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JCVSeq

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We use this protocol and it's working

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Abstract

In order to capture all three segments of the JCV genome, we employed a multiplexed approach to primer design by combining previously developed JCV segment-specific primers and an internal set generated by PrimalScheme. To maximize genome coverage, we developed our own highly multiplexed PCR primers for the medium and large segments using PrimalScheme. Based on publicly available sequences, we chose to make two separate primer sets for the highly divergent A and B lineages, each forming 800 bp amplicons. Because the A and B primers share identical binding sites, they can be mixed together by pool during the sequencing protocol to cover JCV diversity across lineages. The final primer set consists of segment specific small segment UTR primers, segment specific medium and large UTR primers, and internal A and B lineage primers for the medium and large segments.

Guidelines

It is recommended that steps performed up to amplicon generation be performed at a different workstation and with different equipment than steps post-amplicon generation.

Materials

Equipment

- 96-well format Thermocycler (two needed for 49+ sample runs)
- Qubit
- Bioanalyzer
- Magnetic rack fit for a 96-well PCR plate
- Magnetic rack fit for 1.5 / 2-mL microcentrifuge tubes
- Cold block fit for a 96-well PCR plate
- Pipettes (assorted sizes)

Consumables

- 8-strip PCR tubes; may be substituted with PCR plates with heat-sealing film
- 1.5 / 2mL / 5 mL microcentrifuge tubes
- Filtered pipette tips (assorted sizes)
- Reservoirs
- Waste containers

Reagents

- Illumina COVIDSeq Test Kit
- Illumina Nextera XT DNA Library Preparation kit
- New England Biolabs NEBNext ARTIC SARS-CoV-2 Library Prep Kit (Illumina)
- Oxford Nanopore Technologies Rapid barcoding kit

Bioinformatics pipeline

The Grubaugh Lab's virus agnostic analysis pipeline can be adapted to perform consensus assembly and generate quality control plots for JCVSeq. The pipeline can be found here: https://github.com/grubaughlab/DENV_pipeline. To run for this primer scheme and assemble Jamestown Canyon virus genomes, replace reference and BED files with the following files.

Reference genomes

JCVSb.fasta 0B

JCVSa.fasta 0B

JCVMb.fasta 4KB

JCVMa.fasta 4KB

JCVLb.fasta 6KB

JCVLa.fasta 6KB

BED files

JCVSb.bed 0B

JCVSa.bed 0B

JCVMb.bed 0B

JCVMa.bed 0B

JCVLb.bed 1KB

JCVLa.bed 1KB

Troubleshooting

qPCR

1 Briefly vortex and centrifuge all reagents.

2 Prepare 10 µM working stocks of the primers and probes below, by adding 10 µL of 100 µM stock to 90 µL of nuclease-free water.

	Primer/Probe Name	Sequence
	CAES_JCV154_F	TAATGCAG CAAAAGCC AAAG
	CAES_JCV307_R	AAGCCGAT GGATGGTA AGAT
	CAES_JCV181_P	CGCTCGTA AACCGGAG CGGA

qPCR primers developed by the Connecticut Agricultural Experiment Station (CAES) as part of unpublished JCV research

3 On ice, prepare a master mix containing the following:

	Reagent Name	Volume per Sample
	Luna Master Mix	10 µL
	RT	1 µL
	CAES_JCV154 _F	1 µL
	CAES_JCV307 _R	1 µL
	CAES_JCV181 _P	0.5 µL
	Nuclease Free Water	1.5 µL

4 Place the 96-well PCR plate on a cold block and add 15 µL of PCR master mix to each designated well.

5 Add 5 µL of RNA to each designated well. Mix by pipetting, avoiding bubbles.

6 Add 5 µL of positive control and non-template control to the final two wells. Mix by pipetting, avoiding bubbles.

6.1 Our recommended positive control is a gene block targeting the small segment. Its sequence is:

	Name	Sequence
	JCVS_gBlock	CAGATGCA GGGTTTGT GGCATTTAT GGCTGACC ATGGGGAA TCTGTCAG TCTGTCAG CCGTTAGG ATCTTCTT CCTTAATG CAGCAAAA GCCAAGGC TGCTCTCG CTCGTAAA CCGGAGCG GAAAGCTA CTCCTAAA TTTGGAGA GTGGCAGG TGGAAATT GTCAATAAT CATTTTCC TGGAAACA GGAACAAC CCAATTGG TAACAACG ATCTTACC ATCCATCG GCTTTCAG GATATCTAG CTAGATGG GTTCTTGA GCATTTTA CTACAGAT GATGATGA GTCCCAGA GAGAACTC ATAAGGAG CACCATCA TTAATCCA ATTGCAGA GTCCAATG GCATTCAT TGGAACAA TGGCCCAG AGATTTATC TTTCATTCT

	Name	Sequence
		TTCCAGGA ACAGAAAT GTTTTTGG AAATTTTC AAATTCTAT CCCTTGAC CATTGGAA TTTACAGA GTCAAGCA TGGTATGAT GGACCCTC AGTATCTGA AGAAGGCT CTCAGACA GCGC

7 Seal with a transparent plastic PCR seal. Centrifuge in the plate spinner.

8 Set the thermocycler to read FAM.

9 Run the following thermocycler conditions:

	Temperature	Time	Cycles
	55 ºC	10 minutes	
	95 ºC	1 minute	44 cycles
	95 ºC	10 seconds	(included in above 44)
	60 ºC	30 seconds	(inclued in above 44)
		READ PLATE	

Primer Pooling and Preparation

10 Combine the following primers at equal volume into a 20 µM pool. This will be AB1, containing pool 1 of primers covering diversity for both major lineages A and B.

	Primer Name	Sequence
	NYS_JCVSMF1	AGTGTACTACCAAGTATAGAAAACGTTCA
	NYS_JCVSM991R1	AGTAGTGTGCTCCACTGAATACATTTAA
	NYS_JCVMF1	AGTAGTGTACTACCAAGTATAGAAAACGTT
	Yale_JCVM837R1B	GGGTGATAAACTAACCCGCACA
	Yale_JCVM837R1A	GGGTGATACACCAAACCACATAGT
	Yale_JCVM1257F3B	GTGATATGTATCATGAAAAAGCCGGT
	Yale_JCVM1257F3A	GTGACATGTACCATGAAAAAGCTGG
	Yale_JCVM2057R3B	TGTCTTTTCGGGCCATAGCTTC
	Yale_JCVM2057R3A	TATCTTTGCGAGCCATAGCTTCTG
	Yale_JCVM2611F5B	TGGGAACTGTGATGTTCAAGAAAATG
	Yale_JCVM2611F5A	TGGAAATTGCAATGTTCAAGAAAATGATT
	Yale_JCVM3440R5B	TGGGAGTTATAGCATTTAAGTTTGTGC
	Yale_JCVM3439R5A	AGGAGTTACAGCATTT

	Primer Name	Sequence
		AAATTAGTGCAGTA
	Yale_JCVM3680F7B	GACAACGATTACCAAGCTTGCAA
	Yale_JCVM3680F7A	GACAATGATTATCAAGCTTGCAAATTTCT
	NYS_JCVM4510R2	AGTAGTGTGCTACCAAGTATATCTAAATGA
	NYS_JCVLF1	AGTAGTGTACTCCTATTTACAAAACTTACAAATAC
	Yale_JCVL873R1B	GGGCTGTTTGTAATCTCCAGTGG
	Yale_JCVL873R1A	GGGCTGTTTGTAGTTCCCTGT
	Yale_JCVL1177F3	AGCTGGCGACAAGTTATGAATAAGA
	Yale_JCVL2014R3B	GTGCAGGCTCAGTTAGAGATAACA
	Yale_JCVL2014R3A	GTGCAGGCTCAGTTAAGGACAA
	Yale_JCVL2315F5B	CCAAAGGTCTGCATGAGAAGCA
	Yale_JCVL2315F5A	CTAAGGGTTTACATGAGAAACATCATGTT
	Yale_JCVL3121R5B	CCCTATTTTTTTGCCTAGTAGTTTCAACT
	Yale_JCVL3121R5A	CCCTATTCTTTTGTCTT

	Primer Name	Sequence
		GTAGTCTC AAC
	Yale_JCVL3 638F7B	CAATAGTT CAAGATAA GGCCCCTG A
	Yale_JCVL3 638F7A	CGATAGTT CAAGACAA GGCTCCA
	Yale_JCVL4 432R7B	CATTCATG CCTCCTGG TGATGATA
	Yale_JCVL4 432R7A	CATTCATTC CTCCTGGT GATGACA
	Yale_JCVL4 975F9B	GCAGACCC AACAGAGA TGTCAA
	Yale_JCVL4 975F9A	GCTGATCC AACAGAGA TGTCAAGA
	Yale_JCVL5 804R9B	GCCATATTT TCGAATTT TAGACCAT GC
	Yale_JCVL5 804R9A	GCCATATTT TCAAATTT CAAGCCAT GT
	Yale_JCVL6 087F11B	AAAGAAAG CACATTTTA GCAAAATG GTATC
	Yale_JCVL6 087F11A	AAAGAAGG CGCATTTC AGCAAAA
	NYS_JCVL6 957R2	AGTGTGCT CCTATTTAC AAATATATA CTATAAGC

Primers developed using PrimalScheme, in collaboration with New York State Wadsworth Center

11 Combine the following primers at equal volume into a 20 µM pool. This will be AB2, containing pool 1 of primers covering diversity for both major lineages A and B.

	Primer Name	Sequence
	Yale_JCVM582F2B	ACCAACATATGTCATGTGTACGGT
	Yale_JCVM582F2A	ATCAGCATATGTCATGCGTACGAT
	Yale_JCVM1367R2B	TGTGTACTATCAATCCAGTAACATCTTCA
	Yale_JCVM1367R2A	TATGAACCATTAACCCAGTAATGTCTTCA
	Yale_JCVM1910F4B	TGGGATTTTGCAAATGAAATGAAGACA
	Yale_JCVM1910F4A	TGGGATTTCGCAAATGAAATGAAAACT
	Yale_JCVM2747R4B	TACTGCATCTGGGACTAAGGCA
	Yale_JCVM2747R4A	TGTTGCACCTAGGGCTTAGACA
	Yale_JCVM3303F6B	TCTTCGGGTCCTGTCAAGACATTA
	Yale_JCVM3299F6A	TGTGTATTTGGATCCTGTCAAGATATCATA
	Yale_JCVM4134R6B	TGGTCTACTGGTGCAAGCTCTAG
	Yale_JCVM4134R6A	TGGTCAACTGGTGCAAGTTCTAA

	Primer Name	Sequence
	Yale_JCVL7 46F2B	CATATGAAT CAGAAAGA TGGAACAC TAACC
	Yale_JCVL7 46F2A	CATACGAA TCAGAGAG GTGGAACA
	Yale_JCVL15 77R2B	CATTGCCA GAATTTGG TCTCAAAA ATAG
	Yale_JCVL15 77R2A	CACTGCCA GAACCTAG TCTCAAA
	Yale_JCVL16 80F4B	TGCATTAGT TTACCCTT CAGCTGAT
	Yale_JCVL16 80F4A	TGCACTAG TCTACCCA TCTGCA
	Yale_JCVL2 453R4B	AGGATTTG TAAATTAAC TGTTTGCT TGGT
	Yale_JCVL2 453R4A	AGGATCTG CAAATTGA CAGTTTGT TT
	Yale_JCVL2 940F6B	TGTAGGAG AATATGAAG CCAAAATG TGC
	Yale_JCVL2 940F6A	TGTGGGAG AATACGAG GCTAAAAT G
	Yale_JCVL3 744R6	TGTCTTTTT CATATTGG CTTGACAT CC
	Yale_JCVL4 301F8B	TGGGTGAG ACAAGTGA TATGAGGG
	Yale_JCVL4 301F8A	TGGGTGAA ACAAGCGA TATGAGAG

	Primer Name	Sequence
	Yale_JCVL5127R8B	TCTTGTCATTTCTTTCAATTCAAACACAAT
	Yale_JCVL5126R8A	CTCGTCATCTCCTTCAGTTCAAAAAC
	Yale_JCVL5681F10B	TTGGTGAAGACAACAAGCTAACTTATTC
	Yale_JCVL5681F10A	TCGGTGAAGACAATAAGCTAACCTAC
	Yale_JCVL6499R10B	TAGCCTCTAAGCAACCTAAGTTTTCA
	Yale_JCVL6499R10A	TGGCTTCTAAACAACCTAAATTTTCTGG

Primers developed using PrimalScheme, in collaboration with New York State Wadsworth Center

cDNA & Amplification

12 Combine the reagents from the table below to make PCR Master Mix. Multiply each volume by the number of samples (reagent overage already included) and create separate master mixes for each primer pool (AB1 and AB2)

	Reagent Name	Volume per Sample
	IPM	15 µl
	FSM	3.2 µl
	20 µM primer pool	1.2 µl
	Nuclease Free Water	3.6 µl

	Reagent Name	Volume per Sample
	RVT	1.0 µl

13 Add 20 µl of PCR Master Mix to each well of a 96-well plate.

14 Add 5 µl of extracted RNA to each well of the 96-well plate (for a total volume of 25 µl per well)

15 Seal and shake at 1600 rpm for 1 minute.

16 Centrifuge at 280 × g for 1 minute.

17 Place on thermocycler and run the PCR program to generate cDNA and amplify. The thermocycler conditions can be found below

	Temperature	Time	Cycles
	25 ºC	5 minutes	
	50 ºC	10 minutes	
	80 ºC	5 minutes	
	98 ºC	3 minutes	
	98 ºC	15 seconds	35 cycles
	63 ºC	5 minutes	(included in above 35)
	4 ºC	hold	

Hybrid "one-step" cDNA generation and amplification thermocycler conditions

17.1 **This is a safe stopping point, marking the end of day 1 of the protocol. Store all samples at 4 ºC**

Tagment Amplicons and Cleanup

18 Using the Qubit, quantify the last column of samples (including both controls) from both amplicon sets.

19 Prepare the tagmentation master mix:

	Reagent Name	Volume per Sample
	TB1	12 µl
	EBLTS	4 µl
	Nuclease Free Water	20 µl

20 Add 30 µl of the tagmentation master mix to 96 new PCR tubes.

20.1 Keep these new tubes on a cold block.

21 Combine 10 µl of the generation amplicons (pool 1 and 2) into the new tubes.

21.1 Open tubes strip-by-strip and only keep tubes you are immediately using open.

22 Mix for 1 minute at 1400 rpm on the plate mixer (set to 4 ºC) and spin down.

23 Load into the thermocycler and run the following program.

	Temperature	Time
	55 ºC	3 minutes
	10 ºC	hold

24 Once the program has finished, **immediately** remove tubes, spin down, and keep on a cold block.

25 Add 10 µl ST2 to each tube with a multichannel pipette and mix for 1 minute on the plate mixer.

26 Incubate at RT for 5 minutes and spin down.

27 Place on magnetic stand and wait until liquid is clear (a few minutes).

28 Remove and discard all supernatant from each well with a multichannel pipette.

29 Remove from the magnetic stand and add 100 µl TWB to each tube.

29.1 Mix for 1 minute at 1400 rpm on the plate mixer and spin down. Be careful not to introduce bubbles.

30 Repeat steps 27-29, but do not remove the second wash.

Amplify Tagmented Ampicons

31 Prepare the following amplification master mix:

	Reagent Name	Volume per Sample
	EPM	24
	Nuclease Free Water	24

32 Place the tubes with tagmented amplicons on the magnetic stand and wait until the liquid is clear (a few minutes).

33 Working is sets of 3 strips at a time, remove all TWB from each tube.

33.1 Remove any residual TWB from tubes with lower volume pipette.

34 Remove the tubes from the magnetic stand and add 40 µl of master mix to each tube.

35 Clean the foil of the index adapter plate with DNA-Away and 70% ethanol.

36 Add 10 µl index adapters to each tube using a multichannel pipette.

36.1 Make note of which index adapter set was used.

37 Mix for 1 minute at 1400 rpm on the plate mixer and spin down.

38 Load into the thermocycler and run the following program:

	Temperature	Time	Cycles
	72 ºC	3 minutes	
	98 ºC	3 minutes	
	98 ºC	20 seconds	7 cycles
	60 ºC	30 seconds	(included in above 7)
	72 ºC	1 minute	(included in above 7)
	72 ºC	3 minutes	
	10 ºC	hold	

Pool and Clean Up

39 Spin down tubes, place on magnetic stand, and wait until liquid is clear.

40 Transfer 5 µl of each library to new tubes.

	Strips Pooled	Volume per Sample
	1-3	15 µl
	4-5	10 µl
	6-12	5 µl

40.1 Change tips in between tubes; vortex and spin down when finished.

41 Transfer 55 µl (total volume - 5 µl) from each tube to a new 1.5 mL tube

42 Add 0.9 x total pooled volume µl to the tube and vortex.

43 Incubate at RT for 5 minutes

44 Spin down and place on magnetic stand and wait until liquid is clear.

45 Remove and discard supernatant; do not remove tube from magnetic rack.

46 Add 1000 µl of freshly prepared 80% ethanol and incubate for 30 seconds.

47 Repeat steps 45-46.

48 Remove the ethanol wash.

49 Add RSB and vortex to mix. Consult the following table for how much RSB to add:

	Strips Pooled	Volume per Sample
	1-2	30 µl
	3-4	40 µl

	Strips Pooled	Volume per Sample
	5-12	55 µl

50 Incubate at RT for 2 minutes.

51 Transfer total volume - 5 µl of the pooled libraries to a new 1.5 mL tube.

51.1 **This is the final pooled library tube.**

52 Quantify the library using the Qubit.

53 Analyze the fragment distribution using the bioanalyzer.

Protocol references

JCV ecology and evolution. forthcoming.
Bioinformatics pipeline: https://github.com/grubaughlab/DENV_pipeline