

Optimization and Validation of a Plaque Reduction Neutralization Test for the Detection of Neutralizing Antibodies to Four Serotypes of Dengue Virus Used in Support of Dengue Vaccine Development

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Abstract. A dengue plaque reduction neutralization test (PRNT) to measure dengue serotype-specific neutralizing antibodies for all four virus serotypes was developed, optimized, and validated in accordance with guidelines for validation of bioanalytical test methods using human serum samples from dengue-infected persons and persons receiving a dengue vaccine candidate. Production and characterization of dengue challenge viruses used in the assay was standardized. Once virus stocks were characterized, the dengue PRNT₅₀ for each of the four serotypes was optimized according to a factorial design of experiments approach for critical test parameters, including days of cell seeding before testing, percentage of overlay carboxymethylcellulose medium, and days of incubation post-infection to generate a robust assay. The PRNT₅₀ was then validated and demonstrated to be suitable to detect and measure dengue serotype-specific neutralizing antibodies in human serum samples with acceptable intra-assay and inter-assay precision, accuracy/dilutability, specificity, and with a lower limit of quantitation of 10.

INTRODUCTION

Dengue is caused by four closely related but antigenically distinct viruses referred to as dengue virus (DENV) serotypes of the family *Flaviviridae*: DENV-1, DENV-2, DENV-3, and DENV-4.^{1,2} In recent decades, dengue has expanded in tropical and subtropical areas around the world to become one of the most prevalent vector-borne diseases of humans, and a public health priority with approximately 2.5 billion persons living with risk of infection.³ The annual burden of dengue as reported by World Health Organization (WHO) is 50 million dengue infections and 500,000 hospitalizations for dengue hemorrhagic fever, but the true burden may be significantly higher with 70–500 million infections/year, including 2.1 million clinically severe cases.^{3,4}

Many academic laboratories and pharmaceutical companies including Sanofi Pasteur have investigated various conventional and recombinant strategies to develop a safe and effective dengue vaccine. Investigational dengue vaccines aim to induce broad, robust immune response to all four serotypes simultaneously to prevent or limit dengue disease.⁵ Various serologic tests are available to evaluate the humoral response to dengue vaccination and to detect antibodies against dengue, including the hemagglutinin inhibition assay, the complement fixation test, the enzyme-linked immunosorbent assay (ELISA), and viral neutralization assays.^{6–16} However, only neutralizing antibody assays measure the functional dengue serotype-specific antibody response.

The dengue plaque reduction neutralization test (PRNT), which was first developed by Russel and Nisalak in 1967, is widely accepted and considered the laboratory gold standard test by WHO for assessment of dengue vaccine immunogenicity.^{13,15,17} We present the dengue PRNT optimized and validated to support the Sanofi Pasteur dengue vaccine development program, considering the WHO recommendations for use of cell lines, virus, serum-virus neutralization time and temper-

ature, and readout method.^{18–24} Our dengue PRNT uses the 50% neutralization potency estimate (PRNT₅₀) and was optimized and subsequently validated according to the International Conference for Harmonization and Food and Drug Administration guidelines and shown to be suitable to evaluate the immunogenicity of dengue vaccine candidates.^{25–27} Furthermore, best practices were established for the preparation and qualification of reagents used in the dengue PRNT₅₀ to ensure consistent assay performance over time as needed to support large dengue vaccine clinical trials.

MATERIALS AND METHODS

Cell line. Vero cells (CCL-81) were obtained from the American Type Culture Collection (Manassas, VA) and master and working banks of Vero cells were prepared in-house. Cells for dengue PRNT and dengue virus production were maintained in cell culture medium, consisting of minimum essential medium (Invitrogen, Carlsbad, CA) and CMRL-1066 (Invitrogen), respectively, supplemented with 5% heat inactivated fetal bovine serum (FBS; HyClone, Logan UT), 2 mM L-glutamine (Invitrogen), and 1% antibiotic/antimycotic solution (100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B; Invitrogen) and subcultured once a week by splitting one T225 flask (Corning/Costar, Corning, NY) into six new flasks. Cell banks were qualified before use and confirmed to be free of any microbial, mycoplasma and viral contaminants. In addition, each new cell bank was tested for susceptibility to dengue virus infection for each of the four dengue virus serotypes.

Serum samples. Dengue antibody-positive human serum samples from children and adults were obtained from patients with dengue and from persons who had participated in clinical trials of the Sanofi Pasteur recombinant CYD tetravalent dengue vaccine candidate. Yellow fever (YF)- and Japanese encephalitis (JE)-antibody positive human serum samples were obtained from healthy adult donors who received vaccines against YF virus (YF-VAX[®]) or JE virus (JE-VAX[™]), respectively. Dengue antibody-negative human serum samples were obtained from healthy adult donors from non-endemic dengue areas.¹⁹ Sample identifiers were removed and new

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sample identification numbers were issued. Samples were heat inactivated for 30 minutes at 56°C before use. Quality control samples were used in each assay run and demonstrated to be suitable for their intended purpose by consistently performing within the previously established neutralization titer limits, and served to monitor assay performance.

Antibodies. Purified mouse monoclonal antibodies (MAbs) specific to the dengue virus serotype envelope protein are used in the dengue PRNT₅₀ as the primary virus detection antibodies: anti-DENV-1 (D2-1F1-3; Biotem, Le Rivier d'Apprieu, France), anti-DENV-2 (3H5-1-12; Biotem), anti-DENV-3 (8A1-2F12; Biotem), and anti-DENV-4 (1H10-6-7; Biotem).^{28,29} Alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc, West Grove, PA) was used as the secondary antibody. Primary and secondary antibodies were qualified before use by assessing specificity and determining the optimal working dilutions as 1:4,000 and 1:1,000, respectively, for use in the dengue PRNT₅₀.

Dengue viruses. The challenge viruses in the PRNT₅₀ are the parental dengue viruses of the four recombinant CYD vaccine viruses (i.e., DENV-1 strain PUO-359, DENV-2 strain PUO-218, DENV-3 strain PaH881/88, and DENV-4 strain 1228). The source of dengue viruses has been described, and these viruses have been accepted by the WHO as prototype laboratory strains for use in a dengue neutralization assay.^{30,31} For dengue virus production, a multiplicity of infection of 0.001 for DENV-1, DENV-2 and DENV-4 viruses, and a multiplicity of infection of 0.01 for DENV-3 virus was used to infect Vero cells seeded at 5×10^5 cells/flask three days before virus infection. Virus inoculum was removed after 90 minutes incubation at 37°C in an atmosphere of 5% CO₂ and fresh culture medium consisting of CMRL-1066 supplemented with 5% FBS, 1% L-glutamine, 1% antibiotic/antimycotic solution, 50 mM magnesium sulfate (Sigma, St. Louis, MO), and 20 mM HEPES (Sigma Aldrich, St. Louis, MO) was added to each flask and placed in a 37°C incubator in an atmosphere of 5% CO₂ for 3 days.

The flasks were microscopically observed daily post-infection to check for the presence of a cytopathic effect in infected cells and lack of a cytopathic effect in the mock-infected cells. Culture medium was replaced on day 3 post-infection and virus was harvested from day 4 to day 8 post-infection. On the day of harvest, cell culture medium containing virus was collected and centrifuged for 10 minutes at $525 \times g$ and 4°C to remove cell debris. Fetal bovine serum and sorbitol (Sigma) were added to the clarified supernatant to a final concentration of 20% FBS and 10% (w/v) sorbitol to stabilize the virus. Virus was aliquoted, flash-frozen, and transferred to a -70°C freezer for long-term storage.

The above method was used to create master banks of dengue viruses from the original source and to create the virus working lots from the master bank. Viruses were qualified before use in the dengue PRNT₅₀ by assessing the identity, as well as determining the virus concentration and the optimal working dilution to deliver a constant dose of challenge virus.

Dengue plaque assay. Plaque assay (PA) was used to determine the concentration of dengue viruses used in the PRNT₅₀. Twenty four well plates (Corning, Corning, NY) were seeded at 4×10^5 Vero cells/well and placed in a 37°C incubator in an atmosphere of 5% CO₂ for 3 days. Two-fold serial dilutions of virus were inoculated in duplicate on the 24-well plates and incubated at 37°C in an atmosphere of 5% CO₂

for 90 minutes. After virus adsorption, the inoculum was removed and carboxymethylcellulose (CMC) overlay medium consisting of cell culture medium supplemented with 5% heat inactivated FBS, 3% sodium bicarbonate (Invitrogen), 1% antibiotic/antimycotic solution was added. Either 2% or 3% CMC overlay medium was used, depending on the dengue virus serotype. Plates were incubated at 37°C in an atmosphere of 5% CO₂ for an additional 4–6 days, depending on the growth kinetics of each dengue virus serotype. Infected cells were immunostained as described below for the dengue PRNT₅₀. Plaques (dengue virus infected foci) were counted and the virus concentration calculated in plaque-forming units (PFU)/mL. Duplicate titrations were performed for each virus stock, with a minimum of 3 vials/virus lot tested to determine the virus concentration for the different virus banks.

Dengue plaque reduction neutralization test. Vero cells were seeded at 4×10^5 cells/well in 24-well tissue culture plates (Corning/Costar) and incubated for three days at 37°C in an atmosphere of 5% CO₂ until approximately 95% confluency was reached. Test serum and assay quality control serum samples were initially diluted at 1:5 in the first wells of 96-well plates followed by 2-fold serial dilutions up to 12 wells (125 µL/well). Dengue virus was diluted in cell culture media to yield 40–120 plaques/well in the virus control wells. Cell control wells were also included as an assay control, consisting of wells incubated with cell culture media only. An equal volume of dengue virus was added to each diluted serum sample and the virus-serum mixture was incubated at 37°C in an atmosphere of 5% CO₂ for 60 minutes to enable neutralization to occur. The cell culture medium was then aspirated from the 24-well plates with pre-formed Vero cell monolayers, and the virus-serum mixture (200 µL/well) was transferred from the 96-well plates onto the 24-well plates and incubated at 37°C in an atmosphere of 5% CO₂ for 90 minutes to enable non-neutralized dengue virus to adsorb onto Vero cells. The virus-serum inoculum was then aspirated and 1 mL of 2% CMC overlay medium in minimal essential medium supplemented with 5% heat-inactivated FBS, 2 mM L-glutamine and 1% antibiotic/antimycotic solution was added to each well. Plates were returned to the 37°C incubator and incubated in an atmosphere of 5% CO₂ for four days.

The CMC overlay medium was then removed and the cell monolayers were washed twice with wash buffer (0.01 M phosphate-buffered saline, 0.05% Tween-20) to completely remove the CMC overlay medium. Cells were then fixed with cold 80% acetone for 10 minutes at room temperature, rinsed once with wash buffer, and incubated with blocking buffer (2.5% nonfat milk in wash buffer with 0.5% Triton X-100) at 37°C in an atmosphere of 5% CO₂ for 45 minutes. Cells were incubated with dengue virus serotype-specific mouse anti-envelope protein MAbs, followed by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG antibody. Viral plaques were visualized after incubation with nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate substrate. Washing steps were implemented in between each antibody and substrate incubation. Immunostained plates were rinsed with tap water and allowed to air dry before counting plaques.

The presence of dengue virus-infected cells was indicated by the formation of viral plaques (virus-infected foci). The infectivity in PFU per well of dengue challenge virus in the PRNT₅₀ is reduced as a direct result of the presence of

antibodies the test serum samples capable of neutralizing infectious virus. The neutralization titer (PRNT₅₀) of the test serum sample is defined as the reciprocal of the highest test serum dilution for which the virus infectivity is reduced by 50% when compared with the average plaque count of the challenge virus control and is calculated by using a four-point linear regression method. Plaque counts for all 12 serial dilutions of serum are scored to ensure that there is a dose-response. The PRNT₅₀ titer is determined by using the log₁₀-transformed plaque counts from the four selected points, which spanned the 50% neutralization point in that assay run with the two points below and two points above the cutoff, for the regression analysis. The addition of an equal volume of virus to the diluted serum samples was included in the final calculation of the neutralization titers. Because the lowest starting dilution of serum in the assay is 1:5, the theoretical lower limit of quantitation (LLOQ) of the assay is a PRNT₅₀ titer of 10 (reciprocal dilution). The assay run was considered valid only if the following criteria were met: average plaque count for virus control wells fell within the required target of 80 plaques/well and within the acceptable range of 40–120 plaques; the cell control wells showed no plaques; the titers of the two positive assay quality control serum samples were within the pre-established acceptable ranges; and the titer of the negative assay quality control serum sample remained negative.

Optimization of dengue PRNT₅₀. A full factorial design of experiments (DOE) approach was used for the optimization of dengue PRNT₅₀, and each DOE was repeated in at least two independent runs for each of the four dengue virus serotypes. The following conditions were assessed in the DOE: 1) days of cell seeding before use in the PRNT₅₀ (two or three days), 2) percentage of CMC overlay medium (1%, 1.5%, or 2% CMC), 3) days of incubation post-infection (three, four, or five days), 4) working virus dilution, and 5) volume of inoculum. Each test plate was scored on a scale of 1–3 for plaque size and morphology, with 3 being easiest to define and read and 1 the most difficult. The optimal conditions selected from DOE (highest aggregate score from DOE) were further tested to confirm the assay conditions and the virus working dilution. Each virus dilution was tested with two positive and one negative assay quality control serum samples, and each virus serotype was tested in two independent assay runs.

Robustness of dengue PRNT₅₀. Robustness was evaluated by using a full factorial DOE with varying incubation times around the following steps of the assay for each of the four dengue virus serotypes: 1) virus-serum neutralization (60 ± 5 minutes), 2) adsorption on Vero cells (90 ± 5 minutes), 3) blocking (45 ± 10 minutes), 4) incubation with primary antibody (60 ± 10 minutes), and 5) incubation with secondary antibody (60 ± 10 minutes). The incubation times that were tested for robustness were chosen for logistical reasons to enable normal expected variations for these critical parameters. A panel of 12–20 samples was used to assess robustness, and the assay was deemed to be acceptable by demonstrating that replicate titers were within 3-fold of each other for ≥ 80% of samples tested.

Validation of dengue PRNT₅₀. In accordance with International Conference on Harmonization and Food and Drug Administration guidelines, the optimized dengue PRNT₅₀ for each of the four dengue virus serotypes was validated by

assessing the intra-assay and inter-assay precision, accuracy/dilutability, specificity, and LLOQ.^{25–27}

The precision of a test procedure expresses the agreement between a series of multiple results generated with the same homogeneous sample. Intra-assay and inter-assay precision express the precision under the same operating conditions and within laboratory variation, respectively. Intra-assay precision was determined by testing in triplicate in a single assay run, each of 20 human serum samples (pooled or individual samples) with dengue antibody titers covering the range of the assay. Inter-assay precision was determined by testing 20 human serum samples in 3 independent assay runs by at least 2 analysts. Intra-assay and inter-assay precision were considered acceptable if ≥ 90% of the observed results were within one 3-fold difference of the median titer for ≥ 80% of positive samples tested. Because the median titer is a more robust estimate of central trending, and the mean may be skewed by outliers, the median titer is traditionally used in our laboratory for this type of functional assay.

Accuracy/dilutability was assessed by testing five dengue antibody-positive samples (individual or pooled human serum samples) undiluted or diluted (1:4, 1:16, and 1:64) in dengue-negative human serum five times each in the same assay run. The expected titer is the median of the five observed titers of the undiluted sample divided by the dilution factor (4, 16, or 64) of the sample. Accuracy/dilutability was considered acceptable if the absolute difference of the log₂ observed median and the log₂ expected titer was ≤ 1.58 for ≥ 80% of the samples tested at each targeted dilution. Mathematically, this is equivalent to requiring that at least 80% of the samples have an observed and expected PRNT₅₀ titer within 3-fold of each other (log₂3 = 1.58).

Specificity is the ability of the assay to unequivocally assess the analyte in the presence of components that may be expected to be present in the sample matrix. Spiking studies were conducted to evaluate the ability of the assay to specifically measure antibodies to dengue virus in the presence of antibodies to other flaviviruses. Five dengue antibody-positive human serum samples were spiked with YF antibody-positive serum, JE virus antibody-positive serum, or dengue antibody-negative-serum as a control. The titers of dengue virus neutralizing antibodies observed were examined to determine if spiking resulted in a significant change in observed titer as compared with a dengue antibody negative-spiked control. Specificity was considered acceptable if the absolute difference of the log₂ observed titer and the log₂ expected titer was ≤ 1.58 for ≥ 80% of the samples.

The LLOQ was determined as the lowest level of antibody present in a test sample that can be consistently detected with suitable precision and accuracy by applying the test procedure. Ten human serum samples with dengue PRNT₅₀ titers between < 10 and 40 (including at least 2 negative samples) were tested 11 times each in at least two independent assay runs to confirm the theoretical assay LLOQ of 10. Results that were reported as < LLOQ were replaced by half of LLOQ (i.e., 5) for median titer calculation purposes. For any given sample, if more than one-third of the repeat measures was < LLOQ (e.g., 4 of 11), then the sample was not considered a true positive sample and was excluded from this statistical analysis. The percentage of results within one 3-fold difference of the median titer was calculated for each positive sample. The precision for LLOQ samples was considered

acceptable if $\geq 80\%$ of positive samples had $\geq 90\%$ of results within one 3-fold difference of the median titer.

RESULTS

Dengue virus production and qualification for use in PRNT₅₀. To improve virus yield and long-term storage stability during the production of large-scale working banks of dengue virus, MgSO₄ and HEPES were added to the virus cell culture medium, sorbitol and additional FBS were added to the harvest medium, and virus was frozen by using a dry ice/methanol bath.^{32–37} The growth kinetics of dengue virus working banks following this procedure were determined by plaque assay (Figure 1). The concentration of dengue virus steadily increased from day 4 to day 8 post-infection, with a maximum titer in the range of 10^7 PFU/mL at days 6–8 post-infection for all four virus serotypes. For dengue serotypes 1, 2, and 4, the average PFU/mL remained high, above 10^6 PFU/mL and the virus titer modestly increased from 4–8 days post-infection. For dengue serotype 3, the viral titer at day 4 post-infection was approximately 10^4 PFU/mL, then steadily increased to 5×10^6 PFU/mL at day 8 post-infection. The growth kinetics of dengue virus working banks demonstrated that the optimal days of harvest are between day 6 and day 8 post-infection for all virus serotypes. The virus growth kinetics were also determined for the master virus banks and exhibited the same trend.

Long-term virus stability testing demonstrated that the titer in PFU/mL of dengue virus stocks following storage at -70°C for 6, 12, 18, 24, and 36 months did not change more than 3-fold from the titer determined following storage for 1 month after production (within assay variability for plaque assay). The identity of each of the dengue viruses was confirmed by using dengue virus serotype-specific MAbs. Furthermore, using MAb to related flaviviruses such as JE and YF viruses, we demonstrated that our dengue virus master and working banks are free of contamination with other viruses used in our laboratory. The optimal working virus dilution to deliver a virus challenge dose of approximately 80 PFU/well was determined for each virus lot for all four dengue virus serotypes before implementation in PRNT₅₀ testing.

Optimization and robustness of dengue PRNT₅₀. There was no appreciable difference between the plaque counts, morphology, or distribution using Vero cells seeded two or three days

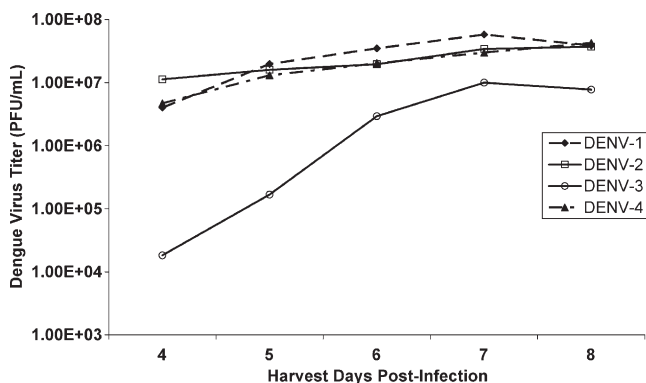


FIGURE 1. Dengue virus growth kinetics. The dengue virus plaque assay titers in plaque-forming units (PFU)/mL are plotted against the harvest days post-infection for each dengue virus (DENV) serotype, illustrating the viral growth kinetics. The concentration of dengue virus steadily increased from day 4 to day 8 post-infection, with a maximum titer in the 10^7 PFU/mL range at days 6–8 post-infection.

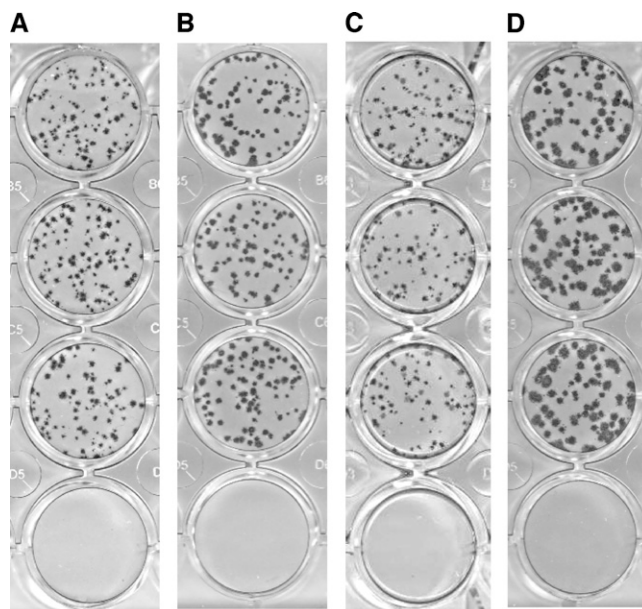


FIGURE 2. Morphology of dengue virus-1 (DENV-1), DENV-2, DENV-3, and DENV-4 plaques. Immunostained DENV-1 (A), DENV-2 (B), DENV-3 (C), and DENV-4 (D) viral plaques developed after incubation with dengue serotype-specific monoclonal antibodies to the envelope protein followed by alkaline phosphatase-conjugated anti-mouse IgG (secondary antibodies) and insoluble nitroblue tetrazolium/5-bromo-4-chloro-3'-indolylphosphate substrate. The first three wells were infected with the challenge virus dose and the fourth well was the uninfected cell control.

before the testing for any of the four dengue serotypes. A noticeable difference was observed in plaque size and morphology between the different days of incubation post-infection at a given percentage of CMC overlay medium. Dengue virus plaques on day 5 post-infection were overlapped greatly, making counting difficult at all CMC concentrations. At days 3 and 4 post-infection, incubation with 1% and 1.5% CMC overlays produced large plaques with undefined borders, making accurate counting difficult. In contrast, incubation with 2% CMC at day 4 post-infection produced plaques for all four serotypes that were easily counted and distributed evenly throughout the wells. The volume of virus-serum inoculum needed per well in 24-well plates was also assessed. Comparable results were observed for virus plaque size, morphology, and distribution of plaques throughout the well using 0.1 or 0.2 mL of inoculum/well.

The results of the optimization experiments demonstrated a linear relationship between virus challenge dose (working dilution) and plaque forming units per well. The neutralization titers for the dengue antibody-positive assay quality control serum samples were within three-fold of each other, and the negative assay quality control serum sample remained negative, demonstrating robustness in this assay. Based on the results of the DOE, the optimal virus working dilution for each dengue virus serotype was targeted to yield 80 PFU/well in the virus control wells.

As a result of the factorial DOE, the optimal dengue PRNT₅₀ conditions for all four serotypes were seeding cells 3 days before testing, 2% CMC overlay medium, 0.2 mL of virus-serum inoculum/well, and incubation for four days post-infection to visualize the virus plaques. Examples of the plaque morphology for each of the four dengue virus serotypes obtained using the optimal assay conditions are shown in Figure 2. Differences

TABLE 1
Results of intra-assay precision of PRNT₅₀ for dengue virus serotypes 1, 2, 3, and 4*

Sample no.†	DENV-1 PRNT ₅₀			DENV-2 PRNT ₅₀			DENV-3 PRNT ₅₀			DENV-4 PRNT ₅₀		
	Titer 1	Titer 2	Titer 3	Titer 1	Titer 2	Titer 3	Titer 1	Titer 2	Titer 3	Titer 1	Titer 2	Titer 3
1	50	32	44	84	96	121	131	140	167	< 10‡	27	38
2	13	21	21	39	87	66	53	56	60	47	83	97
3	16	25	34	31‡	101	96	31	31	26	65	156	129
4	45	31	40	95	104	113	47	NA§	52	45	41	30
5	102	28	37	111	131	104	44	56	46	140	200	90
6	164	70	210	189	98	115	215	191	330	48	42	86
7	152	43	127	200	156	206	183	210	199	213	166	188
8	106	79	192	94	129	122	253	208	238	197	378	159
9	113	67	64	156	139	399	20	47	37	245	267	212
10	68	NA§	88	141	221	184	160	109	128	76	74	72
11	114	280	152	444	666	858	142	183	138	45	79	37
12	352	327	560	435	642	652	190	184	181	608	762	525
13	381	245	270	331	393	423	592	560	459	307	391	286
14	777	356	456	718	468	790	849	469	729	82	77	100
15	751	352	711	844	839	1,481	396	526	478	442	408	332
16	326	521	451	950	1,537	1,595	970	829	961	121	84	141
17	408	782	591	1,322	1,474	1,698	1,380	1,425	1,178	172	72	130
18	2,355	3,307	1,692	4,345	3,848	3,407	2,677	3,647	2,328	1,693	3,096	774
19	1,019	3,124	1,323	1,931	3,870	3,190	1,575	1,732	1,203	593	1,336	744
20	1,799	1,250	1,419	3,896	7,166	6,495	2,462	3,010	2,761	1,578	624	272

*PRNT₅₀ = 50% plaque reduction neutralization test; DENV = dengue virus; NA = not applicable.
 †Four different sample panels; one sample panel per serotype.
 ‡Result is outside ± 3-fold of the median PRNT₅₀ titer of three results.
 §Result was not obtained because the quantity of the sample was not sufficient.

in size and plaque morphology were observed among the dengue virus serotypes. However, all plaques were easily countable under the selected optimized PRNT₅₀ conditions.

The results of robustness experiments also demonstrated that the deliberate variations made to the incubation times did not affect the observed values when data were analyzed at a three-fold difference in PRNT₅₀ titer. Therefore, the dengue PRNT₅₀ for all four serotypes is considered robust in regards to virus-serum neutralization, adsorption on Vero cells, blocking, and incubation with primary and secondary antibodies.

Validation of dengue PRNT₅₀. Once the dengue PRNT₅₀ had been optimized and determined to be sufficiently robust, the

validation of the testing method was demonstrated by determining intra-assay and inter-assay precision, accuracy/dilutability, specificity, and LLOQ, using pre-established acceptance criteria.

The intra-assay precision was confirmed to be acceptable as all samples tested (20 of 20) for serotypes 1 and 3, and 95% (19 of 20) of the samples tested for serotypes 2 and 4 had the three observed values within one 3-fold difference of the median titer (Table 1). Similarly, the inter-assay precision was confirmed to be acceptable because all of the positive samples tested (20 of 20) for serotype 3, 90% (18 of 20) for serotypes 1 and 2, and 85% (17 of 20) for serotype 4, had the three observed values within one 3-fold difference of the median titer (Table 2).

TABLE 2
Results of inter-assay precision of PRNT₅₀ for dengue virus serotypes 1, 2, 3, and 4*

Sample no.†	DENV-1 PRNT ₅₀			DENV-2 PRNT ₅₀			DENV-3 PRNT ₅₀			DENV-4 PRNT ₅₀		
	Titer 1	Titer 2	Titer 3	Titer 1	Titer 2	Titer 3	Titer 1	Titer 2	Titer 3	Titer 1	Titer 2	Titer 3
1	50	45	62	84	81	38	131	93	79	< 10‡	88	50
2	13	38	40	39	55	23	53	100	117	47	134	109
3	16‡	54	58	31	46	17	31	23	11	65	143	176
4	45	38	46	95	86	31	47	67	29	45	12‡	49
5	102	67	24	111	97	37	44	49	37	140	93	113
6	164	129	128	189	135	45	215	171	117	48	57	113
7	152	169	78	200	208	89	183	199	126	213	239	292
8	106	279	161	94	82	51	253	155	162	197	411	359
9	113	432‡	113	156	200	95	20	42	20	245	164	219
10	68	204	202	141	112	74	160	246	96	76	162	78
11	114	268	253	444	386	206	142	126	75	45	205	116
12	352	382	460	435	681	411	190	272	151	608	598	388
13	381	357	217	331	345	203	592	722	379	307	986‡	302
14	777	462	644	718	870	366	849	461	423	82	149	354
15	751	1,242	1,170	844	1,326	652	396	687	431	442	600	666
16	326	911	544	950	728	414	970	1,734	676	121	61	148
17	408	745	708	1,322	1,295	691	1,380	727	781	172	80	309
18	2,355	3,626	4,245	4,345	4,747	1,275‡	2,677	2,722	1,789	1,693	593	1,902
19	1,019	2,919	2,176	1,931	1,449	788	1,575	1,295	819	593	388	1,020
20	1,799	3,406	2,692	3,896	8,180	1,064‡	2,462	6,123	2,535	1,578	395	943

*PRNT₅₀ = 50% plaque reduction neutralization test; DENV = dengue virus.
 †Four different sample panels; one sample panel per serotype.
 ‡Result is outside ± 3-fold of the median PRNT₅₀ titer of three results.

TABLE 3
Results of accuracy/dilutability of PRNT₅₀ for dengue virus serotypes 1, 2, 3, and 4*

Sample no.	Dilution†	DENV-1 PRNT ₅₀			DENV-2 PRNT ₅₀			DENV-3 PRNT ₅₀			DENV-4 PRNT ₅₀		
		Median titer‡	Expected titer§	ABS	Median titer	Expected titer	ABS	Median titer	Expected titer	ABS	Median titer	Expected titer	ABS
1	Undiluted	3,162	NA	NA	3,319	NA	NA	2,317	NA	NA	1,416	NA	NA
	1:4	379	791	1.06	412	830	1.01	658	579	0.18	627	354	0.82
	1:16	139	198	0.51	146	207	0.51	219	145	0.60	124	89	0.49
	1:64	34	49	0.56	101	52	0.95	61	36	0.75	59	22	1.42
2	Undiluted	1,953	NA	NA	3,297	NA	NA	1,999	NA	NA	3,147	NA	NA
	1:4	488	488	0.00	591	824	0.48	401	500	0.32	799	787	0.02
	1:16	123	122	0.01	214	206	0.05	105	125	0.25	229	197	0.22
	1:64	39	31	0.35	49	52	0.07	39	31	0.32	86	49	0.81
3	Undiluted	2,300	NA	NA	3,179	NA	NA	2,918	NA	NA	910	NA	NA
	1:4	812	575	0.50	799	795	0.01	436	730	0.74	210	228	0.12
	1:16	180	144	0.32	264	199	0.41	210	182	0.20	101	57	0.83
	1:64	74	36	1.04	43	50	0.21	52	46	0.19	73	14	2.36
4	Undiluted	3,007	NA	NA	1,347	NA	NA	2,766	NA	NA	1,276	NA	NA
	1:4	416	752	0.85	302	337	0.16	636	692	0.12	249	319	0.36
	1:16	150	188	0.33	105	84	0.31	189	173	0.13	97	80	0.28
	1:64	49	47	0.06	30	21	0.51	78	43	0.85	43	20	1.11
5	Undiluted	3,543	NA	NA	1,367	NA	NA	1,369	NA	NA	1,473	NA	NA
	1:4	503	886	0.82	353	342	0.05	377	342	0.14	308	368	0.26
	1:16	142	221	0.64	84	85	0.03	113	86	0.40	60	92	0.62
	1:64	69	55	0.32	30	21	0.49	45	21	1.07	28	23	0.28

*PRNT₅₀ = 50% plaque reduction neutralization test; DENV = dengue virus; ABS, absolute difference of the log₂ expected titer from the log₂ observed median titer; NA = not applicable.

†Positive sample was tested as undiluted and diluted with negative serum samples 1:4, 1:16 and 1:64 times.

‡Median PRNT₅₀ titers of five results.

§The expected titer (undiluted) was the median of the five results of the undiluted sample within the assay run divided by the dilution factor (4, 16, or 64).

Accuracy and specificity were also confirmed to be acceptable because tested samples met the pre-defined criteria (Tables 3 and 4). The LLOQ of the dengue PRNT₅₀ was confirmed to be a titer of 10 (reciprocal dilution) because 100% (5 of 5), (8 of 8), and (7 of 7) of true positive samples for DENV-2, DENV-3, and DENV-4, respectively, and 83% (5 of 6) of true positive samples for DENV-1 met the acceptance criterion having $\geq 90\%$ of the observed values within three-fold of the median titer (Table 5).

DISCUSSION

Because clinical trials are being conducted worldwide to evaluate dengue vaccine candidates, it is imperative to have reliable methods to evaluate their immunogenicity. Various

serologic tests are available to evaluate humoral responses against dengue. However, their specificity and sensitivity differ. The hemagglutinin inhibition assay is deemed neither sensitive nor specific.^{6,9} The complement fixation test is considered to be specific to the dengue group, but is cumbersome and has been phased out in favor of the readily available and easy to use dengue-specific IgG/IgM ELISAs.^{7,8} The dengue ELISA is not serotype specific and provides only a measure of the binding of antibodies directed against the dengue virus, which may or may not possess neutralizing capability.⁹⁻¹¹ The neutralizing antibody response to the dengue virus is serotype specific, and the PRNT is considered the gold standard to measure dengue virus neutralizing antibodies. Therefore, the successful development and validation of a dengue serotype-specific PRNT and inclusion of qualified

TABLE 4
Results of specificity of PRNT₅₀ for dengue virus serotypes 1, 2, 3, and 4*

Sample no.	Spiking sample†	DENV-1 PRNT ₅₀			DENV-2 PRNT ₅₀			DENV-3 PRNT ₅₀			DENV-4 PRNT ₅₀		
		Observed titer‡	Expected titer§	ABS	Observed titer	Expected titer	ABS	Observed titer	Expected titer	ABS	Observed titer	Expected titer	ABS
1	Negative	99	99	NA	174	174	NA	549	549	NA	79	79	NA
	YF+	116		0.23	168		0.05	399		0.46	59		0.42
	JE+	79		0.33	101		0.78	599		0.13	49		0.69
2	Negative	75	75	NA	95	95	NA	96	96	NA	53	53	NA
	YF+	119		0.67	104		0.13	100		0.06	41		0.37
	JE+	112		0.58	169		0.83	116		0.27	60		0.18
3	Negative	337	337	NA	644	644	NA	445	445	NA	207	207	NA
	YF+	177		0.93	703		0.13	586		0.40	154		0.43
	JE+	364		0.11	889		0.47	438		0.02	140		0.56
4	Negative	443	443	NA	339	339	NA	309	309	NA	85	85	NA
	YF+	398		0.15	447		0.40	220		0.49	76		0.16
	JE+	426		0.06	433		0.35	314		0.02	144		0.76
5	Negative	135	135	NA	317	317	NA	350	350	NA	204	204	NA
	YF+	157		0.22	368		0.22	329		0.09	200		0.03
	JE+	113		0.26	200		0.66	238		0.56	157		0.38

*PRNT₅₀ = 50% plaque reduction neutralization test; DENV = dengue virus; ABS, absolute difference of the log₂ expected titer from the log₂ observed median titer; NA = not applicable.

†Anti-dengue-positive samples were tested after spiking with equal volume of negative, anti-yellow fever virus positive (YF+) and anti-Japanese Encephalitis Virus positive (JE+) human serum samples.

‡Observed PRNT₅₀ titers.

§The expected titer was obtained by spiking of anti-dengue positive sample with equal volume of negative human serum sample.

TABLE 5
Results of lower limit of quantitation of PRNT₅₀ for dengue virus serotypes 1, 2, 3, and 4*

Sample no.	No. repeats†	DENV-1 PRNT ₅₀		DENV-2 PRNT ₅₀		DENV-3 PRNT ₅₀		DENV-4 PRNT ₅₀	
		Median titer‡	%§	Median titer	%	Median titer	%	Median titer	%
1	11	14	NA¶	< 10	NA#	16	100	26	91
2	11	16	100	17	91	23	100	< 10	NA
3	11	16	82	25	91	27	100	21	91
4	11	19	91	20	100	19	100	19	91
5	11	13	NA	< 10	NA	25	91	36	100
6	11	37	91	28	100	20	100	11	100
7	11	32	100	28	100	25	100	24	91
8	11	41	100	10	NA**	33	100	44	100
9	11	< 10	NA	< 10	NA	< 10	NA	< 10	NA
10	11	< 10	NA	< 10	NA	< 10	NA	< 10	NA

*PRNT₅₀ = 50% plaque reduction neutralization test; DENV = dengue virus; NA = not applicable.

†Each sample was tested 11 times in at least 2 independent assay runs.

‡Median PRNT₅₀ titers of 11 values were calculated for each sample.

§Percent of the values within three-fold of the median titer.

¶Samples were excluded from analysis because 45% (5 of 11) and 36% (4 of 11) of the results were negative for DENV-1 PRNT₅₀ samples #1 and #5, respectively, and samples were not considered a true positive sample.

#Samples #9 and #10 for all four serotypes, sample #2 for DENV-4 PRNT₅₀, and samples #2 and #5 for DENV-2 PRNT₅₀ were excluded from statistical analysis because the median titer was < the lower limit of quantitation (i.e., < 10).

**Sample was excluded from analysis because 36% (4 of 11) of the results were negative for DENV-2 PRNT₅₀ sample #8, and the sample was not considered a true positive sample.

assay reagents are key factors in the ability to provide accurate and reliable data crucial for vaccine studies, and especially to ensure the reproducibility of neutralization assay performance over extended periods.

Several variations of the dengue PRNT are used by research laboratories and vaccine developers. They use a variety of cell lines and virus strains, with different readouts to measure dengue vaccine immunogenicity or to study dengue epidemiology and pathogenesis.^{12,16,21,24,38-40} The PRNT endpoint titers are expressed as the reciprocal of the highest test serum dilution showing the desired percent reduction in plaque counts as compared with the virus control counts. A 50% reduction in plaques (PRNT₅₀ titer) is preferred for evaluating human sera after vaccination because it affords acceptable sensitivity and specificity, although greater reduction in plaque counts (≥ 80%) is useful for epidemiologic studies or diagnostic purposes to avoid or reduce cross-reactivity among flaviviruses in dengue-endemic areas.^{18,41}

The dengue PRNT₅₀ presented was developed in view of the WHO guidance for dengue PRNT with some modifications to the classic method. This assay is performed in 24-well plates using Vero cell monolayers. Immunostaining using MAbs directed against dengue serotype-specific envelope protein is used as an alternative to non-specific dyes such as neutral red, amido black, or crystal violet to visualize virus-infected foci and measure the level of dengue virus serotype-specific neutralizing antibodies.

We evaluated several parameters to identify assay conditions that resulted in consistent and reliable test performance, suitable to support the large scale Sanofi Pasteur global dengue vaccine clinical development program. Production of each of the four dengue viruses has been optimized and standardized for use in the PRNT₅₀. In addition, we qualified assay reagents according to reagent-specific procedures based on their intended use and performed side-by-side comparisons between new and qualified lots to demonstrate equivalency. Reagent qualification in this instance is defined as establishing confidence through documented evidence that an assay reagent is suitable for its intended use and consistently performing within the established limits. Acceptance criteria were pre-determined to accept or reject a new lot of

reagent before implementation, and the trending of qualified assay quality controls was applied for a continuous monitoring of assay performance over time.

Our assessment demonstrated successful performance of the assay in that the dengue PRNT₅₀ for each of the four dengue virus serotypes is precise, accurate, and specific, and is suitable for its intended use to detect and measure specific neutralizing antibodies to each of the four dengue virus serotypes in human serum samples with an LLOQ of 10 (reciprocal dilution).

There are several steps of the dengue PRNT₅₀ that may be amenable to automation, including plaque counting, among others, and there have been some recent efforts to develop higher throughput assays: a flow cytometry-based dengue neutralization assay utilizing the Raji human cell line transfected to express DC-SIGN, a receptor or co-receptor used by all four dengue serotypes and microneutralization based formats.^{17,42-47} In conclusion, although new generation tests measuring dengue virus neutralization are being developed, the dengue PRNT remains the most common assay used today, and is considered the gold standard laboratory assay recommended by the WHO against which any new test method will need to be validated.

Received July 30, 2012. Accepted for publication January 25, 2013.

Published online March 4, 2013.

Acknowledgments: We thank the dengue laboratory testing team within the Global Clinical Immunology Department, Sanofi Pasteur, Inc. (Swiftwater, PA) for all their work in supporting the development/validation of the assay and performing clinical trial testing; the Global Clinical Immunology Department biostatistical team for their support in design of the DOE, establishing the acceptance criteria, and analyzing experimental and validation results; and Mark Boaz, Dany DeGrave, Jean Lang, and the dengue project teams for their scientific discussions, for kindly providing clinical human serum samples from the program, and for reviewing the manuscript.

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