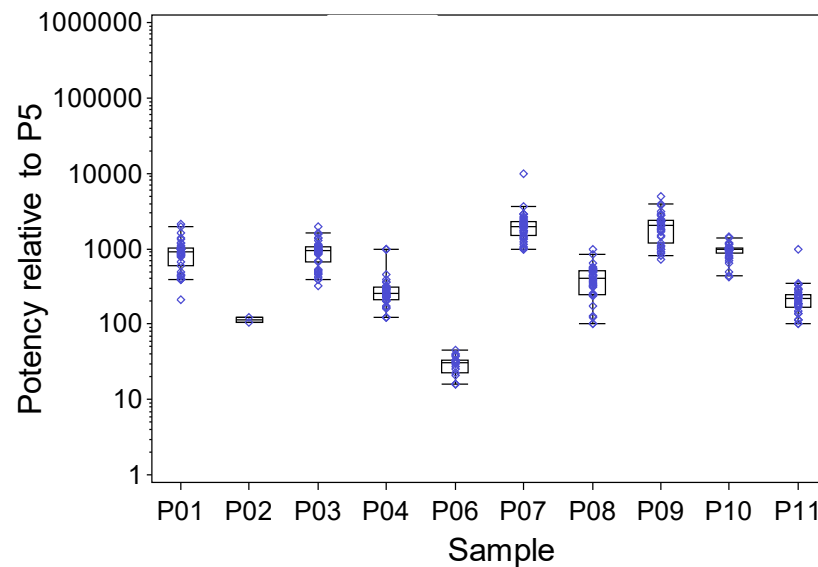
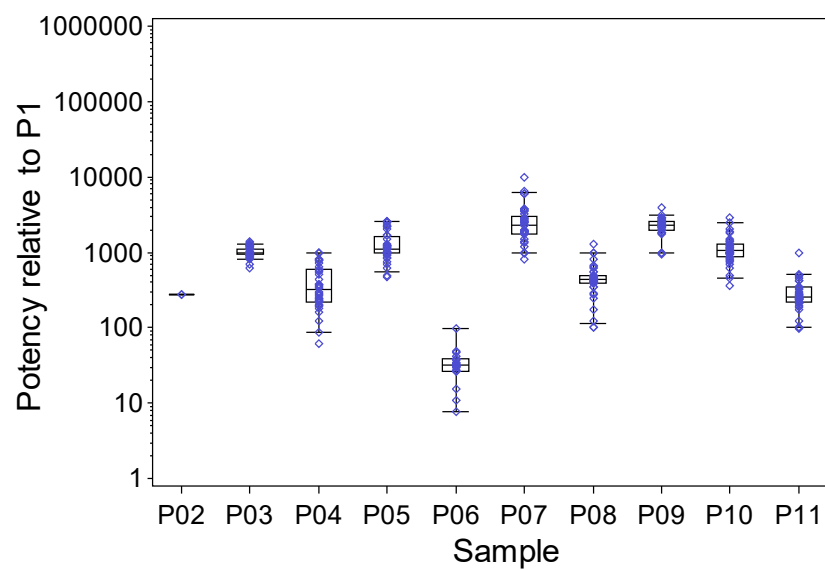
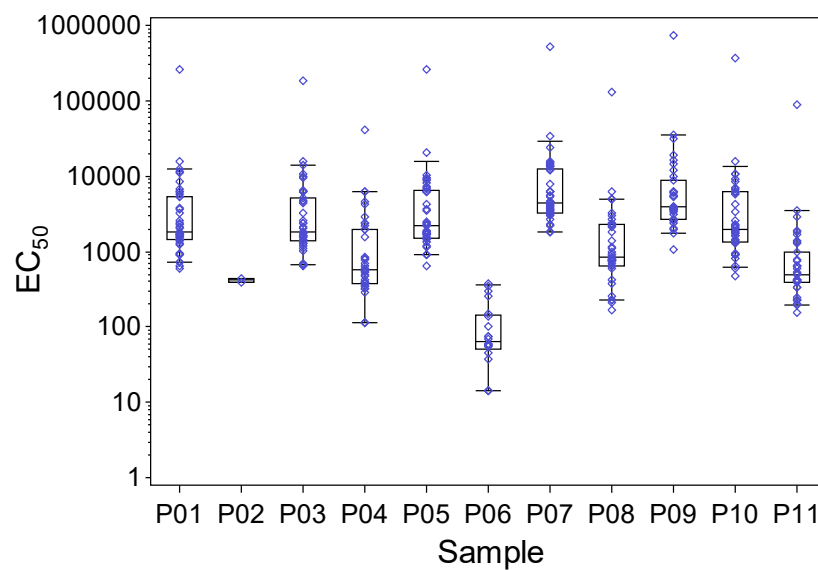
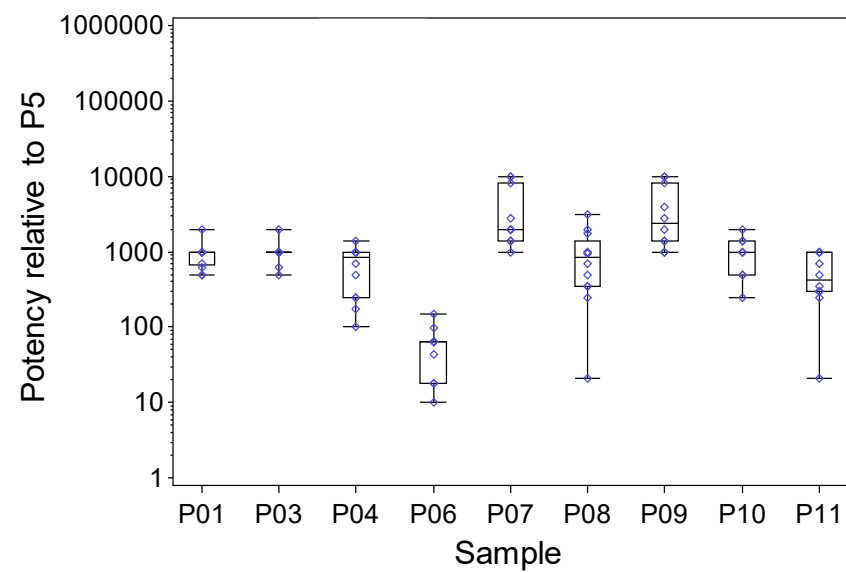
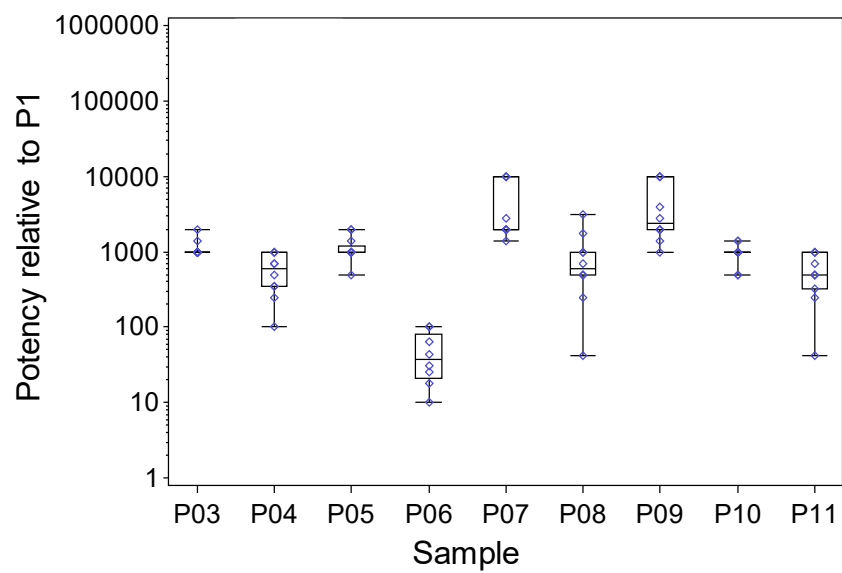
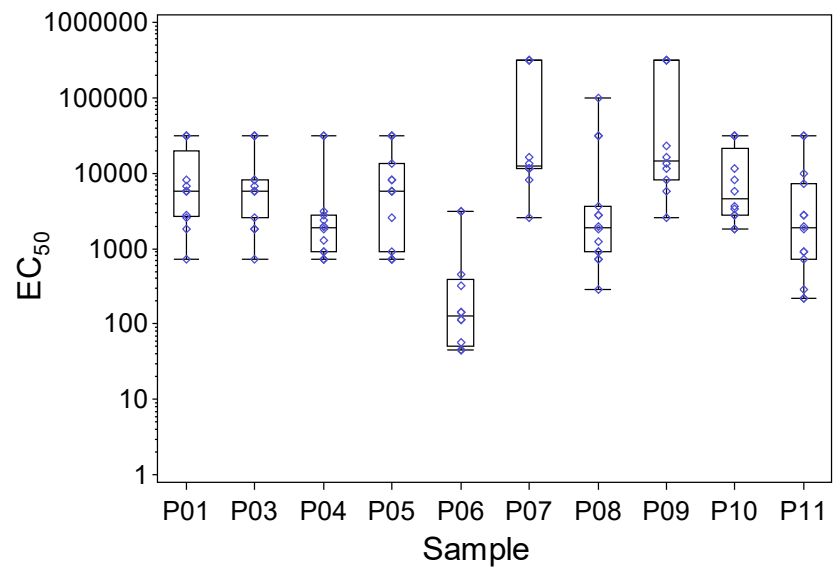


Figure 12C Indirect immunofluorescence assays





**Appendix 1.** List of participating laboratories that returned data (alphabetically according to country/affiliation)

Scientist(s)	Affiliation
David Smith, Suzi McCarthy	PathWest Laboratory Medicine WA Perth, Australia
Carmel Taylor, Peter Moore	Queensland Health Forensic and Scientific Services Coopers Plains, Australia
Maria Farcet, Thomas Kreil	Takeda Manufacturing Austria AG Vienna, Austria
Romana Hochreiter	Valneva Austria GmbH Vienna, Austria
Julien St-Jean	Nixelis, a Q <sup>2</sup> Solutions Company Laval, Canada
Bo Zhang, Ya-Nan Zhang, Xiaodan Li	Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China
Van-Mai Cao-Lormeau, Elsa Dumas- Chastang	Institut Louis Malardé Papeete, Tahiti, French Polynesia
Petra Emmerich#, Jonas Schmidt- Chanasit, Ronald von Posse#	Bernhard-Nocht-Institut für Tropenmedizin Hamburg, Germany; #University of Rostock, Rostock, Germany
Konstanze Stiba, Katja Steinhagen, Erik Lattwein	EUROIMMUN AG Lübeck, Germany
Sarah Schulz, Christina Nölting, Martina Wild	Mikrogen GmbH Neuried, Germany
Ina Brune, Helmut Duchmann	NovaTec Immundiagnostica GmbH Dietzenbach, Germany
Cristina Domingo Carrasco, Andreas Nitsche	Robert-Koch-Institut Berlin, Germany
Anna Maria Eis-Hübinger	Universitätsklinikum Bonn Bonn, Germany
Beate Mareike Kümmerer	Universitätsklinikum Bonn Bonn, Germany
Dipankar Das	Bharat Biotech Intl. Ltd. Hyderabad, India
Asha Mary Abraham, Shoba Mammen, Rajesh Kannangai	Christian Medical College Vellore, India
Rajendra Lingala	Indian Immunologicals Ltd. Hyderabad, India

Concetta Castilletti, Francesca Colavita	National Institute for Infectious Diseases Lazzaro Spallanzani, IRCCS, Rome, Italy
Jamal I-Ching Sam, Chong Long Chua/Yoke Fun Chan	University of Malaya Kuala Lumpur, Malaysia
Inga Szurgot, Peter Liljeström	Karolinska Institute Solna, Sweden
Christine Carrington	The University of the West Indies St. Augustine, Trinidad and Tobago
Ann Powers, Jeremy Ledermann	Centers for Disease Control and Prevention Fort Collins, United States of America
Jason Mendy	Emergent BioSolutions Inc. San Diego, United States of America
Trevor Brasel	University of Texas Medical Branch Galveston, United States of America
Scott Weaver, Grace Rafael	University of Texas Medical Branch Galveston, United States of America
Sergej Franz, Graham Simmons	Vitalant Research Institute San Francisco, United States of America

**Appendix 2.** INSTAND External Quality Assessment Scheme. Group No. 402

N:B. The *recomLine* Tropical Fever IgG and IgM was updated subsequent to this study to increase sensitivity and specificity of the assay.



**INSTAND**



Report on  
External Quality Assessment Scheme  
Group No. 402  
Virus Immunology –  
Chikungunya Virus

Prof. Dr. Heinz Zeichhardt

Dr. Martin Kammel

Issued by:  
**INSTAND**  
Gesellschaft zur Förderung  
der Qualitätssicherung  
in medizinischen Laboratorien e.V.

Düsseldorf/Berlin, Germany, 21.12.2017

## INSTAND EQA schemes in virology

in cooperation with:

Deutsche Vereinigung zur Bekämpfung der Viruskrankheiten e.V. (DVG)  
Gesellschaft für Virologie e.V. (GfV)  
Deutsche Gesellschaft für Hygiene und Mikrobiologie e.V. (DGHM)

### EQAS Adviser:

Prof. i. R. Dr. Heinz Zeichhardt  
Professor of Virology  
Charité - University Medicine Berlin

### Correspondence address:

Prof. Dr. Heinz Zeichhardt  
Institut für Qualitätssicherung in der Virusdiagnostik - IQVD  
Potsdamer Chaussee 80, D-14129 Berlin, Germany  
Tel.: +49-(0)30-81054-300; Fax: +49-(0)30-81054-303  
Email: [Heinz.Zeichhardt@iqvd.de](mailto:Heinz.Zeichhardt@iqvd.de)

### Assistant EQAS Adviser:

Dr. Martin Kammel  
c/o INSTAND e.V.  
Uebierstr. 20, D-40223 Düsseldorf, Germany  
Tel.: +49-(0)30-81054-304; Fax: +49-(0)30-81054-303  
Email: [m.kammel@iqvd.de](mailto:m.kammel@iqvd.de)

*in cooperation with:*

**Bernhard-Nocht-Institut**  
**Nationales Referenzzentrum für tropische Infektionserreger**  
**Abteilung für Virologie**  
**WHO Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research**

### Head:

Prof. Dr. Stephan Günther  
Bernhard-Nocht-Institut, Abteilung für Virologie  
Bernhard-Nocht-Str. 74, 20359 Hamburg  
Tel.: +49-(0)40-42818933; Fax: +49-(0)40-42818400  
Email: [gunther@bni-hamburg.de](mailto:gunther@bni-hamburg.de)

### Deputies:

Dr. Petra Emmerich  
Prof. Dr. Dr. Jonas Schmidt-Chanasit  
Bernhard-Nocht-Institut, Abteilung für Virologie  
Bernhard-Nocht-Str. 74, 20359 Hamburg  
Tel.: +49-(0)40-42818942; Fax: +49-(0)40-42818941  
Email: [jonassi@gmx.de](mailto:jonassi@gmx.de); [schmidt-chanasit@bni-hamburg.de](mailto:schmidt-chanasit@bni-hamburg.de)  
[emmerich@bni-hamburg.de](mailto:emmerich@bni-hamburg.de)

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#### INSTAND Expert Laboratories

- Bernhard-Nocht-Institut, Nationales Referenzzentrum für tropische Infektionserreger, Abteilung für Virologie, WHO Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research, Hamburg: Prof. Dr. S. Günther, Dr. P. Emmerich, Prof. Dr. Dr. J. Schmidt-Chanasit
- Institut für Mikrobiologie der Bundeswehr (IMB), Nationales Konsiliarlaboratorium für Frühsommer-Meningoenzephalitis (FSME), München: PD Dr. G. Dobler, PD Dr. J. J. Bugert
- Paul-Ehrlich-Institut, Bundesinstitut für Impfstoffe und biomedizinische Arzneimittel, WHO Collaborating Centre for Quality Assurance of Blood Products and in vitro Diagnostic Devices, Abt. Virologie, Langen: Dr. M. Chudy, Dr. S. A. Baylis, Dr. J. Kreß
- Universität Leipzig, Institut für Virologie: Prof. Dr. U. G. Liebert, Dr. M. Maier
- Universitätsklinikum Bonn, Institut für Virologie: Prof. Dr. A.-M. Eis-Hübinge
- Universitätsklinikum Düsseldorf, Institut für Virologie: Prof. Dr. J. Timm, Prof. Dr. O. Adams

Carried out by:

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INSTAND e.V.  
Ublerstr. 20  
40223 Düsseldorf, Germany  
Tel.: +49 (0)211 - 1592 13 0  
Fax: +49 (0)211 - 1592 1330  
Email: [instand@instand-ev.de](mailto:instand@instand-ev.de)  
Internet: [www.instand-ev.de](http://www.instand-ev.de)

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## Notes to the evaluation

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### *Guidelines of the German Medical Association (RiliBÄK)*

The External Quality Assessment (EQA) scheme "Virus Immunology – Chikungunya Virus" (402) is not listed in the new Guidelines of the German Medical Association on quality assurance in medical laboratory testing (Bundesärztekammer/ RiliBÄK = Richtlinie der Bundesärztekammer zur Qualitätssicherung laboratoriumsmedizinischer Untersuchungen). However, it is performed by INSTAND e.V. in accordance to the requirements of the specified RiliBÄK Section B2 "Qualitative medical laboratory testing".

For details on the new RiliBÄK, please refer to

- the English version of the guideline translated by INSTAND e.V. with the consent of the Executive Board of the German Medical Association published in "German Medical Science"



### *Release of reports of EQA schemes in virus diagnostics*

Each participant of this EQA scheme receives an email with a table allowing to directly open and/or save the report of the corresponding EQA scheme by clicking the respective download button.

Furthermore, each report of a defined EQA scheme will be released on the INSTAND homepage immediately after completion as PDF file under

"EQAS Online / Service for EQA tests / EQA area (Virus Immunology)"

in English language (<http://www.instand-ev.de/en/eqas-online/service-for-eqa-tests.html>) and

in German language (<http://www.instand-ev.de/ringversuche-online/ringversuche-service.html>).

## Certificate

---

Participation documents are sent out by post for this EQA scheme "Virus Immunology - Chikungunya Virus" (402) as follows:

- certificate of successful participation,
- confirmation of participation,
- statement of individual results.

The certificate of successful participation of this EQA scheme lists the respective measurands/tests, assigned to defined test categories, for which the requirements of the EQA scheme are met.

Each test category is individually evaluated for the certificate of successful participation and separately listed in all participation and evaluation documents.

The EQA scheme "Virus Immunology - Chikungunya Virus" (402) comprises the following test categories:

### *Test categories*

(10) Testing for anti-CHIKV-IgG or anti-CHIKV total

(11) Testing for avidity of anti-CHIKV-IgG

(20) Testing for anti-CHIKV-IgM

***Receiving a certificate of successful participation***

The INSTAND EQA scheme "Virus Immunology - Chikungunya Virus" (402) is not listed in the RiliBÄK, however, it is performed by INSTAND e.V. in accordance to the requirements of the specified RiliBÄK Section B 2.

The evaluation criteria for the results of EQA schemes for the detection of virus specific antibodies follow the new Guidelines of the German Medical Association, RiliBÄK, Specified Section E 2 "Special requirements for round robin testing of qualitative medical laboratory tests" under (3) Analysing the round robin test results, sentence 1:

"Analysis is performed based on the target results. The evaluation criteria must be fulfilled for all samples."

For receiving a certificate of successful participation for a defined EQA scheme it is required that you analyzed all samples of the sample set correctly with the same method in the corresponding test categories (100% correct results according to the target values).

***Example - Program "Virus Immunology - Chikungunya Virus" (402):***

All samples of the sample set have to be tested correctly with the same method in test category 10 "Testing for anti-CHIKV-IgG or anti-CHIKV-total". The same applies for test categories 11 and 20, respectively, of this EQA scheme.

**Frequency of participation to this EQA scheme and validity of the certificates**

The INSTAND EQA scheme "Virus Immunology - Chikungunya Virus" (402) is not listed in the RiliBÄK, however, it is performed by INSTAND e.V. in accordance to the requirements of the specified RiliBÄK Section B 2.

According to the INSTAND program the EQA scheme "Virus Immunology - Chikungunya Virus" (402) is performed once a year.

As practiced for EQA schemes regulated in the Specified RiliBÄK Section E 2, the validity period for the EQA scheme "Virus Immunology - Chikungunya Virus" (402) is 24 months. The validity of the certificate starts with the closing date of the EQA scheme (deadline for the receipt of data). This date is printed on top of the certificates.

**Statement of individual results**

For this EQA scheme "Virus Immunology - Chikungunya Virus" (402) a statement of individual results is sent out by post together with the certificate of successful participation and statement of participation.

The statement of individual results lists for each measurand/test, assigned to defined test categories, the "correct result" with the target value or target value interval as "valid results" as well as the reported result of the laboratory as "your results". This information is given line by line for each sample analyzed.

In addition a "+"-symbol indicates that a certificate of successful participation is issued for a given test category if the laboratory reached 100% correct results for all samples of a sample set according to the target values or target value intervals.

**Overview of results**

A summary of results is given for each of the samples in a table with a specification by test categories. A success rate is depicted for each of the samples reflecting the portion of "correct" results (expressed as "percent" correct results and as "number of correct results per number of total results reported"). In addition an overall success rate - based on the results for all samples of a sample set - is given for each of the test categories.

See Table 2 of the annotation to this report.

### Deployed EQA samples

Anti-CHIKV-positive sera or plasmas of patients are deployed in the EQA scheme "Virus Immunology - Chikungunya virus" (402). The positive samples are diluted in a negative serum/plasma pool of healthy blood donors.

Negative samples are from a negative sera of healthy blood donors.

### Target values

The evaluation of this EQA scheme is based on the determination of target values or target value intervals for each of the samples analyzed.

**Please note:** Reference measurement methods for the determination of target values are not applicable for virus diagnostics.

The target value of a given EQA scheme sample - preset by the EQA scheme adviser - is confirmed by the INSTAND Expert Laboratories prior to the distribution of the samples to the participants of this EQA scheme. The above mentioned INSTAND Expert Laboratories test the samples for a second time during the course of the EQA scheme as regular participants. The final target value for a given sample is derived from the consensus value from all qualitative results and consensus value from all avidity results in percent. For this the results reported by the INSTAND Expert Laboratories before and during the EQA scheme are considered.

#### *Accepted statements of results according to the respective sample property*

- **Qualitative results**
  - "positive", "negative" or "borderline" (nominal characteristics)  
for anti-CHIKV-IgG or anti-CHIKV-total as well as anti-CHIKV-IgM
  - "high", "low" or "intermediate/no avidity/no statement possible/not done" (nominal characteristics)  
for avidity testing of anti-CHIKV-IgG  
The statement "not done" is allowed, when an analysis was not required for an anti-CHIKV-IgG-negative sample.
  - "not done" or "no avidity" for negative samples
- **Results in percent**  
for avidity testing of anti-CHIKV-IgG  
the qualitative results and the avidity results in percent are separately evaluated. The results in percent have to be linked with a qualitative statement. The statement "not done" is allowed, when an analysis was not required for an anti-CHIKV-IgG-negative sample.
- **Results in titer values or in U/ml**  
Results in titer values or U/ml are depicted in the figures "without target value" only for orientation without disadvantage for the certificate of successful participation. See annex 3.2 to this report.

If the results of a given EQA scheme deviate from the preset target value, it will be investigated whether the deviating results are due to the test performance in the laboratory or to test immanent problems of commercial or in-house-tests. This investigation is performed together with the INSTAND Expert Laboratories under the auspices of the EQA scheme adviser and in cooperation with the Joint Diagnostic Council of DVV and GfV.

### Determination of evaluation intervals

For anti-CHIKV-IgG or anti-CHIKV-total as well as anti-CHIKV-IgM:  
not applicable

For avidity testing of anti-CHIKV-IgG:

Results from avidity testing are semi-quantitative statements. Therefore a final target value derived from a consensus value from all results in percent cannot be assigned to the results for avidity testing of anti-CHIKV-IgG.

In this case the EQA scheme adviser defines - depending on the sample properties - an evaluation interval with lower and upper percent values for avidity for each of the anti-CHIKV-IgG-positive samples. The reported results of a laboratory will be evaluated as "correct" or "false" result in accordance to this defined evaluation interval.

### Annotation of the EQAS Adviser

Dear colleagues,

Below please find a detailed comment on the EQA scheme

"Virus Immunology - Chikungunya Virus" (402) September 2017 with:

- Information about test categories, statement of results and evaluation criteria,
- Summary of sample properties, target values, results and success rates as well as
- Annexes with detailed description of all reported qualitative results, titer values, values in U/ml, as well as values in percent for avidity including differentiation according to test formats, manufacturers and names of test kits.

Number of participants in this EQA scheme: 39 laboratories

#### 1 Test categories, statement of results and evaluation criteria for this EQA scheme

The following statements of results were requested for each of the test categories in this EQA scheme which were the basis of evaluation (see Table 1):

Table 1: Test categories, statement of results and evaluation criteria

Test categories	Statement of results the following statements of results were requested	Evaluation criteria no. of correctly determined samples for receiving a certificate of successful participation
(10) Testing for anti-CHIKV-IgG or anti-CHIKV total	positive or negative or borderline	5 of 5 samples
(11) Testing for avidity of anti-CHIKV-IgG	<u>positive samples</u> high or low or intermediate or no statement possible if applicable results in percent  <u>negative samples</u> not done or no avidity	5 of 5 samples
(20) Testing for anti-CHIKV-IgM	positive or negative or borderline	5 of 5 samples

#### Reporting of results in the protocol sheets

Only statements on qualitative results as stated in Table 1 are considered for receiving a certificate of successful participation. Results in titer values or U/ml are depicted in the figures "without target value" only for orientation without disadvantage for the certificate of successful participation. See annex 3.2 to this report.

The simultaneous reporting of different results obtained with one and the same test cannot be accepted and will be evaluated as a missing value, e.g. the simultaneous reporting of a "positive" and "borderline" result for one and the same sample will not be accepted.

A result has not been considered for evaluation in case you had specified that this result should only be taken as additional information and ignored as valid result.

We ask you to report also the raw data of your test results (e.g. s/co, index etc.). We will start to show the evaluations of these raw data in due time.

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## 2 Summary of sample properties, target values, results and success rates

Table 2: Summary of sample properties, target values, results and success rates

Sample No.	Sample properties		Considered as "correct" results (target values / target value intervals)			Success rates for all methods per sample		
	Sample source	Dilution	IgG/ total test cat. 10	Avidity test cat. 11	IgM test cat. 20	IgG/ total test cat. 10	Avidity test category 11	IgM test category 20
402005	Serum of a patient with an acute chikungunya virus infection; diluted with a negative serum of a healthy blood donor, chikungunya virus RNA negative; traveler returned from Mancora / Peru; clinical signs: mosquito bite, fever, exanthema. stomach pains. blood collected: approx. 24 days after onset of disease	1 : 1.19	positive	qualitative: low percent: 0 – 49%	positive	91.1% (41/45)	qualitative: 100% (1/1) percent: 100% (1/1)	93.0% <sup>b</sup> (40/43)
402006* = 402007	Serum of a patient with a past chikungunya virus infection; diluted with a negative serum of a healthy blood donor, chikungunya virus RNA not tested; traveler returned from French Guiana; clinical signs: Exanthema; aching limbs and massive joint pain; blood collected: approx. 10 months after onset of disease	1 : 1.32	positive	qualitative: high percent: 50 - 100%	negative / borderline	100% (45/45)	qualitative: 100% (1/1) percent: 100% (1/1)	93.0% (40/43)
402007* = 402006						100% (45/45)	100% (1/1) percent: 100% (1/1)	90.7% (39/43)
402008	Negative serum of a healthy blood donor without signs of a chikungunya virus infection	----	negative	qualitative: no avidity percent: ---	negative	100% (45/45)	qualitative: 100% (1/1) percent: ---	100% (43/43)
402009 <sup>b</sup>	Plasma pool (three blood collections during convalescence period) from one patient with a past chikungunya virus infection; chikungunya virus RNA negative; traveler returned from Brazil; clinical signs: arthralgia blood collected: within 8-11 months after onset of disease  <i>The plasma pool represents a candidate for an anti-chikungunya-IgG WHO international standard, provided by Paul-Ehrlich-Institut, WHO Collaborating Centre for Quality Assurance of Blood Products and in vitro Diagnostic Devices, Langen.</i>	----	positive	qualitative: high percent: 50 - 100%	not evaluated <sup>a</sup>	100% (45/45)	qualitative: 100% (1/1) percent: 100% (1/1)	not evaluated <sup>a</sup>
Success rate for all samples in test categories 10, 11 and 20, respectively <sup>a</sup>						89.7% <sup>a</sup> (35/39) <sup>a</sup>	100% <sup>a</sup> (1/1) <sup>a</sup>	84.6% <sup>a</sup> (33/39) <sup>a</sup>

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Legend to Table 2:

- <sup>a</sup> The success rates for all samples in test categories 10, 11 and 20, respectively, refer to the number of participating laboratories. Laboratories having reported results obtained by several methods are recorded only once in the corresponding test category.
- <sup>\*</sup> Samples 402006 and 402007 (identical samples): Some tests of different manufacturers for the detection of anti-CHIKV-IgM (test category 20) yielded unexpected results. It remains to be clarified whether the discrepant results are due to reduced analytical specificity of some of the tests or virus specific IgM is persisting.
- <sup>\*</sup> Sample 402009: The results for this sample in test category 20 (testing for anti-CHIKV-IgM) have not been evaluated (without disadvantage for the certificate) due to inconsistent results.
- <sup>§</sup> We thank Dr. Sally A. Baylis und Dr. Constanze Yue from Paul-Ehrlich-Institut, WHO Collaborating Centre for Quality Assurance of Blood Products and in vitro Diagnostic Devices, Langen, for having provided the plasma pool for sample 402009.

Results in titer values or U/ml are depicted in the figures "without target value" (without disadvantage for the certificate of successful participation) (see annexes).

A detailed description of the results for all samples of this EQA scheme "Virus Immunology - Chikungunya Virus" (402) including a differentiation according to the test formats, manufacturers and names of test kits is given in the annexes to this report (see Section 3).

**3 Annexes - Tables and figures including differentiation according to test formats, manufacturers and names of test kits**

- 3.1 Qualitative testing for anti-CHIKV  
(Test categories 10, 11 and 20)
- 3.2 Testing for anti-CHIKV including statements on titer values, results in U/ml  
and percent values for avidity, respectively  
(Test categories 10, 11 and 20)

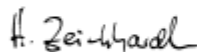
We gratefully acknowledge the excellent cooperation with Prof. Dr. Stephan Günther, Dr. Petra Emmerich and Prof. Dr. Dr. Jonas Schmidt-Chanasit of the Bernhard-Nocht-Institut (Nationales Referenzzentrum für tropische Infektionserreger, Abteilung für Virologie, WHO Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research) as well as with the INSTAND-Expert Laboratories.

We thank Dr. Sally A. Baylis und Dr. Constanze Yue from Paul-Ehrlich-Institut, WHO Collaborating Centre for Quality Assurance of Blood Products and in vitro Diagnostic Devices, Langen, for having provided the plasma pool for sample 402009.

Surplus samples of the current and previous EQA schemes in virus diagnostics are available for test assessment of your virus diagnostics. Please contact INSTAND e.V. for details.

Thank you very much for your kind cooperation.

Sincerely yours,



Prof. Dr. Heinz Zeichhardt  
EQAS Adviser



Dr. Martin Kammel  
Assistant EQAS Adviser

INSTAND e.V. - Gesellschaft zur Förderung der Qualitätssicherung in medizinischen  
Laboratorien e.V. - [www.instandev.de](http://www.instandev.de)  
in cooperation with  
Deutsche Vereinigung zur Bekämpfung der Viruskrankheiten e.V. (DVV)  
Gesellschaft für Virologie e.V. (GfV)  
Deutsche Gesellschaft für Hygiene und Mikrobiologie e.V. (DGHM)

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## **Annex**

### **3.1 Qualitative testing for anti-CHIKV**

**Anti-CHIKV-IgG**  
(test category 10)

**Avidity of anti-CHIKV-IgG**  
(test category 11)

**Anti-CHIKV-IgM**  
(test category 20)

*Differentiation according to method, manufacturer and test name*

Gesellschaft zur Förderung der Qualitätssicherung in medizinischen Laboratorien e. V. (INSTITUT)  
 in cooperation with  
 Deutsche Vereinigung zur Bekämpfung der Viruskrankheiten e. V. (DVV)  
 Gesellschaft für Virologie e. V. (GfV)  
 Deutsche Gesellschaft für Hygiene und Mikrobiologie e. V. (DGHM)

## EQAS Virology September 2017

## Virus immunology Chikungunya virus (402)

## Qualitative results for sample 402005

## 10. Testing for anti-CHIKV-IgG or anti-CHIKV total : positive

Total test	total	positive	borderline	negative	Success rate
	45	41	1	3	91.1%
Method / Manufacturer	total	positive	borderline	negative	Success rate
<b>ELISA-anti-CHIKV-IgG (10)</b>	21	21	0	0	100.0%
EuroImmuno - Anti-CHIKV-ELISA (IgG)	17	17			100.0%
NovaTec - ELISA Chikungunya IgG	3	3			100.0%
IBL - Chikungunya IgG capture ELISA	1	1			100.0%
<b>IFT-anti-CHIKV-IgG (30)</b>	18	18	0	0	100.0%
EuroImmuno - Anti-CHIKV-IIFT (IgG)	13	13			100.0%
EuroImmuno - Arboviren-Fieber-Mosaik 1 (IgG)	2	2			100.0%
in house	2	2			100.0%
EuroImmuno - Arboviren-Profil 3 (IgG)	1	1			100.0%
<b>Line-IA-anti-CHIKV-IgG (51)</b>	5	1	1	3	20.0%
Mikrogen - recomLine Tropical Fever IgG	5	1	1	3	20.0%
<b>other anti-CHIKV-total (99)</b>	1	1	0	0	100.0%
in house	1	1			100.0%

## Qualitative results for sample 402005

## 11. Testing for avidity of anti-CHIKV-IgG : low

Total test	total	high	intermed.	low	Success rate
	1	0	0	1	100.0%
Method / Manufacturer	total	high	intermed.	low	Success rate
<b>ELISA-anti-CHIKV-IgG (10)</b>	1	0	0	1	100.0%
EuroImmuno - Anti-CHIKV- IgG Avidität	1			1	100.0%

### Qualitative results for sample 402005

20. Testing for anti-CHIKV-IgM : positive

Total test	total	positive	borderline	negative	Success rate
	43	40	0	3	93.0%
Method / Manufacturer	total	positive	borderline	negative	Success rate
<b>ELISA-anti-CHIKV-IgM (10)</b>	22	22	0	0	100.0%
EuroImmun - Anti-CHIKV-ELISA (IgM)	17	17			100.0%
NovaTec - ELISA Chikungunya IgM #capture	4	4			100.0%
IBL - Chikungunya IgM #capture ELISA	1	1			100.0%
<b>IFT-anti-CHIKV-IgM (30)</b>	16	15	0	1	93.8%
EuroImmun - Anti-CHIKV-IIFT (IgM)	12	12			100.0%
in house	2	2			100.0%
EuroImmun - Arboviren-Fieber-Mosaik 1 (IgM)	1			1	0.0%
EuroImmun - Arboviren-Profil 3 (IgM)	1	1			100.0%
<b>Line-IA-anti-CHIKV-IgM (51)</b>	5	3	0	2	60.0%
Mikrogen - recomLine Tropical Fever IgM	5	3		2	60.0%

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## Qualitative results for sample 402006

## 10. Testing for anti-CHIKV-IgG or anti-CHIKV total : positive

Total test	total	positive	borderline	negative	Success rate
	45	45	0	0	100.0%
Method / Manufacturer	total	positive	borderline	negative	Success rate
<b>ELISA-anti-CHIKV-IgG (10)</b>	21	21	0	0	100.0%
EuroImmun - Anti-CHIKV-ELISA (IgG)	17	17			100.0%
NovaTec - ELISA Chikungunya IgG	3	3			100.0%
IBL - Chikungunya IgG capture ELISA	1	1			100.0%
<b>IFT-anti-CHIKV-IgG (30)</b>	18	18	0	0	100.0%
EuroImmun - Anti-CHIKV-IIIFT (IgG)	13	13			100.0%
in house	2	2			100.0%
EuroImmun - Arboviren-Fieber-Mosaik 1 (IgG)	2	2			100.0%
EuroImmun - Arboviren-Profil 3 (IgG)	1	1			100.0%
<b>Line-IA-anti-CHIKV-IgG (51)</b>	5	5	0	0	100.0%
Mikrogen - recomLine Tropical Fever IgG	5	5			100.0%
<b>other anti-CHIKV-total (99)</b>	1	1	0	0	100.0%
in house	1	1			100.0%

## Qualitative results for sample 402006

## 11. Testing for avidity of anti-CHIKV-IgG : high

Total test	total	high	intermed.	low	Success rate
	1	1	0	0	100.0%
Method / Manufacturer	total	high	intermed.	low	Success rate
<b>ELISA-anti-CHIKV-IgG (10)</b>	1	1	0	0	100.0%
EuroImmun - Anti-CHIKV- IgG Avidität	1	1			100.0%

## Qualitative results for sample 402006

## 20. Testing for anti-CHIKV-IgM : negative,borderline

Total test	total	positive	borderline	negative	Success rate
	43	3	2	38	93.0%
Method / Manufacturer	total	positive	borderline	negative	Success rate
<b>ELISA-anti-CHIKV-IgM (10)</b>	22	0	1	21	100.0%
EuroImmun - Anti-CHIKV-ELISA (IgM)	17		1	16	100.0%
NovaTec - ELISA Chikungunya IgM #capture	4			4	100.0%
IBL - Chikungunya IgM #capture ELISA	1			1	100.0%
<b>IFT-anti-CHIKV-IgM (30)</b>	16	3	0	13	81.2%
EuroImmun - Anti-CHIKV-IIIFT (IgM)	12	2		10	83.3%
in house	2			2	100.0%
EuroImmun - Arboviren-Profil 3 (IgM)	1			1	100.0%
EuroImmun - Arboviren-Fieber-Mosaik 1 (IgM)	1	1			0.0%

## 20. Testing for anti-CHIKV-IgM

Method / Manufacturer	total	positive	borderline	negative	Success rate
Line-IA-anti-CHIKV-IgM (51)	5	0	1	4	100.0%
Mikrogen - recomLine Tropical Fever IgM	5		1	4	100.0%

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### Qualitative results for sample 402007

10. Testing for anti-CHIKV-IgG or anti-CHIKV total : **positive**

Total test	total	positive	borderline	negative	Success rate
	45	45	0	0	100.0%
Method / Manufacturer	total	positive	borderline	negative	Success rate
<b>ELISA-anti-CHIKV-IgG (10)</b>	21	21	0	0	100.0%
EuroImmun - Anti-CHIKV-ELISA (IgG)	17	17			100.0%
NovaTec - ELISA Chikungunya IgG	3	3			100.0%
IBL - Chikungunya IgG capture ELISA	1	1			100.0%
<b>IFT-anti-CHIKV-IgG (30)</b>	18	18	0	0	100.0%
EuroImmun - Anti-CHIKV-IIIFT (IgG)	13	13			100.0%
in house	2	2			100.0%
EuroImmun - Arboviren-Fieber-Mosaik 1 (IgG)	2	2			100.0%
EuroImmun - Arboviren-Profil 3 (IgG)	1	1			100.0%
<b>Line-IA-anti-CHIKV-IgG (51)</b>	5	5	0	0	100.0%
Mikrogen - recomLine Tropical Fever IgG	5	5			100.0%
<b>other anti-CHIKV-total (99)</b>	1	1	0	0	100.0%
in house	1	1			100.0%

### Qualitative results for sample 402007

## 11. Testing for avidity of anti-CHIKV-IgG : high

<b>Total test</b>	<b>total</b>	<b>high</b>	<b>intermed.</b>	<b>low</b>	<b>Success rate</b>
	<b>1</b>	<b>1</b>	<b>0</b>	<b>0</b>	<b>100.0%</b>
<b>Method / Manufacturer</b>	<b>total</b>	<b>high</b>	<b>intermed.</b>	<b>low</b>	<b>Success rate</b>
<b>ELISA-anti-CHIKV-IgG (10)</b>	<b>1</b>	<b>1</b>	<b>0</b>	<b>0</b>	<b>100.0%</b>
<b>Eurolmmun - Anti-CHIKV- IgG Avidität</b>	<b>1</b>	<b>1</b>			<b>100.0%</b>

### Qualitative results for sample 402007

20. Testing for anti-CHIKV-IgM : **negative, borderline**

Total test	total	positive	borderline	negative	Success rate
	43	4	5	34	90.7%
Method / Manufacturer	total	positive	borderline	negative	Success rate
<b>ELISA-anti-CHIKV-IgM (10)</b>	22	0	2	20	100.0%
EuroImmun - Anti-CHIKV-ELISA (IgM)	17		2	15	100.0%
NovaTec - ELISA Chikungunya IgM #capture	4			4	100.0%
IBL - Chikungunya IgM #capture ELISA	1			1	100.0%
<b>IFT-anti-CHIKV-IgM (30)</b>	16	4	2	10	75.0%
EuroImmun - Anti-CHIKV-IIFT (IgM)	12	3	2	7	75.0%
in house	2			2	100.0%
EuroImmun - Arboviren-Profil 3 (IgM)	1			1	100.0%
EuroImmun - Arboviren-Fieber-Mosaik 1 (IgM)	1	1			0.0%

## 20. Testing for anti-CHIKV-IgM

Method / Manufacturer	total	positive	borderline	negative	Success rate
Line-IA-anti-CHIKV-IgM (51)	5	0	1	4	100.0%
Mikrogen - recomLine Tropical Fever IgM	5		1	4	100.0%

### Qualitative results for sample 402008

Total test	total	positive	borderline	negative	Success rate
	45	0	0	45	100.0%
Method / Manufacturer	total	positive	borderline	negative	Success rate
<b>ELISA-anti-CHIKV-IgG (10)</b>	21	0	0	21	100.0%
Eurolmmun - Anti-CHIKV-ELISA (IgG)	17			17	100.0%
NovaTec - ELISA Chikungunya IgG	3			3	100.0%
IBL - Chikungunya IgG capture ELISA	1			1	100.0%
<b>IFT-anti-CHIKV-IgG (30)</b>	18	0	0	18	100.0%
Eurolmmun - Anti-CHIKV-IIFT (IgG)	13			13	100.0%
Eurolmmun - Arboviren-Fieber-Mosak 1 (IgG)	2			2	100.0%
in house	2			2	100.0%
Eurolmmun - Arboviren-Profil 3 (IgG)	1			1	100.0%
<b>Line-IA-anti-CHIKV-IgG (51)</b>	5	0	0	5	100.0%
Mikrogen - recomLine Tropical Fever IgG	5			5	100.0%
<b>other anti-CHIKV-total (99)</b>	1	0	0	1	100.0%
in house	1			1	100.0%

20. Testing for anti-CHIKV-IgM : negative

Total test	total	positive	borderline	negative	Success rate
	43	0	0	43	100.0%
Method / Manufacturer	total	positive	borderline	negative	Success rate
<b>ELISA-anti-CHIKV-IgM (10)</b>	22	0	0	22	100.0%
Eurolmmun - Anti-CHIKV-ELISA (IgM)	17			17	100.0%
NovaTec - ELISA Chikungunya IgM #-capture	4			4	100.0%
IBL - Chikungunya IgM #-capture ELISA	1			1	100.0%
<b>IFT-anti-CHIKV-IgM (30)</b>	16	0	0	16	100.0%
Eurolmmun - Anti-CHIKV-IIFT (IgM)	12			12	100.0%
in house	2			2	100.0%
Eurolmmun - Arboviren-Fieber-Mosak 1 (IgM)	1			1	100.0%
Eurolmmun - Arboviren-Profil 3 (IgM)	1			1	100.0%
<b>Line-IA-anti-CHIKV-IgM (51)</b>	5	0	0	5	100.0%
Mikrogen - recomLine Tropical Fever IgM	5			5	100.0%

### Qualitative results for sample 402009

10. Testing for anti-CHIKV-IgG or anti-CHIKV total : positive

Total test	total	positive	borderline	negative	Success rate
	45	45	0	0	100.0%
Method / Manufacturer	total	positive	borderline	negative	Success rate
<b>ELISA-anti-CHIKV-IgG (10)</b>	21	21	0	0	100.0%
EuroImmun - Anti-CHIKV-ELISA (IgG)	17	17			100.0%
NovaTec - ELISA Chikungunya IgG	3	3			100.0%
IBL - Chikungunya IgG capture ELISA	1	1			100.0%
<b>IFT-anti-CHIKV-IgG (30)</b>	18	18	0	0	100.0%
EuroImmun - Anti-CHIKV-IIFT (IgG)	13	13			100.0%
EuroImmun - Arboviren-Fieber-Mosaik 1 (IgG)	2	2			100.0%
in house	2	2			100.0%
EuroImmun - Arboviren-Profil 3 (IgG)	1	1			100.0%
<b>Line-IA-anti-CHIKV-IgG (51)</b>	5	5	0	0	100.0%
Mikrogen - recomLine Tropical Fever IgG	5	5			100.0%
<b>other anti-CHIKV-total (99)</b>	1	1	0	0	100.0%
in house	1	1			100.0%

### Qualitative results for sample 402009

11. Testing for avidity of anti-CHIKV-IgG : **high**

Total test	total	high	intermed.	low	Success rate
	1	1	0	0	100.0%
Method / Manufacturer	total	high	intermed.	low	Success rate
ELISA-anti-CHIKV-IgG (10)	1	1	0	0	100.0%
Eurolmmun - Anti-CHIKV- IgG Avidität	1	1			100.0%

### Qualitative results for sample 402009

20. Testing for anti-CHIKV-IgM : no evaluation

Total test	total	positive	borderline	negative	Success rate
	43	15	9	19	
Method / Manufacturer	total	positive	borderline	negative	Success rate
<b>ELISA-anti-CHIKV-IgM (10)</b>	22	9	8	5	
EuroImmun - Anti-CHIKV-ELISA (IgM)	17	5	8	4	
NovaTec - ELISA Chikungunya IgM #capture	4	3		1	
IBL - Chikungunya IgM #capture ELISA	1	1			
<b>IFT-anti-CHIKV-IgM (30)</b>	16	6	1	9	
EuroImmun - Anti-CHIKV-IIFT (IgM)	12	6	1	5	
in house	2			2	
EuroImmun - Arboviren-Fieber-Mosaik 1 (IgM)	1			1	
EuroImmun - Arboviren-Profil 3 (IgM)	1			1	

### Qualitative results for sample 402009

## 20. Testing for anti-CHIKV-IgM

Method / Manufacturer	total	positive	borderline	negative	Success rate
Line-IA-anti-CHIKV-IgM (51)	5	0	0	5	
Mikrogen - recomLine Tropical Fever IgM	5			5	

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## Annex

### 3.2 Testing for anti-CHIKV

#### Anti-CHIKV-IgG

(test category 10)

*Titer values and results as IU/ml*

#### Avidity of anti-CHIKV-IgG

(test category 11)

*Percent values*

#### Anti-CHIKV-IgM

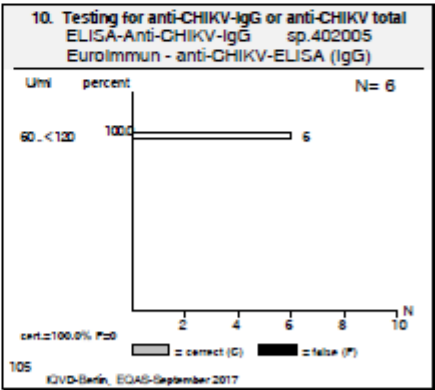
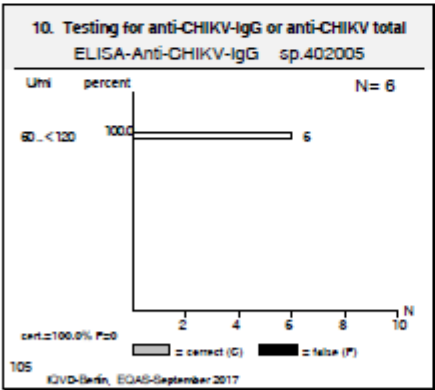
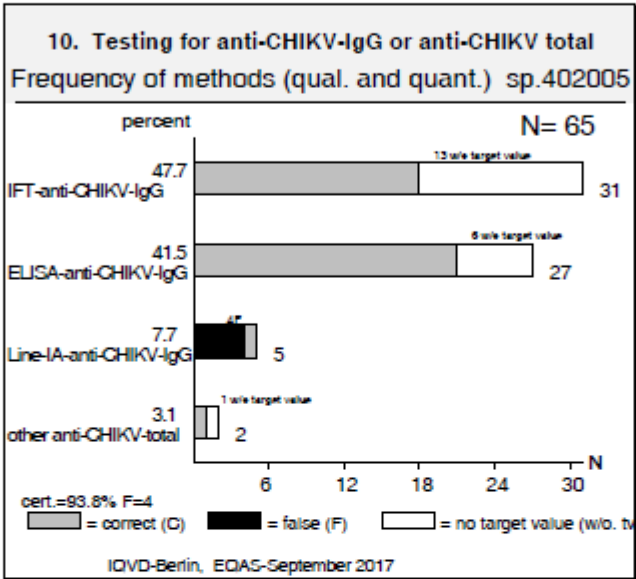
(test category 20)

*Titer values and results as U/ml*

*Differentiation according to method, manufacturer and test name*

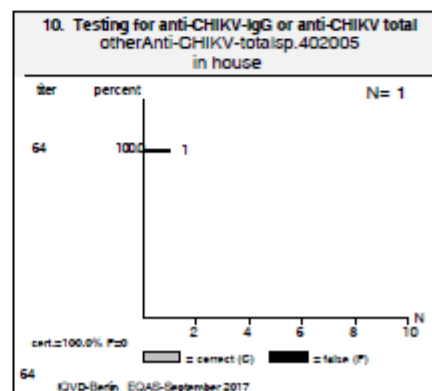
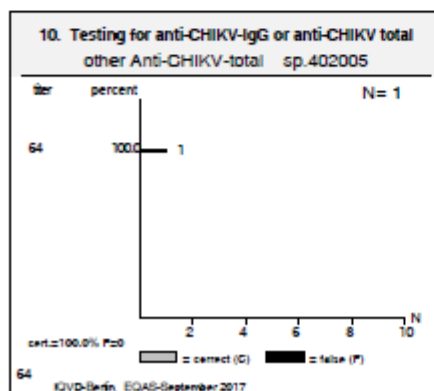
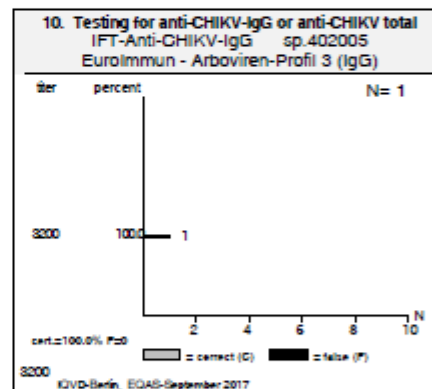
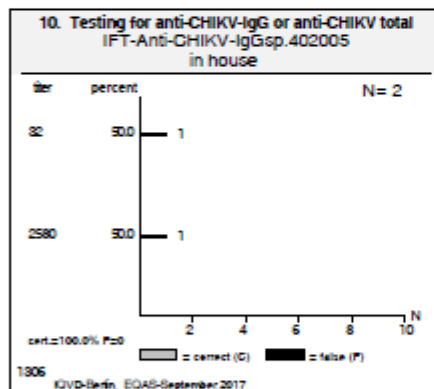
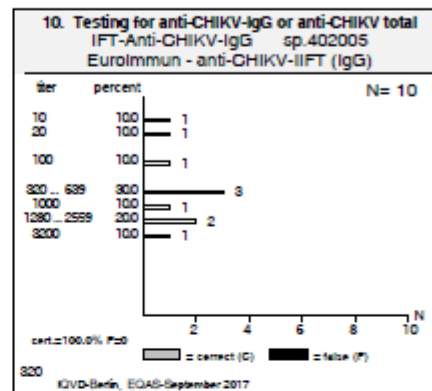
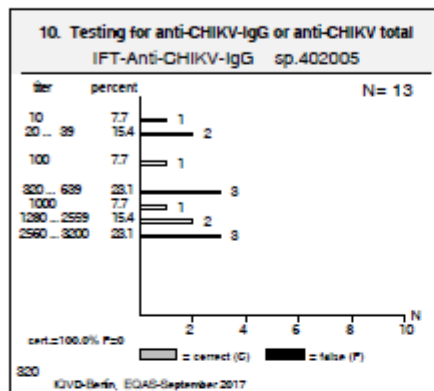
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Virusimmunology Chikungunya virus (402) September 2017  
sample 402005 , anti-CHIKV-IgG or anti-CHIKV total : positive

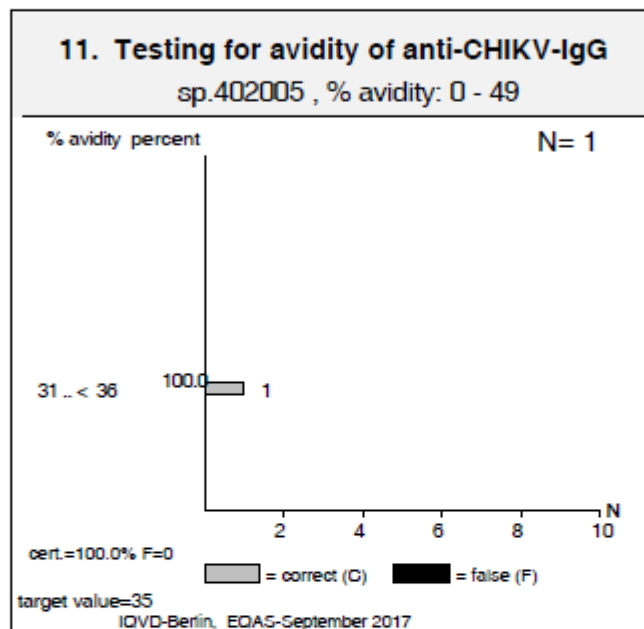


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Virusimmunology Chikungunya virus (402) September 2017  
sample 402005, anti-CHIKV-IgG or anti-CHIKV total : positive

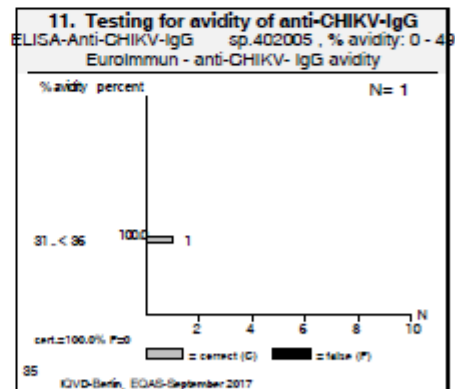
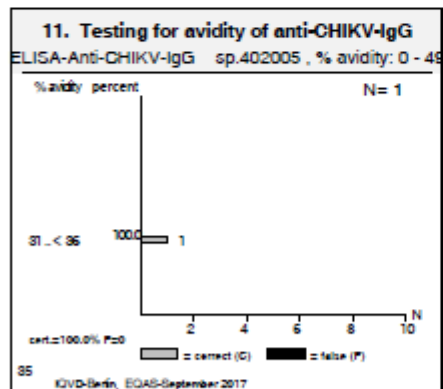
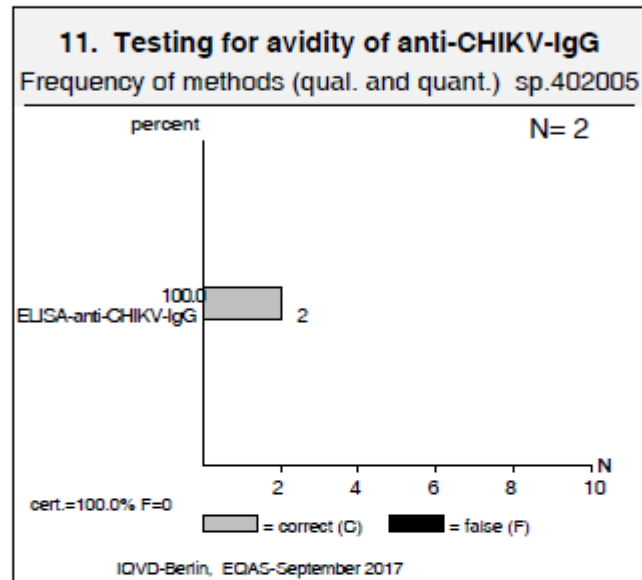


Virusimmunology Chikungunya virus (402) September 2017  
sample 402005 , avidity of anti-CHIKV-IgG : low , % avidity: 0 - 49



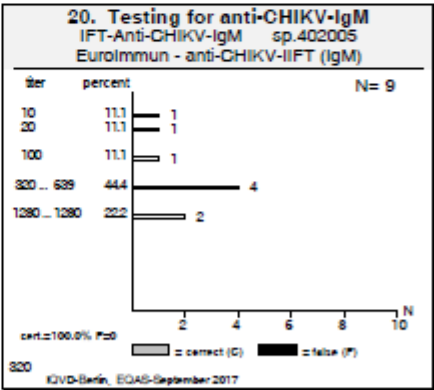
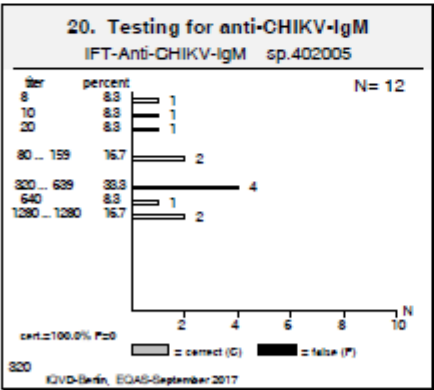
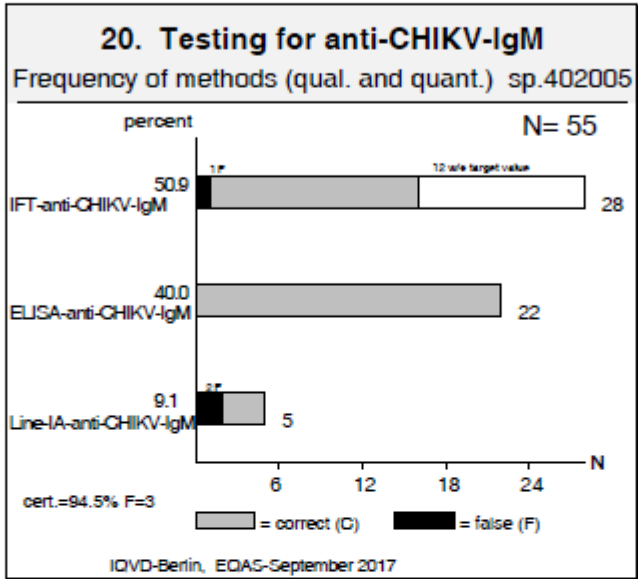
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Virusimmunology Chikungunya virus (402) September 2017  
 sample 402005 , avidity of anti-CHIKV-IgG : low , % avidity: 0 - 49



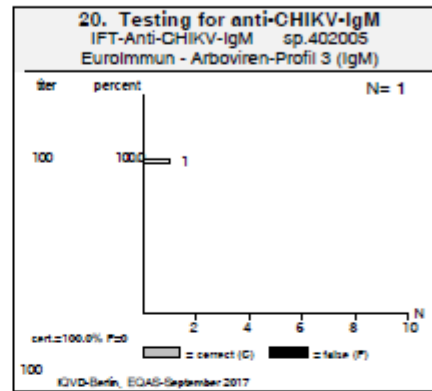
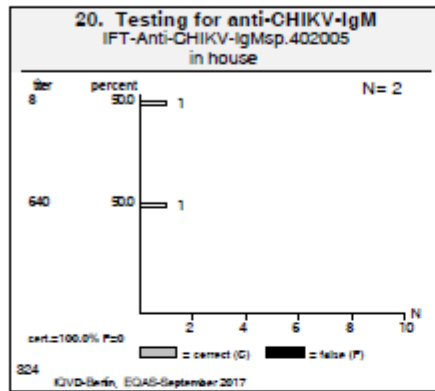
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Virusimmunology Chikungunya virus (402) September 2017  
sample 402005, anti-CHIKV-IgM : positive



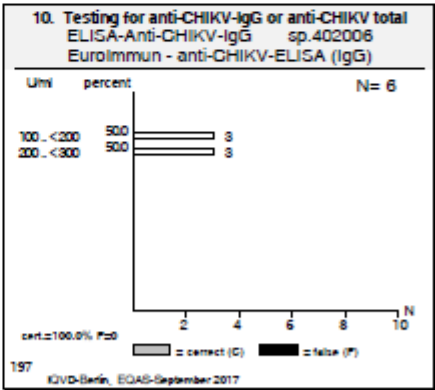
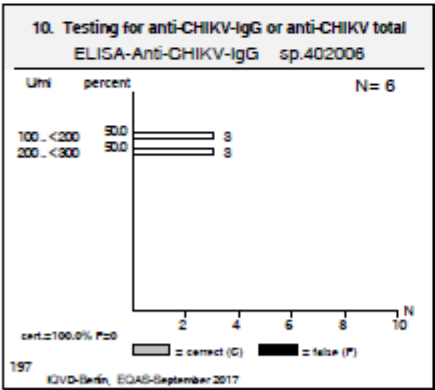
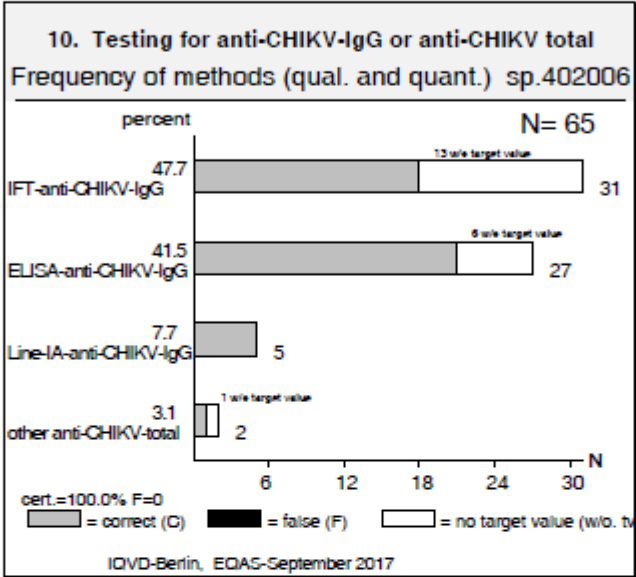
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Virusimmunology Chikungunya virus (402) September 2017  
 sample 402005, anti-CHIKV-IgM : positive



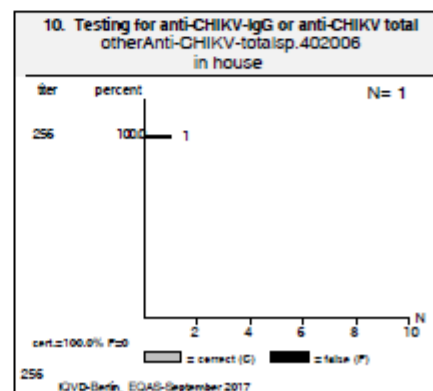
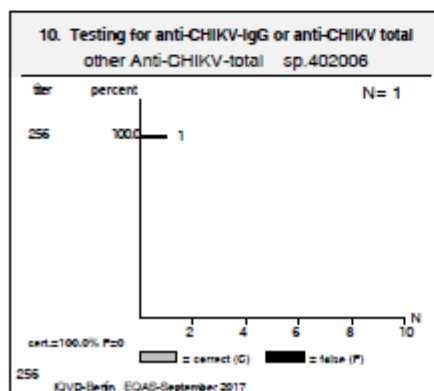
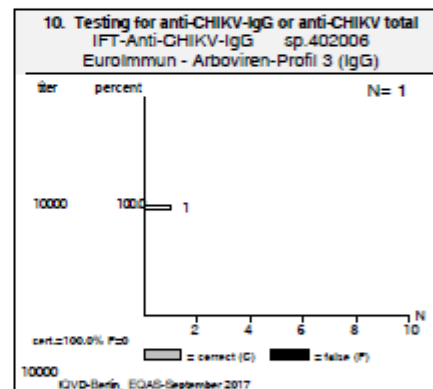
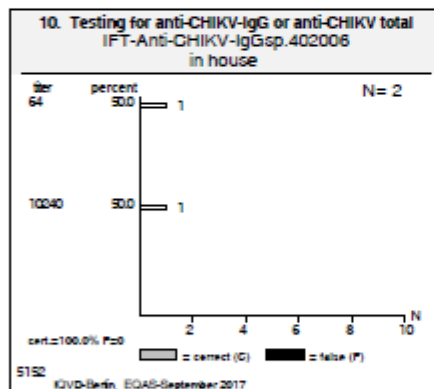
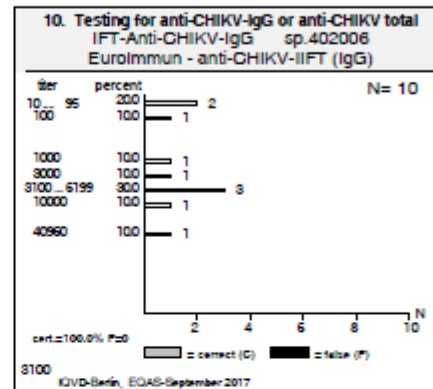
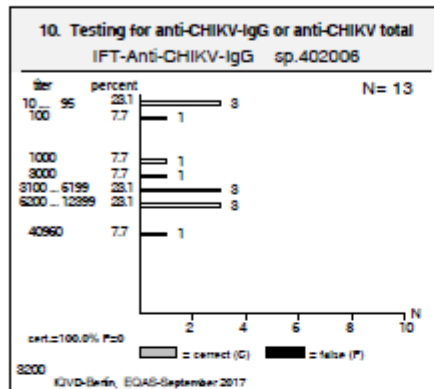
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Virusimmunology Chikungunya virus (402) September 2017  
sample 402006 , anti-CHIKV-IgG or anti-CHIKV total : positive



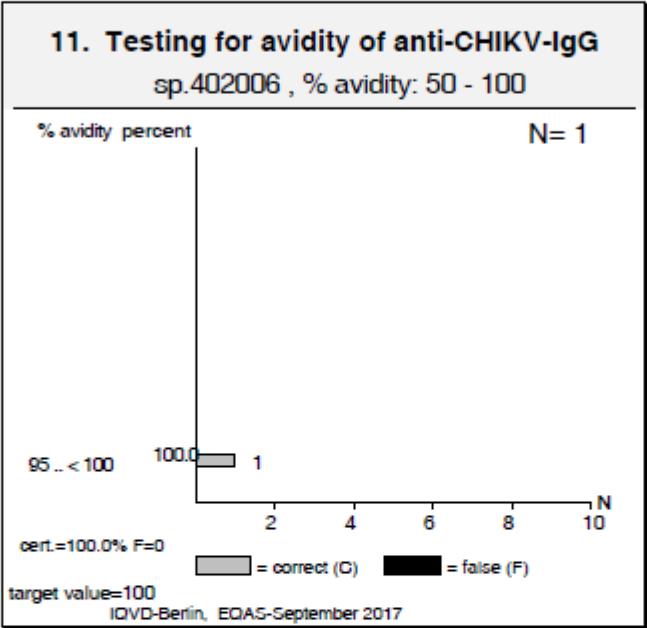
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Virusimmunology Chikungunya virus (402) September 2017  
sample 402006, anti-CHIKV-IgG or anti-CHIKV total : positive



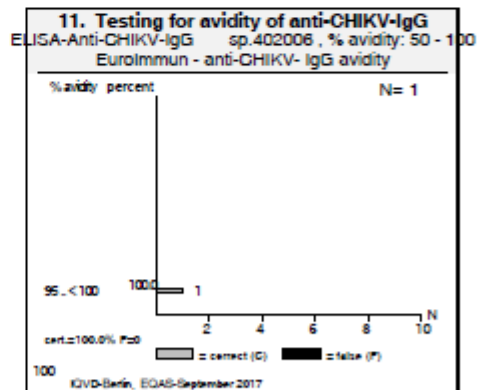
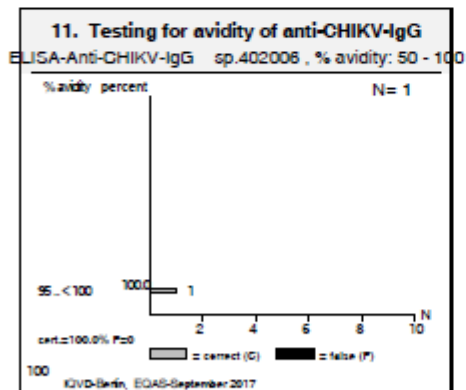
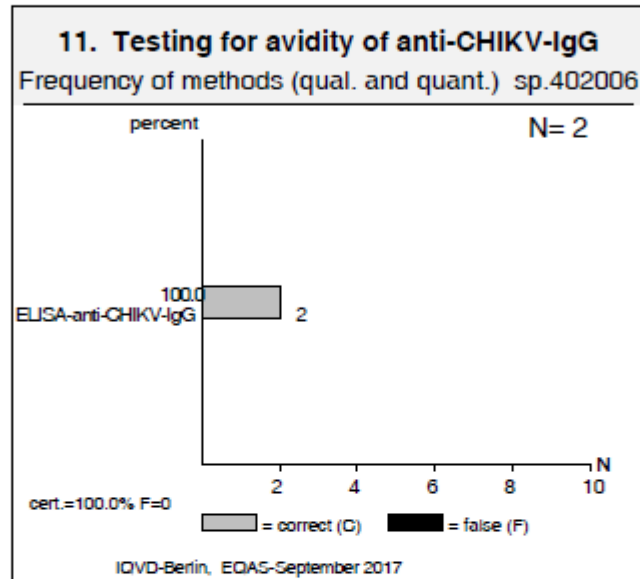
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Virusimmunology Chikungunya virus (402) September 2017  
sample 402006 , avidity of anti-CHIKV-IgG : high , % avidity: 50 - 100



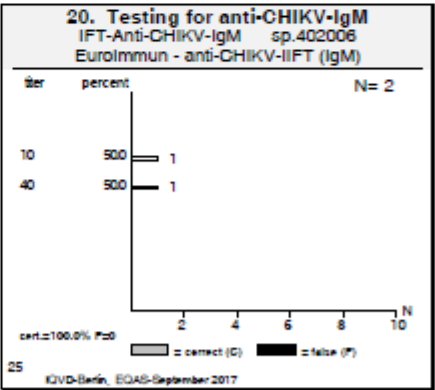
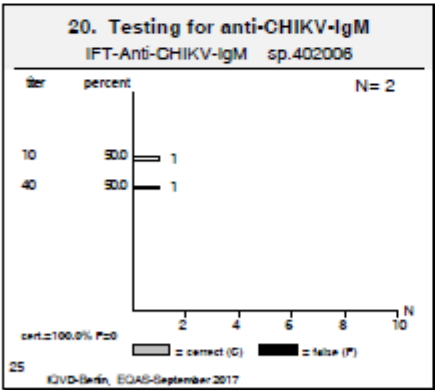
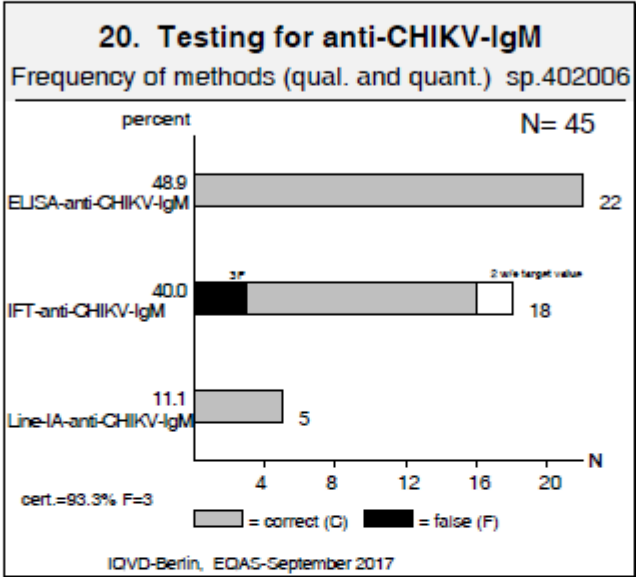
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Virusimmunology Chikungunya virus (402) September 2017  
 sample 402006 , avidity of anti-CHIKV-IgG : high , % avidity: 50 - 100



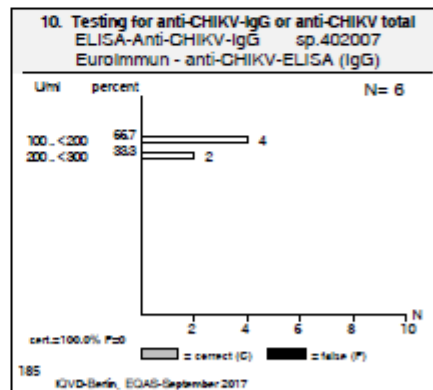
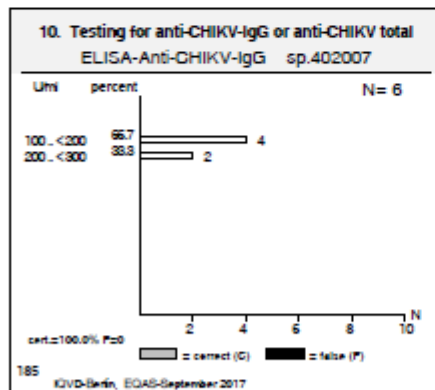
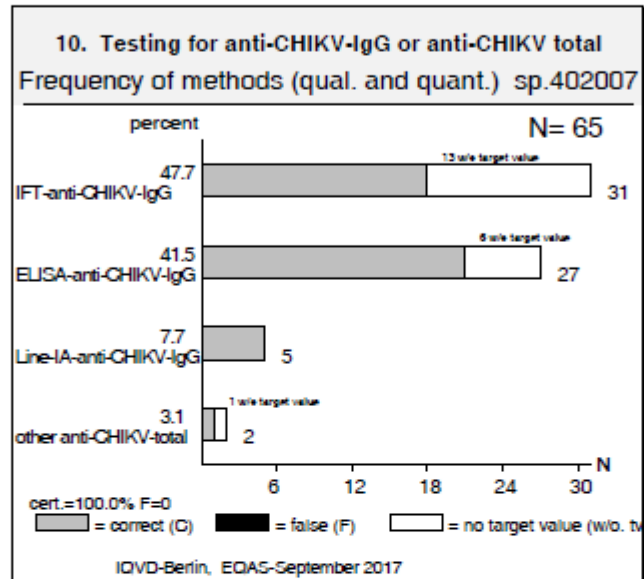
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Virusimmunology Chikungunya virus (402) September 2017  
sample 402006 , anti-CHIKV-IgM : negative, borderline



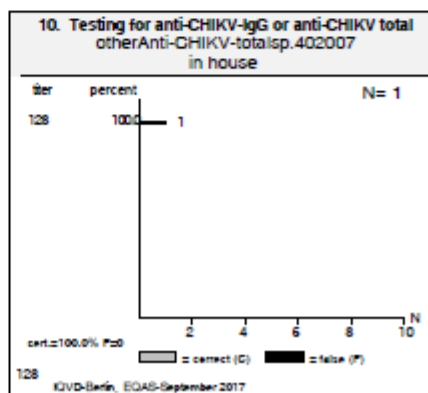
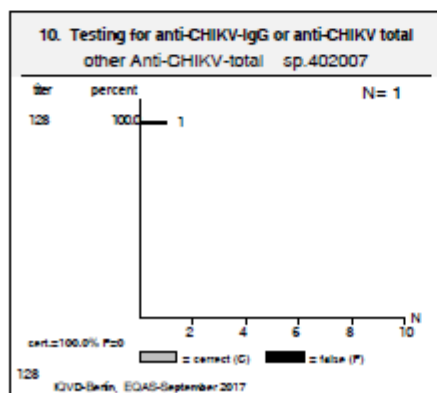
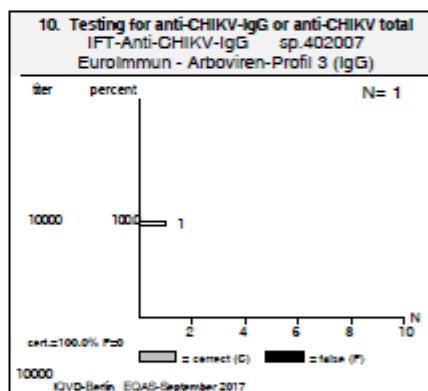
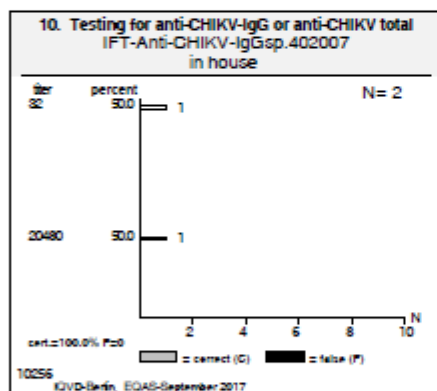
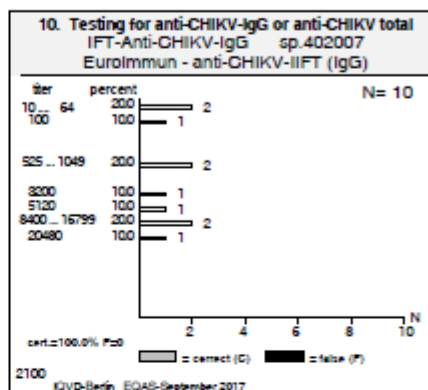
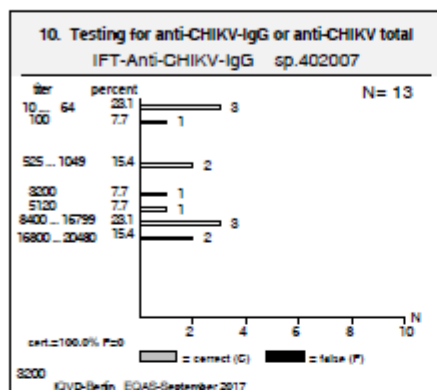
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Virusimmunology Chikungunya virus (402) September 2017  
sample 402007, anti-CHIKV-IgG or anti-CHIKV total : positive



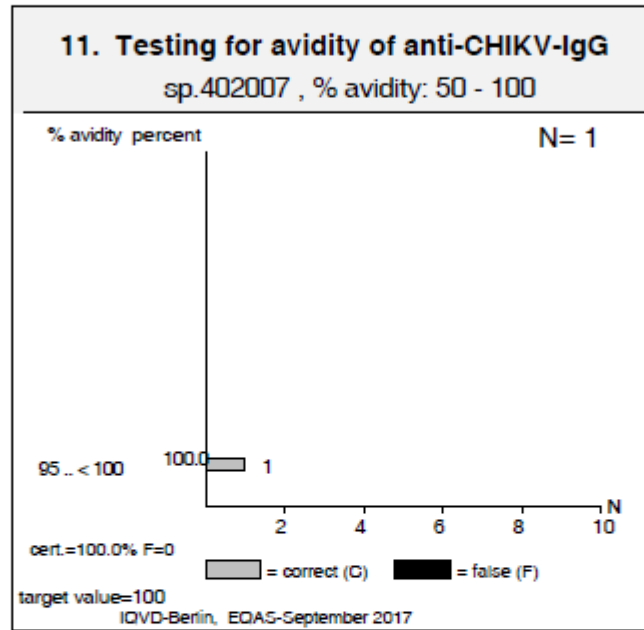
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Virusimmunology Chikungunya virus (402) September 2017  
sample 402007, anti-CHIKV-IgG or anti-CHIKV total : positive



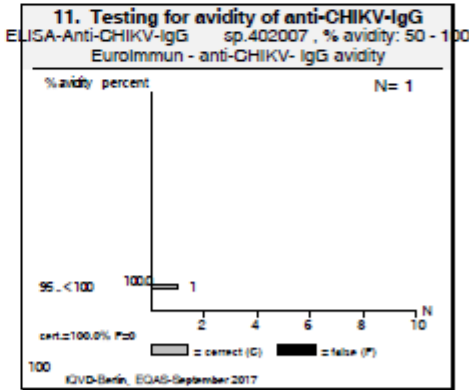
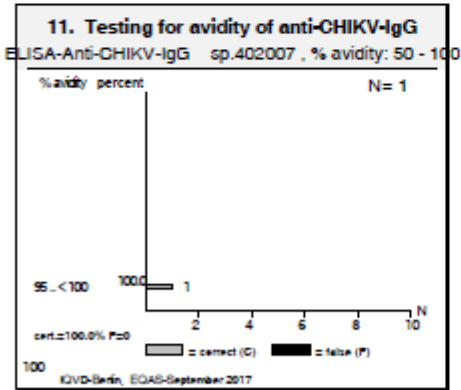
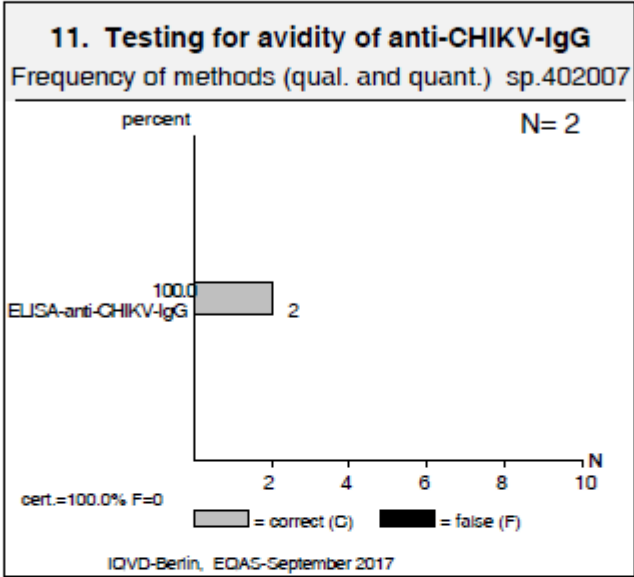
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Virusimmunology Chikungunya virus (402) September 2017  
 sample 402007, avidity of anti-CHIKV-IgG : high, % avidity: 50 - 100



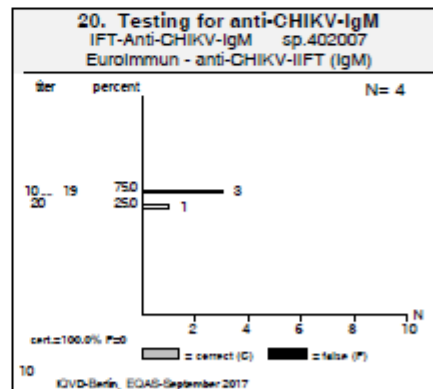
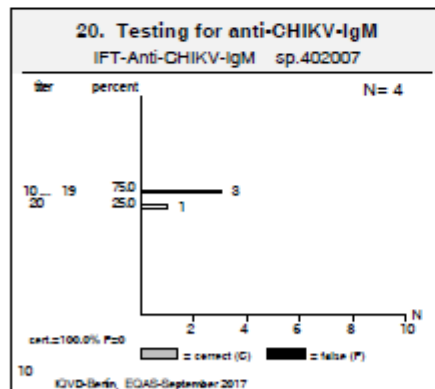
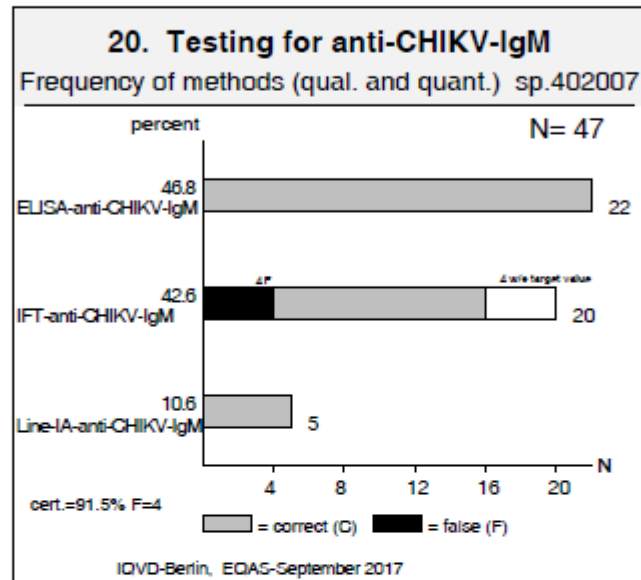
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Virusimmunology Chikungunya virus (402) September 2017  
sample 402007 , avidity of anti-CHIKV-IgG : high , % avidity: 50 - 100



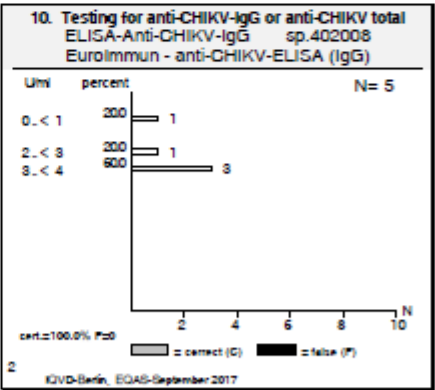
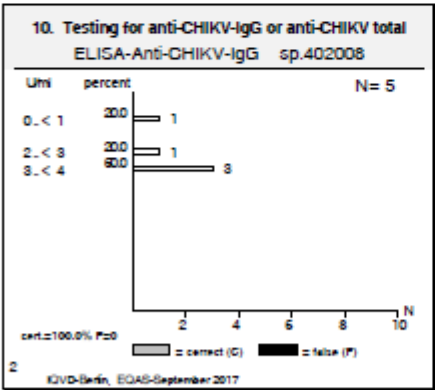
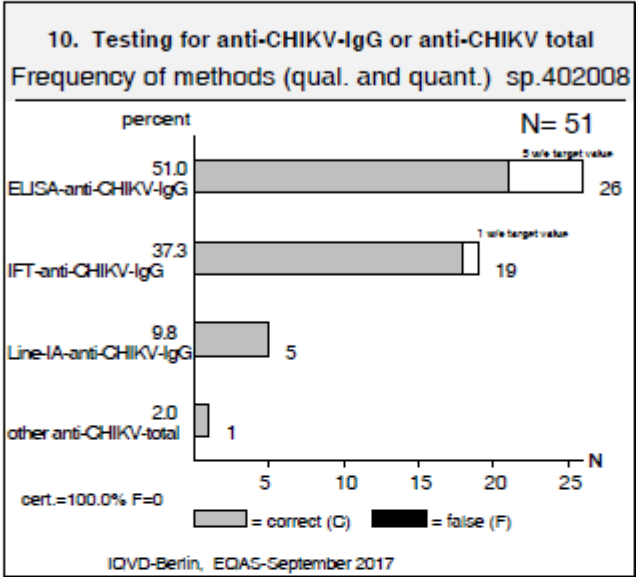
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Virusimmunology Chikungunya virus (402) September 2017  
 sample 402007, anti-CHIKV-IgM : negative, borderline



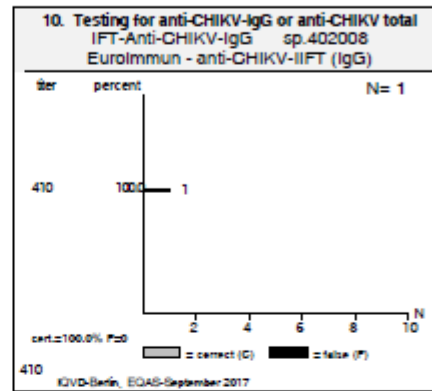
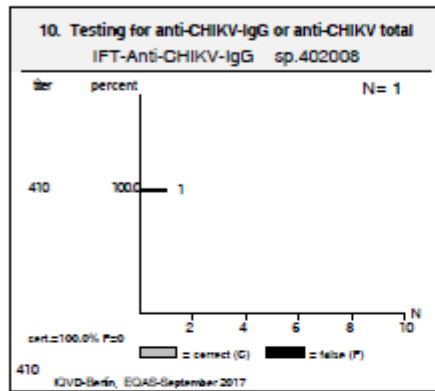
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sample 402008 , anti-CHIKV-IgG or anti-CHIKV total : negative



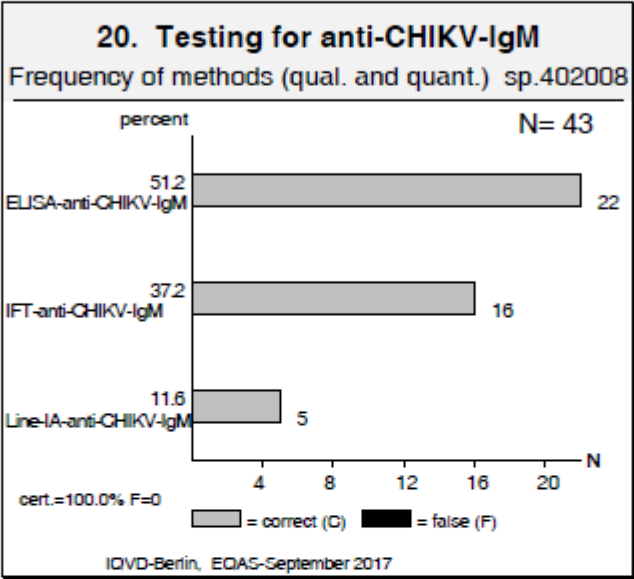
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Virusimmunology Chikungunya virus (402) September 2017  
 sample 402008, anti-CHIKV-IgG or anti-CHIKV total : negative



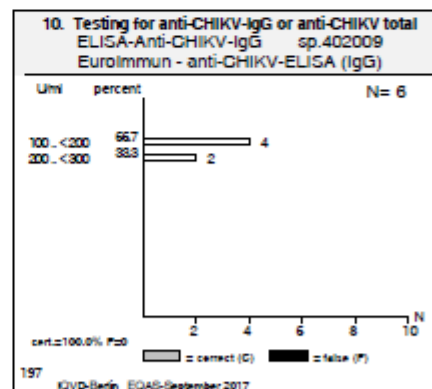
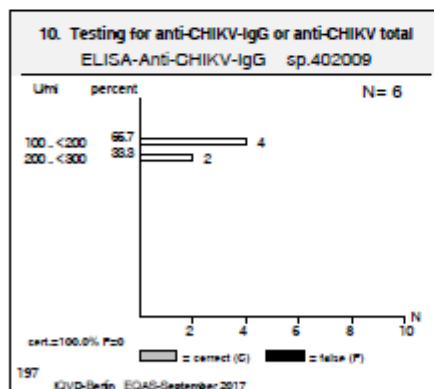
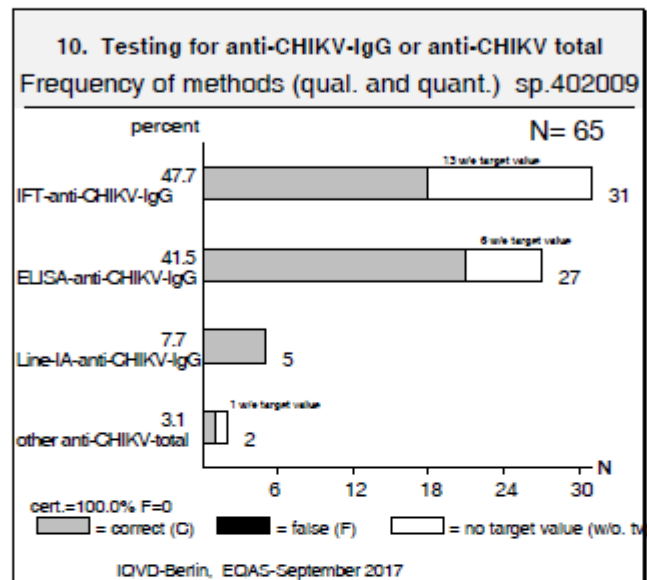
Gesellschaft zur Förderung der Qualitätssicherung in medizinischen Laboratorien e. V. (INSTAND)  
in cooperation with  
Deutsche Vereinigung zur Bekämpfung der Viruskrankheiten e. V. (DVV)  
Gesellschaft für Virologie e. V. (GfV)  
Deutsche Gesellschaft für Hygiene und Mikrobiologie e. V. (DGHM)

Virusimmunology Chikungunya virus (402) September 2017  
sample 402008 , anti-CHIKV-IgM : negative



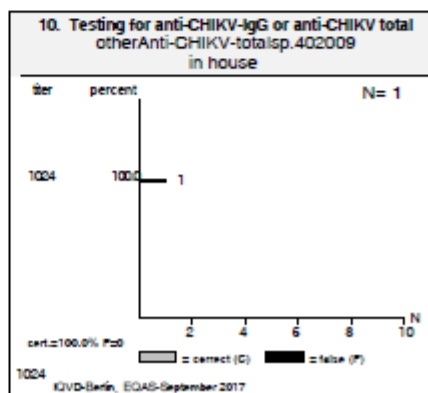
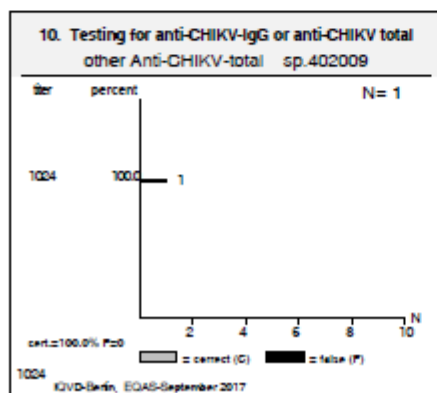
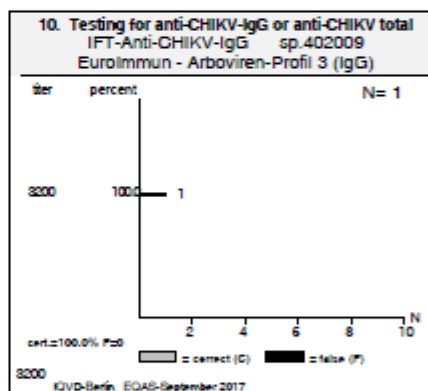
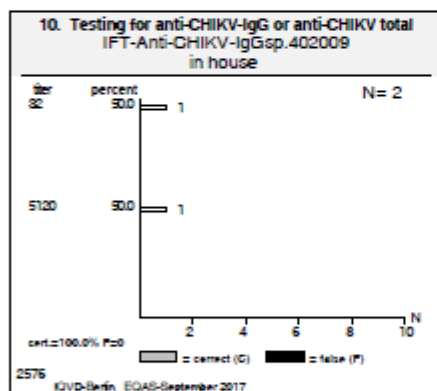
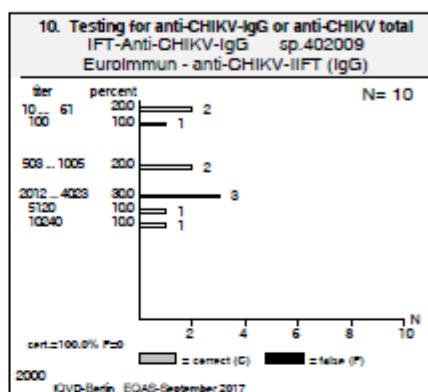
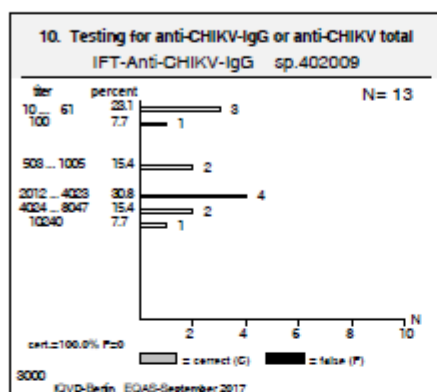
Gesellschaft zur Förderung der Qualitätssicherung in medizinischen Laboratorien e. V. (INISTAND)  
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Gesellschaft für Virologie e. V. (GfV)  
Deutsche Gesellschaft für Hygiene und Mikrobiologie e. V. (DGHM)

Virusimmunology Chikungunya virus (402) September 2017  
sample 402009, anti-CHIKV-IgG or anti-CHIKV total : positive



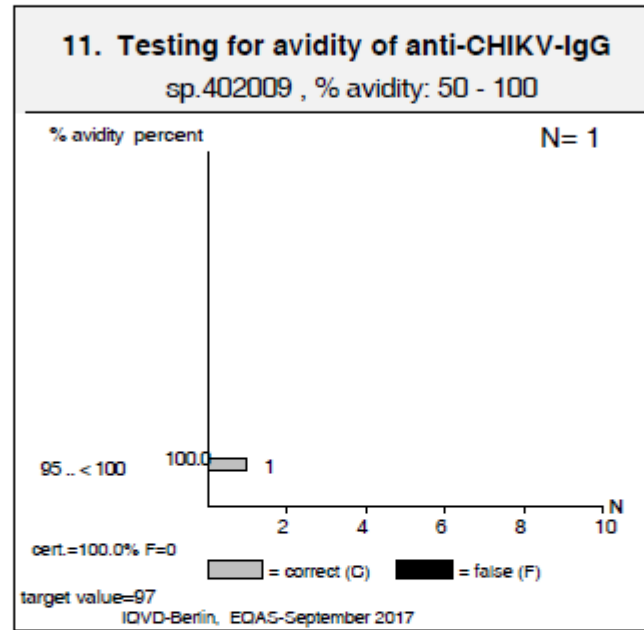
Gesellschaft zur Förderung der Qualitätssicherung in medizinischen Laboratorien e. V. (INSTAND)  
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Deutsche Gesellschaft für Hygiene und Mikrobiologie e. V. (DGHM)

Virusimmunology Chikungunya virus (402) September 2017  
sample 402009, anti-CHIKV-IgG or anti-CHIKV total : positive



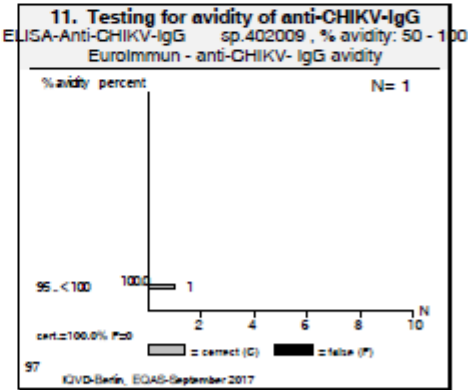
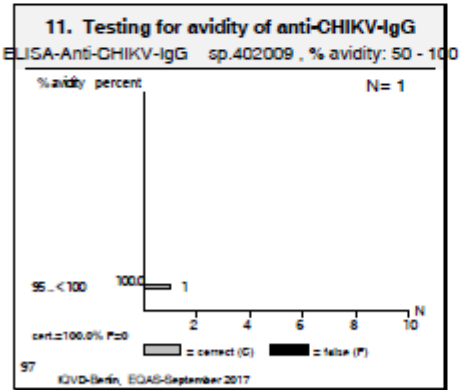
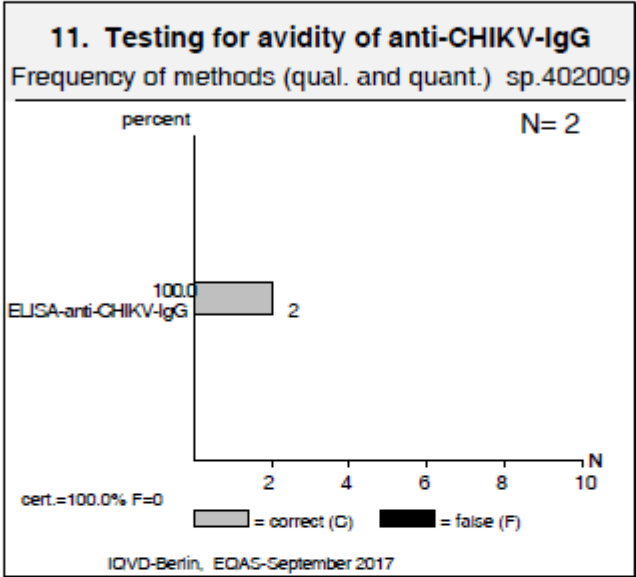
Gesellschaft zur Förderung der Qualitätssicherung in medizinischen Laboratorien e. V. (INSTAND)  
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 Deutsche Gesellschaft für Hygiene und Mikrobiologie e. V. (DGHM)

Virusimmunology Chikungunya virus (402) September 2017  
 sample 402009 , avidity of anti-CHIKV-IgG : high , % avidity: 50 - 100



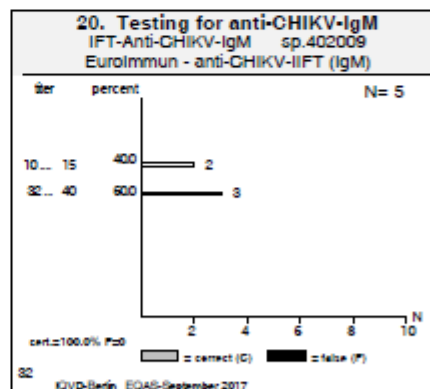
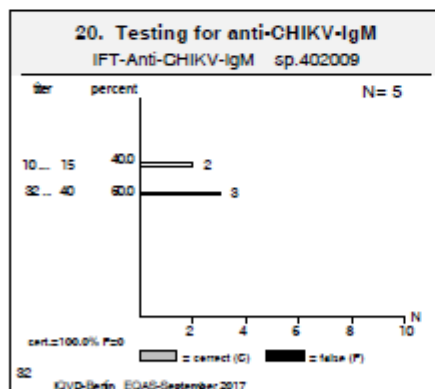
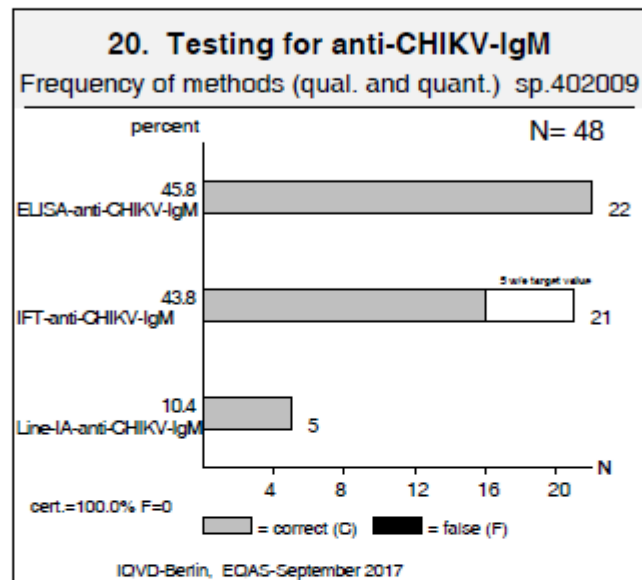
Gesellschaft zur Förderung der Qualitätssicherung in medizinischen Laboratorien e. V. (INSTAND)  
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Virusimmunology Chikungunya virus (402) September 2017  
sample 402009 , avidity of anti-CHIKV-IgG : high , % avidity: 50 - 100



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 Gesellschaft für Virologie e. V. (GfV)  
 Deutsche Gesellschaft für Hygiene und Mikrobiologie e. V. (DGHM)

Virusimmunology Chikungunya virus (402) September 2017  
 sample 402009, anti-CHIKV-IgM :



## Appendix 3. Study protocol

Bundesinstitut für Impfstoffe und biomedizinische Arzneimittel  
Federal Institute for Vaccines and Biomedicines

Paul-Ehrlich-Institut 

### Study Protocol

#### **Collaborative Study to Evaluate a Candidate World Health Organization International Standard for Chikungunya Virus Antibodies**

##### Background

Chikungunya virus (CHIKV) is an arthropod-transmitted virus of the *Togaviridae* family, genus Alphavirus. CHIKV causes chikungunya fever which is characterized by an acute infection with high fever, rash, myalgia and polyarthralgia. Currently, no international standard (IS) for the determination of the potency of antibodies developed following infection with CHIKV is available. Anti-CHIKV antibody potency determination is important to determine the analytical sensitivity of serological assays, to enable discrimination of cross-reactive CHIKV antibodies from other anti-alphavirus antibodies, to attempt to define clinical parameters such as protective levels of antibody and to in general achieve better comparability of results obtained in different laboratories.

The Paul-Ehrlich-Institut (PEI), Federal Institute for Vaccines and Biomedicines, as a WHO Collaborating Centre for both the quality assurance of blood products and *in vitro* diagnostic devices and for the standardization and evaluation of vaccines, developed two candidate anti-CHIKV antibody preparations for testing and comparison across assays and laboratories to evaluate its suitability as a WHOIS.

##### Objective

The study will evaluate the potency of the two proposed candidate materials for a WHO IS for anti-CHIKV antibodies in parallel with other antibody preparations using assays in routine use in the participants' laboratories. The aim is to select the most suitable candidate standard for assay harmonization and agree an internationally assigned unitage for the candidate standard following statistical analysis of the study data at the PEI.

##### Samples

The candidate materials are plasma pools obtained from infected patients:

1. Plasma obtained from a convalescent German patient with a past Chikungunya virus infection.
2. Plasma pool obtained from Puerto Rican blood donors with a past Chikungunya virus infection.

Additional liquid/frozen plasma samples from convalescent chikungunya patients and donors as well as negative controls are provided to test for commutability as well as specificity of assays used in the participating laboratories.

None of the lyophilized samples nor the liquid/frozen plasma samples have been heat-inactivated. Assays requiring heat inactivation should ensure that all samples are appropriately heat-inactivated.

All materials are not for administration to humans or animals. They are not for *in vitro* diagnostic use; they are for evaluation purposes only and should not be used to determine the validity of assays for anti-CHIKV antibodies. Eleven samples are provided and coded P1-P11. Three sets of samples are provided per assay – sufficient for three assay runs. A new set of samples should be used for each assay run. Laboratories that have indicated that they perform more than one type of assay have been sent additional samples for each respective assay.

#### Shipment and Storage of Samples

All samples are shipped on dry ice and should be stored at  $\leq -20^{\circ}\text{C}$ , upon receipt.

Participants are asked to confirm receipt of samples and to report any anomaly on the "Acknowledgment of Receipt" form accompanying the plasma samples.

#### Study Protocol

Participants are requested to use their preferred methods indicated in the PEI questionnaire for the detection of anti-CHIKV antibodies according to the listed requirements:

- **Please note, that the provided liquid/frozen plasma materials were NOT heat-inactivated**
- Please perform three independent assays for anti-CHIKV antibodies on three different days. Each Laboratory will receive 3 sets of samples for 3 independent test repeats. If more than one method is to be performed, additional sets of samples have been provided.
- **The lyophilized samples should be reconstituted in 0.5 mL of sterile, molecular-grade water. Use a fresh vial for each assay run.**
- Include all samples in each assay to allow for comparison of the antibody potency. If this is not feasible, please record which samples were tested concurrently.
- Please prepare a series of dilutions for samples P1 – P11 in each independent. If possible, at least two independent replicate series of dilutions (not two samplings from a single dilution series) should be assayed.
- For the dilution series it is important to cover the range of quantifiable antibody concentrations (at least 4 steps) including at least one step beyond the end-point dilution. For experiment 1, we suggested a starting dilution which is noted in the reporting sheet. Please adjust dilutions in the subsequent assays if needed and record the change in the Excel reporting sheet.
- **Please only perform testing for IgG.**
- An Excel reporting sheet has been provided for recording all essential information. Only use the reporting sheet for recording the results to help perform the statistical analysis at PEI.
- Please use the Excel reporting sheet to document the results for each dilution. We will use the raw data for statistical analysis. For neutralization assays, please calculate the neutralization titer, if possible. However, please also provide with the raw values.
- Note the cut-off value in the designated field and include for each sample dilution tested if it is considered positive or negative according to the assay specifications.
- For better comparability, please specify in the reporting sheet the conditions of the assay used (diluent type, virus strain and titer, incubation time, cell seeding conditions used for the specific assay) under "Remarks"
- Please note all deviations from the assay protocol in the Excel reporting sheets.

#### Reporting of Results

Participants are requested to report the results as soon as possible after receipt of the plasma samples, latest on 31<sup>st</sup> December 2019. All completed forms (Participant Questionnaire and **Results Reporting form**) should be returned by e-mail to [Sally.Baylis@pei.de](mailto:Sally.Baylis@pei.de) and [Hanna.Roth@pei.de](mailto:Hanna.Roth@pei.de). Please advise the study organizers if you are unable to complete the study in the prescribed time frame.

#### Data Analysis

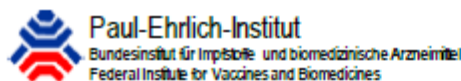
The confidentiality of each laboratory will be ensured with each participant being anonymous to the other laboratories. All data resulting from the collaborative study will be analyzed at PEI by an experienced biometrician. The analysis will assess the potency of all provided materials relative to each other and the sensitivities of the different assay methods.

Participants will receive a copy of the drafted study report including data analysis, proposed conclusions and recommendations to the WHO ECBS on the use, selection and ~~unitage~~ of the WHO IS for anti-CHIKV antibodies. It is intended that the finalized report will be submitted to ECBS in 2020 for review by the Expert Committee on Biological Standardization and decision of establishment of one of the candidate ISs.

Participation in the WHO collaborative study is further dependent on the following conditions:

- Data obtained in the assays are not to be published or cited before formal decision of the ECBS about the WHO IS for anti-CHIKV antibody and without permission of the study organizer.
- Participants should not use the study materials for purposes other than conducting the collaborative study. Some of the study materials are covered by material transfer agreements which permit the use of the materials only in the context of the collaborative study and the samples should not be used for independent research.
- Individual participant's data will be coded and reported "blind" to other participants during the preparation of the study report, and also subsequent publications.
- Participants accept responsibility for safe handling and disposal of the materials provided.

## Appendix 4. Draft instructions for use



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



1<sup>st</sup> World Health Organization International Standard for  
Anti-Chikungunya Virus Immunoglobulin G

PE code 1602/19

(Version 3.0, March 2023)

#### 1. INTENDED USE

The World Health Organization (WHO) International Standard for anti-chikungunya virus (CHIKV) immunoglobulin G (human) was developed from a pool of three plasma donations from a convalescent chikungunya patient and evaluated in an international collaborative study. The principal use of the International Standard is for the calibration and harmonization of serological assays for the quantification of anti-CHIKV neutralizing IgG. The standard can be used as reagent for control for immunoassay performance. Further details of the collaborative study are available in the report (1).

#### 2. UNITAGE

The International Standard has been assigned a unitage of 1,000 International Units (IU)/ml after reconstitution in 0.5 ml sterile, cell culture grade water. The IU relates to antibody (IgG) neutralization activity for virus neutralization assays. For other types of immunoassay, the standard may be used as a control reagent (with no assigned unitage) following dilution (dilution to be determined by user and is assay-dependent). The application of the standard is applicable to detection of specific CHIKV antigenic targets such as E1; E1/E2, whole virus – it should not be used to compare between groups of assays of different specificity.

#### 3. CONTENTS

Each vial contains the freeze-dried residue of 0.5 ml of human plasma.

#### 4. CAUTION

##### THIS PREPARATION IS NOT FOR ADMINISTRATION TO HUMANS.

As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. The plasma has been found negative for hepatitis B virus, hepatitis C virus as well as human immunodeficiency virus by NAT testing.

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. For virus neutralization assays, the reconstituted material should be heat-inactivated prior to use.

#### 6. STABILITY

Paul-Ehrlich-Institut  
Paul-Ehrlich-Str. 61-69  
63226 Langen, Germany

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 expiry 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 (1). 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

1. Collaborative study to evaluate a candidate World Health Organization International Standard for antibodies to chikungunya virus. 2022. WHO Expert Committee on Biological Standardization. WHO/BS/2022.2432.

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.

#### 8. ACKNOWLEDGEMENTS

We are grateful to the anonymous donor who provided plasma and to all collaborative study participants.

#### 9. FURTHER INFORMATION

Further information for this material can be obtained as follows: [whocclvd@pei.de](mailto:whocclvd@pei.de) or  
WHO Biological Reference Preparations:  
<http://www.who.int/biologicals/en/>

#### 10. 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 [whocclvd@pei.de](mailto:whocclvd@pei.de).

#### 11. CITATION

In any circumstance where the recipient publishes a reference to PEI materials, it is important that the correct name of the preparation, the PEI code number, the name and the address of PEI are cited correctly.

Email: [whocclvd@pei.de](mailto:whocclvd@pei.de)  
Web: <http://www.pei.de>



## 12. MATERIAL SAFETY SHEET

Physical properties (at room temperature)		
Physical appearance	Lyophilized powder	
Fire hazard	None	
Chemical properties		
Stable	Yes	Corrosive: No
Hygroscopic	No	Oxidizing: No
Flammable	No	Irritant: No
Other (specify)	Material of human origin	
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.		

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.

## 13. 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.

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Paul-Ehrlich-Str. 61-69  
63226 Langen, Germany

Email: [whocclva@pei.de](mailto:whocclva@pei.de)  
Web: <http://www.pei.de>