

Online Resource 1. Materials and Methods (Detailed)

Cells and virus

African Green Monkey kidney (Vero) cells were obtained from the American Type Culture Collection (ATCC number: CCL-81) and maintained at 37°C, 5% CO₂ atmosphere and 95% relative humidity in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Carlsbad, CA), supplemented with 2-10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. C6/36 cells, derived from whole larvae of *Aedes albopictus* (ATCC number: CRL-1660) were cultured in Leibovitz's L-15 medium (Gibco BRL, Carlsbad, CA), supplemented with 2-10% FBS and 20 mg/mL tryptose phosphate broth, and incubated at 28°C, 5% CO₂ atmosphere and 95% relative humidity. The DENV-2 infectious clone (pBAC-DENV-FL), derived from the New Guinea C prototype strain (DENV-2 NGC M2) [1] was chosen over DENV-2 NGC N2 because the first one showed a very attenuated phenotype in Vero cells, ensuring biosafety for subsequent applications as a backbone for the development of a novel viral vector. This DENV infectious clone was used for the generation of a DNA-launched DENV replicon (pBAC-DENV-REP-EGFP) and a replication-deficient mutant (pBAC-DENV-REP-GVD) which have a nonsynonymous mutation leading to the inactivating amino acid change Asp (D) → Val (V) in the active site GDD of the viral RNA-dependent RNA Polymerase (RdRP) or NS5 [2].

Plasmids and bacterial strains

The pBAC-DENV-FL plasmid consisting in the DENV full-length genome cloned in pBeloBAC11 [3,4] under the control of the CMV promoter was used for the construction of pBAC-DENV-REP-EGFP and pBAC-DENV-REP-GVD. BAC propagation and amplification were performed in *E. coli* DH10B (Gibco BRL, Carlsbad, CA) as previously described [5]. *EGFP* gene was amplified from plasmid pEGFP-N2 (BD Biosciences Clontech). The plasmid pJET1.2/blunt was used for direct cloning of PCR products. Plasmid DNA purification was performed by using QIAprep Spin Miniprep and QIAGEN Large-Construct Kits (QIAGEN GmbH, Germany) for standard and BAC plasmids, respectively. For DNA purification from agarose gels, QIAEX II or QIAquick Gel

Extraction kits (QIAGEN GmbH, Germany) were used according to the DNA fragment size.

Cloning strategy of a DNA-launched DENV replicon expressing the reporter EGFP gene

For the construction of the DENV replicon expressing EGFP, the region comprising the *E/NS1* junction (cleavage site for the host cell signalase) [6], *NS1*, *NS2*, and partial *NS3* (nt 4701) genes was generated by PCR amplification, and cloned into pBeloBAC11 by using the *Sfo* I and *Sph* I restriction sites to generate the plasmid pBAC-NS1/3. An *Nsi* I restriction site is located 27 nt upstream of the *Sph* I in the DENV NS1/3 fragment. The forward oligonucleotide was designed to incorporate an *Mlu* I restriction site downstream of the *Sfo* I restriction site, which allowed the cloning of a second PCR fragment comprising the CMV promoter, 5'UTR, and 63 nt of the capsid gene [7], generating the plasmid pBAC-dSTRUCT. Subsequently, the reporter *EGFP* gene was cloned into the *Mlu* I restriction site, in frame with the viral ORF to generate the plasmid pBAC-dSTRUCT-EGFP. Finally, the *Sfo* I-*Nsi* I fragment was subcloned into the previously constructed infectious clone pBAC-DENV-FL to generate the plasmid pBAC-DENV-REP-EGFP (Figure 1, Table 1). Additionally, the active site (GDD) of the RdRP in the DENV replicon was mutated (GVD) by mean of overlapping PCR using mutagenic oligonucleotides (Figure 2) to disable its replicase activity, generating pBAC-DENV-REP-GVD, which could transcribe and translate from the CMV promoter, but not autonomously replicate. The genetic integrity of the cloned DNAs was verified throughout the assembly process by sequencing using specific oligonucleotides (Online Resources 2 to 4).

Transfection and confocal microscopy

Vero cells were grown on coverslips to 90% confluence in 24-well plates and subsequently mock-transfected or transfected with 2 µg of the EGFP-containing constructs by using 2 µL of LipofectamineTM 2000 (Life Technologies Corp., Carlsbad, CA, USA) according to manufacturer's instructions. After 6 h of incubation at 37 °C, the transfection media were replaced and cells were incubated at 37°C for different times post-transfection, until cell supernatants and lysates were collected or the cell cultures were fixed with

paraformaldehyde 3.8%. Cells were stained with Hoechst (nuclei) and NS3 protein was detected with the monoclonal antibody E1D8, kindly provided by Dr. Eva Harris. Secondary antibodies conjugated to AlexaFluor®488 and AlexaFluor®594 dyes (Life Technologies Corp., USA) were used in this study. Images were obtained in a motorized inverted research microscope IX-81 (Olympus Corporation, Tokyo, Japan) equipped with an IX2-DSU (disk scanning unit) module (Olympus Europa Holding GmbH, Hamburg, Germany) and a digital CCD camera ORCA-R² (Hamamatsu Photonics K. K., Japan), and analyzed by mean of the Xcellence Pro 1.2 software (Olympus Soft Imaging Solutions GmbH).

Conventional RT-PCR and two-step qRT-PCR

Viral RNA from cellular lysates of transfected cells was extracted with the RNeasy minikit (Qiagen®, Chatsworth, CA, USA), following the manufacturer instructions. The RNA extracts were further treated with RQ1 RNase-free DNase I (Promega Corp. USA) and used for cDNA synthesis with the GoScript reverse transcriptase (Promega Corp. USA), according the manufacturer instructions. For conventional RT-PCR, the oligonucleotides NS3_F and NS3_R (Table 1) were used separately at the RT step to evaluate the presence of negative and positive strands of viral RNA in cellular lysates of transfected cells, respectively. For RT-qPCR, DENV-specific forward oligonucleotide NS5_F (Table 1) was used at the RT step for detection of negative-sense viral RNA. The oligonucleotides NS5_F, NS5_R and the TaqMan® MGB™ probe NS5_DENV (Table 1) were used for quantitative amplification by qPCR, as previously described [1]. The Rotor-Gene® Q Real-Time PCR system (Qiagen®, Chatsworth, CA, USA) was used for amplification and quantitative analyses. For relative quantification of viral RNA levels in cellular lysates, the endogenous 18S ribosomal RNA (Applied Biosystems, CA, USA) was used as a reference gene to correct the level of starting genetic material.

Statistical analysis

For quantitative analyses, experiments were carried out independently three times with three replicates included for each experimental point. Results were expressed as the mean +/- standard error of the mean (SEM). To determine the significance of the mean

differences, two-tailed *t*-student tests were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California, USA).

REFERENCES

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Online Resource 2. Oligonucleotides for sequencing of pBAC-NS1/3.

Name	Length	Sequence (5' – 3')	Position ^a	Tm
Primer1F ^b	18	CGGGTGTTCGCGGGTGTC	25-42	59.2
Primer4F(1385-4701) ^c	22	GACAGGAGACATCAAAGGAATC	457-478	49.4
Primer5F(1385-4701) ^c	24	GGTGACTGAGGACTGTGGAAATAG	1048-1071	53.5
Primer6F(1385-4701) ^c	21	CTGACTGATGCGTTAGCCTTG	1613-1633	52.7
Primer7F(1385-4701) ^c	23	GGAAGAACAACACTGACCATAC	2170-2192	49.7
Primer1R ^b	24	TTTATGCTTCCGGCTCGTATGTTG	2617-2594	59.1
Primer2R(1385-4701) ^c	20	GAGCCCTCCAGCCACTAATG	2011-1992	53.7
Primer3R(1385-4701) ^c	24	GTCATAGTAGCGCCCACCATAACC	1440-1417	57.7
Primer4R(1385-4701) ^c	23	GTGTATGGTAGCCTGGTCTGTAG	977-955	50.0
Primer5R(1385-4701) ^c	22	ATGATTCCCTTGATGTCTCCTG	480-459	49.9

^a Position respect to the *in silico* construct of pBAC-NS1/3. ^b Oligonucleotides previously used for sequencing of pBAC-CMV-DEN2 5'. ^c Oligonucleotides previously used for sequencing of pBAC-DENV1385-4701.

NOTE: The DENV insert spreads from nucleotides 148 to 2496 in the construct.

Online Resource 3. Oligonucleotides for sequencing of pBAC-dSTRUCT.

Name	Length	Sequence (5' – 3')	Position ^a	Tm
Primer1F ^b	18	CGGGTGTGGCGGGTGTC	25-42	59.2
Primer5R(1385-4701) ^c	22	ATGATTCCTTTGATGTCTCCTG	1236-1215	49.9

^a Position respect to the *in silico* construct of pBAC-dSTRUCT. ^b Oligonucleotide previously used for sequencing of pBAC-CMV-DEN2 5'. ^c Oligonucleotide previously used for sequencing of pBAC-DENV1385-4701. NOTE: The DENV insert spreads from nucleotides 148 to 3252 in the construct.

Online Resource 4. Oligonucleotides for sequencing of pBAC-dSTRUCT-EGFP and pBAC-DENV-REP-EGFP.

Name	Length	Sequence (5' – 3')	Position ^a	Tm
Primer1F ^b	18	CGGGTGTGGCGGGTGTCTC	25-42	59.2
Primer2F ^b	22	CGGTTTGACTCACGGGGATTTC	572-593	59.2
EGFP-F	21	ATGGTGAGCAAGGGCGAGGAG	917-937	60.0
Primer5R(1385-4701) ^c	22	ATGATTCCTTTGATGTCTCCTG	1959-1938	49.9
EGFP-R	18	CTTGTAACAGCTCGTCCAT	1633-1616	42.2
3R_dSTRUCT_GFP	21	ACGCCGTAGGTCAGGGTGGTC	1122-1102	59.7
Primer4R ^b	22	ATGGAAAAGCCGATTGAACG	588-567	59.3

^a Position respect to the *in silico* construct of pBAC-dSTRUCT-EGFP. ^b Oligonucleotides previously used for sequencing of pBAC-CMV-DEN2 5'. ^c Oligonucleotide previously used for sequencing of pBAC-DENV1385-4701. NOTE: The DENV insert spreads from nucleotides 148 to 3975 in the construct.