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

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We use this protocol and it's working

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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.

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

	A	B	C
			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	ACCTAGATATYATTGG	55.9ºC	0,030uM	10µl	1

	A	B	C	D	E	F
		CCAGAGGA				
	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
	DENV1SA_29_RIGHT	GCYCCTTCGGGATCACTCTCAT	59.7ºC	0,030uM	10µl	1
	DENV1SA_2_LEFT	TGTTGAACATAATRAACAGGAGGAA AAGA	55.9ºC	0,01125uM	7,5µl	2
	DENV1SA_2_RIGHT	GAATCCTGGGTGTCKCAAAGCC	59.5ºC	0,01125uM	7,5µl	2
	DENV1SA_4_LEFT	ACTGTGCATTGAAGCTAAAATATCAA ACA	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

	A	B	C	D	E	F
	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
	DENV1SA_18_LEFT	CAGAAGGGATCATCCCAGCCCT	60.9ºC	0,0075uM	2,5µl	2
	DENV1SA_18_RIGHT	CCTCCTTGTTCGGAATTGTGCA	57.9ºC	0,0075uM	2,5µl	2
	DENV1SA_20_LEFT	GCTGCTCATTCCAGARCCAGAC	59.2ºC	0,015uM	5µl	2
	DENV1SA_20_RIGHT	ATGGGTTCACCTGGGAATAGCA	58.4ºC	0,015uM	5µl	2
	DENV1SA_22_LEFT	TCCATCACACTGGCTACTGGAC	58.6ºC	0,015uM	5µl	2

	A	B	C	D	E	F
	DENV1SA_22_RIGHT	CCCACAACCGAGGTCTATGACT	58.4ºC	0,015uM	5µl	2
	DENV1SA_24_LEFT	GCTYAGAGGAAACCAATTCTGCA	56.5ºC	0,030uM	10µl	2
	DENV1SA_24_RIGHT	TGATCCTGATGGYTTGACCTCA	54.7ºC	0,030uM	10µl	2
	DENV1SA_26_LEFT	CTGCACAAGAGAGGAGTTCACA	56.8ºC	0,015uM	5µl	2
	DENV1SA_26_RIGHT	TATTCTTGTGTCCCATCCGGCT	58.3ºC	0,015uM	5µl	2
	DENV1SA_28_LEFT	GAAACCCCCAAYCTAGCTRAGA	56.4ºC	0,030uM	10µl	2
	DENV1SA_28_RIGHT	TAGCCGCTAGTCTCAGGTCTCT	58.8ºC	0,030uM	10µl	2
	DENV1SA_30_LEFT	GGGCCACYAATATACAAGTAGCCA	57.6ºC	0,030uM	10µl	2
	DENV1SA_30_RIGHT	CCCGCTGCTGCGTTATGTCT	60.4ºC	0,015uM	10µl	2
	DENV1SA_31_RIGHT	CCTGTTGATTCAACAGCACCATTCCA	59.7ºC	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