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

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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 CHIKV serotype strains.

MATERIALS

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

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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	2 µL	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 469µl and pool 2 of 460µl.

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
	Primer	Sequence	Concentration inside of the pool *	Volume of primer within the pool	Pool
	400_1_LEFT_1	TGACACACGTAGCCTACCAGTT	0,015uM	10ul	1
	400_1_RIGHT_1	CGCATCGGGCAAACGCAGTGGTA	0,015uM	10ul	1
	400_3_LEFT_3	GCAGACGTCGCGATATACCAAG	0,015uM	10ul	1
	400_3_RIGHT_3	CCAGCTCTTAAGTAGCATGCGG	0,015uM	10ul	1
	400_5_LEFT_0	GATGTGCAAGACTACCGACACG	0,015uM	10ul	1
	400_5_RIGHT_0	GACTGGGTATCAGGCCTCTTGT	0,015uM	10ul	1
	400_7_LEFT_4	CAAGAAGCCCAGGATGCTGAAA	0,015uM	10ul	1
	400_7_RIGHT_4	GCTATGCGTACACGTCTTCACT	0,015uM	10ul	1
	400_9_LEFT_4	GCAGAGAGGACAGAACACGAGT	0,015uM	10ul	1
	400_9_RIGHT_4	CTCTCTGTCTCATCACGTCGGT	0,015uM	10ul	1

	A	B	C	D	E
	400_11_LEFT_0	AGCAGTGCGGCTTCTTCAATAT	0,015uM	10ul	1
	400_11_RIGHT_0	TGCCTAACTGCGTAAACTCCTTT	0,015uM	10ul	1
	400_13_LEFT_2	ATTAAGGAGTGGGAGGTGGAGC	0,015uM	10ul	1
	400_13_RIGHT_2	TCTAGAATGGACGCTGCCTCAG	0,015uM	10ul	1
	400_15_LEFT_4	GGGGAAAGAATGGAATGGCTGG	0,015uM	10ul	1
	400_15_RIGHT_4	CGTTCACTGGTTCTATCTGCGT	0,015uM	10ul	1
	400_17_LEFT_0	AACTGAACGCAGCCTTTGTAGG	0,015uM	10ul	1
	400_17_RIGHT_0	ACACCTGTGGAGAGGAGAGGTA	0,015uM	10ul	1
	400_19_LEFT_1	CATACAGATGCGGACCCAAGTG	0,015uM	10ul	1
	400_19_RIGHT_1	GTTCAGGAGTCATGGCATAACGG	0,015uM	10ul	1
	400_21_LEFT_2	GCGCGTAAGTCCAAGGGAATAC	0,015uM	10ul	1
	400_21_RIGHT_2	GTCTCCGCTGTTTCTTGTACGG	0,015uM	10ul	1
	400_23_LEFT_1	ACTTTCGGAGACTTCCTACCCG	0,015uM	10ul	1
	400_23_RIGHT_1	ACAGCCTCTCTTTAGTCTCTGGA	0,015uM	10ul	1
	400_25_LEFT_2	ACCAAATCACCGATGAGTATGATGC	0,015uM	10ul	1
	400_25_RIGHT_2	TCGTTATCCTGATAGGGCTGGC	0,015uM	10ul	1
	400_27_LEFT_4	AGGCCTAAGGTGCAGGTTATACA	0,015uM	10ul	1
	400_27_RIGHT_4	GCAGGTGACAGCTGGAAATCTC	0,015uM	10ul	1

	A	B	C	D	E
	400_29_LEFT_0	CGATGAATTGATGGCAGCCAGA	0,015uM	10ul	1
	400_29_RIGHT_0	GCAAAGGTGGCCATGGACATTA	0,015uM	10ul	1
	400_31_LEFT_1	TTCTACAATAGGAGGTACCAGCCT	0,015uM	10ul	1
	400_31_RIGHT_1	TTCATGCACATTCTCTCTCTGCG	0,015uM	10ul	1
	400_33_LEFT_3	GATACCCGTGCACATGAAGTCC	0,015uM	10ul	1
	400_33_RIGHT_3	TTTTTCGTAGCAGCAGGGTGTG	0,015uM	10ul	1
	400_35_LEFT_0	CCACAAGACCGTACCTAGCTCA	0,015uM	10ul	1
	400_35_RIGHT_0	TGGTGAAATGGGTGCGTACATG	0,015uM	10ul	1
	400_37_LEFT_3	AATGTCACAACAGTCCGGCAAT	0,015uM	10ul	1
	400_37_RIGHT_3	TTGGGTGGTCAGGATACAGCAA	0,015uM	10ul	1
	400_39_LEFT_1	GGCCACCCGCATGAGATAATTC	0,015uM	10ul	1
	400_39_RIGHT_1	ATAGGACAATCAGGGCTGCCAG	0,015uM	10ul	1
	400_41_LEFT_0	CTTGGAACCAACGCTATCGCTT	0,015uM	10ul	1
	400_41_RIGHT_0	AGCAGCCACAGTGATATTATTTCCT	0,015uM	10ul	1
	400_43_LEFT_0	ACCAGGACAATTTGGCGACATC	0,015uM	10ul	1
	400_43_RIGHT_0	ATACCTCACACGACATGTCCGT	0,015uM	10ul	1
	400_45_LEFT_3	CTACACAAGTACACTGTGCAGCC	0,015uM	10ul	1
	400_45_RIGHT_3	TGTTATTCAGGGGTTGTTCAGCC	0,015uM	10ul	1

	A	B	C	D	E
	400_2_LEFT_0	CCAGCAAGGAGGATGATGTCGGAC	0,015uM	10ul	2
	400_2_RIGHT_0	TGTGTCGAACCCTACCCAGTAC	0,015uM	10ul	2
	400_4_LEFT_0	TGTTCTCAGTAGGGTCAACGCT	0,015uM	10ul	2
	400_4_RIGHT_0	GGATGCCGGTCATTTGATCACA	0,015uM	10ul	2
	400_6_LEFT_1	TGAGAAGCTTTTGGGGGTCAGA	0,015uM	10ul	2
	400_6_RIGHT_1	ACATCTTCCTGTGCTGCCTGTA	0,015uM	10ul	2
	400_8_LEFT_0	ACTTTCCCCGCAGACCGTATTA	0,015uM	10ul	2
	400_8_RIGHT_0	CAGCTTCTTCCTTCTTGCAGCA	0,015uM	10ul	2
	400_10_LEFT_0	ACCTGGTGACTAGCGGAAAGAA	0,015uM	10ul	2
	400_10_RIGHT_0	GACGACACAATGGCAGTCACAG	0,015uM	10ul	2
	400_12_LEFT_0	GAGGGTGGGTTAAACAACTGCA	0,015uM	10ul	2
	400_12_RIGHT_0	TTATCCCCGCTGTTTCGAGGAT	0,015uM	10ul	2
	400_14_LEFT_1	ACGCGGATAACCACTGGGATAA	0,015uM	10ul	2
	400_14_RIGHT_1	TTATAGCCGCTAACCAGGAGCA	0,015uM	10ul	2
	400_16_LEFT_0	AGGTGACTCACTGAGACTGCTC	0,015uM	10ul	2
	400_16_RIGHT_0	ATCGTTCTTCGCGATGTCCATG	0,015uM	10ul	2
	400_18_LEFT_2	GGACCAAACTTCTCAAATTACACGGA	0,015uM	10ul	2
	400_18_RIGHT_2	CCAAACTACTGTCAGGGTGCAC	0,015uM	10ul	2

	A	B	C	D	E
	400_20_LEFT_4	CAGAAATGCCCGGTGGATGATG	0,015uM	10ul	2
	400_20_RIGHT_4	ATCGGCGCTTAGATCAAACTGAC	0,015uM	10ul	2
	400_22_LEFT_0	GAGGGAGAAACCTGACCGTGAT	0,015uM	10ul	2
	400_22_RIGHT_0	AGTCATAACTCGTCGTCCGTGT	0,015uM	10ul	2
	400_24_LEFT_4	CACGGCCAATAGAAGCAGGTATC	0,015uM	10ul	2
	400_24_RIGHT_4	TTGACGGATTGAATGTCGCTCG	0,015uM	10ul	2
	400_26_LEFT_0	ACCCACTTTGGACTCAGCAGTA	0,015uM	10ul	2
	400_26_RIGHT_0	AGGACGGCGTTCAATCTCCTAA	0,015uM	10ul	2
	400_28_LEFT_0	CCAGGATGATTCACTTGCGCTT	0,015uM	10ul	2
	400_28_RIGHT_0	GGAGCTTTCTGGGATACAACTGC	0,015uM	10ul	2
	400_30_LEFT_1	GATGGCAACGAACAGGGCTAAT	0,015uM	10ul	2
	400_30_RIGHT_1	GGTCTGGGTCTGATGACTTGGA	0,015uM	10ul	2
	400_32_LEFT_3	CCCCCAAAAAGAAACCGGTTCA	0,015uM	10ul	2
	400_32_RIGHT_3	GAGTACTGTACTGCTCCGTGGT	0,015uM	10ul	2
	400_34_LEFT_0	CGTCACGAAAATCACCCCTGAG	0,015uM	10ul	2
	400_34_RIGHT_0	TCTGTCGCTTCGTTTCTGATGC	0,015uM	10ul	2
	400_36_LEFT_0	CCGTGCACGATTACTGGAACAA	0,015uM	10ul	2
	400_36_RIGHT_0	CACAATTGCACTTGTACCGCAC	0,015uM	10ul	2

	A	B	C	D	E
	400_38_LEFT_1	TCCTCTGGCAAATGTGACATGC	0,015uM	10ul	2
	400_38_RIGHT_1	CACCCACCATCGACAGGAGTAT	0,015uM	10ul	2
	400_40_LEFT_1	TATACCTGTGGAACGAGCAGCA	0,015uM	10ul	2
	400_40_RIGHT_1	TGTACCGCAGCATTTCACGTAC	0,015uM	10ul	2
	400_42_LEFT_4	TCAGCATACAGGGCTCATACCG	0,015uM	10ul	2
	400_42_RIGHT_4	GACGGTCTCTGCAGTACCAGTT	0,015uM	10ul	2
	400_44_LEFT_0	ATCTCCATCGACATACCGGACG	0,015uM	10ul	2
	400_44_RIGHT_0	TGTGACGCCGGGTAATTGACTA	0,015uM	10ul	2
	400_46_LEFT_1	TCCCTAAAGAGACACACCGCAT	0,015uM	10ul	2
	400_46_RIGHT_1	TCTTAGCTATATATGGTGTGTCTCTTAGGG	0,015uM	10ul	2

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

Note: The primers were designed using the https://primalscheme.com based on the KP164576, KP164571, KP164572, KP164568, KP164570 and KP164569 reference genomes (Machado, 2019).

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 ou pool2)

	A	B	C	D
	Q5 Master Mix High fidelity 2X	12,5 µl	12,5 µl	1.225 µl
	Pool primers (Pool1 or Pool2) /Use concentration	1,7 µl	1,7 µl	166,6 µl
	Ultra Pure Water	8,3 µl	8,3 µl	813,3 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