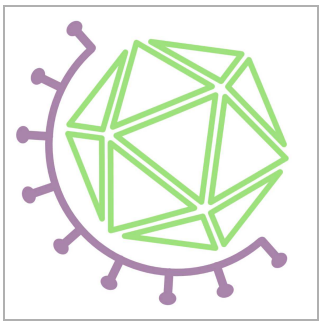

DEC 13, 2022

WORKS FOR ME 1

Hepatitis C Virus (HCV) subtype 1b sequencing protocol v.1

COMMENTS 0

DOI
dx.doi.org/10.17504/protocols.io.5qpvorkkzv4o/v1

Alí F Ruiz-Higareda[1], Kame Galan[1], Julio C Garza-Cabello[1], Ana G. Rivas-Estilla[1]

[1]Centro de Investigación e Innovación en Virología Médica, Departamento de Bioquímica y Medicina Molecular, Facultad de Medicina de la Universidad Autónoma de Nuevo León

Alí F Ruiz-Higareda
Centro de Investigación e Innovación en Virología Médica, De...

ABSTRACT

Amplicon sequencing protocol for Hepatitis C virus subtype 1b using Oxford Nanopore Technologies.

DOI

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PROTOCOL CITATION

Alí F Ruiz-Higareda, Kame Galan, Julio C Garza-Cabello, Ana G. Rivas-Estilla 2022. Hepatitis C Virus (HCV) subtype 1b sequencing protocol v.1. **protocols.io** https://dx.doi.org/10.17504/protocols.io.5qpvorkkzv4o/v1

CREATED

Nov 29, 2022

LAST MODIFIED

Dec 13, 2022

PROTOCOL INTEGER ID

73340

MATERIALS TEXT

Component	Supplier	Part Number
AMPure XP beads	Beckman	A63881
SuperScript IV Reverse Transcriptase	Invitrogen	18090010
RNaseOUT Recombinant Ribonuclease Inhibitor	Invitrogen	10777019

Component	Supplier	Part Number
Random hexamers	Invitrogen	N8080127
Q5 Hot Start High-Fidelity Polymerase or	NEB	M0493
Q5 Hot Start High-Fidelity 2X Master Mix	NEB	M0494
Nuclease-free water (100 mL)	NEB	B1500
NEBNext Ultra II End Repair/dA-tailing module	NEB	E7546
NEBNext Quick Ligation Module	NEB	E6056S
dNTP Solution Mix (10 mM ea.)	NEB	N0447
Blunt/TA Ligase Master Mix	NEB	M0367
Short Fragment Buffer Expansion Kit	ONT	EXP-SFB001
R9.4.1 flow cells	ONT	FLO-MIN106
Native Barcoding Expansion Kit 96	ONT	EXP-NBD196
Flow Cell Wash Kit (optional)	ONT	EXP-WSH003
Flow Cell Priming Kit	ONT	EXP-FLP002

Primers used:

PRIMER NAME
TAGTATGAGTGTCGTGCAGCCT 1L
GATCCGGAGTAACTGCGACA 4R
CACATCAACAGAACTGCCCTGA 6L
ACCACCAAGTTCTCTAAGGCGG 8R
CATCAAGGGCAGGCTGGTCCC 9.5L
AGGTCCTGGTCTACATTGGTGT 12R
TTCCAAGTGGCCCATCTACAC 15L
CAGCCGACATACATGCCATGAT 18R
TACATCGAACAGGGAATGCAGC 20L
GGAACCGTTTTTGACATGTCCG 22R
TCCATGCTCACCGACCCCTCC 26L
CTTTGATTTCCAGGCATTCACCA 28R
TCACGAACGACTGCTCCAAC 4L
TTCATCCATGTACAGCCGAACC 6R
GACGTGCTGCTCCTCAACAA 7L
TGAGCGGACCGAGTATGGCGA 9.5R
TTAACGGCGTGTGTTGGACTG 13L
TCAGGACGAGTACCTTGTACCC 14R
TGTGTGAGTGCTATGACGCG 18L
ATGTCCACAAGCACCTTCCCA 20R
TCATGTCAACGTGGGTACAAGG 23L
TCTTTCTCCGTGGAGGTGGT 25R
AAGCCAGCTCGCCTTATCGT 29L
AGATGCCTACCCCTACAGAAAGT 32R

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Pool	Primers
1	1L, 4R, 6L, 8R, 9.5L, 12R, 15L, 18R, 20L, 22R, 26L, 28R
2	7L, 9.5R, 13L, 14R, 18L, 20R
3	4L, 6R, 23L, 25R, 15L, 18R, 29L, 32R

Final concentration for each pool should be 10µM

cDNA synthesis

1 Prepare the RNA-primer Mix following these instructions:

2m

1.1 Mix the following components in a 0.2 mL tube:

Component	Volume
10 mM dNTP mix (10mM each)	1 µL
50 µM random hexamers	1 µL
Template RNA	11 µL

30s

1.2 Mix and briefly centrifugate the components.

6m

1.3 Heat the RNA-primer mix at 65 °C for 00:05:00 and then incubate On ice for at least 00:01:00

2 Prepare the RT reaction mix following the next steps:

2.1 Vortex and briefly centrifugate the 5X SSIV Buffer.

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2.2 Combine the following components in a tube:

Component	Volume
5X SSIV Buffer	4 µL
100 mM DTT	1 µL
RNaseOUT Recombinant RNase Inhibitor	1 µL
SuperScript IV Reverse Transcriptase (200 U/µL)	1 µL

2.3 Combine and the RNA-primer and RT reaction mixes in a 0.2 mL tube, mix by pipetting and pulse spin the tube to collect liquid at the bottom of the tube.

20m

3 Incubate the reaction as follows:
00:10:00 at 55 °C
00:10:00 at 80 °C

3h

Genome amplification

4 The primer pools used in this section are described in "MATERIALS" Section.
In the mastermix hood set up the multiplex PCR reactions as follows in 0.2mL 8 PCR tubes:

Component	Pool 1	Pool 2	Pool 3
Q5 Hot Start DNA Polymerase	0.25 µL	0.25 µL	0.25 µL
10 mM dNTPs	0.5 µL	0.5 µL	0.5 µL
Primer Pool 1, 3 or 2 (10µM)	1 µL	1 µL	1 µL
5X Q5 Reaction Buffer	5 µL	5 µL	5 µL
Nuclease-free water	15.75 µL	15.75 µL	15.75 µL

5 Add 2.5 µL cDNA to each tube and mix well by pipetting.

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6 Pulse centrifuge the tubes to collect the contents at the bottom of the tube.

3h

7 Set-up the following program on the thermal cycler:

Step	Temperature	Time	Cycles
Heat Activation	94 °C	00:03:00	1
Denaturation	94 °C	00:00:30	35
Annealing	64 °C	00:00:45	35
Extension	72 °C	00:02:00	35
Final Extension	72 °C	00:10:00	1
Hold	8 °C	∞	1

8 PCR products can be verified using 1% agarose gels.

*

1h 20m

Sequencing library preparation

30m

9 **DNA repair and end-prep.**

Incubate for 00:15:00 at 20 °C

1 µg of DNA* in 24 µL of nuclease free water
1.75 µL of NEB NextFFPE DNA Repair Buffer
1.75 µL of Ultra II End-prep reaction buffer
1 µL of NEB Next FFPE DNA Repair Mix
1 µL of NEB Next FFPE DNA Repair Mix
*DNA from previous step(PCR Products)
Inactivate the reaction for 00:15:00 at 65 °C.

30m

10 **Barcode ligation**

Incubate for 00:20:00 at 25 °C:
1.25 µL of Native Barcode
5 µL of Blunt/TA Ligase Master Mix
3.75 µL of end-prepped DNA

Inactivate the reaction for 00:10:00 at 65 °C.

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Note

11 Mix all the barcoded samples into a single 1.5 ml tube.

Purify products with AMPureXP beads:

11.1 Add resuspended AMPure XP beads to the pooled barcoded sample in a 1:1 relation, mix by flicking the tube.

11.2 Incubate on a Hula mixer for 00:05:00 at Room temperature 5m

11.3 Pellet on a magnetic rack until eluate is clear and colorless, with the cap open. Keep the tube on the magnet, and pipette off the supernatant.

Note

11.4 Wash the beads with 200 µL of SFB.

11.5 Pellet on a magnetic rack until eluate is clear and colorless, with the cap open. Keep the tube on the magnet, and pipette off the supernatant.

Note

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11.6 Resuspend the pellet with 30 µL of nuclease free water and incubate it for 00:02:00 at Room temperature 2m

11.7 Pellet on a magnetic rack until eluate is clear and colorless, for at least 00:01:00. 1m

11.8 Remove and retain 30 µL of the elute in a 1.5 mL tube.

12 Adapter Ligation 40m

Incubate for 00:20:00 at Room temperature:

30 µL of pooled barcoded sample from previous step.
5 µL of Adapter Mix (AMII).
10 µL NEB NextQuick Ligation Reaction Buffer (X5).
5 µL Quick T4 DNA Ligase.

Incubate reaction for 00:20:00 at Room temperature.

13 Purify product with AMPureXP beads:

13.1 Add 50 µL of resuspended AMPure XP beads to the reaction, mix by flicking the tube.

13.2 Incubate on a Hula mixer for 00:05:00 at Room temperature

13.3 Pellet on a magnetic rack until eluate is clear and colorless, with the cap open. Keep the tube on the magnet, and pipette off the supernatant.

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Note

13.4 Wash the beads with 250 µL of SFB.

13.5 Pellet on a magnetic rack until eluate is clear and colorless, with the cap open. Keep the tube on the magnet, and pipette off the supernatant.

Note

10m

13.6 Resuspend the pellet with 13 µL of elution buffer(ONT) and incubate it for 00:10:00 at Room temperature

13.7 Pellet on a magnetic rack until eluate is clear and colorless, for at least 00:01:00 .

13.8 Remove and retain 13 µL of the elute in a 1.5 mL tube.

Priming and loading the SpotON flow cell.

14

Note

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Add 30 µl of Flush Tether (FLT) directly to the tube of the Flush Buffer (FB), and pulse spin the tube to collect liquid at the bottom of the tube.

15 Open the MinION device lid and slide the flow cell under the clip.

16 Slide the priming port cover to open the priming port.

17 Check for a small air bubble under the cover. Draw back a small volume to remove any bubbles (a few µL):

17.1 Set a P1000 pipette to 200 µl.

17.2 Insert the tip into the priming port.

17.3 Turn the wheel 220-230 µL, to draw back 20-30 µL, or until you can see a small volume of buffer entering the pipette tip.

18 Load 800 µl of the priming mix into the flow cell via the priming port.

Note

19 Wait for 5 minutes.

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20 While waiting prepare the library as follows:

Component	Volume
DNA library	12 µL
Loading Beads (LB), mixed immediately before use	25.5 µL
Sequencing Buffer (SQB)	37.5 µL

21 Gently lift the SpotON sample port cover.

22 Load 200 µl of the priming mix into the flow cell via the priming port.

Note

23 Mix the prepared library gently by pipetting up and down.

24 Add 75 µL of sample to the flow cell via the SpotON port in a dropwise fashion.

Note

25 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION device lid.

MinION Software

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26 While starting the sequencing run using MinKNOW, the following parameters are to be selected:

26.1 **Kit:** SQK-LSK109.

Barcode kit: EXP-NBD196, turn on Live basecalling, ensure to turn on double-ended barcoding in the basecalling settings

Run length: Set the run length to a minimum of 12 hours (you can stop the run once sufficient data has been collected or you can select more time if deemed necessary).

Basecalling: On and select 'fast basecalling'. High accuracy can be selected, however it would require to basecall more time since it is more demanding task for the computer.

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