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Development and characterization of a stable luciferase dengue virus for high-throughput screening

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ABSTRACT

To facilitate dengue virus (DENV) drug discovery, we developed a stable luciferase reporter DENV-2. A *renilla* luciferase gene was engineered into the capsid-coding region of an infectious cDNA clone of DENV-2. Transfection of BHK-21 cells with the cDNA clone-derived RNA generated high titers (>10^6 PFU/ml) of luciferase reporter DENV-2. The reporter virus was infectious to a variety of cells, producing robust luciferase signals. Compared with wild-type virus, the reporter virus replicated slower in both mammalian Vero and mosquito C6/36 cells. To examine the stability of the reporter virus, we continuously passaged the virus on Vero cells for five rounds. All passaged viruses stably maintained the luciferase gene, demonstrating the stability of the reporter virus. Furthermore, we found that the passaged virus accumulated a mutation (T108M) in viral NS4B gene that could enhance viral RNA replication in a cell-type specific manner. Using the reporter virus, we developed a HTS assay in a 384-well format. The HTS assay was validated with known DENV inhibitors and showed a robust *Z′* factor of 0.79. The Luc-DENV-2 HTS assay allows screening for inhibitors of all steps of the viral life cycle. The reporter virus will also be a useful tool for studying DENV replication and pathogenesis.

1. Introduction

Dengue virus (DENV) is a mosquito-borne, enveloped RNA virus. It belongs to the genus *Flavivirus* in the family *Flaviviridae* (Chambers et al., 1990). The four genetically related but serologically distinct serotypes of DENV (DENV-1 to -4) cause the most common arthropod-borne viral infection worldwide. Approximately 2.5 billion people are at risk of DENV infection worldwide. Word Health Organization estimated 50–100 million human infections annually, leading to about 20,000 deaths (Gubler et al., 2007; Guzman et al., 2010). Primary DENV infection typically results in mild dengue fever (DF) and provides lifelong immunity to the infecting virus serotype. However, secondary infection with different DENV serotypes increases the risk of developing dengue hemorrhagic fever (DHF), which can lead to life-threatening dengue shock syndrome (DSS) (Gubler et al., 2007). Despite the urgent need to control dengue infections, neither specific antiviral therapies nor licensed vaccines are currently available (Whitehead et al., 2007). The development of therapies for DENV requires both insights into the viral life cycle and rational strategies for identifying inhibitors.

Dengue virion is approximately 50 nm in diameter and contains a single plus-sense RNA that is approximately 10.6 kb in length (Chambers et al., 1990; Kuhn et al., 2002). The genomic RNA contains a single long open reading frame (ORF) that is flanked by 5′ and 3′ untranslated region (UTR) (Lindenbach et al., 2007). Virus infection was initiated by attachment to cell surface. The virus enters the cell by receptor-mediated endocytosis (van der Schaar et al., 2007). Following internalization, the low pH of the endosome triggers structural rearrangements of the envelope protein, leading to fusion of the viral and cellular membranes. After the release of nucleocapsid into the cytoplasm, the genomic RNA is translated into a single polyprotein which is co- and post-translationally processed by viral and cellular proteases into 10 mature proteins: three structural proteins (capsid [C], premembrane or membrane [M], and envelope [E] proteins) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Lindenbach et al., 2007). The non-structural proteins amplify viral RNA in replication complexes associated with endoplasmic reticulum (ER) membranes (Mackenzie et al., 2001). Newly synthesized RNA and capsid protein are enveloped by prM and E, generating immature virions that bud into the lumen of ER. The immature viral particles

Abbreviations: DENV, dengue virus; HTS, high-throughput screen; C, capsid protein; prM, premembrane; E, envelope; NS, non-structural protein; VLP, virus-like particle; Rluc, *Renilla* luciferase; FMDV2A, foot-and-mouth disease virus 2A autocleavage site.

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are transported through the Golgi apparatus and exocytosis pathway. Host protease furin cleaves prM into pr and M, resulting in infectious mature virions (Elshuber et al., 2003; Li et al., 2008; Yu et al., 2008). Small-molecular inhibitors capable of blocking any of these replication steps could be potentially developed for antiviral therapy.

Both target-based and cell-based approaches have been employed in DENV drug discovery (Noble et al., 2010). Target-based approach usually uses recombinant protein for functional assays. Cell-based approach uses viral infection to screen for inhibitors. For DENV, two cell-based high-throughput screening (HTS) assays have been reported: a replicon-containing cell line assay (Ng et al., 2007; Puig-Basagoiti et al., 2006) and a virus-like particle (VLP) assay (Qing et al., 2010). The VLP contains a replicon that lacks viral structural genes; infection of cells with VLP leads to a single round viral entry and RNA synthesis without virion assembly. Neither of the two assays covers the complete replication cycle. This limit could be overcome by a reporter DENV infection assay. Reporter flaviviruses were previously reported for JEV (Yun et al., 2003), WNV (Deas et al., 2005), and DENV (Kaptein et al., 2010) by engineering the IRES (internal ribosomal entry site)-reporter gene at the 3′ UTR of viral genome. Such reporter viruses were not stable since the reporter gene was deleted after one to two rounds of viral infection cycles (Deas et al., 2005). Frolov and colleagues first reported that YFV with a reporter gene engineered at the capsid gene was stable in cell culture (Shustov et al., 2007). Taking a similar approach, Gamarnik and coworkers recently reported a reporter DENV (Samsa et al., 2009). However, none of the stable reporter flaviviruses have been developed and characterized for antiviral screening.

In this study, we report the development and characterization of a stable luciferase DENV-2. The reporter DENV-2 was developed into a 384-well format HTS assay. The new HTS assay was robust with an average Z' factor of 0.79, and was validated with various known DENV inhibitors. Statistical criteria for plate uniformity assessment indicate that the assay is ready for compound library screening. To our knowledge, this is the first study that a reporter flavivirus was applied to HTS assay, and the system should greatly facilitate dengue drug discovery.

2. Materials and methods

2.1. Cell lines, viruses, antibodies, and compounds

Mosquito C6/36 cells were cultured in RPMI medium (Invitrogen) with 10% fetal bovine serum (FBS; Thermo Scientific HyClone) at 28 °C. All other cells were grown at 37 °C with 5% CO_2. BHK-21 (baby hamster kidney fibroblast), Vero (African green monkey kidney), and HuH-7 (human liver) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 10% FBS and 100 U/ml penicillin–streptomycin (PS). LLC-MK2 (Rhesus monkey kidney epithelial) cells were cultured in M199 Earle's medium (Invitrogen) with 10% FBS and 100 U/ml PS. The A549 (human lung) cells were cultured in F-12 medium (Invitrogen) supplemented with 10% FBS and 2 mM L-glutamine. BHK-21 cells containing persistently replicating DENV-2 TSV01 replicon with a neomycin phosphotransferase gene were cultured in DMEM with 10% FBS plus 1 mg/ml of Geneticin (G418; Invitrogen). DENV-2 virus (strain TSV01; GenBank accession No. AY037116) was derived from an infectious cDNA clone (Zou et al., 2011). Dengue specific anti-E monoclonal antibody 4G2 was prepared from hybridoma cell lines purchased from the American Type Culture Collection (ATCC) (Rajamanonmani et al., 2009). DENV entry inhibitor compound 6 (Wang et al., 2009) and nucleoside analogue inhibitor NITD008 (Yin et al., 2009) were synthesized in house. α-Glucosidase inhibitor castanospermine (Whitby et al., 2005) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All compounds were dissolved in 90% DMSO for antiviral experiments.

2.2. Plasmid construction

As shown in Fig. 1, an infectious cDNA clone of DENV-2 (designated as pACYC FLTSV) and a cDNA clone of *renilla* luciferase reporter replicon (pACYC Rluc2A TSV Rep) were used to construct a cDNA clone of luciferase reporter DENV-2 (pACYC Rluc2A TSV). All constructs were derived from DENV-2 strain TSV01. The construction of pACYC FLTSV was recently reported (Zou et al., 2011). The pACYC Rluc2A TSV Rep was constructed by replacing the structural genes with *renilla* luciferase gene followed by FMDV2A. In the replicon, the coding sequences of 38 amino acids at the N-terminus of capsid protein and 31 amino acids at the C-terminus of E protein were retained to preserve correct processing and translocation of NS1 as well as correct topology of the remaining nonstructural polyprotein across the membrane of ER.

A two-step cloning strategy was used to construct pACYC Rluc2A TSV plasmid. First, a standard overlap PCR was used to create a cassette containing "SacII-T7 promoter-5′ UTR-N-terminal 38 amino acids of capsid protein-*renilla* luciferase gene-FMDV2A-authentic initiation codon of capsid protein to the unique BsrGI site in E protein". Fragment covering "SacII to FMDV2A" was amplified with the primer pACYC SacII-F and FMDV2A Capsid-R using the pACYC Rluc2A TSV Rep as a template. Fragment spanning "the authentic initiation codon of capsid protein to BsrGI unique site in E protein" (located at nucleotide position 1840 of the viral genome) was amplified with primer FMDV2A Capsid-F and TSV 1948-R using the pACYC FLTSV as a template. The primer sequences are presented in Table 1. The two fragments were fused together and ligated into pCR-Blunt II-TOPO with Zero Blunt TOPO PCR Cloning Kit according to the Manufacturer's instructions (Invitrogen). Next, the fragment from SacII to BsrGI was engineered at the corresponding sites into pACYC FLTSV, resulting in plasmid pACYC Rluc2A TSV. Compared with the wild-type pACYC FLTSV, pACYC Rluc2A TSV contained an extra fragment (representing the first 38 amino acids of C protein-a *renilla* luciferase gene-FMDV2A) between the 5′ UTR and the complete ORF of the viral genome. A non-replicative NS5 mutation W859A (Zou et al., 2011) was introduced into the pACYC Rluc2A TSV by site-directed mutagenesis. All the constructs were verified by DNA sequencing.

2.3. In vitro transcription, RNA transfection, immunofluorescence assay (IFA), and plaque assay

The genome-length RNA of wild-type DENV-2 TSV01 virus (designated as WT DENV) and luciferase reporter DENV-2 TSV01 virus (designated as Luc-DENV) were *in vitro* transcribed from corresponding cDNA plasmids that were linearized with ClaI. A T7 mMESSAGE mMACHINE kit (Ambion) was used for RNA synthesis as described previous (Zhou et al., 2007). The RNAs were electroporated into BHK-21 cells as previously described (Shi et al., 2002). After transfection of genome-length RNA, cells were cultured at 37 °C for the first 24 h and then transferred to 30 °C. Supernatants were collected every 24 h until day 5 post-transfection. The culture medium containing viruses were aliquoted and stored at −80 °C.

IFA were performed as previously described (Hsieh et al., 2011). Plaque assays were performed as previously described, except that the plate incubation period (after the addition of the overlay agar) was extended for two extra days (Poh et al., 2009). This modification was required to compare the plaque morphology of the Luc-DENV and the WT DENV.

2.4. Transient transfection analysis of the Luc-DENV RNA

A transient transfection assay of the Luc-DENV RNA was used to quantify viral translation and RNA replication. Ten micrograms of wild-type Luc-DENV (designated as WT Luc-DENV) and NS5

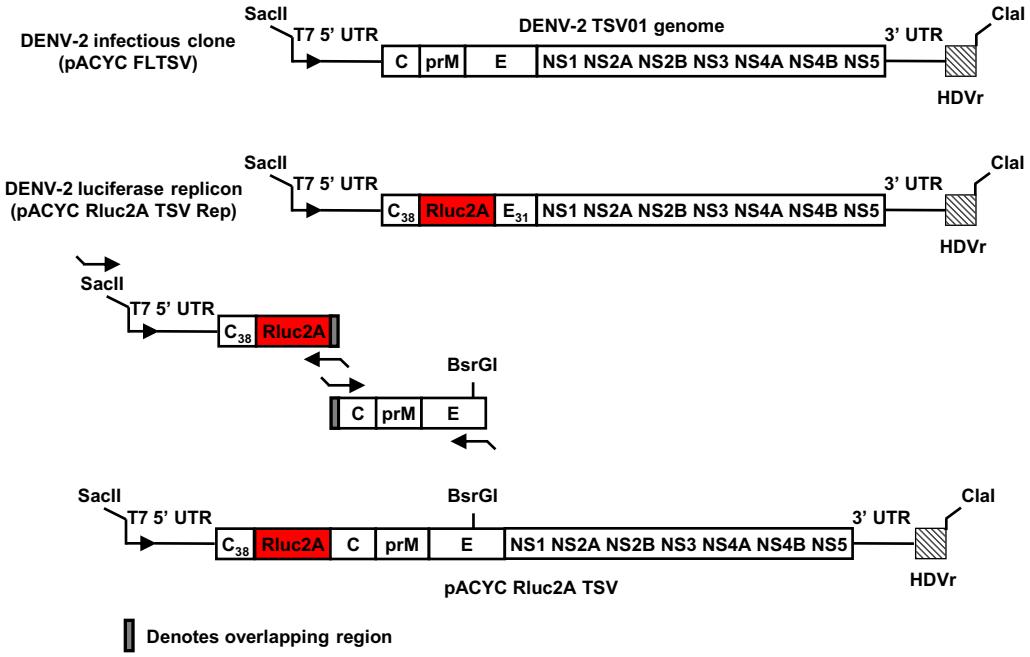


Fig. 1. Schematic of the cDNA clone of DENV-2 TSV01 containing *renilla* luciferase reporter gene. An infectious cDNA clone of DENV-2 TSV01 (pACYC FLTSV) was used as the backbone for construction of the cDNA clone of dengue virus containing *renilla* luciferase reporter. A *renilla* luciferase reporting replicon of DENV-2 TSV01 (pACYC Rluc2A TSV Rep) was used as a template for overlap PCR. The replicon retains N-terminal 38 amino acids of the capsid protein (C_{38}) and C-terminal 31 amino acids of E protein (E_{31}). A cassette containing "SacII-T7 promoter-5′ UTR-C_{38}-*renilla* luciferase reporter gene-FMDV2A-start codon of capsid protein to BsrGI unique site in E protein" was produced by overlapping PCR as described in Section 2. The cassette was ligated into pACYC FLTSV at SacII and BsrGI to form the cDNA clone of luciferase reporter dengue virus (pACYC Rluc2A TSV).

Table 1
Primer sequences.

Primer	Sequence 5′–3′
pACYC SacII-F	CCGCGGCCCTCTCACTTCCCTGTTAAGTATC
FMDV2A Capsid-R	CGCCTTTTTCCGTTGGTTATTCATTGGCCCAGGG TTGGACTCGAC
FMDV2A Capsid-F	GTCGAGTCCAACCCTGGGCCAATGAATAACCAACGG AAAAAGGCG
TSV 1948-R	CAAAAGGGATCTTGCACGGAGAACC
Rluc 664V	GAGATCCCTCTCGTTAAGGGAG
TSV 674C	GTCCCATAAGTTACCCATGTAGACG

F, Forward; R, Reverse.

W859A mutant Luc-DENV (designated as MT Luc-DENV) RNA was electroporated into 8×10^6 BHK-21. The transfected cells were resuspended in 25 ml of DMEM with 10% FBS; 1, 1, 1, 1, 0.5, 0.4, 0.3, and 0.25 ml of the transfected cells were seeded into 12-well plates; and the cells were assayed for luciferase activities at 2, 4, 6, 24, 48, 72, 96, and 120 h post-transfection (p.t.), respectively. Triplicate wells were seeded for each data point. For harvesting of lysates at early time points (2, 4, and 6 h p.t.), 12-well plates were spun at 700*g* for 5 min at 4 °C to ensure adherence of the transfected cells. The medium was then removed, and the cells were washed with PBS and spun for an additional 5 min at 700*g*. For samples collected at later time points ($\geqslant$24 h p.t.), cells had already adhered to plates and were washed once with PBS without centrifugation. After the PBS washing, cells were lysed with 250 μl of lysis buffer on a shaker for 20 min. Luciferase assays were performed as described below.

2.5. Luciferase assay

Two types of luciferase assay were performed. One assay utilizes lysis approach, the cells must be lysed before addition of luciferase substrate, and luciferase activity was measured by mixing 20 μl lysates with 50 μl assay reagent (Promega). The other assay utilizes live cell substrate (Enduren or ViviRen; Promega) without cell lysis. The HTS assay was performed using the latter assay. For both assays, luciferase activities were measured using a Clarity luminescence microplate reader (BioTek).

2.6. RNA extraction and standard RT-PCR

To test the stability of reporter virus (Luc-DENV), we passaged the virus on Vero cells for five rounds. For each round, Vero cells (1×10^6 cells) in a T-25 flask were infected with 100 μl of culture supernatant derived from the previous round of infection and cultured in DMEM with 2% FBS for 3 days. Viral RNAs were extracted from culture fluids using RNeasy kits (Qiagen). Viral RNAs were amplified by RT-PCR using SuperScript III one-step RT-PCR kits (Invitrogen) with primer targeting the luciferase gene and a primer targeting capsid gene (forward primer: Rluc 664 V and reverse primer: TSV 674C were listed in Table 1). The resulting 914-bp DNA product was analyzed on a 1% agarose gel. The passage 5 (P5) reporter virus was subjected to complete genome-length sequencing.

2.7. Preparation of reporter virus stock

Ten T-175 flasks of 90% confluence of BHK-21 cells were transfected with Luc-DENV RNA as previously described (Shi et al., 2002). After incubation for 24 h at 37 °C, cells were transferred to 30 °C. Culture medium was removed and replaced with 40 ml of fresh phenol red-free DMEM medium (Invitrogen) with 2% FBS on day 3 post-transfection (p.t.). From day 4 to 9 p.t., culture medium was harvested and replenished with 40 ml of fresh medium every 24 h. Apparent cytopathic effect (CPE) was observed on day 9 p.t.. The supernatants were pooled together, filtered through a 0.22 μm filter, aliquoted, and stored at −80 °C. The amount of infectious Luc-DENV in the stock was titrated by plaque assay.

2.8. Validation of reporter virus assay by antiviral compounds

Vero cells were seeded at 2×10^4 cells per well in 96-well plates. After incubation overnight, the cells were infected with Luc-DENV at an MOI of 0.5 in the presence of reference compounds. Compound 6 is a small-molecular entry inhibitor (Wang et al., 2009); NITD008 is an adenosine analogue (Yin et al., 2009); castanospermine is an α-glucosidase inhibitor (Courageot et al., 2000; Whitby et al., 2005). At day 2 p.i., luciferase activity was measured using Enduren, and cell viability was estimated in an MTS assay as previously described (Wang et al., 2009). Each experiment was performed in quadruplicate. Meanwhile, the reference compounds were also tested by a VLP infection assay and a DENV-2 TSV01 subgenomic replicon-containing cell line assay. The VLP assay was performed as previously reported (Qing et al., 2010). For replicon-containing cell line assay, BHK-21 cells containing replicon of DENV-2 TSV01 were seeded at 1×10^4 cells per well in 96-well plates in DMEM with 5% FBS without G418. Compounds were added to the medium at 16 h post-seeding. After 48 h of compound-treatment, the cells were lysed and assayed for luciferase activities as described above.

2.9. Plate uniformity assessment

Plate uniformity assessments were performed to determine whether our assay was suitable for adaptation to a high-throughput format. We followed the recommendations of the National Institutes of Health Chemical Genomics Center's Assay Guidance Manual Version 5.0 for plate uniformity assessments (Eli Lilly and Company, 2008). Three separate experiments consisting of three 384-well plates each were performed on three different days. Briefly, 5×10^3 Vero cells were seeded into each well of 384-well plate and cultured at 37 °C overnight. The cells were infected with Luc-DENV at an MOI of 0.5. Cells treated with 0.1% DMSO represented the maximum signal value, and cells treated with 1 μM and 25 μM NITD008 represented middle and minimal signal value, respectively.

2.10. Data analysis and statistics

Data analyses for luciferase activity and cell viability were performed using the Prism software (GraphPadPrism4, San Diego, CA). The luciferase data were presented as means plus standard deviations. To evaluate the performance of the HTS assay, the signal-to-noise ratio (S/N), coefficient of variation (CV), and Z' factor values were calculated. Z' factor between 0.5 and 1 indicates an excellent assay with good separation between controls (Zhang et al., 1999). For plate uniformity assessment, all calculations were performed using the Assay Guidance Manual's spreadsheet (Eli Lilly and Company, 2008).

3. Results

3.1. Construction of the cDNA clone of dengue virus that encodes renilla luciferase

We constructed a cDNA clone of DENV-2 that encodes a *renilla* luciferase reporter gene. As depicted in Fig. 1, a fragment encompassing the first 38 amino acids of C protein, a *renilla* luciferase gene, and a FMDV2A sequence was inserted at the junction between 5′ UTR and the ORF of viral genome. The duplication of the N-terminal 38 amino acids of C protein is to maintain RNA elements that are required for genome cyclization. As reported previously, genome cyclization is essential for DENV replication (Alvarez et al., 2005, 2008; Friebe and Harris, 2010). The FMDV2A sequence was engineered to ensure that the luciferase protein was properly processed.

3.2. Characterization of Luc-DENV

Equal amounts of wild-type (WT) DENV-2 and Luc-DENV genome-length RNAs were electroporated into BHK-21 cells. Viral protein synthesis, plaque morphology, and virus production were compared between the WT and Luc-DENV RNAs. For viral protein synthesis, IFA (detecting viral E protein) showed increasing numbers of E-positive cells from day 1 to 3 for both WT DENV and Luc-DENV (Fig. 2A). However, at each time point, the Luc-DENV transfection showed fewer IFA-positive cells than WT DENV transfection. For example, on day 3 p.t., only 45% of the Luc-DENV-transfected cells were IFA-positive, whereas 80% of the WT DENV-transfected cells were IFA-positive. The Luc-DENV displayed smaller plaque size than WT DENV (Fig. 2B). As measured by plaque assays, the Luc-DENV RNA-transfected cells produced increasing amounts of viruses from day 2 to 5; the viral titer peak of WT DENV were about >10-fold higher than that of Luc-DENV (Fig. 2C); the titer of Luc-DENV reached 1×10^5 to 1×10^6 PFU/ml on day 5 post-transfection. In agreement with the kinetics of virus production, the Luc-DENV RNA-transfected cells exhibited increasing amounts of luciferase signal from day 1 to 5 p.t. (Fig. 2D). As a negative control, a mutant (MT) Luc-DENV RNA containing a lethal RdRp mutation (W859A) (Zou et al., 2011) was also transfected into BHK-21 cells; the luciferase signals dropped to background level from 6 to 48 h p.t. (Fig. 2D). We also compared the growth kinetics of WT DENV and Luc-DENV on Vero (Fig. 2E) and C6/36 cells (Fig. 2F) by plaque assay; the Luc-DENV grew slower than WT DENV did on both cells. Overall, the results demonstrate that the reporter virus has a slower replication kinetic than the WT virus.

3.3. Stability of the Luc-DENV in cell culture

To examine the stability of Luc-DENV, we passaged the virus on Vero cells for five rounds. Vero cells in a T-25 flask were infected with Luc-DENV (derived from RNA transfection, defined as P0) at an MOI of 0.1. At 72 h p.i., 100 μl of culture fluid was transferred to a new T-25 flask containing naive Vero cells in 5 ml of culture medium. After five rounds of such passaging, viruses from each passage (P0–P5) were used to infect Vero cells at an MOI of 0.3. The infected cells were assayed for luciferase activities at 48 h post-infection. As shown in Fig. 3A, comparable luciferase signals (above 2×10^6 units) were observed from the cells infected with the first two rounds of passaged viruses, and luciferase signals increased from P3 to P5. These results indicate that the luciferase reporter was stably maintained during passaging; furthermore, adaptive mutation(s) may have accumulated in viral genome to enhance replication (see detailed analysis below). To confirm the stability of reporter virus, we extracted viral RNA from each passage, and performed RT-PCR to amplify the junction region between the luciferase and capsid genes. Each of the P1 to P5 viral RNAs produced an expected RT-PCR product of 914-bp (Fig. 3B).

Plaque assays showed that the plaque size of Luc-DENV increased marginally after five rounds of passaging (Fig. 3C). Sequencing the complete genome of P5 virus revealed two adaptive mutations: a C → T change at nucleotide position 7148, leading to amino acid T108 M change in NS4B; and a T → A change (mixed in chromas) at nucleotide position 7962, resulting in amino acid D131E change in the methyltransferase domain of NS5. A previous study showed that the D131E change in NS5 increased the N7 methylation activity by 37% (Kroschewski et al., 2008). To examine the effect of adaptations on viral replication, we engineered the C7148T and T7962A mutation alone or in combination

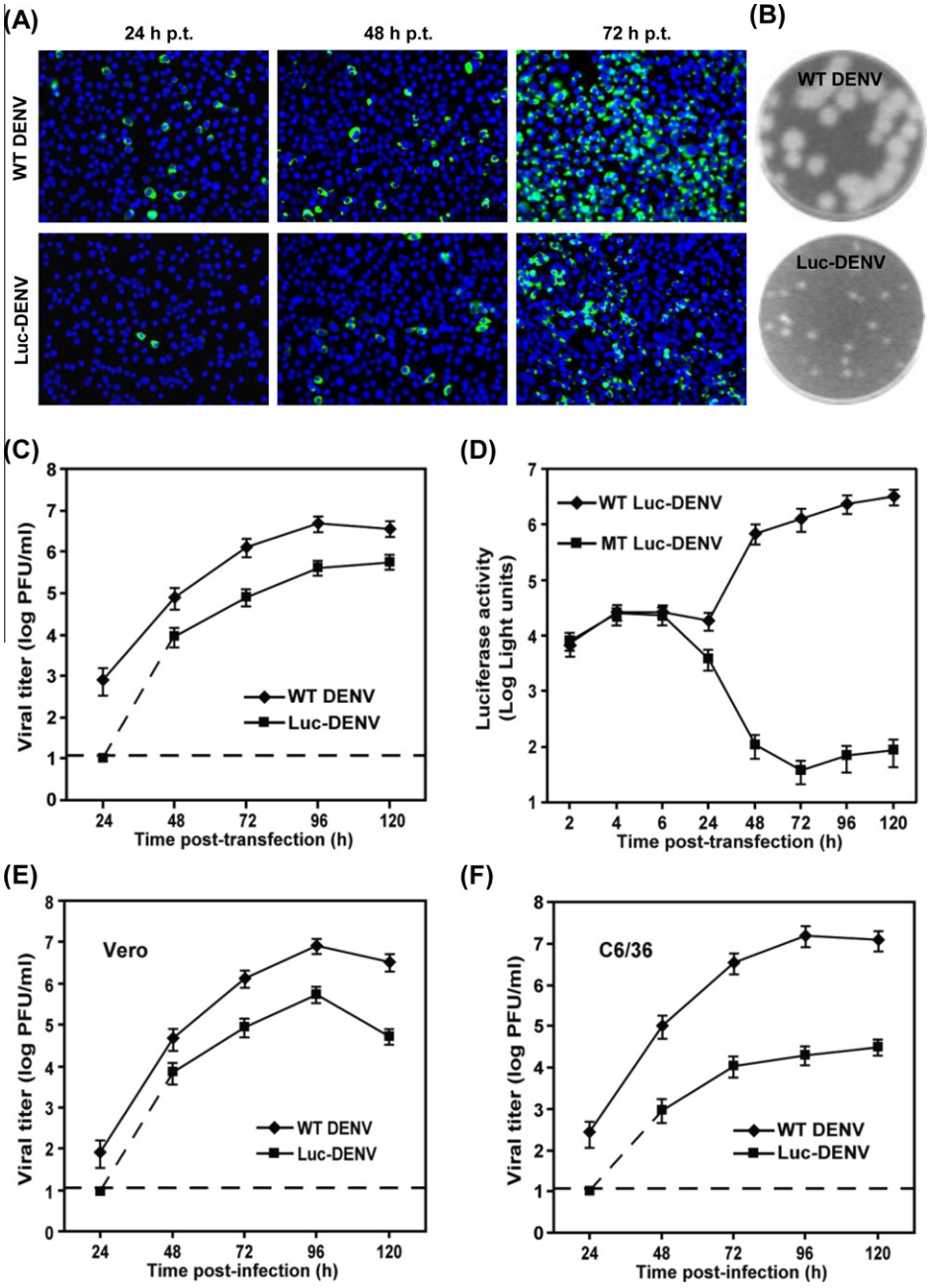


Fig. 2. Characterization of the Luc-DENV. (A) IFA of viral protein expression in cells transfected with full-length wild-type DENV-2 TSV01 virus (WT DENV) and luciferase reporter DENV-2 TSV01 virus (Luc-DENV) RNA transcript. The transfected cells were analyzed by IFA (detecting viral E protein expression) on day 1, day 2 and day 3 post-transfection. (B) Plaque morphology of WT DENV and Luc-DENV. (C) Yield of Luc-DENV after transfection. BHK-21 cells were transfected with 10 μg of WT DENV and Luc-DENV RNA transcript, viral titers from culture supernatants at indicated time points were determined by plaque assay as described in Section 2. Error bars indicate the standard deviations from two independent experiments; dashed line, limit of sensitivity of the plaque assay. (D) Transient transfection analysis of wild-type Luc-DENV (WT Luc-DENV) and a non-replicative NS5 W859A mutant (MT Luc-DENV) RNA. Luciferase activity in the transfected cells was measured at the indicated time points post-transfection. Error bars indicate the standard deviations from three independent experiments. (E and F) Comparison of growth kinetic of WT DENV and Luc-DENV on Vero and C6/36, respectively. 3×10^5 Vero and 8×10^5 C6/36 cells were seeded into each well of a 12-well plate, respectively. After incubation overnight, the cells were infected with WT DENV and Luc-DENV at an MOI of 0.1 for 1 h, and then the virus was removed and the cells were washed three times by medium, 1 ml of fresh medium was added to the cells after washing. Viral titer from culture supernatants at indicated time points were determined by plaque assay. Error bars indicate the standard deviations from two independent experiments; dashed line, limit of sensitivity of the plaque assay.

into a luciferase replicon (pACYC Rluc2A TSV Rep). Equal amounts of WT or mutant (containing C7148T alone, T7962A alone, or C7148T + T7962A) replicon RNAs were transfected into Vero cells. The transfected cells were assayed for luciferase activities at various time points after transfection. As shown in Fig. 3D, similar levels of luciferase activities were observed at 2, 4, and 6 h p.t. for all replicons, suggesting that an equivalent transfection efficiency was achieved for each construct. In contrast, the luciferase signals from C7148T replicon were 17.2- and 6.5-fold higher than those of the WT at 24 and 30 h p.t., respectively; the T7962A replicon generated a luciferase profile similar to that of the WT; and the double mutant C7148T + T7962A replicon yielded a luciferase curve similar to that of the C7148T replicon. These results demonstrate that the single T108M mutation in NS4B is responsible for the enhanced viral replication. Interestingly, we found that the NS4B T108M mutation-mediated replication enhancement was restricted to

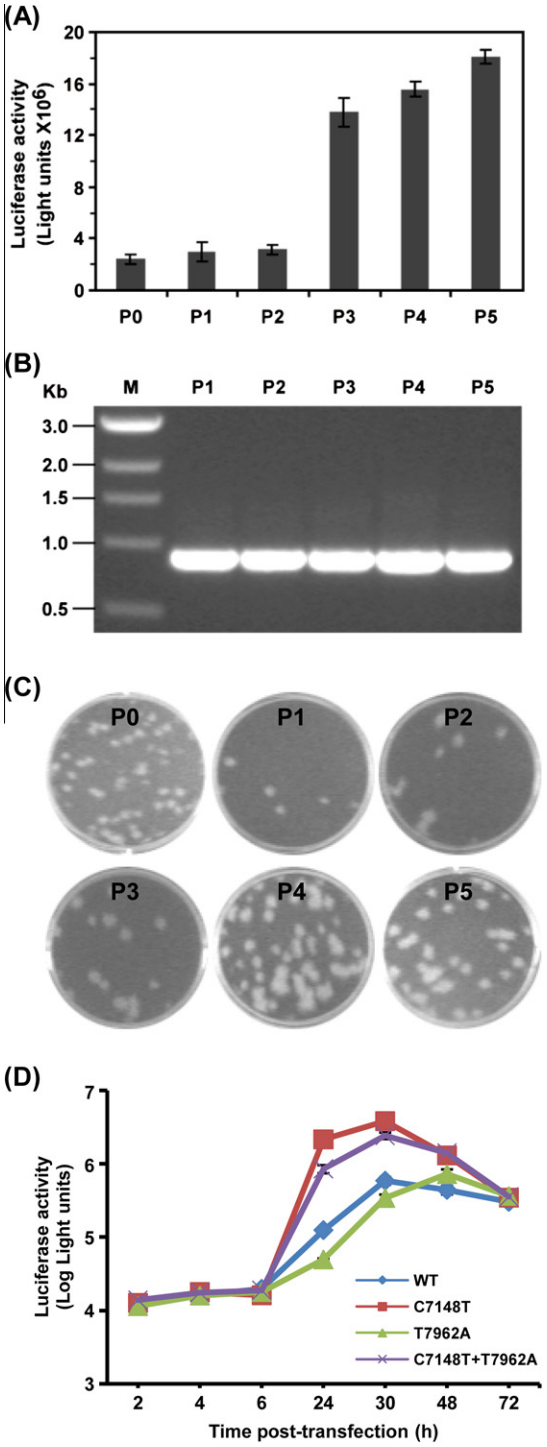


Fig. 3. Stability of the Luc-DENV in cell culture. (A) Luc-DENV was passaged on Vero cells for five rounds. Viruses from each passage (P1–P5) were used to infect 2×10^4 naive Vero cells seeded in 96-well plate at an MOI of 0.3. At 48 h post-infection, cells were lysed and luciferase activity was measured. Error bars represent the standard deviation of triplicate measurements; one representative of two experiments is shown. (B) Detection of the *renilla* luciferase reporter during virus passage. Viral RNA was extracted from culture supernatants of each passage, RT-PCR was performed with a primer set that targeted the junction region of *renilla* luciferase reporter gene and capsid protein. The resulting RT-PCR products were resolved by 1% agarose gel electrophoresis. (C) Plaque morphologies of Luc-DENV from each passage. Virus from culture supernatants was subjected to plaque assay on BHK-21 cells. (D) C7148T adaptive mutation could enhance viral replication on Vero cells. Ten micrograms of WT and mutant (C7148T alone, T7962A alone, or C7148T + T7962A) replicon RNAs were transfected into Vero cells, luciferase activities were measured at the indicated time points. Average result of triplicate measurements is shown.

Vero cells; no enhancement of replicon replication was observed in BHK-21 cells (data not shown). Collectively, these results demonstrate that (i) the luciferase reporter virus is stable; and (ii) continuous passaging of the virus in Vero cells accumulates an NS4B mutation that could enhance viral replication in a cell-type specific manner.

3.4. Optimization of the Luc-DENV infection assay for HTS

To develop the Luc-DENV into a HTS assay, we first identified an appropriate cell line for Luc-DENV infection. A549, BHK-21, Huh-7, LLC-MK2, or Vero cells (0–80,000; 2-fold serial dilutions) grown in 80 μl of medium plus 2% FBS were infected with 20 μl Luc-DENV (3.5×10^5 PFU/ml) in a 96-well plate. The cells were assayed for luciferase activities from 48 to 72 h after infection. Fig. 4A shows the highest luciferase signals for each cell line (chosen from the

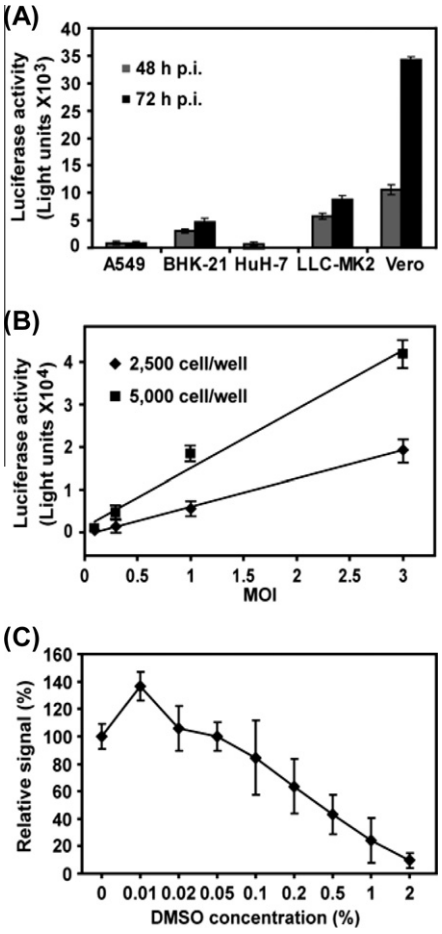


Fig. 4. Optimization of the Luc-DENV infection assay into HTS format. (A) Luc-DENV infection in different cell lines, and time course of infection. Twenty microliters of Luc-DENV (3.5×10^5 PFU/ml) were used to infect indicated cell lines at various cell numbers (0–80,000). Infection was scored as a function of luciferase activity at indicated time points. The highest signal in each cell line among the various cell numbers is represented. Error bars indicate the standard derivations from two independent experiments. (B) Correlation between assay signal and MOI. A total of 2500 or 5000 Vero cells in 384-well plate were infected by Luc-DENV at indicated MOI, luciferase signal was measured at 48 h p.i.. Average results from two independent experiments are presented. (C) The effect of DMSO in Luc-DENV infection. Five thousand Vero cells per well in 384-well plate were infected by Luc-DENV at an MOI of 0.5, and different concentrations of DMSO were added. Luciferase activity was measured at 48 h p.i., the percentages of luciferase signals from the DMSO-treated infection were compared with those from without DMSO-treated infection control (set to 100%). Error bars reflect the standard derivations of two independent experiments.

Table 2
EC_{50} values (μM) of three distinct inhibitors of DENV in four cell-based antiviral assays.

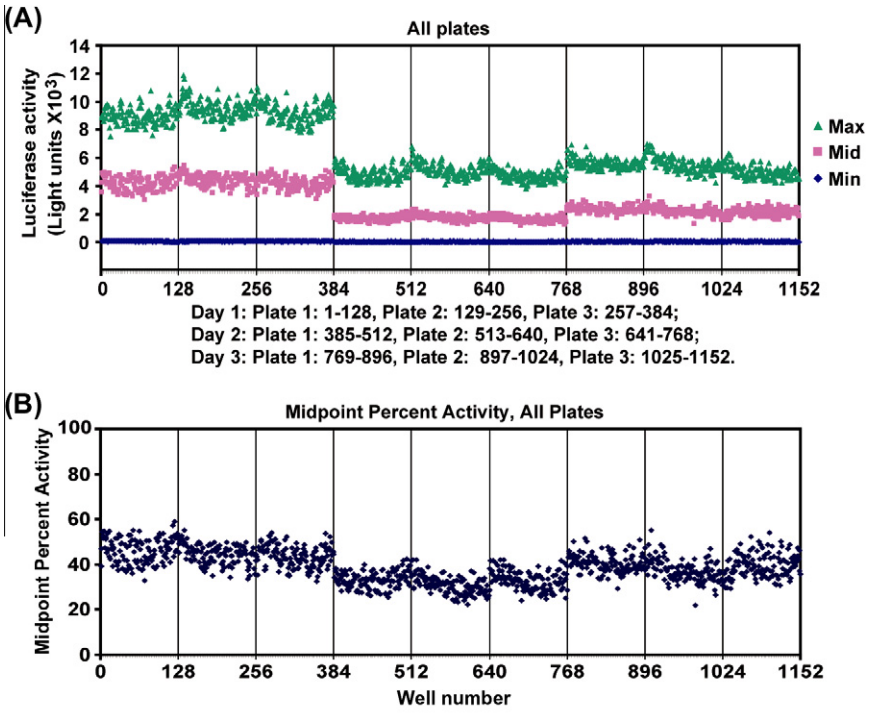
Compound[a] EC_{50}(μM)[b]	Entry inhibitor Compound 6	RNA synthesis inhibitor NITD008	Assembly inhibitor Castanospermine
Assay system			
Replicon-containing cell line	>10	1.1	>10
VLP	0.2	3.0	>10
Luc-DENV	0.12	1.2	8.8
Viral titer reduction	0.2	0.9	6.3

[a] All the compounds were tested at a concentration that did not show cytotoxicity as measured by an MTS assay (Wang et al., 2009).
[b] EC_{50} (μM) was calculated from dose–response curves by Prism software. The results represent the average of two or more independent experiments.

optimal group of seeded cell number). The signals increased over 72 h in BHK-21, LLC-MK2, and Vero cells; similar levels of luciferase activities were observed from 48 to 72 h in A549 cells; the signals in Huh-7 cells decreased from 48 to 72 h. These results suggest that the susceptibility to Luc-DENV infection is different among different cell lines, among which Vero cells yielded highest luciferase signals upon infection.

We then chose Vero cells for optimization in a 384-well format. Vero cells (2500 or 5000) were seeded into each well of 384-well plate, and infected with Luc-DENV at varying MOI. At 48 h p.i., the cells were assayed for luciferase activity by adding 5 μM final concentration of Enduren substrate. As shown in Fig. 4B, the assay signal correlated well with the MOI, suggesting that infection of 5000 Vero cells with 0.5 MOI of Luc-DENV could yield signals high enough for HTS assay. Thus, we chose this condition for further development. To minimize the interference of FBS and phenol red in culture medium with luciferase readout (Qing et al., 2010), we prepared the Luc-DENV stock and performed the assay in phenol red-free medium with 2% FBS. In addition, we found that luciferase substrate ViviRen improved signal by 2–3 folds than substrate Enduren (data not shown); therefore, ViviRen (5 μM final concentration) was included in the final assay.

Since compounds are usually dissolved in DMSO, we tested the effect of DMSO on Luc-DENV infection in 96-well plate. Increasing amount of DMSO decreased luciferase signals (Fig. 4C). Up to 0.1%



(C) Validation Checklist

Intra-Plate Tests	Meets Criterion?
1. Check for drift and edge effects in all plates	Yes
2. All max (HI) signal CV's < 20%	Yes
3. All mid signal (unnormalized) CV's < 20%	Yes
4. All normalized mid signal (mid %) SD's < 20	Yes
5. All min (LO) SD's < Min(max (HI) SD, mid SD)	Yes
6. All SW's >2	Yes
7. All Z' Factors > 0.4 (and < 1 ; must pass one of 6 or 7)	Yes
Inter-Plate Tests	
1. All within-day fold shifts < 2	Yes
2. All Average (between-)Day fold shifts < 2	Yes

Fig. 5. 384-well plate uniformity assessment of the Luc-DENV infection assay. (A) Three independent experiments consisting of three 384-well plates each were performed on three different days according to the recommendations of the National Institutes of Health Chemical Genomics Center's Assay Guidance Manual Version 5.0. "Max" signal derived from infected cells treated with 0.1% DMSO. "Mid" and "Min" signal derived from infected cells treated with 1 and 25 μM NITD008, respectively. Luciferase signal from all plates are presented. (B) Midpoint percent activity of all plates derived from (A) are shown. (C) Summary of the assay validation for Luc-DENV infection assay. All calculations were performed using the Assay Guidance Manual's spreadsheet (Eli Lilly and Company, 2008).

DMSO was well tolerated, but 0.5% DMSO reduced the luciferase signal by 50%. Therefore, we chose 0.1% DMSO as our final assay concentration.

3.5. Validation of the Luc-DENV assay by known DENV inhibitors

We verified the Luc-DENV assay using three distinct DENV inhibitors. (i) Compound 6 is a small-molecular entry inhibitor (Wang et al., 2009); (ii) NITD008 is an adenosine analogue which functions as a chain terminator during DENV RNA synthesis (Yin et al., 2009); (iii) castanospermine is an α-glucosidase inhibitor that suppresses DENV assembly through affecting E glycoprotein processing (Courageot et al., 2000; Whitby et al., 2005). These inhibitors were tested in three different cell-based assays at non-toxic concentrations: replicon-containing cell line assay which only covers viral translation and RNA synthesis; VLP infection assay which covers viral entry, translation, and RNA synthesis, but not virus assembly; Luc-DENV infection assay which includes the complete viral infection cycle. As a positive control, the compounds were also tested in the viral titer reduction assay using WT DENV-2 virus. Table 2 summarizes the EC_{50} values (μM) of the inhibitors in these antiviral assays. As expected, entry inhibitor compound 6 did not show any activity in the replicon cell line assay; RNA synthesis inhibitor NITD008 showed activity in all assays; virion assembly inhibitor castanospermine did not show any activity in the replicon cell line and VLP infection assays. These results validate that the Luc-DENV assay allows screening for inhibitors of viral entry, replication, and virion assembly.

3.6. Statistical evaluation of the Luc-DENV HTS assay

The signal to noise ratio (S/N) was calculated using the absolute positive signal (Luc-DENV-infected cells) divided by absolute background signal (uninfected cells). It ranged from 126- to 186-fold. The assay quality was consistently good in all three independent experiments of plate uniformity assessment (Fig. 5A and B). All of the statistical criteria for both intra-plate and inter-plate assessment were met in 384-well plate uniformity assessment (Fig. 5C). The assay showed an average Z' of 0.79 (HTS assays with $Z' \geqslant 0.5$ are considered robust). These results demonstrate that the Luc-DENV assay is ready for HTS.

4. Discussion

A reliable screening assay is critical for drug discovery. The goal of this study was to develop a stable luciferase DENV for HTS. Fig. 6 summarizes the assay scheme in a 384-well format. The current Luc-DENV infection assay is homogeneous, and requires 3 days to complete. In contrast, the traditional plaque reduction assay is labor intensive, and requires at least 6 days (2-day compound treatment plus 4-day plaque assay). Application of the Luc-DENV for HTS assay should exclude the "hits" targeting the *renilla* luciferase reporter; this problem could be solved by a secondary screen against *renilla* luciferase activity. In addition, the insertion of FMDV2A in the reporter virus may also reduce the specificity of anti-DENV drug screening, as compounds that interfere with the function of FMDV2A could lead to false positives. Thus, "hits" derived from the primary HTS assay should be reconfirmed by other assays such as viral titer reduction assay.

One major shortcoming of the current reporter DENV-2 (strain TSV01) assay is the high sensitivity to DMSO. The assay could tolerate up to 0.1% DMSO; DMSO at this concentration reduced luciferase activity by less than 20%. However, 0.5% DMSO reduced the luciferase signal by approximately 50% (Fig. 4C). More recently, we found that the issue of DMSO sensitivity is virus strain-dependent. Another luciferase reporter virus, derived from DENV-2 NGC strain, showed better tolerance to DMSO treatment. Using the assay procedure described in Fig. 6, we found that 1% DMSO treatment did not decrease the luciferase signal derived from the cells infected with the NGC reporter virus (data not shown). The difference in DMSO sensitivity could be due to the fact that the NGC strain is a well adapted laboratory virus, whereas the TSV01 strain is a clinical isolate which has not been fully adapted in cell culture.

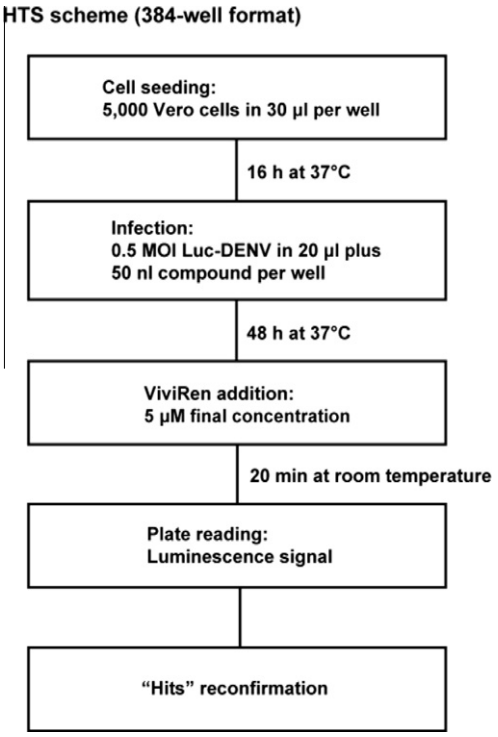


Fig. 6. Flowchart of the HTS assay using the Luc-DENV. Briefly, 5000 Vero cells were seeded into each well of 384-well plates, infected with Luc-DENV at an MOI of 0.5, and incubated with compounds for 48 h, and assayed for luciferase activities.

Insertion of a *renilla* luciferase into the viral genome attenuated virus replication in cell culture. Upon passaging of the reporter virus on Vero cell, the virus accumulated a T108M mutation in NS4B that could enhance its replication. Interestingly, the NS4B T108M-mediated enhancement of viral replication is host cell-dependent. The enhancement was observed only in Vero cells, but not in other cell lines (such as BHK-21). A similar, but distinct observation was previously reported that an NS4B P101L mutation in DENV-4 increased viral replication in mammalian cells, but decreased viral replication in C6/36 cell (Hanley et al., 2003). The results suggest that these NS4B mutations exert their enhancement of RNA synthesis through interaction with a cellular factor that varies among different hosts.

It is worth noting that although the *renilla* luciferase reporter DENV-2 was stable, a GFP reporter DENV-2 using the same construction strategy was unstable. The GFP reporter was partially lost during five rounds of passaging on Vero cells (data not shown). One possible explanation is that certain RNA elements within the GFP gene may interfere with viral replication. Thus, continuous passaging of the GFP virus led to the deletion of GFP reporter.

In summary, the luciferase reporter DENV-2 assay developed in this study should be useful for screening novel anti-DENV agents. The reporter virus will also be useful for studying DENV replication and pathogenesis.

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