

Reverse transcription, primer pools preparation and multiplex PCR steps for CHIKV 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 CHIKV 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 469µl and pool 2 of 460µl.

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	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
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



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400_15_LEFT_4	GGGGAAAGAATGGAATGGCTGG	0,015uM	10ul	1
400_15_RIGHT_4	CGTTCACCTGGTTCTATCTGCGT	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	GTTTCAGGAGTCATGGCATAACGG	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
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	TTCATGCACATTCTCTCTGCG	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	AGCAGCCACAGTGATATTATTCCT	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	TGTTATTACAGGGGTGTTAGCC	0,015uM	10ul	1
400_2_LEFT_0	CCAGCAAGGAGGATGATGTCGGAC	0,015uM	10ul	2
400_2_RIGHT_0	TGTGTGCAACCCTACCCAGTAC	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



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400_8_LEFT_0	ACTTTCCCCGCAGACCGTATTA	0,015uM	10ul	2
400_8_RIGHT_0	CAGCTTCTTCCTTCTTGCAGCA	0,015uM	10ul	2
400_10_LEFT_0	ACCTGGTGA CTAGCGGAAAGAA	0,015uM	10ul	2
400_10_RIGHT_0	GACGACACAATGGCAGTCACAG	0,015uM	10ul	2
400_12_LEFT_0	GAGGGTGGGTAAACA ACTGCA	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	GGACCAA ACTTCTCAAATTACACGGA	0,015uM	10ul	2
400_18_RIGHT_2	CCAAACTACTGTCAGGGTGCAC	0,015uM	10ul	2
400_20_LEFT_4	CAGAAATGCCCGGTGGATGATG	0,015uM	10ul	2
400_20_RIGHT_4	ATCGGCGCTTAGATCAA ACTGAC	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	ACCCACTTTGGA CTACAGCAGTA	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	TCTGTGCTTCGTTTCTGATGC	0,015uM	10ul	2
400_36_LEFT_0	CCGTGCACGATTACTGGAACAA	0,015uM	10ul	2
400_36_RIGHT_0	CACAATTGCACTTG TACCGCAC	0,015uM	10ul	2
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	TGTACCGCAGCATTTACGTAC	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	TCTTAGCTATATATGGTGTGTCTCTTAG GG	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).

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/	1,7 µl	1,7 µl	166,6 µl
Água Ultra Pura	8,3 µl	8,3 µl	813,3 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:

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:

Machado, Laís Ceschini, et al. “Genome Sequencing Reveals Coinfection by Multiple Chikungunya Virus Genotypes in a Recent Outbreak in Brazil.” *PLOS Neglected Tropical Diseases*, edited by Fabrice Simon, vol. 13, no. 5, May 2019, p. e0007332, doi:<https://doi.org/10.1371/journal.pntd.0007332>.