

Reverse transcription, primer pools preparation and multiplex PCR steps for MAYV 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 MAYV 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 310µl and pool 2 of 280µl.

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
MAYV_1_LEFT	GCTCTTCCTCTGCATTGCAAGA	61.2	0,015uM	10ul	1
MAYV_1_RIGHT	ACTCCTTTCAGTGCCTGGAAGT	61.56	0,015uM	10ul	1
MAYV_3_LEFT	CGATGCCTGCTAAAGAGTTGGC	62.13	0,015uM	10ul	1
MAYV_3_RIGHT	GTCAAGCCGATACTCTTTCGCC	61.62	0,015uM	10ul	1
MAYV_5_LEFT	GGAAGCAGAAGAAACACTGGCA	61.33	0,015uM	10ul	1
MAYV_5_RIGHT	CGTATTCGGCGTCTGTCCCTTC	61.6	0,015uM	10ul	1
MAYV_7_LEFT	TACAGAGATTGGACATCACTGCA	60.02	0,015uM	10ul	1
MAYV_7_RIGHT	CAGCTGTCATGACTTCGTGTCC	61.49	0,015uM	10ul	1
MAYV_9_LEFT	TTGGTACCCGTTTTGGAAACCG	61.58	0,015uM	10ul	1
MAYV_9_RIGHT	ATGAGGTTGTACTCGCTGACCA	61.41	0,015uM	10ul	1
MAYV_11_LEFT	TCAGAGCGGTTAGACCTCCATG	61.53	0,015uM	10ul	1
MAYV_11_RIGHT	GACAGTCGGTTAATCTCCGCTG	61.05	0,015uM	10ul	1
MAYV_13_LEFT	ACGCCCAGGATACAGTACCACT	62.88	0,015uM	10ul	1



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MAYV_13_RIGHT	AGGGAGCTGCTGTCAGATGTAAT	61.46	0,015uM	10ul	1
MAYV_15_LEFT	CGCCTAGAAGATGTGTGCCAAG	61.57	0,015uM	10ul	1
MAYV_15_RIGHT	AGTCTTTGCATTACCGGCACAG	61.45	0,015uM	10ul	1
MAYV_17_LEFT	CAACGTTACCCAGATGCGTGAA	61.44	0,015uM	10ul	1
MAYV_17_RIGHT	CCGGGCTTGAAATGCTCTGATAT	61.06	0,015uM	10ul	1
MAYV_19_LEFT	TAACTAATTCTGCCTGTGCCGC	61.26	0,015uM	10ul	1
MAYV_19_RIGHT	AGAGCACCTATTTAGGACCGCC	61.87	0,015uM	10ul	1
MAYV_21_LEFT	CGCATGTGCATGAAGATTGAGC	61.11	0,015uM	10ul	1
MAYV_21_RIGHT	GCCTTCCGGTGTAATCTTGGTG	61.51	0,015uM	10ul	1
MAYV_23_LEFT	TAGCCTACTGTGCAGACTGTGG	61.73	0,015uM	10ul	1
MAYV_23_RIGHT	CGGCATGTGCATGTCAATCTCT	61.58	0,015uM	10ul	1
MAYV_25_LEFT	GTCAGAAGCGGTAAACGGGAAG	61.5	0,015uM	10ul	1
MAYV_25_RIGHT	GGTCCAGTCAACTCCATCCAGA	61.42	0,015uM	10ul	1
MAYV_27_LEFT	TCACCTTACGTAAAGTGCTGCG	61.18	0,015uM	10ul	1
MAYV_27_RIGHT	TGTCTGGGTATAGGGAACGTGG	61.22	0,015uM	10ul	1
MAYV_29_LEFT	CGGCAACATACCCGTTTCGATG	62.42	0,015uM	10ul	1
MAYV_29_RIGHT	ACTGCCAGAGCTATCAGTAACCC	61.76	0,015uM	10ul	1
MAYV_30_RIGHT	TCACCTTCTCATAGTTATGCAAGTAA CTA	55.9	0,015uM	10ul	1
MAYV_2_LEFT	AGATCAGACATGCAGGACTCCA	60.89	0,015uM	10ul	2
MAYV_2_RIGHT	TAACGACGTAGCCCTCACATGA	61.2	0,015uM	10ul	2
MAYV_4_LEFT	AGAGGATTGTCTGTAACGGGAG	62.04	0,015uM	10ul	2
MAYV_4_RIGHT	ACAACACCTGCTCCTGCTCTAA	61.61	0,015uM	10ul	2
MAYV_6_LEFT	AACCGTAAATTGTACCACATAGCCG	61.65	0,015uM	10ul	2
MAYV_6_RIGHT	CGAAAGCCTCATCGACGTACAA	60.98	0,015uM	10ul	2
MAYV_8_LEFT	TGACACCACAGGCCAGACTAAG	61.93	0,015uM	10ul	2
MAYV_8_RIGHT	GGTGAATAAGCCCTGTCTCCT	61.49	0,015uM	10ul	2
MAYV_10_LEFT	TGGTCACGGAATACCACCCAAT	61.69	0,015uM	10ul	2
MAYV_10_RIGHT	TACCATTTGTAGGGTGCAGGGT	61.37	0,015uM	10ul	2
MAYV_12_LEFT	ACGCGGTGGGTCCAAATTTTAA	61.34	0,015uM	10ul	2
MAYV_12_RIGHT	CCACAAAGTCGTGATCTCAGCC	61.5	0,015uM	10ul	2
MAYV_14_LEFT	ACCAGCCTCTGTAGTCCTGTG	61.67	0,015uM	10ul	2
MAYV_14_RIGHT	ATGTCACCTCCTCTTCGGTCG	61.52	0,015uM	10ul	2
MAYV_16_LEFT	CTGAAACAGGGCAGTGCATCTT	61.4	0,015uM	10ul	2
MAYV_16_RIGHT	TCCCAGTATTCATTGTTGCAGGC	61.44	0,015uM	10ul	2
MAYV_18_LEFT	CCGCCTATCTGTGTGGAATCCA	62.13	0,015uM	10ul	2
MAYV_18_RIGHT	CCATGTTAACCCAAGTGGCACA	61.34	0,015uM	10ul	2
MAYV_20_LEFT	ACTGGCTATGTCCACCTTTGCA	62.22	0,015uM	10ul	2

MAYV_20_RIGHT	CCGGCTTCATTACCTTGTACACC	61.51	0,015uM	10ul	2
MAYV_22_LEFT	CCCATCTTTGACAACAAGGGCC	61.99	0,015uM	10ul	2
MAYV_22_RIGHT	AGGCCAATTTGGGAAGCAAACCT	61.29	0,015uM	10ul	2
MAYV_24_LEFT	GATTTACGGTGCGACCACATCA	61.25	0,015uM	10ul	2
MAYV_24_RIGHT	CTGTTCATCAAAGACGGGGCTCC	61.51	0,015uM	10ul	2
MAYV_26_LEFT	TCTATCGGGGTGTTGTGCTGTG	62.56	0,015uM	10ul	2
MAYV_26_RIGHT	AACAGTATGCACCTCCCCACAT	61.7	0,015uM	10ul	2
MAYV_28_LEFT	TCCAGAGTAGGACGTTGGACAG	61.14	0,015uM	10ul	2
MAYV_28_RIGHT	TGGATCACTGATCGACCTTCCG	61.85	0,015uM	10ul	2

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

Note: The primers were designed using <https://primalscheme.com> based on the Y618127 and KP842812 reference genomes (Vieira 2020).

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/	1,2 µl	1,2 µl	117,6 µl
Água Ultra Pura	8,8 µl	8,8 µl	862,4 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



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Step2: (45 cycles)

98°C ---- 15 seconds

61°C ---- 30 seconds

72°C ---- 5 minutes

Step3:

72°C ---- 2 minutes

Hold 4°C

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References:

Julia da Silva Pessoa Vieira, Carla, et al. "The Emergence of Chikungunya ECSA Lineage in a Mayaro Endemic Region on the Southern Border of the Amazon Forest." *Tropical Medicine and Infectious Disease*, vol. 5, no. 2, June 2020, p. 105,

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