

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

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.

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, H2O Ultrapure, primers described in table 1.

Procedures:

1. Reverse transcription

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

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 cada)	1µL	98µL
Total	2µL	194µL

B) 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:

65°C ---- 5 minutes

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

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

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 ou RNase Inhibitor	1µL	98µL
SSIV Reverse Transcriptase	1µL	98µL
Total	7µL	686µL

E) 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:

42°C ---- 50 minutes

70°C ---- 10 minutes

4°C ---- Hold

F) Store the cDNA at -20°C.

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.

2. Pools of primers

- A) Select two 0,6mL tubes for each pool.
- B) Using the original 100uM primer solution eluted individually, put them together following the table below containing each primer volume.
- C) Pool 1 will have a final volume of 130µl and pool 2 of 170µl.
- D) 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.

Primer	Sequence	Tm	Concentration inside of the pool *	volume of primer within the pool	POOL
DENV1SA_1_LEFT	TACGTGGACCGACAAGAACAGT	58.1°C	0,0075uM	2,5µl	1
DENV1SA_1_RIGHT	ACTATCATRTGTGGCTCTCCCC	57.2°C	0,0075uM	2,5µl	1
DENV1SA_3_LEFT	CACACGTGGGACTTGGTCTAGA	58.4°C	0,01125uM	7,5µl	1
DENV1SA_3_RIGHT	ACACACAAAGTTCGCGTCTTGT	57.6°C	0,01125uM	7,5µl	1
DENV1SA_5_LEFT	CCTCACATTGGACTGCTCACCT	59.1°C	0,01125uM	7,5µl	1
DENV1SA_5_RIGHT	TGCACTARRACAGTTCATGCT	56.6°C	0,01125uM	7,5µl	1
DENV1SA_7_LEFT	CGAGGAGCACGAAGGATGGC	60.6°C	0,0075uM	2,5µl	1
DENV1SA_7_RIGHT	ATGATGTTCTCAAGACGCGTGG	57.5°C	0,0075uM	2,5µl	1
DENV1SA_9_LEFT	TGGGAAGTTGAGGACTAYGGGT	58.7°C	0,015uM	5µl	1
DENV1SA_9_RIGHT	TGTRGTTCTGAGRGATGGACCTC	57.8°C	0,015uM	5µl	1
DENV1SA_11_LEFT	GATGACTGGAACACTGGCTGTT	57.4°C	0,030uM	10µl	1
DENV1SA_11_RIGHT	CACCGGAAGCCATGTTGTTTTT	56.7°C	0,030uM	10µl	1
DENV1SA_13_LEFT	AASAAGAAGCAGAACACTCCGG	57.3°C	0,015uM	5µl	1
DENV1SA_13_RIGHT	ACTGGCCCAGCTTGTTCCAG	62.4°C	0,015uM	5µl	1



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DENV1SA_15_LEFT	ATGGAGTGGTGACAACAAGTGG	57.4°C	0,015uM	5µl	1
DENV1SA_15_RIGHT	GCTGGATCGGTAAARTGTGCTTC	57.4°C	0,015uM	5µl	1
DENV1SA_17_LEFT	ACGGGTRATYCAAYTGAGCAGRA	58.5°C	0,01125uM	7,5µl	1
DENV1SA_17_RIGHT	CCTCTTCTCATGAGCTCCACA	56.3°C	0,01125uM	7,5µl	1
DENV1SA_19_LEFT	AGTGTCTCAGGTGACCTAATATTGGA	57°C	0,0075uM	2,5µl	1
DENV1SA_19_RIGHT	RGCTGCCACTGTCAGTATCATG	57.5°C	0,0075uM	2,5µl	1
DENV1SA_21_LEFT	YGCAAAYCAGGCWGCYATATTGAT	57.6°C	0,015uM	5µl	1
DENV1SA_21_RIGHT	GATGTTTGCCATGGACACTGCT	58.2°C	0,015uM	5µl	1
DENV1SA_23_LEFT	ACAACCAAACATGCAGTGTCTGA	57.5°C	0,015uM	5µl	1
DENV1SA_23_RIGHT	TTTCGCACTAGCATCCCTCCAT	58.5°C	0,015uM	5µl	1
DENV1SA_25_LEFT	ACCTAGATATYATTGGCCAGAGGA	55.9°C	0,030uM	10µl	1
DENV1SA_25_RIGHT	ACCTTTCGTCTTCCACTGCTTC	57.3°C	0,030uM	10µl	1
DENV1SA_27_LEFT	TGGAAGGAGAAGGACTGCACAA	58.4°C	0,030uM	10µl	1
DENV1SA_27_RIGHT	CACRCAATCATCTCCGCTRATT	55.5°C	0,030uM	10µl	1
DENV1SA_29_LEFT	ATGGAGCCTGAGAGAACTGCT	58.2°C	0,030uM	10µl	1
DENV1SA_29_RIGHT	GCYCCTTCGGGATCACTCTCAT	59.7°C	0,030uM	10µl	1
DENV1SA_2_LEFT	TGTTGAACATAATRAACAGGAGGAA AAGA	55.9°C	0,01125uM	7,5µl	2
DENV1SA_2_RIGHT	GAATCCTGGGTGTCKCAAAGCC	59.5°C	0,01125uM	7,5µl	2
DENV1SA_4_LEFT	ACTGTGCATTGAAGCTAAAATATCAA ACA	56°C	0,015uM	5µl	2
DENV1SA_4_RIGHT	ACCATTGTTTGTGGACAAGCCA	57.7°C	0,015uM	5µl	2
DENV1SA_6_LEFT	AAACTGACYTTARAGGGGATGTCAT	56.1°C	0,015uM	5µl	2
DENV1SA_6_RIGHT	ATATGCRGTCCCAAAAACCTGG	56.7°C	0,015uM	5µl	2
DENV1SA_8_LEFT	AGGCTGACTCCCCAAAAGACT	58.5°C	0,015uM	5µl	2
DENV1SA_8_RIGHT	TTGATGGCAGCTGACATTAGCC	57.8°C	0,015uM	5µl	2
DENV1SA_10_LEFT	GCAGGGCCATGGCACCTAGG	63.5°C	0,0075uM	2,5µl	2
DENV1SA_10_RIGHT	TCCCCATCCTGTCTGAAGCATT	58.4°C	0,0075uM	2,5µl	2
DENV1SA_12_LEFT	GGATTATGCATGGAARACAAYGGC	56.9°C	0,030uM	10µl	2
DENV1SA_12_RIGHT	GTGAGTGTRTCATCCCTYTCTTCA	56.2°C	0,030uM	10µl	2
DENV1SA_14_LEFT	AGGTCCCAAGTAGGAGTGGGAGT	61.2°C	0,015uM	5µl	2
DENV1SA_14_RIGHT	CACCTCRCTCAATCTCTGGT	57.2°C	0,015uM	5µl	2
DENV1SA_16_LEFT	GGGAGATAGTTGACCTCATGTGCCA	60.3°C	0,015uM	5µl	2
DENV1SA_16_RIGHT	CCTGTCCGCCCCGAAATTTC	61.7°C	0,015uM	5µl	2
DENV1SA_18_LEFT	CAGAAGGGATCATCCCAGCCCT	60.9°C	0,0075uM	2,5µl	2
DENV1SA_18_RIGHT	CCTCCTTGTTCCGAATTGTGCA	57.9°C	0,0075uM	2,5µl	2
DENV1SA_20_LEFT	GCTGCTCATTCCAGARCCAGAC	59.2°C	0,015uM	5µl	2
DENV1SA_20_RIGHT	ATGGGTTACCTGGGAATAGCA	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	CCCCTGCTGCGTTATGTCT	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.

3. Multiplex PCR

A) 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 1 Multiplex 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

B) 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:



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98°C ---- 30 seconds

Step2: (45 cycles)

98°C ---- 15 seconds

58°C ---- 30 seconds

72°C ---- 5 minutes

Step3:

72°C ---- 2 minutes

Hold 4°C

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References:

Brito, Anderson Fernandes, et al. "Lying in Wait: The Resurgence of Dengue Virus after the Zika Epidemic in Brazil." *Nature Communications*, vol. 12, no. 1, May 2021, p. 2619, doi:<https://doi.org/10.1038/s41467-021-22921-7>.