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Guidelines for plaque reduction neutralization testing of human antibodies to dengue viruses

Immunization, Vaccines and Biologicals

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World Health Organization
Department of Immunization, Vaccines and Biologicals
CH-1211 Geneva 27, Switzerland
• *Fax:* + 41 22 791 4227 • *Email:* vaccines@who.int •

Contents

Abbreviations and acronyms

ADE	Antibody dependent enhancement
BSL	Biological safety level
CMC	Carboxymethyl cellulose
CPE	Cytopathic effect
DEN	Dengue
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue haemorrhagic fever
DME	Dulbecco's modified MEM
DSS	Dengue shock syndrome
ELISA	Enzyme-linked immunosorbent assay
LNI	Log serum neutralization index
MC	Methylcellulose
MEM	Minimal essential medium
PDVI	Paediatric dengue vaccine initiative
PFU	Plaque forming unit
PRNT	Plaque reduction neutralization test
RNA	Ribonucleic acid
WN	West nile
WNV	West nile virus

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Preface and background

Through the Steering Committee on Dengue and other Flavivirus Vaccines, the World Health Organization (WHO) has had a long-standing commitment to facilitate and to guide research and development of vaccines for medically important flaviviruses. Recently, the Paediatric Dengue Vaccine Initiative (PDVI) was formed to accelerate the development, testing, and introduction of dengue (DEN) vaccines worldwide, partnering with WHO in this important public health effort. There are now a variety of DEN vaccines in various stages of the developmental pipeline. In an attempt to make inter-laboratory information more directly comparable, WHO and PDVI initiated a program to harmonize the procedures used for the plaque-reduction neutralization test (PRNT). The PRNT is the most common assay used to measure neutralizing antibody. The presence of antibody is believed to be most relevant for determining protective anti-DEN virus (DENV) immunity. While other neutralizing antibody assays are being considered for use in large scale vaccine field trials, the PRNT is still considered to be the laboratory standard against which other neutralizing antibody assays should be compared (Martin et al., 2006; Vorndam and Beltran, 2002). The need for PRNT harmonization has been identified at several consultations of WHO and PDVI.

In addition to these meetings, WHO has conducted a collaborative study with the aim of establishing a reference panel of dengue infection-immune sera. These data revealed considerable inter-laboratory variability due to different methods employed to perform and analyse the PRNT data, corroborating the need for establishing a more harmonized approach to DENV PRNT.

These guidelines are a direct result of all of these efforts.

Purpose and scope of guidelines

The levels of flavivirus-neutralizing antibody titters in the serum of vaccinated, or infection-immune individuals, correlates best with protection from subsequent virus infection. However, the lack of a standardized PRNT poses a hurdle for comparing data between vaccine trials and testing laboratories, and defining a threshold value to use as a true serological correlate of protection. The purpose of these guidelines is to:

1) provide scientific insight into the biology of the flavivirus neutralization test;

2) provide guidelines for test harmonization, and;

3) provide minimal recommendations for a test protocol for laboratories which might be interested in establishing the test. It must be remembered that the PRNT is, above all, a biological assay, and as such will always have a certain degree of inter-laboratory variation. Due to this and other lab-to-lab variability, such as specific vaccine design approaches, strains used for vaccine development, and other unique manufacturing requirements, these guidelines will not mandate the use of a single standardized protocol. Ultimately, the definition of a protective level of vaccine-induced neutralizing antibody by whatever suitable assay will need to be validated in a vaccine efficacy trial.

1. General Information

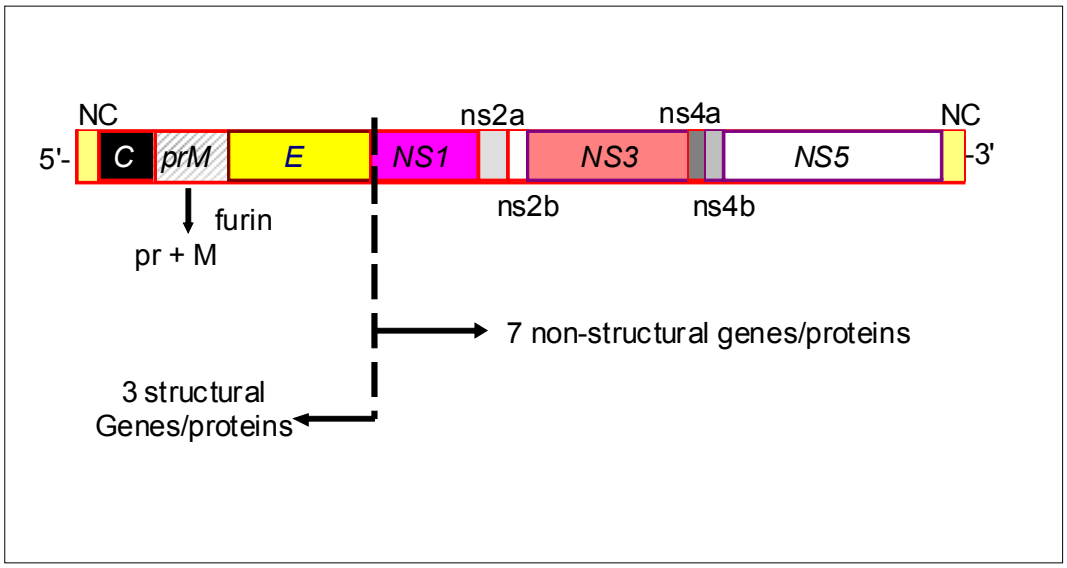
1.1 Introduction

The DENVs are members of the family Flaviviridae, genus flavivirus, are mosquito borne, and represent a major public health problem throughout the tropical world. The DENVs are a set of 4 different serotypes of viruses (DENV 1-4). Each of these DENVs is similar to one another, but serologically distinct enough that infection with one serotype will not protect against infection with another serotype. For this and other reasons to be discussed later in this document, an effective DENV vaccine should induce an immune response against the four serotypes. Although now somewhat dated, the review book on DEN compiled by Gubler and Kuno still serves as an excellent source for information on DEN (Gubler and Kuno, 1997).

Additionally, there are no laboratory animal models that reliably mimic human infection with DENV. The lack of a suitable animal model makes it difficult to assess protective capacities of vaccine candidates and correlates of protection in vivo. In the absence of correlates, the protective capacity of any vaccine candidate will be finally defined as its ability to protect humans from DENV infection. Laboratory studies with DENVs and other flaviviruses have indicated, however, that protection of small animals from virus infection is best correlated to levels of virus-neutralizing antibodies (An et al., 1999; Huang et al., 2003; Johnson and Roehrig, 1999; Kinney et al., 1997). Similar studies with DENV in sub-human primate models have confirmed these observations (Blaney et al., 2005; Guirakhoo et al., 2004; Robert Putnak et al., 2005; Whitehead et al., 2003). The virus PRNT remains the most widely accepted approach to measuring virus-neutralizing and protective antibodies. Newer assays measuring virus-neutralizing antibodies are being developed and will be briefly discussed later in this document.

All flaviviruses are simple positive-sense, single-stranded, RNA viruses, approximately 55 nm in diameter. The genome is approximately 11,000 base pairs long, with 5' capped and 3'-end usually not polyadenylated. The genome encodes 10 proteins in a single open-reading frame (Figure 1). There are three structural proteins encoded in the 5'-one third of the viral genome: the capsid (C) protein forms the nucleocapsid shell protecting the viral genome, and the premembrane (prM), and envelope (E) proteins, both virion surface proteins embedded in the virion envelope. Seven non-structural (NS) proteins are encoded in the 3'-two thirds of the viral genome: NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5. Each of the NS proteins has specific viral functions, but for the purpose of these guidelines, will not be discussed in further detail.

Figure 1: Organization of the flaviviral genome



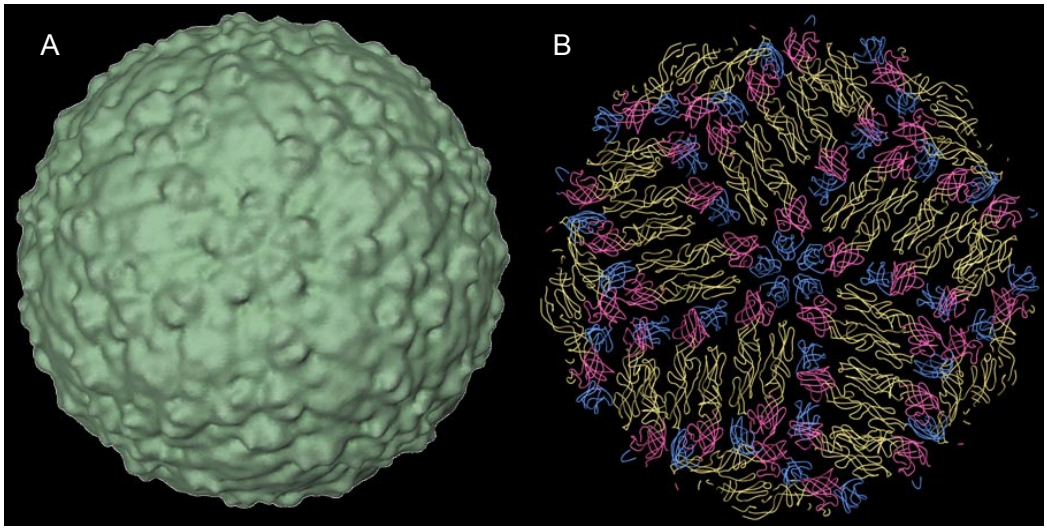
1.2 Flavivirus immunochemistry

Antibody-mediated virus neutralization is defined as the interaction of virus and antibody resulting in inactivation of virus such that it is no longer able to infect and replicate in cell cultures or animals. Virus-neutralizing antibody is the primary protective antibody type elicited by flaviviral vaccines. The flaviviral E glycoprotein is a Class II fusion protein and is responsible for viral attachment to host-cell receptors, and virus-mediated cell membrane fusion (Stiasny and Heinz, 2006). As such, it is the most important viral protein in regard to viral infectivity and, therefore, elicits essentially all virus-specific neutralizing antibody. It is primarily anti-E glycoprotein antibody that is measured in the current PRNT. Other non-E glycoprotein specific antibodies (e.g., anti-NS1 antibody) can demonstrate virus protective effects in vivo in small animal models, however these effects are not mediated by virion-antibody interactions (Calvert et al., 2006; Costa, Freire, and Alves, 2005; Costa et al., 2006; Schlesinger, Brandriss, and Walsh, 1987; Zhang et al., 1988).

Great progress in understanding the structure and function of the flaviviral E glycoprotein has recently been made (Kuhn et al., 2002; Modis et al., 2003; Modis et al., 2005; Zhang et al., 2003a; Zhang et al., 2003b). The E glycoprotein exists as 90 "head-to-tail" homodimers on the virion surface (Figure 2). The E glycoprotein monomer can be divided into three structural domains: DI, DII, and DIII (Figure 3). The DII (also known as the dimerization domain) is a long finger-like structure that contains the hydrophobic membrane-fusion sequence at its tip. In the homodimer, the fusion tip is protected during replication by a combination of DIII of the associated monomer, E protein glycosylation, and the prM protein (Rey et al., 1995). The DIII has been shown with DENV to be involved in virus attachment to Vero cells in culture (Crill and Roehrig, 2001). These binding characteristics have been confirmed using expressed DIII (Chu et al., 2005). The DI contains the E glycoprotein molecular hinge. As a Class II fusion protein, the E glycoprotein can undergo an acid-catalysed oligomeric reorganization to a fusogenic homotrimer (Bressanelli et al., 2004; Modis et al., 2004; Stiasny et al.,

2004; Zhang et al., 2004). It is believed that this event occurs in the endosome, allowing the viral nucleocapsid to escape into the cytoplasm and initiate RNA and protein synthesis. Similarly, it is believed that the prM protein functions as a chaperone protein for the E glycoprotein during viral maturation, helping maintain the E glycoprotein structure until viral morphogenesis is complete and the virion escapes the acidic exocytic vesicles (Stiasny and Heinz, 2006).

Figure 2: Structure of the flavivirion.



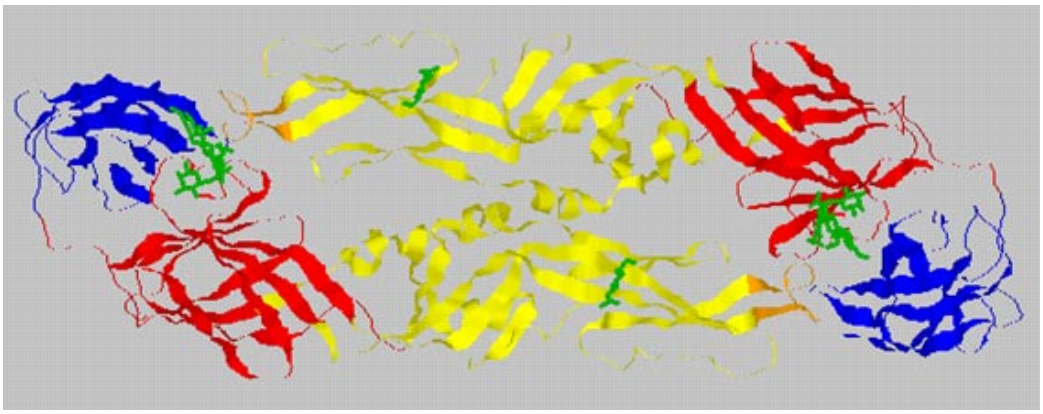
Cryoelectron microscopic structure of the West Nile virus.

Panel A, surface shaded view of the West Nile virion looking down the five-fold axis.

Panel B, C-alpha trace showing the fitted E protein heterodimers looking down the 5-fold axis. Structural domains of monomers identified by colour: red, Domain I; yellow, Domain II; blue, domain III.

Courtesy of Baerbel Kaufmann and Richard Kuhn, Purdue University.

Figure 3: Structure of the E glycoprotein homodimer



Domains I, II, and III, are colour coded red, yellow, and blue. Asparagine -linked carbohydrates are shown in green. The fusion tip is shown in orange.

The structural domains I, II, and III, map to previously defined antigenic domains; C, A, and B, respectively (Roehrig, 2003; Roehrig, Bolin, and Kelly, 1998). Antibodies to both DII and DIII have been shown to neutralize virus. Anti-DIII antibodies tend to be powerful neutralizing antibodies, and be more virus type-specific. Anti-DII antibodies are more virus cross-reactive, and while they can neutralize virus infectivity, they are usually less potent than anti-DIII antibodies (Crill and Chang, 2004). Two mechanisms of flaviviral neutralization (blocking attachment of virus to cells, and blocking the virus fusion process) have been identified (Crill and Roehrig, 2001; Gollins and Porterfield, 1986). It is not known at this time how many antibody molecules are needed to neutralize the infectivity of a single virion.

Some evidence suggests that the prM protein might also elicit virus-neutralizing antibodies (Kaufman et al., 1989). The ability of anti-prM antibodies to attach to the virion is directly related to the extent of "prM →M" processing, which is mediated by furin-like cell-associated enzymes and therefore is cell-type specific. As mentioned above, the purpose of the prM protein appears to be as a chaperone for the E glycoprotein during virion morphogenesis, and it does not function as an attachment protein, per se. It has been noted that DENVs grown in invertebrate cell culture often have a large amount of prM protein in the extracellular virion which can reduce viral infectivity and change epitope expression (Elshuber et al., 2003; Guirakhoo, Bolin, and Roehrig, 1992; Randolph, Winkler, and Stollar, 1990). Whether or not this phenomenon occurs in the mosquito vector has not been determined.

1.3 Dengue virus immunology

The adaptive, protective immune response to DENV infection is driven by the presence or absence of virus neutralizing antibodies and T-cell responses involved in helping antibody synthesis. Upon infection DENV elicits IgM, IgG, and IgA antibody responses. The IgM response begins early, frequently before onset of symptoms. IgM is usually detectable in serologic assays by 7-8 days post-onset of symptoms (Sa-Ngasang et al., 2006). IgA antibodies are also detectable and have half-lives similar to IgM (Groen et al., 1999). IgG antibodies are detectable soon after infection and are maintained for years (Halstead, 1974). Infection with any given DENV serotype results in immunity to that particular serotype, however there is no long-term protection against infection with any of the other three DEN serotype viruses (Sabin, 1959).

Re-infection of individuals with a distinct second or third serotype of DENV may result in dengue fever, or a more severe infection resulting in dengue hemorrhagic fever (DHF) and/or dengue shock syndrome (DSS). There have been a number of hypotheses presented that might explain the more severe manifestation of disease following secondary DENV infections. Detailed discussions on these mechanisms are beyond the scope of this document. Regardless of the hypothesized mechanism, pre-existing DENV-reactive antibody appears to be one factor involved in mediating DHF/DSS (Burke and Kliks, 2006; Halstead, Chow, and Marchette, 1973; Halstead and O'Rourke, 1977a; Halstead and O'Rourke, 1977b; Halstead, O'Rourke, and Allison, 1977; Halstead and Papaevangelou, 1980; Kliks, 1990; Kliks et al., 1988; Kliks et al., 1989; Kouri et al., 1989). The antibodies that are most likely involved are those that cross-react with, but do not neutralize multiple serotypes

of DENV. Non-neutralized DENV-antibody complexes can be ushered into DENV-susceptible cells via surface expressed Fc-receptors (Brown et al., 2006; Littaua, Kurane, and Ennis, 1990; Rodrigo et al., 2006; Schlesinger and Chapman, 1999). This phenomenon is known as antibody-dependent enhancement (ADE) of DENV replication.

The concept of ADE of DENV infections has been studied for a number of years, and remains a concern for vaccine developers. While the biological relevance of in vitro ADE remains ill-defined, our understanding of the antibody specificities that lead to protection or enhancement is improving. It is known that antibodies elicited by DIII of the E glycoprotein are more virus-type specific and neutralizing. Because of their high virus-neutralizing potential, these antibodies are highly protective in animal models of infection. On the other hand, antibodies elicited by either DI or DII are more cross-reactive among viruses, and demonstrate lower or no virus-neutralizing capacity. Recent evidence from West Nile virus (WNV) primary-infected humans, whose lymphocytes were used to prepare human monoclonal antibodies, indicates that the early antibody response may be directed towards DII (Throsby et al., 2006). If the early antibody response to DENV is similar, then the primary humoral response will likely consist of cross-reactive antibodies, non-neutralizing antibodies. It is also possible that upon secondary infection with a different DENV serotype, there will be a rapid memory response that consists of DENV-cross reactive antibodies due to epitopes shared between DENV serotypes and the abundance of memory B-cells specific for DI/DII cross-reactive epitopes. The theoretical possibility of ADE in post-vaccinal DENV infections resulting in DHF/DSS dictates that any vaccine candidate should best elicit only serotype-specific virus-neutralizing antibodies against all four serotypes and therefore should be tetravalent, and include viruses from all four DENV serotypes. Because of our limited knowledge of how to create a DENV serotype-specific vaccine, vaccine developers are creating tetravalent DENV vaccines (Blaney et al., 2005; Guirakhoo et al., 2004; Kanesa-thasan et al., 2001; Kochel et al., 2002; Sun et al., 2003).

1.4 Methods to measure virus-antibody interactions

A variety of serological tests have been used to measure anti-flaviviral antibody. These tests include the hemagglutination-inhibition test, complement fixation test, fluorescent antibody test, enzyme-linked immunosorbent assay (ELISA), and PRNT. Each of these tests measures different antibody activities. Only the PRNT measures the biological parameter of in vitro virus neutralization and is the most serologically virus-specific test among flaviviruses, and serotype-specific test among dengue viruses, correlating well to serum levels of protection from virus infection. Newer tests measuring virus neutralization are being developed, but PRNT remains the laboratory standard against which these tests will need to be validated.

1.5 The plaque-reduction neutralization test (PRNT)

The virus PRNT assay was first described in the 1950s, and was later adapted to DENV (Russell et al., 1967). The basic design of the PRNT allows for virus-antibody interaction to occur in a test tube or microtiter plate, and then measuring antibody effects on viral infectivity by plating the mixture on virus-susceptible cells. The cells are overlaid with a semi-solid media that restricts spread of progeny virus. Each virus that initiates a productive infection produces a localized area of infection (a plaque), that can be detected in a variety of ways. Plaques are counted and compared back to the starting concentration of virus to determine the percent reduction in total virus infectivity. In the PRNT, the serum specimen being tested is usually subjected to serial dilutions prior to mixing with a standardized amount of virus. The concentration of virus is held constant such that, when added to susceptible cells and overlaid with semi-solid media, individual plaques can be discerned and counted. In this way, PRNT end-point titers can be calculated for each serum specimen at any selected percent reduction of virus activity. A disadvantage of the PRNT is that it is labour intensive and therefore not readily amenable to high throughput, making it difficult to use for large-scale surveillance and vaccine trials.

A variation of the virus-neutralization assay - the log serum neutralization index (LNI) - has also been used to evaluate the potential of a serum specimen to neutralize virus infectivity. In this test, the concentration of serum is held constant, while the challenge dose of virus is increased. The attractive aspect of this approach is that it may better measure the capacity of a serum to protect from virus infection because serum is usually being tested undiluted. Because the serum is used undiluted, routinely securing volumes of serum sufficient to perform this test is difficult and mitigates against general implementation of this assay. Additionally, even though a given undiluted serum specimen may neutralize a large amount of virus, e.g., 1 x 107 plaque-forming units (PFUs), in nature the virus dose delivered by a mosquito during a blood meal rarely exceeds 1 x 104 PFUs.

2. Test conditions for the PRNT with DENVs

2.1 Cell lines and growth of viral stocks

DENVs grow in many different cell lines derived from both vertebrate and invertebrate sources. The cell line used for virus amplification needs to be harmonized between laboratories. Because DENV vaccines are targeted for humans, and because the processing of the prM protein is altered in C6/36 cell grown DENV, a cell line of mammalian origin, such as the continuous African green monkey-derived Vero cells is recommended by WHO to produce seed viruses and for use in the DENV PRNT. To avoid the problems of cell-culture adaptation of virus, low-passage virus stock banks should be developed and employed for viral growth. While the number of virus passages deemed to be acceptable in the PRNT has never been experimentally determined, using viruses with no more than 5-10 cell culture passages beyond the banking passage should be attainable if the master and working seed stock approach is used.

The conditions for virus amplification/preparation should be standardized with the use of an appropriate multiplicity of infection (around 10-2 to 10-3). Virus should be harvested during the middle to end of the exponential phase of growth, to avoid high concentrations of inactivated particles in viral preparations. The supernatant should be clarified by low-speed centrifugation and stabilized with a cryo-protective agent (e.g., fetal calf serum >20%) before aliquoting and storage of virus at -70°C. Lyophilisation is an alternative for long-term storage. Virus working banks should be appropriately qualified for sterility (bacteria, fungi, and mycoplasma), potency (virus titration on the cell line used for PRNT), and if possible identity (using serotype-specific monoclonal antibodies).

2.2 Cell line for plaquing viruses

DENVs will plaque in a variety of cell lines. Currently two mammalian cell lines are used most widely, Vero cells or monkey kidney derived, LLC-MK2 cells. Each of these cell types have advantages, however, only Vero cells have been produced in a fashion that have allowed them to be used for production of live-attenuated vaccines and are therefore, recommended for use in the PRNT. Master and working cell banks should be prepared (as done for vaccine production). Doing so will limit the number of passages and prevent any drift of sensitivity to virus infection. Quality control protocols for the cell banks that monitor sterility (bacteria, mycoplasma and fungi) and susceptibility of cells to viral infection should be included.

Vero cell monolayers should be prepared two to three days before use. Sub-confluent or just-confluent monolayers should be used to avoid any alteration or loss of cells during the course of the assay. Quality of the cell monolayer is critical for plaque development and therefore to generate accurate results.

2.3 Viruses used in the test

Currently there are a variety of DENVs on which vaccines are being based (Table 1). Because the well-established dengue laboratory strains used for PRNT implementation were isolated many years ago and have been amplified by several passages in mosquito or various other cell lines, there is no clear rationale to choose one set of viruses over another.

Table 1. Viruses currently used in DENV vaccine development or reference labs

	Vaccine Developer or Reference Lab				
Virus serotype	**1**	**2**	**3**	**4**	**5**
DEN1	Hawaii	16007	16007 / PUO-359	WP or PR94	WestPac
DEN2	NGC	16681	16681 / PUO-218	NGC or Tonga 74	S16803
DEN3	H87	16562	16562 / PaH881/88	Sleman 78	CH53489
DEN4	H241	1036	1036 / 1228	814669 (Dom/81)	TVP360 or 341750

Plaquing efficiency is an important parameter in determining PRNT results. Using virus stocks containing large amounts of inactive virions could result in falsely low PRNT titers. There are a variety of ways to analyse the contents of a viral preparation. These approaches include, but are not limited to: the quantisation of viral genomic copies by RT-PCR; the quantisation of the total envelope protein mass (by ELISA or other methods); or direct particle counting in an electron microscope. Each of these techniques has limitations. The best practical approach to developing a virus stock with high infectivity is to carefully monitor the viral growth conditions and harvest at the appropriate time post-infection as described in section 2.1.

In the context of assessing vaccine immunogenicity (i.e., serological surrogates of protection), it is advisable to determine PRNT activity against a variety of virus strains such as other wild-type or virulent viruses (low passage virus or viruses isolated from human cases of Dengue Fever (DF) or Dengue Haemorrhagic Fever (DHF), or recent viral isolates from different dengue endemic areas (Endy et al., 2004; Kochel et al., 2002). These results should be compared to PRNT results using the prototype laboratory strain(s). This being said, there is little evidence of antigenic drift within a given DENV serotype that would result in a strain resistant to a post-vaccinal response to that serotype. Ongoing comparisons of the DEN E protein structure and cross-reaction analyses between laboratory strains and recent field isolates should yield helpful data to develop future recommendations. Regulatory authorities may also choose to specify other strains that might be used in assays measuring vaccine-induced immunity in addition to the strains used currently by vaccine developers.

2.4 Media

Fetal calf or bovine serum used for virus and cell growth, and for virus and sample dilutions should be heat-inactivated at 56oC for 30 minutes, and used at a low final concentration (2-5%) in the PRNT. The media used for cell growth should be compatible with the cell type used. For Vero cells, Minimal Essential Medium (MEM), Dulbecco's Modified MEM (DME), M199, or equivalent media, are generally sufficient.

2.5 Plaque forming unit target and vessel size

The challenge virus dose and the number of repeats tested for each dilution (serum or virus) are key factors for achieving accurate measurements. The challenge dose should be modified based on the surface area of the cell monolayer (e.g., 6-well versus 24-well plates), to get readily discernable plaques and minimize plaque overlap. The target number of plaques per well can vary by virus strain, however 40-60 pfu per 35 mm dish should permit accurate titrations while minimizing plaque-overlap. Plaque overlap results from crowding of plaques in an individual well. Comparing plaque counts in the test system versus input virus that has been "back-titrated" is the most acceptable way to rule out plaque-overlap. In order to reach an acceptable precision for the plaque counts, it is recommended that at least three repeat wells for a challenge dose of 50 PFU/reaction or less be used.

2.6 Heat inactivation of serum specimen

The technique of heat inactivation at 56oC for 30 minutes of serum specimens targeted for serological evaluation was introduced to limit the effects that complement or adventitious virus may have on the final results. This practice is routine in most laboratories and should be employed.

2.7 Addition of complement

Neutralization of DENVs does not require complement so addition of exogenous sources of complement to the PRNT is not necessary.

2.8 Filtration of specimen

Filtration of serum specimens to remove particulates is not necessary.

2.9 Dilution of specimen

In functional assays intended to assess vaccinal immunogenicity, the serum sample dilution series for antibody titration should ideally start below the "seroprotective" threshold titer. Regarding DENV neutralizing antibodies, the seroprotection threshold remains unknown; but numerous laboratories in the context of vaccine immunogenicity assessment consider a seropositivity threshold to be 1:10. A 1:5 starting dilution would be preferred over 1:10 in this context, however that should be balanced with the increased sample volume required for the test.

The number of dilutions can vary according to the objective of the testing. For screening purpose, sera can be processed using a dilution corresponding to the seropositive threshold titer, however the use of at least three dilutions is recommended due to possible cell toxicity or presence of non-specific inhibitors of virus replication in the first serum dilution. For endpoint titration, 2- to 4-fold serum dilutions should be used. These dilution series lead to a more precise estimate of the endpoint titer than higher dilutions factors. The number of dilutions used depends on the dilution series and what is sufficient to establish the endpoint titer in post-vaccinal samples. The appropriate dilution range can be previously determined by preliminary titration from ten-fold serum dilutions.

2.10 Virus-antibody incubation periods

Antigen-antibody reactions are quite rapid, with a period of 1-2 hours at 37°C sufficient for the PRNT. Extending this period could result in partial virus inactivation. An overnight incubation at 4°C is also acceptable, however switching between a variety of incubation periods within a given laboratory should be avoided

2.11 Virus adsorption to cells

Virus adsorption to Vero cells occurs rapidly at 37°C, with 90 percent of the infectious virus attaching within the first 30 minutes (Crill and Roehrig, 2001). Longer adsorption periods, while not necessarily detrimental to infection, are not needed for maximal viral adsorption. It is important to ensure coverage of the cell monolayer with media during viral adsorption. This is accomplished by tilting the plates at a recurring interval to ensure consistent media coverage of each well.

2.12 Days of incubation

The days of incubation for the plaquing plates will depend on the growth characteristics of the virus strains used. Typically DENV requires 4 to 7 days for plaques to be visible.

2.13 Temperature of incubation

Temperature of incubation for the plaquing plates should be compatible with the cells used. For Vero and LLC-MK2 cells, an incubation temperature of 37°C is appropriate, although lower temperatures, e.g., 35°C, are acceptable.

2.14 Overlay

In the case of conventional PRNT an overlay is added onto cell monolayer to limit the virus diffusion within the plate which permits plaque formation. The overlay can be added to the cell monolayer either after aspiration of the serum/virus mixtures or without elimination of the mixtures (e.g., yellow fever PRNT). The approach used should be consistent and documented within each laboratory. Semi-solid medium such as carboxymethyl cellulose (CMC), methylcellulose (MC), and agarose are acceptable. Lower grade agar solutions may contain charged inhibitors that may inhibit plaque formation. If these types of chemicals are used, they should first be tested to determine the presence of such inhibitors. Since the quality of the overlay medium is critical to ensure data reliability, a new batch of commercial reagent should

be qualified by comparison with a previous batch for plaque forming efficacy. Selection of the overlay media is based upon the techniques used for plaque visualization and whether or not the overlay must be removed for staining. In the latter case, CMC is used at 2-3% and MC is used at 0.8-1.5% final concentration. Agarose solutions are typically used at 1-2% final concentrations, and are primarily used when plaque are visualized with a vital dye such as neutral red. For flaviviruses, a two-overlay approach is preferred. The first overlay is added after the virus adsorption period, and does not contain neutral red. After an appropriate growth period, a second overlay containing 0.5% neutral red is added to visualize plaques.

2.15 Plaque visualization

2.15.1 Direct staining of cells

There are a variety of ways to reveal plaques for enumeration in the conventional PRNT. Cell coloration with vital dyes added in the first or second overlay (such as neutral red) permits monitoring the development of viral plaques as uncoloured holes in the cell monolayer. However this method has some limitations. Neutral red is cytotoxic at high concentrations and light sensitive, therefore the dye concentration in the overlay is necessarily limited, and plates stained with neutral red should be kept in light-tight containers or incubators. This is especially important for the single overlay technique. Because of this the contrast between the coloured cell monolayer and uncoloured plaques may be weak and thus affects the plaque enumeration accuracy. On the other hand, keeping cells alive with a neutral red overlay makes it possible to introduce flexibility into incubation time that may sharpen plaques or even bring out new plaques. Other dyes (such as amido-black or crystal violet) can be used to improve the cell monolayer staining and to allow possible automation for counting plaques. These stains cannot be added directly to the overlay and require overlay removal for staining. Even though dying the cell monolayers requires an additional coloration step after plaque development and before plaque counting, this approach has some other advantages:

1) The area of the cell monolayer required to reach the minimal recommended plaque count can be reduced because of the increased capacity to visualize smaller plaques;

2) The strong contrast between the cell sheet and the clear plaque permits photographic capture of plate images, and storage of images as raw data records for compliance purposes;

3) Chemical fixation of cells inactivates DENV so the staining and counting steps can be implemented under the Biological Safety Level (BSL) 1 containment (instead of BSL2 or 3 for dengue vaccine or wild-type viruses);

4) Plaques do not have to be counted immediately.

2.15.2 Direct staining of viral plaques

Viral plaques can also be immunostained using DENV-reactive polyclonal antisera or monoclonal antibodies. Staining plaques with DENV serotype-specific monoclonal antibodies allows an additional identity test of the viral serotype used in the assay. Immunostaining also permits the detection of viruses that plaque poorly, or the use of cell lines in which the virus does not demonstrate cytophathic effect (CPE), e.g., C6/36 cells. As with staining of the cell sheet with amido black or crystal violet, immunostaining requires chemical fixation of cells that inactivates DENV. Subsequent counting can be implemented under BSL1 containment. Plaques do not have to be counted immediately, and the strong contrast between the cell sheet and the stained plaque permits photographic capture of plate images and storage of images as raw data records for compliance purposes.

2.16 Counting plaques

Regardless of the method of visualization, plaques are usually counted manually. Depending on the visualization method, plaques can be counted immediately or later as in the case of immunostaining or cell monolayer coloration .

2.17 Data analysis

2.17.1 Acceptance criteria for a valid assay and a valid sample titer

The criteria needed to validate an individual test are:

1) Integrity of uninfected cell monolayer control;
2) Appropriate plaque counts per well as determined by back-titration of input virus;
3) Little or no reduction in plaque counts with negative serum control;
4) Appropriate PRNT titer of positive control sera, and;
5) No serum toxicity observed with low serum dilutions.

2.17.2 End-point calculations

PRNT end-point titers are expressed as the reciprocal of the last serum dilution showing the desired percent reduction in plaque counts. Currently no international reference sera are available for routine testing. The PRNT titer should be calculated based on a 50% or greater reduction in plaque counts (PRNT50). A PRNT50 titer is preferred over titers using higher cut-offs (e.g., PRNT90) for vaccinee sera, providing more accurate results from the linear portion of the titration curve. However, PRNT50 titers are more variable. The more stringent PRNT90 titers are more useful in DENV endemic areas for epidemiological studies or diagnostic purposes, by decreasing the background serum cross-reactivities among flaviviruses.

There are several ways to calculate PRNT titers. The simplest and most widely used way to calculate titers is to count plaques and report the titer as the reciprocal of the last serum dilution to show ≥50% reduction of the input plaque count as based on the back-titration of input plaques (see above). One area for titer variability between labs is whether or not the dilution made by mixing virus and antibody (usually the equal amount which results in a 1:2 antibody dilution) is included in the final titer calculations. Because there is no consistency in whether the 1:2 antigen-antibody dilution is included in the final titer, reporting of the data should explicitly state how this dilution was handled. Use of curve fitting methods from several serum dilutions may permit calculation of a more precise result. There are a variety of computer analysis programs available for this (e.g., SPSS or GraphPad Prism). That being said, consistency in the interpretation method is as important as the method chosen for analysis.

3. Minimal requirements for the PRNT

For laboratories just beginning DENV PRNT testing, the following protocol, while not exhaustive, is offered as a minimal recommended format for establishing the PRNT. This procedure assumes that the conditions of cell and virus growth have been predetermined and used prior to performing the actual PRNT.

Materials:

- Pipette aid
- Micropipettor: 0-100 µl, 0-1000µl
- Sterile micropipette tips: 0-200 µl, 0-1000 µl
- Serological pipettes: 1 ml, 2 ml, 5 ml
- 6-well plates
- Bio safety cabinet (laminar flow hood)
- Incubator: 37°C, 5% CO2
- Autoclave
- Sterile dilution tubes: 10 X 100 mm, capped
- Water bath: 37°C, 56°C
- Light box
- Vortex mixer

Reagents:

- 2X EMEM
- Fetal Bovine Serum (FBS)
- Penicillin/Streptomycin
- L-Glutamine
- Vero Cells
- Sodium Bicarbonate
- HEPES
- Non-essential Amino Acids
- Low melting point agarose
- Neutral red vital stain

Note: Perform this first to make sure agar is cool. Weigh out agarose (1 g per 100 ml total volume of first overlay). Add agarose to enough sterile water for dissolved agarose solution to constitute one half (1/2) of the total volume of the first overlay (This 2X agarose solution will be mixed with the equal amount of 2X medium). Microwave until dissolved and place into a water bath and allow to cool to 40°C.

Procedure:

1) Heat inactivate all sera to be assayed in a 56°C water bath for 30 minutes.

2) Prepare serial two-fold dilutions of test sera and positive and negative control sera as follows:

   a) Arrange pre-determined number of sterile, capped dilution tubes in a test tube rack. Generally, six tubes are used in each dilution series are sufficient.

   b) Using a 1000 µl pipettor and sterile tips, add 450 µl of serum diluent (EMEM with 2% heat-inactivated FBS, 1% Penn/strep, 1% L-glutamine, and 1% sodium bicarbonate) to the first tube of each dilution series and 250 µl of diluent to each of the five other tubes in the series.

   c) Using a 100 µl pipettor and sterile tips, add 50 µl of heat-inactivated serum to the tube containing 450 µl of diluent, thus making an initial serum dilution of 1:10 (final 1:20).

   d) Perform serial two-fold dilutions of each of the test sera and the positive and negative control sera by using a sterile pipette tip to transfer 250 µl of the 1:10 serum dilution to the next tube in the dilution series containing 250 µl of serum diluent. After each transfer, mix the tube by gentle vortexing; change pipette tips and continue the dilution series to the end of the required range. After the final transfer, discard 250 µl or store for future use from the last tube in the dilution series so that each tube of the dilution series contains 250 µl. Internal positive control sera should be used until a WHO standard is developed.

   e) To one additional tube add 250 µl of serum diluent. This will be used as a diluent only control.

3) Prepare dilutions for the virus plaque dose in diluent.

   a) Rapidly thaw, in a 37°C water bath, a previously unthawed vial of virus of the appropriate serotype. Place immediately on ice.

   b) Dilute the virus stock to the dilution previously determined.

4) Using a sterile repeating pipettor add an equal volume (e.g., 250 µl) of the diluted virus stock to each of the serum dilution tubes, including controls for a final virus concentration of approximately 50 PFU/ 0.2 ml. Add virus to the diluent only control first, then negative control, then to the test sera, and finally to the positive controls; avoid contaminating the pipette with sera. Mix the tubes well by gentle vortexing or shaking.

5) Incubate the tubes in a 37°C water bath for 1 hour.

6) Label 6-well plates containing confluent Vero cell monolayers for inoculation (two 6-well plates will be required for each serum dilution series). Using a vacuum aspirator or pipette, remove all but about 0.2 ml of the culture supernatant fluid from each well, leaving enough residual fluid so that the cell monolayers do not become dry.

7) Using a sterile tip add 200 µl of the virus-serum mixture and inoculate into each of the two wells of a properly prepared 6-well plate. Discard the pipette tip. Evenly distribute the inoculum by rocking the plate back and forth and from side to side. With a fresh tip, continue inoculation until all plates have been inoculated.

8) Incubate the inoculated plates at 37°C for 1 hour in a 5% CO2 incubator to allow for virus absorption. Be sure that the plates are level so that the cell monolayers do not become dry. Rock the plates at regular intervals to maintain moisture on the cell sheets.

9) After the incubation period, remove the inoculum and add 2.5 ml of agarose-containing overlay medium to each well using a pipette or Cornwall syringe or manostat.

First Overlay medium

2X EMEM	200 ml
FBS	20 ml
NAA	4 ml
P/S	4 ml
L-glutamine	2 ml
HEPES	4 ml

Combine this solution with an equal volume of 2% agarose solution (see above) immediately prior to use. Be sure to allow the agarose/media solution to cool somewhat prior to adding to wells. Hot solutions kill cells.

10) Set the plates at room temperature for 15-20 minutes to allow the agarose to solidify.

11) Incubate the plates upside down to minimize water condensation in the wells in a 37°C, 5% CO2 incubator for 5 days (depending on virus strain) to allow virus plaques to develop.

12) After the previously determined incubation time (e.g., 5 days) prepare the second overlay containing the vital stain neutral red. Add 2 ml of the second overlay to each well. The composition of the second overlay is the same as the first overlay (media and agarose). In addition add neutral red to a final concentration of 0.5%. Allow the agar solution to cool as with the first overlay, then add the neutral red (which should be at room temperature) right before use.

13) Set the plates at room temperature for 15-20 minutes to allow the agarose to solidify.

14) Incubate the plates, same environment as above, for a minimum of 18 hours or up to 48 hours to allow the cells to maximally take up stain. Incubate plates upside down to minimize water condensation in the wells.

15) Place the stained 6-well plates on a light box. Using a counting pen, count the plaques (these will be clear areas in a red background) and record the results. For a valid assay the negative control should contain a minimum of 30 plaques. Plaque overlap as would be caused by excessive numbers of plaques must be avoided.

16) Determine the PRNT by linear regression (probit) analysis using either probit paper or a computer software program, or determine highest dilution that results in ≤50% of input plaque count.

SAFETY ISSUES: UNIVERSAL PRECAUTIONS REMAIN IN EFFECT AT TIMES WHEN HANDLING HUMAN SERA. PLACE ALL WASTE IN THE BIOHAZARD WASTE CONTAINERS. WIPE DOWN AREA WITH 70 % ALCOHOL

4. New tests to measure virus neutralization

Modifications in the standard PRNT assays are being investigated to shorten the incubation period required to see plaques, stain infected cell cultures to enhance plaque visibility, eliminate the plaque enumeration step, or reduce or eliminate the reliance on cell culture. Any new approach to assessing virus-neutralizing antibodies will have to be validated against the standard PRNT, as outlined in this document, so that the relationship and equivalence of the new and old test is fully understood. The first modification in the PRNT is the reduction of the size of vessel used for plaquing. Twenty-four well plates seem to be the smallest reasonably sized vessel for plaquing when conventional cell staining techniques are used.

Immunostaining of virus-infected cells, instead of directly staining them with histochemical stains or vital dyes offers some advantages over these standard procedures. Immunostaining relies on adding enzyme conjugated anti-viral antibody (direct test) or non-conjugated antiviral antibody (later detected with an enzyme conjugated anti-species antibody - indirect test) to virus-infected cell cultures. The binding of the enzyme conjugate to the cell sheet is detected with an immunohistochemical stain specific for the enzyme used (usually horseradish peroxidase or alkaline phosphatase). The availability of DEN virus serotype specific Mabs permits enumeration of plaques within complex mixtures of serotypes using this approach. While using mixtures of DEN viruses in this way may reduce the total number of plates needed to quantitate virus plaques, issues of plaque overlap still need to be considered. Use of immunochemical staining which results in signal amplification because of the presence of the enzyme-conjugate may also facilitate identification of plaques at an earlier stage post-infection, resulting in reduced plaquing incubation times.

Perhaps the most promising technique is to quantitate infectious virus using small numbers of virions and a 96-well plate format (micro neutralization assay) (Vorndam and Beltran, 2002). In this assay, individual plaques are not enumerated, but rather the viral growth measured in any given well is related to the optical density observed in that well using a soluble enzyme substrate. The most difficult parameters to control using this assay design are determining the appropriate amount of input virus and the incubation times between virus-infection and ELISA detection. Since viral spread is not limited by semisolid overlays, waiting too long before staining can result in overgrowth of virus. Since not all viruses grow at the same rate, the incubation periods will be virus-specific. Standardizing this assay for four virus serotypes is doable, however if cross-reactivities need to be measured against a variety of strains, the task becomes more difficult. It is also possible to convert this assay from an immunochemical assay to a nucleic acid detection assay. In this way, viral RNA produced by non-neutralized virus can be detected in a quantitative assay (from culture supernatants and/or cell monolayers). The issues of input virus and time of incubations is still relevant for this process.

Another new approach to measuring virus neutralizing antibodies is to dispense with virus completely and use an "infectious" vector that expresses the E protein or E/prM proteins with or without genes that encode "reporter" molecules (Pierson et al., 2005; Pierson et al., 2006). These assays are based upon the observation that essentially all virus-neutralizing antibodies are elicited by the E protein. A variety of reporter molecules (e.g., green fluorescent protein) can be used to detect residual "virus" activity. However, such approaches suffer the same drawbacks as the micro neutralization assays. In addition this system requires the construction of a new vector for each strain to be tested.

5. Summary

The PRNT is the current laboratory standard for measuring DENV neutralizing antibody. Standardization of the procedure using appropriate reference material, followed by each laboratory's own qualification and validation may result in better inter-laboratory comparisons of results. These guidelines outline the variables that are important to consider in performing this biological assay, and suggest a procedure that can be used by those laboratories interested in using the PRNT. It is important to note that virus neutralizing antibody titer, as determined in the PRNT, is considered to be the best immune correlate of protection for flaviviral infections. The fact remains, however that a true definition of protective levels of vaccine-induced DENV neutralizing antibody will require vaccine-efficacy trials using fully validated assays.

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The World Health Organization has managed cooperation with its Member States and provided technical support in the field of vaccine-preventable diseases since 1975. In 2003, the office carrying out this function was renamed the WHO Department of Immunization, Vaccines and Biologicals.

The Department's goal is the achievement of a world in which all people at risk are protected against vaccine-preventable diseases. Work towards this goal can be visualized as occurring along a continuum. The range of activities spans from research, development and evaluation of vaccines to implementation and evaluation of immunization programmes in countries.

WHO facilitates and coordinates research and development on new vaccines and immunization-related technologies for viral, bacterial and parasitic diseases. Existing life-saving vaccines are further improved and new vaccines targeted at public health crises, such as HIV/AIDS and SARS, are discovered and tested *(Initiative for Vaccine Research).*

The quality and safety of vaccines and other biological medicines is ensured through the development and establishment of global norms and standards *(Quality Assurance and Safety of Biologicals).*

The evaluation of the impact of vaccine-preventable diseases informs decisions to introduce new vaccines. Optimal strategies and activities for reducing morbidity and mortality through the use of vaccines are implemented *(Vaccine Assessment and Monitoring).*

Efforts are directed towards reducing financial and technical barriers to the introduction of new and established vaccines and immunization-related technologies *(Access to Technologies).*

Under the guidance of its Member States, WHO, in conjunction with outside world experts, develops and promotes policies and strategies to maximize the use and delivery of vaccines of public health importance. Countries are supported so that they acquire the technical and managerial skills, competence and infrastructure needed to achieve disease control and/or elimination and eradication objectives *(Expanded Programme on Immunization).*

Department of Immunization, Vaccines and Biologicals
Family and Community Health

World Health Organization
CH-1211 Geneva 27
Switzerland
Fax: +41 22 791 4227
Email: vaccines@who.int
or visit our web site at: http://www.who.int/vaccines-documents

World Health Organization

Guidelines for plaque-reduction neutralization testing of human antibodies to dengue viruses. Published Date: June 2008.Â ThePRNT is the most common assay used to measure neutralizing antibody. The presence of antibody is believed to be most relevant means of determining protective anti-DEN virus (DENV) immunity. While other neutralizing antibody assays are being considered for use in large-scale vaccine field trials, the PRNT is still considered to be the laboratory standard against which other neutralizing antibody assays should be compared. The need for PRNT coordination has been identified at several consultations between the WHO and PDVI. â€œGuidelines for Plaque Reduction Neutralization Testing of Human antibodies to Dengue virusâ€ Initiative for Vaccine Research of the Department of Immunization, Vaccines and Biologicals. World Health Organization. Department of Immunization, Vaccines and Biologicals. http://whqlibdoc.who.int/hq/2007/WHO_IVB_07.07_eng.pdf.