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DengueSeq: A pan-serotype whole genome amplicon sequencing protocol for dengue virus V.3

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We use this protocol and it's working

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ABSTRACT

Version 3 updates:

- Updated abstract
- Included link to preprint and bioinformatics pipeline
- Added links to additional library prep kits
- Included DENV2 and DENV4 sylvatic reference genomes and BED files
- Changed primer names in BED files

DengueSeq preprint:
https://www.medrxiv.org/content/10.1101/2023.10.13.23296997v1
Bioinformatics pipeline: https://github.com/grubaughlab/DENV_pipeline/tree/v1.0

Background
The increasing burden of dengue virus on public health due to more explosive and frequent outbreaks highlights the need for improved surveillance and control. Genomic surveillance of dengue virus not only provides important insights into the emergence and spread of genetically diverse serotypes and genotypes, but it is also critical to monitor the effectiveness of newly implemented control strategies. Here, we present DengueSeq, an amplicon sequencing protocol, which enables whole-genome sequencing of all four dengue virus serotypes.

Results
We developed primer schemes for the four dengue virus serotypes, which can be combined into a pan-serotype approach. We validated both approaches using genetically diverse virus stocks and clinical specimens that contained a range of virus copies. High genome coverage (>95%) was achieved for all genotypes, except DENV2 (genotype VI) and DENV 4 (genotype IV) sylvatics, with similar performance of the serotype-specific and pan-serotype approaches. The limit of detection to reach 70% coverage was 10-100 RNA copies/µL for all four serotypes, which is similar to other commonly used primer schemes. DengueSeq facilitates the sequencing of samples without known serotypes, allows the detection of multiple serotypes in the same sample, and can be used with a variety of library prep kits and sequencing instruments.

Conclusions
DengueSeq was systematically evaluated with virus stocks and clinical specimens spanning the genetic diversity within each of the four dengue virus serotypes. The primer schemes can be plugged into existing amplicon sequencing workflows to facilitate the global need for expanded dengue virus genomic surveillance.

Link to DengueSeq preprint.

GUIDELINES

It is recommended that steps performed up to amplicon generation be performed at a different workstation and with different equipment than steps post-amplicon generation.

MATERIALS

DengueSeq preprint
https://www.medrxiv.org/content/10.1101/2023.10.13.23296997v1

Primers
Primer schemes for the four dengue virus serotypes (1-4) can be used individually as serotype-specific or combined as a single pan-serotype approach. Primers may be ordered from any oligonucleotide company. For primer preparation instructions for the serotype-specific and pan-serotype approaches, see Step 1 of the protocol. See files below for primer sequences.

Primer Schemes:

DENV1_Primer-Scheme.xlsx
DENV2_Primer-Scheme.xlsx
DENV3_Primer-Scheme.xlsx
DENV4_Primer-Scheme.xlsx

Equipment

- 96-well format Thermocycler (two needed for 49+ sample runs)
- Qubit
- Bioanalyzer
- Magnetic rack fit for a 96-well PCR plate
- Magnetic rack fit for 1.5 / 2-mL microcentrifuge tubes
- Cold block fit for a 96-well PCR plate
- Pipettes (assorted sizes)

Consumables

- 8-strip PCR tubes; may be substituted with PCR plates with heat-sealing film
- 1.5 / 2mL / 5 mL microcentrifuge tubes
- Filtered pipette tips (assorted sizes)
- Reservoirs
- Waste containers

Reagents

- Illumina COVIDSeq Test Kit
- Illumina Nextera XT DNA Library Preparation kit
- New England Biolabs NEBNext ARTIC SARS-CoV-2 Library Prep Kit (Illumina)

- Oxford Nanopore Technologies Rapid barcoding kit

Bioinformatics pipeline
https://github.com/grubaughlab/DENV_pipeline/tree/v1.0

Reference genomes
DENV1.fasta
DENV2.fasta
DENV2_sylvatic.fasta
DENV3.fasta
DENV4.fasta
DENV4_sylvatic.fasta

BED files
DENV1.bed
DENV2.bed
DENV2_sylvatic.bed
DENV3.bed
DENV4.bed
DENV4_sylvatic.bed

SAFETY WARNINGS

Before starting work with dengue virus samples, please contact your local EHS (environment, health and safety) or biosafety office for proper guidance on how to work with these samples in your laboratory.

BEFORE START INSTRUCTIONS

If substituting 8-strip PCR tubes for PCR plates, plates must be heat-sealed before each thermocycling step.

Dengue Serotype Identification / Plate Setup

1 There are options available for use: Serotype-specific and Pan-serotype approach.

Various library prep kits can be used with the DengueSeq primers, following the manufacturer's protocol. Below we describe detailed steps for the Illumina COVIDSeq Test Kit (RUO version), but other library prep kits for amplicon sequencing can be used as well.

For example:

- Illumina COVIDSeq Test Kit
- Illumina Nextera XT DNA Library Preparation kit
- New England Biolabs NEBNext ARTIC SARS-CoV-2 Library Prep Kit (Illumina)
- Oxford Nanopore Technologies Rapid barcoding kit

Prepare Working Primer Solutions

STEP CASE

Serotype-specific approach 61 steps

Identify the serotype of all samples being sequenced.

We recommend using the CDC DENV-1-4 Real-Time RT-PCR Multiplex Assay for serotype identification if an in-house assay is not available.

Organize samples by serotype. Step 4 requires a serotype-specific master mix to be added to the samples; order the samples to best accommodate this.

Each serotype-specific master mix will require its own negative template controls, one cDNA control (added in Step 3) and one PCR control (added in step 4). Be sure to account for these extra controls in the plate design.

2

Briefly centrifuge all primers

2.1 Within each serotype, organize odd and even-numbered primers into two separate groups:
Odd-numbered primers = Pool 1
Even-numbered primers = Pool 2

> **Note**
>
> Recommended to organize numerically, i.e. 1_left, 1_right, 3-left, 3 right...

2.2 If primers were ordered lyophilized, resuspend to
[M] 100 micromolar (µM) in nuclease-free water

2.3 Add 90 µL nuclease-free water to two sets of 8-strip PCR tubes for each serotype:

Serotype	Tubes for Pool 1	Tubes for Pool 2
DENV1	36	34
DENV2	38	36

	Serotype	Tubes for Pool 1	Tubes for Pool 2
	DENV3	36	34
	DENV4	36	34

2.4 Label the tops of each tube with the corresponding primer that will be added

- For readability, recommended this format: D1 1L, D1 1R, D1 3L, D2 3R...

2.5 Add 10 µL 100 µM primer to each corresponding tube to reach [M] 10 micromolar (µM) /primer

- Recommend to either mix tubes with a new pipette tip or gently vortex

2.6 Working pool-by-pool, transfer 10 µL 10µM primer into 1.5mL microcentrifuge tubes

- 8 tubes total, with 2 tubes (Pool 1 and Pool 2) per serotype
- Recommended to label each tube with serotype, concentration, pool #, and date of dilution
- This will be the working primer solution that will be added during the amplicon generation step

2.7 Store all primers at -20ºC until use

cDNA Synthesis

3 Prepare the following reagents:

	Reagent	Storage	Instructions
	EPH3 HT	-20°C	Thaw at room temperature, invert to mix
	FSM HT	-20°C	Thaw at room temperature, invert to mix
	RVT HT	-20°C	Invert to mix, keep on ice
	Nuclease-free water	Room Temp.	Keep at room temperature

3.1 Add 8.5 µL EPH3 HT to new PCR tubes according to the number of samples

- Include one additional reaction for a cDNA NTC for each serotype-specific master mix (NTC), which will be included through the entirety of the protocol

3.2 Add 8.5 µL RNA to each tube

Add 8.5 µL nuclease-free water to the cDNA NTC

- Mix by pipetting up and down 10 times
- Briefly centrifuge tubes

3.3 Load tubes into thermocycler and run the following program to generate first-strand cDNA:

	Temperature	Time
	65°C	3 minutes
	4°C	Hold

17µL reaction, lid temp = 105°C, preheat lid = on

3.4 In a new 1.5mL tube, prepare the following master mix according to the number of samples:

	Reagent	µL per Sample
	FSM HT	7.2
	RVT HT	0.8
	Total	8

Note

All master mix volumes in this protocol are for one reaction and do not account for lost volume due to pipetting. Multiply volumes by reaction number accordingly.

3.5 Add 8 µL Master Mix to each tube

Mix by pipetting up and down 10 times

Briefly centrifuge tubes

3.6 Load tubes into thermocycler and run the following program to generate second-strand cDNA:

	Temperature	Time
	25°C	5 minutes
	50°C	10 minutes
	80°C	5 minutes
	4°C	∞

25µL reaction, lid temp = 105°C, preheat lid = on

3.7 Remove tubes from thermocycler and briefly centrifuge

Note

This is a safe stopping point. cDNA can be stored long-term at -20°C.

Amplicon Generation

4 Prepare the following reagents:

	Reagent	Storage	Instructions
	Primer Pool 1	-20°C	Thaw at room temperature, vortex to mix
	Primer Pool 2	-20°C	Thaw at room temperature, vortex to mix
	IPM HT	-20°C	Thaw at room temperature, invert to mix
	Nuclease-free water	Room temp.	Keep at room temperature

4.1 In two separate tubes, prepare the following master mixes according to the number of samples:

- **If processing more than one serotype in a run, prepare separate master mixes for each serotype, using the proper primer pools.**
- Include one additional reaction for a PCR NTC for each serotype-specific master mix, which will be included through the entirety of the protocol.

Pool 1

	Reagent	µL per Sample

	Reagent	µL per Sample
	IPM HT	12.5
	Primer Pool 1	3.6
	Nuclease-free Water	3.9
	Total	20

Pool 2

	Reagent	µL per Sample
	IPM HT	12.5
	Primer Pool 2	3.6
	Nuclease-free Water	3.9
	Total	20

4.2 Distribute the master mixes:

Add 20 µL Pool 1 Master Mix to a new set of PCR tubes, according to the number of samples

Add 20 µL Pool 2 Master Mix to a new set of PCR tubes, according to the number of samples

- Include one additional tube at the end of each set for the PCR NTC
- **Be sure to match the serotype of each master mix to the previously-identified serotype of each sample**

4.3 Add 5 µL cDNA to each tube in both Pool 1 and Pool 2.

Add 5 µL nuclease-free water to the PCR NTC in both Pool 1 and Pool 2.

- Mix by pipetting up and down 10 times
- Briefly centrifuge tubes

4.4 Load tubes into thermocycler and run the following program:

	Step(s)	Temperature	Time	Cycles
	Initial denaturation	98°C	3 minutes	1
	Denaturation	98°C	15 seconds	35
	Anneal and extension	63°C	5 minutes	
	Hold	4°C	∞	∞

25µL reaction, lid temp = 105°C, preheat lid = on

4.5 When program is complete, samples can remain in the thermocycler at 4°C or be stored at -20°C

- Briefly centrifuge tubes before use

Note

This is a safe stopping point. Amplicons can be stored long-term at -20°C.

Amplicon Tagmentation and Cleanup

5 Prepare the following reagents:

	Reagent	Storage	Instructions
	EBLTS HT	4°C	Vortex to mix
	TB1 HT	-20°C	Thaw at room temperature, vortex to mix
	ST2 HT	Room temp.	Vortex before use
	TWB HT	4°C	Vortex before use
	Nuclease-free water	Room temp.	Keep at room temperature

5.1 Quantify samples on a Qubit to confirm proper amplification in both amplicon pools

5.2 Prepare the following master mix:

	Reagent	µL per sample
	TB1 HT	10
	EBLTS HT	3.3
	Nuclease-free water	16.7
	Total	30

5.3 Add 30 µL Master Mix to a new set of PCR tubes, according to the number of samples

5.4 For each sample, combine amplicon pools:
Add 10 µL Pool 1 to the Master Mix tubes
Add 10 µL Pool 2 to the Master Mix tubes
- Mix by pipetting up and down 10 times
- Briefly centrifuge tubes

Note

Remaining amplicon pools can be stored long-term at -20°C

5.5 Load tubes into thermocycler and run the following program:

	Temperature	Time
	55°C	3 minutes
	10°C	∞

50µL reaction, lid temp = 105°C, preheat lid = on

5.6 When the program is complete, **immediately** remove the tubes from the thermocycler
- Briefly centrifuge tubes

5.7 Add 10 µL ST2 to each tube
- Mix by pipetting up and down
- Briefly centrifuge tubes

5.8 Incubate at Room temperature for 5 minutes

5.9 Place tubes on a magnetic stand and wait until the liquid is clear before continuing

5.10 Remove and discard all supernatant
- Do not allow pipette tip to come into contact with the inner walls of the tube, as it can

scrape away the magnetic beads

5.11 Remove tubes from the magnetic stand and add 100 µL TWB HT to each tube (first wash)

- Mix by pipetting up and down 10 times
- Briefly centrifuge tubes
- Be careful not to introduce bubbles

5.12 Repeat steps 5.9 - 5.11 (second wash)

- Do not remove supernatant after the second wash at this point

5.13 Place the tubes on the magnetic stand

Amplify Tagmented Amplicons

6 Prepare the following reagents:

	Reagent	Storage	Instructions
	EPM HT	-20°C	Thaw at room temperature, invert to mix
	Index adapters	-20°C	Thaw at room temperature, vortex to mix
	Nuclease-free water	Room temp.	Keep at room temperature

6.1 Prepare the following Master Mix:

	Reagent	µL per sample
	EPM	20
	Nuclease-free water	20
	Total	40

6.2 When the tubes with tagmented amplicons (on the magnetic stand) are clear, remove and discard all supernatant

- Remove any remaining supernatant with a smaller-sized pipette.

6.3 Remove tubes from the magnetic stand and add 40 µL Master Mix to each tube

6.4 Briefly centrifuge the plate containing the dual-barcoded index adapters
Clean the surface of the sealing foil with RNase Away
Remove residual RNase away by wiping with 70% EtOH

6.5 Add 10 µL dual-coded index adapters to each well

- Mix by pipetting up and down 10 times
- Briefly centrifuge tubes

6.6 Load tubes into thermocycler and run the following program:

	Temperature	Time	Cycles
	72°C	3 minutes	1
	98°C	3 minutes	1
	98°C	20 seconds	7
	60°C	30 seconds	
	72°C	1 minute	
	72°C	3 minutes	1
	10°C	∞	∞

When program is complete, samples can remain in the thermocycler at 10°C or be stored at -20°C.

Note

This is a safe stopping point. Individual libraries can be stored long-term at -20°C.

Pooling and Cleanup

7 Prepare the following reagents:

	Reagent	Storage	Instructions
	ITB HT	Room temp.	Vortex to mix

	Reagent	Storage	Instructions
	RSB HT	4°C	Keep at room temperature, vortex to mix
	80% EtOH	Room temp.	Prepare 2.5 - 3mL using nuclease free water and molecular-grade 100% EtOH immediately before use

7.1 Place tubes on a magnetic stand and wait until the liquid is clear

7.2 Pool libraries together in a new 1.5mL microcentrifuge tube by equal volume:

	Number of samples	µL per sample
	1 - 24	15
	25 - 48	10
	49 - 96	5

Note

For easier pooling of a large number of samples, it is recommended to multichannel the libraries into a new 8-strip of 0.2mL PCR tubes, then transfer the total volume to the microcentrifuge tube.

7.3 Calculate the volume of ITB needed to reach **0.9x ITB : total pooled volume**

Note

Example:
34 samples; 10µL of each sample pooled
*34 * 10 = 340µL total pooled volume*
*340µL * 0.9 = 306µL ITB*

7.4 Add the calculated volume of ITB to the pooled sample tube

- Vortex for 10 seconds to mix
- Briefly centrifuge

7.5 Incubate at Room temperature for 5 minutes

7.6 Place tube on a single-tube magnetic stand and wait until the liquid is clear

7.7 Remove and discard all supernatant

7.8 Add 1000 µL 80% EtOH to the tube and incubate at Room temperature for 30 seconds

7.9 Repeat steps 7.7 - 7.8 and remove and discard supernatant after the second wash

- Remove any residual EtOH with a smaller-sized pipette

7.10 Add RSB HT to the tube according to the number of samples pooled:

- For 1 - 24 samples add 30 µL RSB HT
- For 25 - 48 samples add 40 µL RSB HT
- For 49 - 96 samples add 50 µL RSB HT

7.11 Vortex tube to mix
Briefly centrifuge tube

7.12 Incubate at Room temperature 2 minutes

7.13 Place on the magnetic stand and wait until the liquid is clear

7.14 Transfer **[total volume RSB] - 5µL** to a new 1.5mL microcentrifuge tube

7.15 Quantify library on a Qubit and obtain fragment distribution using a Bioanalyzer/Tape Station

Sequencing

8 Protocol validated on the Illumina NovaSeq (2x150).

> **Note**
>
> For sequencing we recommend generating at least 1 million reads per sample for optimal sequencing coverage. Sequencing may be performed on Illumina and Oxford Nanopore Technologies sequencing platforms following standard protocols.

Bioinformatics/Analysis

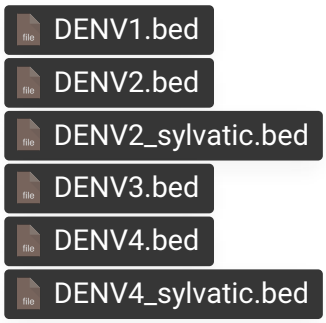
9 Sequencing results may be analyzed utilizing a standard amplicon sequencing bioinformatics pipeline, including those employed for SARS-CoV-2 sequencing (e.g. iVar).

Bioinformatics pipeline: https://github.com/grubaughlab/DENV_pipeline/tree/v1.0

Reference Sequences:

DENV1.fasta
DENV2.fasta
DENV2_sylvatic.fasta
DENV3.fasta
DENV4.fasta
DENV4_sylvatic.fasta

BED files:


DENV1.bed
DENV2.bed
DENV2_sylvatic.bed
DENV3.bed
DENV4.bed
DENV4_sylvatic.bed