JUL 14, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.4r3l2ob2pv1y/v1

Protocol Citation: Laís Ceschini, Carla Julia da Silva Pessoa Vieira, Gustavo Lima, Luisa Maria Inácio da Silva, Raul Emídio Lima, Tiago Graf, Gabriel Wallau 2023. Reverse transcription, primer pools preparation and multiplex PCR steps for DENV2 serotype. **protocols.io** https://dx.doi.org/10.17504/protocols.io.4r3l2ob2pv1y/v1

Protocol status: Working
We use this protocol and it's working

Created: May 30, 2022

Last Modified: Jul 14, 2023

PROTOCOL integer ID:
63519

Reverse transcription, primer pools preparation and multiplex PCR steps for DENV2 serotype

Laís Ceschini[1], Carla Julia da Silva Pessoa Vieira[1], Gustavo Lima[2], Luisa Maria Inácio da Silva[1], Raul Emídio Lima[2], Tiago Graf[3], Gabriel Wallau[1]

[1]Departamento de Entomologia, Instituto Aggeu Magalhães (IAM);
[2]Núcleo de Plataformas Tecnológicas (NPT), Instituto Aggeu Magalhães (IAM);
[3]Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador

Laís Ceschini

ABSTRACT

This step-by-step protocol describes the cDNA synthesis, primer pools preparation and multiplex PCR conditions with the main goal to sequence the complete genome of DENV2 serotype strains.

MATERIALS

SuperScript™ IV First-Strand Synthesis System. (200 reactions) Cat: 18091200 Invitrogen
Q5® High-Fidelity 2X Master Mix. Cat: M0492L NEB, H20 Ultre Pure, primers described in table 1

Reverse transcription

1 Using a 2mL tube prepare the **Mix 1** described below for 96 samples:

	A	B	C
	Random Hexamers (50µM)	1µL	98µL
	dNTPs mix (10mM cada)	1µL	98µL
	Mix 1Reverse transcription	Vol. (1x)	96 samples (+2 = 98 to keep some extra due to pipetting issues)
	Total	2µL	194µL

2 1. Using 0,2mL PCR tubes or 96 wells plates add 11-16µL of extracted RNA from RT-PCR positive samples. Add **2µL** of Mix 1 to the tube/well and take it to the thermocycler with the following set up:

65ºC ---- 5 minutes

3 Take the tubes/wells to ice for 1 minute. (you can prepare a water bath with ice cubes to have a uniform temperature distribution)

4 1. Using a 2mL tube prepare **Mix 2:**

	Mix 2Reverse Transcription	Vol. (1x)	96 samples (+2 = 98 to keep some extra due to pipetting issues)
	5x SSIV Buffer	4µL	392µL
	100mM DTT	1µL	98µL

	RNaseOUT ouRNase Inhibitor	1µL	98µL
	SSIV Reverse Transcriptase	1µL	98µL
	Total	7µL	686µL

5 1. Add **7µL** of **Mix 2** to the tubes containing the **Mix 1** plus RNA and take it to the thermocycler following the set up below:

Step1:
42ºC ---- 50 minutes
70ºC ---- 10 minutes
4ºC ---- Hold

6 Store the cDNA at -20ºC.

Observation:. As a suggestion, to improve the final results only samples RT-PCR positive showing a Ct value of < 30 should be used for cDNA conversion and genomic amplification

Pools of primers

7 Select two 0,6mL tubes for each pool.

8 Using the original 100uM primer solution eluted individually, put them together following the table below containing each primer volume.

TABLE 1: Primers and pool order.

	A	B	C	D	E
	Primer	Sequence	Tm	Concentration inside of the pool *	POOL
	DENV2_1_LEFT	CAGTTGACACGCGGTTTCTCTC	0,030uM	10ul	1
	DENV2_1_RIGHT	TTAGGAAACGAAGGAACGCCAC	0,030uM	10ul	1

	A	B	C	D	E
	DENV2_3_LEFT	TGGCGTTCCATTTAACCACACG	0,030uM	10ul	1
	DENV2_3_RIGHT	CGTTCCTATGGTGTATGCCAGG	0,030uM	10ul	1
	DENV2_5_LEFT	TGTGTGACGACGATGGCAAAAA	0,015uM	5ul	1
	DENV2_5_RIGHT	CCTTGCCATGYTTTCCTGTGTCA	0,015uM	5ul	1
	DENV2_7_LEFT	GCACAGGCAATGGTTCCTAGAC	0,0075uM	2,5ul	1
	DENV2_7_RIGHT	AGGGATCTTACATGGAGAACCGT	0,0075uM	2,5ul	1
	DENV2_9_LEFT	ACAGAAAAAGATAGCCCAGTCAACA	0,015uM	5ul	1
	DENV2_9_RIGHT	GTCACGACTCCCACCAATACTAG	0,015uM	5ul	1
	DENV2_11_LEFT	CTGATGTGGAAACAAATAACACCAGA	0,015uM	5ul	1
	DENV2_11_RIGHT	CATGGACGGCTCTGTTGTCTTT	0,015uM	5ul	1
	DENV2_13_LEFT	ACAGACCAGGCTACCATACACA	0,015uM	5ul	1
	DENV2_13_RIGHT	GCATGTTTCGTTCCTACTCGGG	0,015uM	5ul	1
	DENV2_15_LEFT	TGCAGCTGGACTACTCTTGAGA	0,015uM	5ul	1
	DENV2_15_RIGHT	AAAATGCTCACCATCCCGACTG	0,015uM	5ul	1
	DENV2_17_LEFT	AATCCTGTCAATAACAATATCAGAAGATGG	0,030uM	10ul	1
	DENV2_17_RIGHT	GCTTCCAGCCTCCTCCATATGA	0,030uM	10ul	1
	DENV2_19_LEFT	AGGAAAAGTTGTGGGTCTTTATGGT	0,030uM	10ul	1
	DENV2_19_RIGHT	ACTGGTGATAGCAGCCTCATAGT	0,030uM	10ul	1
	DENV2_21_LEFT	TGGGTCACGGATTTTAAAGGGAA	0,015uM	5ul	1
	DENV2_21_RIGHT	GCTTCTTTCCAGTGTGCACAGT	0,015uM	5ul	1
	DENV2_23_LEFT	CCTTTGTGGACCTAATGAGAAGAGG	0,015uM	5ul	1
	DENV2_23_RIGHT	CGGCAGTTCACTGAGAGCATGA	0,015uM	5ul	1
	DENV2_25_LEFT	CCACACTGGATAGCAGCTTCAA	0,030uM	10ul	1
	DENV2_25_RIGHT	CCCAAGACCCATTAGCACTGT	0,030uM	10ul	1

	A	B	C	D	E
	DENV2_27_LEFT	GGATGCTACTCACAAGTCAACCC	0,015uM	5ul	1
	DENV2_27_RIGHT	GAAAAGAGAAGTCCAGCTCCGG	0,015uM	5ul	1
	DENV2_29_LEFT	ACTGAGATGGTTCGTCGAGAGA	0,015uM	5ul	1
	DENV2_29_RIGHT	GTGGATTCCTCACTAAGGCTCCT	0,015uM	5ul	1
	DENV2_31_LEFT	CGCAACATCGGAATTGAAAGTGA	0,015uM	5ul	1
	DENV2_31_RIGHT	CCAAGGCTGCATTGCTTCTCAC	0,015uM	5ul	1
	DENV2_33_LEFT	GCTTGGAGCACGCTTCTTAGAG	0,015uM	5ul	1
	DENV2_33_RIGHT	TGGGCTTCCATATTGGTGAAAGT	0,015uM	5ul	1
	DENV2_35_LEFT	AGAGGATGGAACGATTGGACACA	0,015uM	5ul	1
	DENV2_35_RIGHT	CCACTGGAGTTTTGTCTTCCATCC	0,015uM	5ul	1
	DENV2_37_LEFT	GAAGAGGAAGAGGCAGGWGTCC	0,015uM	5ul	1
	DENV2_37_RIGHT	CTGGAATGATGCTGAGGAGACAG	0,015uM	5ul	1
	DENV2_2_LEFT	ATGCTGAAACGCGAGAGAAACC	0,030uM	10ul	2
	DENV2_2_RIGHT	CATGGCCATGAGGGTACACATG	0,030uM	10ul	2
	DENV2_4_LEFT	CATGGATGTCATCAGAAGGGGC	0,015uM	5ul	2
	DENV2_4_RIGHT	TCTGTTGTTGTGTTGGTCAGCT	0,015uM	5ul	2
	DENV2_6_LEFT	GGCATTGTGACCTGTGCTATGT	0,015uM	5ul	2
	DENV2_6_RIGHT	GGGGATTTTTGAAAGTGACCAATGT	0,015uM	5ul	2
	DENV2_8_LEFT	ACAGCTCAAAGGAATGTCATACTCT	0,015uM	5ul	2
	DENV2_8_RIGHT	TCCTCCCAGGGATCCAAAATCC	0,015uM	5ul	2
	DENV2_10_LEFT	AGTGGGGTCTCATGGACTATGA	0,015uM	5ul	2
	DENV2_10_RIGHT	GATCGTTTTCCTGCCTGCATGA	0,015uM	5ul	2
	DENV2_12_LEFT	CCCAACACAAACAGAGCTTGGA	0,0075uM	2,5ul	2
	DENV2_12_RIGHT	TTTCCACAGTCCTCAGTCACCA	0,0075uM	2,5ul	2

	A	B	C	D	E
	DENV2_14_LEFT	CGGACATGGGCAGATTGACAAC	0,015uM	5ul	2
	DENV2_14_RIGHT	CCAAGGCTAACGCATCAGTCAG	0,015uM	5ul	2
	DENV2_16_LEFT	GCAGAAAGCGGATTGGATACCA	0,030uM	10ul	2
	DENV2_16_RIGHT	ATGCTGCTGCCGTGATTGGTAT	0,030uM	10ul	2
	DENV2_18_LEFT	AGATCGGAGCCGGAGTTTACAA	0,030uM	10ul	2
	DENV2_18_RIGHT	TGTCATCTTCGATCTCTGGATTGTC	0,030uM	10ul	2
	DENV2_20_LEFT	ATACCAAACCCCAGCCATCAGA	0,015uM	5ul	2
	DENV2_20_RIGHT	CCTACTGAGTTGTATCACTTTCTTTCCA	0,015uM	5ul	2
	DENV2_22_LEFT	ATGCCAGTGACCCACTCTAGTG	0,015uM	5ul	2
	DENV2_22_RIGHT	CCTTTCCCCTTCTTTTGTCCAGA	0,015uM	5ul	2
	DENV2_24_LEFT	TCCAACTTTCATGACTCAGAAGGC	0,015uM	5ul	2
	DENV2_24_RIGHT	GGAAACCCATCTCGTTTGCCAT	0,015uM	5ul	2
	DENV2_26_LEFT	ACATCCTGGACATAGATCTACGTCC	0,030uM	10ul	2
	DENV2_26_RIGHT	AGGTCAATCACTGTTATTCCATCGAC	0,030uM	10ul	2
	DENV2_28_LEFT	TGTGGGAAGGAAATCCAGGGAG	0,0075uM	2,5ul	2
	DENV2_28_RIGHT	CCCCACAATAGTATGACCAGCC	0,0075uM	2,5ul	2
	DENV2_30_LEFT	AAGCAGGACGAACACTCAGAGT	0,015uM	5ul	2
	DENV2_30_RIGHT	CCCACGTTTTGTATGGGTGGTC	0,015uM	5ul	2
	DENV2_32_LEFT	GGCAGAGTGGCTTTGGAAAGAA	0,015uM	5ul	2
	DENV2_32_RIGHT	CCTTCTCCTTCCACTCCACTCA	0,015uM	5ul	2
	DENV2_34_LEFT	AAAGACCAACACCAAGAGGCAC	0,015uM	5ul	2
	DENV2_34_RIGHT	CAGTTCATCTTGGTTTCTGCATGG	0,015uM	5ul	2
	DENV2_36_LEFT	GAACAACCTGGTCCATACACGC	0,0075uM	2,5ul	2
	DENV2_36_RIGHT	GGGGCTCACAGGTAGCATAGTT	0,0075uM	2,5ul	2

*approximate concentration of each primer in the 25µl PCR reaction.

Note: The primers were designed using the https://primalscheme.com based on the FJ467493, KY923048, KX274130, MG189962, KT187556, KU365903, KU517845, KY794785, KU948303, KU517847, KX372564, KX452038, KX380815, KU509277, KY627762, KY427085, KJ830750, MG779196, KY937188, EU660415, KF955402, MF459663, KU509273, FJ906969, KU094070, KM587709, HQ891023, HQ541799, HQ541798, KJ734727, KU509267, KJ189308, KY474331, KX702404, JX286526, KP188554, KP188555, JX669479, KP188551, KP188550 reference genomes.

9 Pool 1 will have a final volume of 230µl and pool 2 of 190µl.

10 In order to prepare the solution to use in the Multiplex PCR, dilute each pool 1:10. That is, 10µl of pool 1 and 90µl of ultrapure water.

Multiplex PCR

11 1. Prepare the **Mix 1** for a Multiplex PCR for each **Pool 1** e **Pool 2** using a Falcon tube of 15mL (~96 amostras) or a 2mL tube.

	Mix 1Multiplex PCR	Vol. Pool 1(1x)	Vol. Pool 2(1x)	96 amostras (+2) (pool1 ou pool2)
	Q5 Master Mix High fidelity 2X	12,5 µl	12,5 µl	1.225 µl
	Conjunto de primers (Pool1 ou Pool2) /concentração de uso/	1,5 µl	1,5 µl	147 µl
	Água Ultra Pura	8,5 µl	8,5 µl	833 µl
	Total	22,5µl	22,5µl	2205µl

12 1. Add **2,5µl of cDNA** (totalling 5µl) in 22,5µl of the pool1 and pool2 reaction and take it to the thermocycler following the conditions bellow:

Step1:
98ºC ---- 30 seconds
Step2: (45 cycles)
98ºC ---- 15 seconds
58ºC ---- 30 seconds
72ºC ---- 5 minutes
Step3:
72ºC ---- 2 minutes
Hold 4ºC