Pathogen Whole Genome Sequencing - Multiplexed amplicon sequencing

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1 *Works for me*

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HEMOCENTRO DE RIBEIRAO PRETO

ABSTRACT

This protocol outlines a method for complete genome sequence of emergent virus usign COVIDSeq Test (Illumina). The validation was done for Dengue virus (DENV), Chikungunya virus (CHIKV), and Monkeypox virus (MPXV). DENV and CHIKV primers were adapted from Brazil-UK Centre for Arbovirus Discovery, Diagnosis, Genomics, and Epidemiology.

EXTERNAL LINK

https://support.illumina.com/downloads/illumina-covidseq-test-instructions-for-use-1000000128490.html

PROTOCOL INFO

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Illumina COVIDSeq Test Instructions for Use

KEYWORDS

Dengue Virus, Chikungunya Virus, Monkeypox viruses, CovidSeqTM

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Dilute and pool primers

1 ****

DENV1 Primer Preparation

Reagents:

A	B	C
Reagent	**Storage**	**Instructions**
DENV1 Primer Pairs (100µM)	-20ºC	Thaw at RT
Nuclease-free water	RT	

Primers should be ordered lyophilized or resuspended (100 µM; recommended). Lyophilized primers should be resuspended to 100 µM in nuclease-free water.

1.1 Separate odd and even numbered primer pairs into two separate boxes. These will constitute the two pools.

For example:
Primer Pool 1: *1 forward, 1 reverse, 3 forward, 3 reverse...*
Primer Pool 2: *2 forward, 2 reverse, 4 forward, 4 reverse...*

1.2 Label 30 tubes with the corresponding primer name (e.g. 1 forward).

1.3 To each tube add **90 µL** of nuclease-free water.

1.4 For each primer tube:
- Vortex;
- Spin down;
- Add **10µL** to the corresponding labeled tube;
- Pippete 10 times to mix.

1.5 ⚠

After all 30 primers have been aliquoted, combine 10µL from all odd-numbered primer pairs into a 2mL tube, **except primer pairs 3.** For this pair, add 25µL. This will be the odd-numbered primer pool (Pool 1).

1.6 ⚠

Combine 10µL from all even-numbered primer pairs into a 2mL tube, **except primer pairs 14.** For this pair, add 25µL. This will be the even-numbered primer pool (Pool 2).

1.7 ⏸

For all **26 DENV-2, 30 DENV-3 and 32 DENV-4 primers** aliquoted, combine 10µl from all odd-numbered primer pairs into a 2mL tube. This will be the odd-numbered primers (Pool 1). The same must be done for even-numbered primers (Pool 2).

Safe stopping point: pooled primers can be stored at -20º.

Anneal RNA

2

LIBRARY PREPARATION METHOD
Illumina® COVIDSeq Test™

In this process the RNA is annealed using random hexamers to prepare for cDNA synthesis.

Reagent:

A	B	C
Reagent	**Storage**	**Instructions**
EPH3 HT (Elution Prime Fragment 3HC Mix)	-20°C	Thaw at RT

2.1. Label PCR tube/plate CDNA and add the following:

8.5 µL EPH3 HT to each reaction.

8.5 µL RNA sample to each reaction.

2.2. Briefly centrifugue tubes/plate and place on the programmed thermal cycler.

A	B	C	D
PCR program			
Steps	**Temperature**	**Time**	**Cycles**
Anneal	65°C	3 minutes	1
Hold	4°C	Hold	Hold
Volume amount: 17µl		*Lid: 105°C*	

Synthesize First Strand cDNA

3

This step reverse transcribes the RNA fragments primed with random hexamers into first

strand cDNA using reverse transcriptase.

Reagents:

A	B	C
Reagent	**Storage**	**Instruction**
FSM HT (First Strand Mix HT)	-20°C	Thaw at RT
RVT HT (Reverse Transcriptase HT)	-20°C	Keep on ice

3.1. In a tube, prepare the following master mix:

8 µL FSM HT

1 µL RVT HT

**** Reagent overage is included to account for small pipetting errors.***

3.2. Add 8µl master mix to each tube/well of the cDNA plate/tube and place on the programmed thermal cycler.

A	B	C	D
PCR Program			
Steps	**Temperature**	**Time**	**Cycles**
Anneal	25°C	5 minutes	1
Extension	50°C	10 minutes	1
Enzyme inactivation	80°C	5 minutes	1
Hold	4°C	Hold	Hold
Volume amount: 25µl		*Lid: 105°C*	

Safe stopping point: storage at -20°C for up to 7 days.

Amplicon Generation

4

In this step uses two separate PCR reactions.

Reagents:

A	B	C
Reagent	**Storage**	**Instructions**
IPM HT (Illumina PCR Mix HT)	-20°C	Thaw at RT
DENV-1, DENV-2, DENV-3 or DENV-4 (Primers Pool 1)	-20°C	Thaw at RT
DENV-1, DENV-2, DENV-3 or DENV-4 (Primers Pool 2)	-20°C	Thaw at RT
Nuclease free water		

4.1. In two separate tubes, prepare the following master mixes:

A	B	C	D
Pool 1		*Pool 2*	
Reagent	**Volume in 20µl master mix**	**Reagent**	**Volume in 20µl master mix**
IPM HT	15	IPM HT	15
DENV-1, DENV-2, DENV-3 or DENV4 Pool 1 (10µM)	4,3	DENV-1, DENV-2, DENV-3 or DENV-4 Pool 2 (10µM)	4,3
Nuclease free water	4,7	Nuclease free water	4,7

**** Reagent overage is included to account for small pipetting errors.***

4.2. Label two steps of PCR plates/tubes for Pool 1 and Pool 2.

4.3. Add the following:

20.0 µL Pool 1 master mix to each Pool 1 well//tube.

20.0 µL Pool 2 master mix to each Pool 2 well/tube.

5 µL First strand cDNA to each tube in both sets.

4.4. Place on thermal cycler and run the following program:

A	B	C	D
PCR Program			
Steps	**Temperature**	**Time**	**Cycles**
Initial denaturation	98°C	3 minutes	1
Denaturation	98°C	15 seconds	35 times
Anneal and extension	63°C	5 minutes	
Hold	4°C	Hold	Hold
Volume amount: 25µl		*Lid: 105°C*	

Safe stopping point: storage at -20°C for up to 3 days.

Amplicon Tagmentation

5

This is a step to tagment PCR amplicons, wich is a process that fragment and tags the PCR amplicons with adapter sequences.

Reagents:

A	B	C
Reagent	**Storage**	**Instructions**
EBLTS HT (Enrichment BLT HT)	2°C to 8°C	Thaw at RT
TB1 HT (Tagmentation Buffer 1 HT)	-20°	Thaw at RT
Nuclease free water		

5.1. Prepare the following master mix:

A	B
Reagent	**Volume in 30µl master mix**
TB1 HT	12µl
EBLTS HT	4µl
Nuclease free water	20µl

**** Reagent overage is included to account for small pipetting errors.***

5.2. Combine **10.0 µL Pool 1 amplicons** and **10.0 µL Pool 2 amplicons** in each PCR plate/tube and **add 30µl master mix**.

Pooling of amplicons should be conducted on a dedicated post-PCR area to prevent contamination.

5.3. Place on thermal cycler and run the following program:

A	B
Temperature	**Time**
55°C	3 minutes
10°C	Hold
Volume amount: 50µl	*Lid: 105°C*

Amplicon Tagmentation Clean up

6

This step washes the adapter-tagged amplicons before PCR amplification.

Reagents:

A	B	C
Reagents	**Storage**	**Instructions**
ST2 HT (Stop Tagmentation Buffer 2 HT)	RT	RT - vortex before use
TWB HT (Tagment Wash Buffer HT)	2°C to 8°C	Vortex before use

**Dispense ST2 HT and TWB HT slowly to minimize foaming.*

6.1. Add **10.0 µL ST2 HT** to each well/tube, mix by pippetting up and down, and briefly centrigue.

6.2. Incubate at **room temperature** for **5 minutes**.

6.3. Place on magnetic stand and wait until liquid is clear (~ 3 minutes).

6.4. Remove and discard all supernatant.

6.5. Remove plates/tubes from magnetic stand.

6.6. Wash beads as follows:

- Add **100 µL TWB HT** to each well/tube.
- Mix by shake 1600 rpm for 1 minute.
- Centrifuge briefly.
- Place on the magnetic stand and wait until liquid is clear (~3 minutes).
- Remove and discard all supernatant.

6.7. Repeat steps 6.5 and 6.6.

***Leave the supernatant after the second wash so that the beads don't dry out.**

Amplify tagmented amplicons

7 This step amplifies the tagmented amplicons using a PCR program. The PCR steps adds prepaired base pair index adapters, and sequences required for sequencing cluster generation.

Reagents:

A	B	C
Reagent	**Storage**	**Instructions**
EPM HT (Enhanced PCR Mix HT)	-20°C	Invert to mix
Index adapters	-20°C	Thaw at RT; Centrifugue briefly
Nuclease free water		

7.1. Prepare the following master mix:

A	B
Reagent	**Volume master mix**
EPM HT	24µl
Nuclease free water	24µl

**** Reagent overage is included to account for small pipetting errors.***

7.2. Place the plate/tubes on magnetic stand and remove any remaining TWB HT.

7.3. Remove the plate/tubes from the magnetic stand e add **40.0 µL master mix** to each well/tube.

7.4. Add **10.0 µL index adapters** to each well/tube and pipette up and down to mix and spin down.

Pooled and Clean Up

8

This step combines libraries from each tubes/96-well sample into one tube (1.5 mL).

Reagents:

A	B	C
Reagent	**Storage**	**Instructions**
ITB (Illumina Tube Beads)	RT	Vortex thoroughly to mix.
RSB HT (Ressuspension Buffer HT)	4°C	Bring to RT; Vortex to mix.
80% Ethanol (EtOH)	RT	

8.1. Briefly centrifugue tubes/plate and place on magnetic stand. Wait until the liquid is clear (~ 3 minutes).

8.2. Pool libraries by equal volume:

A	B
Number of infividual samples	**Volume to pool per sample**
1-24	40µl
25-48	20µl
49-72	10µl
73-96	5µl

8.2.1. Transfer the volume pooled library from each tube/well to a new PCR tube (1.5 mL).

8.3. Pipette up and down to mix pooled libraries and briefly spin down.

8.4. Add ITB using the resulting volume of pooled samples **multiplied by 0.9**.

8.5. Vortex to mix and incube at RT for 5 minutes.

8.6. Place on magnetic stand and wait until the liquid is clear (~5 minutes).

8.7. Discard all supernatant and wash beads as follows:

- add **1000 µL 80% Ethanol** to each tube and wait 30 seconds.
- Remove and **discard all supernatant**.

- **Wash beads a second time**.

8.8. Remove all residual ethanol and add RSB HT by equal volume:

A	B
Number of individual samples	**Volume to pool per sample**
24	13,75µl
48	27,5µl
72	41,25µl
96	55µl

8.9. Briefly centrifugue and vortex, and then incubate at RT for 2 minutes.

8.10. Place on magnetic stand and wait until liquid is clear (~2 minutes). Transfer the supernatant to a new microcentrifuge tube.

Safe stopping point: storage at -20°C for up to 30 days.

Normalize libraries

9

9.1. Quantify library on Qubit and obtain fragment distribution using a Bioanalyzer.
9.2. Calculate the molarity value using the formula:

$$\frac{\textit{Library concentration (ng/µl)}}{\textit{660 g/mol x average library size (bp)}} \times 10e6 = \text{Molarity (nM)}$$

9.3. Dilute each library pool to a minimum of **30 µL** at a **normalized concentration** **4 nanomolar (nM)** using Rsb HT.

Sequencing

10

After normalized, libraries are ready to be denatured and diluted to the final loading concentration (8pM).

- Protocol validated on the **MiSeq** (*v2 Reagent kit 300 cycles*).

Step 10 includes a Step case.
CHIKV
INFLA A
HTLV

step case

CHIKV

11 For sequencing of *Chickungunya virus* **optimized the first-strand cDNA synthesis reaction.**

SuperScript™ IV First-Strand cDNA Synthesis Reaction

cDNA synthesis reaction

11.1 ▪ **Anneal primer to template RNA**

1. Combine the following components in a PCR reaction tube.
Reagents:

A	B
Reagent	**Volume**
Random hexamers	1µl
10mM dNTP mix (10mM each)	1µl
RNA	8µl
Nuclease free water	3µl

2. Mix by pipetting and briefly centrifugue the components.
3. Place on thermocycler and run the following program:

A	B
Temperature	**Time**
65°C	5 minutes

11.2 ▪ **RT reaction mix**