Reverse transcription, primer pools preparation and multiplex PCR steps for DENV1 serotype for genomic sequencing

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

Luisa Maria Inácio da Silva

AUG 11, 2023

OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.5jyl8pjyrg2w/v1

Protocol Citation: Laís Ceschini, Carla Julia da Silva Pessoa Vieira, Luisa Maria Inácio da Silva, Gustavo Lima, Raul Emídio, Tiago Graf, Gabriel Luz Wallau 2023. Reverse transcription, primer pools preparation and multiplex PCR steps for DENV1 serotype for genomic sequencing. **protocols.io** https://dx.doi.org/10.17504/protocols.io.5jyl8pjyrg2w/v1

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 DENV1 serotype strains.

MATERIALS

Reagents:

Reverse transcription: SuperScript™ IV First-Strand Synthesis System. (200 reactions) Cat:
18091200 Invitrogen

Multiplex PCR: Q5® High-Fidelity 2X Master Mix. Cat: M0492L NEB, H20 Ultre Pure, primers described in table 1.

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

Created: Aug 10, 2023

Last Modified: Aug 11, 2023

PROTOCOL integer ID: 86328

Reverse transcription

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

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

2 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 Using a 2mL tube prepare **Mix 2**:

	A	B	C
	Mix 2 Reverse 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 or RNase Inhibitor	1µL	98µL
	SSIV Reverse Transcriptase	1µL	98µL
	Total	7µL	686µL

5 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.

9 Pool 1 will have a final volume of 130µl and pool 2 of 170µl.

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.

TABLE 1: Primers and pool order

	A	B	C	D	E	F
	Primer	Sequence	Tm	Concentration inside of the pool *	Volume of primer within the pool	POOL
	DENV1SA_1_LEFT	TACGTGGACCGACAAGAACAGT	58.1ºC	0,0075uM	2,5µl	1
	DENV1SA_1_RIGHT	ACTATCATRTGTGGCTCTCCCC	57.2ºC	0,0075uM	2,5µl	1
	DENV1SA_3_LEFT	CACACGTGGGACTTGGTCTAGA	58.4ºC	0,01125uM	7,5µl	1
	DENV1SA_3_RIGHT	ACACACAAAGTTCGCGTCTTGT	57.6ºC	0,01125uM	7,5µl	1
	DENV1SA_5_LEFT	CCTCACATTGGACTGCTCACCT	59.1ºC	0,01125uM	7,5µl	1
	DENV1SA_5_RIGHT	TGCACTARRACAGTTCCATGCT	56.6ºC	0,01125uM	7,5µl	1
	DENV1SA_7_LEFT	CGAGGAGCACGAAGGATGGC	60.6ºC	0,0075uM	2,5µl	1
	DENV1SA_7_RIGHT	ATGATGTTCTCAAGACGCGTGG	57.5ºC	0,0075uM	2,5µl	1
	DENV1SA_9_LEFT	TGGGAAGTTGAGGACTAYGGGT	58.7ºC	0,015uM	5µl	1
	DENV1SA_9_RIGHT	TGTRGTTCTGAGRGATGGACCTC	57.8ºC	0,015uM	5µl	1
	DENV1SA_11_LEFT	GATGACTGGAACACTGGCTGTT	57.4ºC	0,030uM	10µl	1

	A	B	C	D	E	F
	DENV1SA_11_RIGHDENV1SA_11_RIGHT	CACCGGAAGCCATGTTGTTTTT	56.7ºC	0,030uM	10µl	1
	DENV1SA_13_LEFT	AASAAGAAGCAGAACACTCCGG	57.3ºC	0,015uM	5µl	1
	DENV1SA_13_RIGHT	ACTGGCCCAGCTTGGTTCCAG	62.4ºC	0,015uM	5µl	1
	DENV1SA_15_LEFT	ATGGAGTGGTGACAACAAGTGG	57.4ºC	0,015uM	5µl	1
	DENV1SA_15_RIGHT	GCTGGATCGGTAAARTGTGCTTC	57.4ºC	0,015uM	5µl	1
	DENV1SA_17_LEFT	ACGGGTRATYCAAYTGAGCAGRA	58.5ºC	0,01125uM	7,5µl	1
	DENV1SA_17_RIGHT	CCTCTTCTCATGAGCTCCACA	56.3ºC	0,01125uM	7,5µl	1
	DENV1SA_19_LEFT	AGTGTCTCAGGTGACCTAATATTGGA	57ºC	0,0075uM	2,5µl	1
	DENV1SA_19_RIGHT	RGCTGCCACTGTCAGTATCATG	57.5ºC	0,0075uM	2,5µl	1
	DENV1SA_21_LEFT	YGCAAAYCAGGCWGCYATATTGAT	57.6ºC	0,015uM	5µl	1
	DENV1SA_21_RIGHT	GATGTTTGCCATGGACACTGCT	58.2ºC	0,015uM	5µl	1
	DENV1SA_23_LEFT	ACAACCAAACATGCAGTGTCGA	57.5ºC	0,015uM	5µl	1
	DENV1SA_23_RIGHT	TTTCGCACTAGCATCCCTCCAT	58.5ºC	0,015uM	5µl	1
	DENV1SA_25_LEFT	ACCTAGATATYATTGGCCAGAGGA	55.9ºC	0,030uM	10µl	1
	DENV1SA_25_RIGHT	ACCTTTCGTCTTCCACTGCTTC	57.3ºC	0,030uM	10µl	1
	DENV1SA_27_LEFT	TGGAAGGAGAAGGACTGCACAA	58.4ºC	0,030uM	10µl	1
	DENV1SA_27_RIGHT	CACRCAATCATCTCCGCTRATT	55.5ºC	0,030uM	10µl	1
	DENV1SA_29_LEFT	ATGGAGCCTGAGAGAAACTGCT	58.2ºC	0,030uM	10µl	1

	A	B	C	D	E	F
	DENV1SA_29_RIGHT	GCYCCTTCGGGATCACTCTCAT	59.7ºC	0,030uM	10µl	1
	DENV1SA_2_LEFT	TGTTGAACATAATRAACAGGAGGAAAAGA	55.9ºC	0,01125uM	7,5µl	2
	DENV1SA_2_RIGHT	GAATCCTGGGTGTCKCAAAGCC	59.5ºC	0,01125uM	7,5µl	2
	DENV1SA_4_LEFT	ACTGTGCATTGAAGCTAAAATATCAAACA	56ºC	0,015uM	5µl	2
	DENV1SA_4_RIGHT	ACCATTGTTTGTGGACAAGCCA	57.7ºC	0,015uM	5µl	2
	DENV1SA_6_LEFT	AAACTGACYTTARAGGGGATGTCAT	56.1ºC	0,015uM	5µl	2
	DENV1SA_6_RIGHT	ATATGCRGTCCCAAAAACCTGG	56.7ºC	0,015uM	5µl	2
	DENV1SA_8_LEFT	AGGCTGACTCCCCAAAAAGACT	58.5ºC	0,015uM	5µl	2
	DENV1SA_8_RIGHT	TTGATGGCAGCTGACATTAGCC	57.8ºC	0,015uM	5µl	2
	DENV1SA_10_LEFT	GCAGGGCCATGGCACCTAGG	63.5ºC	0,0075uM	2,5µl	2
	DENV1SA_10_RIGHT	TCCCCATCCTGTCTGAAGCATT	58.4ºC	0,0075uM	2,5µl	2
	DENV1SA_12_LEFT	GGATTATGCATGGAARACAAYGGC	56.9ºC	0,030uM	10µl	2
	DENV1SA_12_RIGHT	GTGAGTGTRTCATCCCTYTCTTCA	56.2ºC	0,030uM	10µl	2
	DENV1SA_14_LEFT	AGGTCCCAAGTAGGAGTGGGAGT	61.2ºC	0,015uM	5µl	2
	DENV1SA_14_RIGHT	CACCTCRTCCTCAATCTCTGGT	57.2ºC	0,015uM	5µl	2
	DENV1SA_16_LEFT	GGGAGATAGTTGACCTCATGTGCCA	60.3ºC	0,015uM	5µl	2
	DENV1SA_16_RIGHT	CCTGTCGGCCCGGAAATTTGC	61.7ºC	0,015uM	5µl	2

	A	B	C	D	E	F
	DENV1SA_18_LEFT	CAGAAGGGATCATCCCAGCCCT	60.9°C	0,0075uM	2,5µl	2
	DENV1SA_18_RIGHT	CCTCCTTGTTCGGAATTGTGCA	57.9°C	0,0075uM	2,5µl	2
	DENV1SA_20_LEFT	GCTGCTCATTCCAGARCCAGAC	59.2°C	0,015uM	5µl	2
	DENV1SA_20_RIGHT	ATGGGTTCACCTGGGAATAGCA	58.4°C	0,015uM	5µl	2
	DENV1SA_22_LEFT	TCCATCACACTGGCTACTGGAC	58.6°C	0,015uM	5µl	2
	DENV1SA_22_RIGHT	CCCACAACCGAGGTCTATGACT	58.4°C	0,015uM	5µl	2
	DENV1SA_24_LEFT	GCTYAGAGGAAACCAATTCTGCA	56.5°C	0,030uM	10µl	2
	DENV1SA_24_RIGHT	TGATCCTGATGGYTTGACCTCA	54.7°C	0,030uM	10µl	2
	DENV1SA_26_LEFT	CTGCACAAGAGAGGAGTTCACA	56.8°C	0,015uM	5µl	2
	DENV1SA_26_RIGHT	TATTCTTGTGTCCCATCCGGCT	58.3°C	0,015uM	5µl	2
	DENV1SA_28_LEFT	GAAACCCCCAAYCTAGCTRAGA	56.4°C	0,030uM	10µl	2
	DENV1SA_28_RIGHT	TAGCCGCTAGTCTCAGGTCTCT	58.8°C	0,030uM	10µl	2
	DENV1SA_30_LEFT	GGGCCACYAATATACAAGTAGCCA	57.6°C	0,030uM	10µl	2
	DENV1SA_30_RIGHT	CCCGCTGCTGCGTTATGTCT	60.4°C	0,015uM	10µl	2
	DENV1SA_31_RIGHT	CCTGTTGATTCAACAGCACCATTCCA	59.7°C	0,015uM	10µl	2

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

Note: The primers were designed using the https://primalscheme.com (Brito, 2021) based on the JX669463.1 and KP188568 reference genomes.

Multiplex PCR

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

	A	B	C	D
	Mix 1 Multiplex PCR	Vol. Pool 1 (1x)	Vol. Pool 2 (1x)	96 samples (+2) (pool1 or pool2)
	Q5 Master Mix High fidelity 2X	12,5 µl	12,5 µl	1.225 µl
	Pool primers (Pool1 ou Pool2) /Use concentration /	1,5 µl	1,5 µl	147 µl
	Ultra Pure Water	8,5 µl	8,5 µl	833 µl
	Total	22,5µl	22,5µl	2205µl

12 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