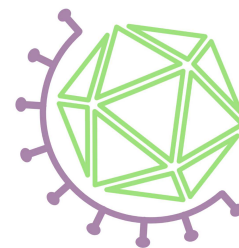


Dec 13, 2022

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

DOI

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



Ali F Ruiz-Higareda¹, Kame Galan¹, Julio C Garza-Cabello¹, Ana G. Rivas-Estilla¹

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



Ali F Ruiz-Higareda

Centro de Investigación e Innovación en Virología Médica, De...

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.5qpvorkkzv4o/v1>

Protocol Citation: Ali 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>

License: This is an open access protocol distributed under the terms of the **Creative Commons Attribution License**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working

Created: November 29, 2022

Last Modified: December 13, 2022

Protocol Integer ID: 73340

Keywords: hcv, sequencing protocol, using oxford nanopore technology, oxford nanopore technology

Abstract

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

Materials

Component	Supplier	Part Number
AMPure XP beads	Beckman	A63881
SuperScript IV Reverse Transcriptase	Invitrogen	18090010
RNaseOUT Recombinant Ribonuclease Inhibitor	Invitrogen	10777019
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
TAGTATGAGTGTCTGTCGAGCCT	1L
GATCCGGAGTAACTGCGACA	4R
CACATCAACAGAACTGCCCTGA	6L
ACCACCAAGTTCTCTAAGGCGG	8R
CATCAAGGGCAGGCTGGTCCC	9.5L
AGGTCCTGGTCTACATTGGTGT	12R
TTCCAAGTGGCCCATCTACAC	15L
CAGCCGACATACATGCCATGAT	18R

```

TACATCGAACAGGGAATGCAGC 20L
GGAACCGTTTTTGACATGTCCG 22R
TCCATGCTCACCGACCCCTCC 26L
CTTTGATTTCCAGGCATTACCA 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

```

	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

Troubleshooting



cDNA synthesis

1 Prepare the RNA-primer Mix following these instructions:

1.1 Mix the following components in a 0.2 mL tube:

2m

Component	Volume
10 mM dNTP mix (10mM each)	1 μ L
50 μ M random hexamers	1 μ L
Template RNA	11 μ L

1.2 Mix and briefly centrifugate the components.

30s

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

6m

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.

2.2 Combine the following components in a tube:

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



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.

3 Incubate the reaction as follows:

00:10:00 at 55 °C

00:10:00 at 80 °C

20m

Genome amplification

3h

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.

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

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

3h

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.

Sequencing library preparation

1h 20m

9 DNA repair and end-prep.

30m

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 .

10 Barcode ligation

30m

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 .

Note

Use one native barcode from the EXP-NBD196 per sample.



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

Not to touch the pellet while discarding the supernatant is very important.

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

Do not touch the pellet while discarding the supernatant.

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.

Note

Not to touch the pellet while discarding the supernatant is very important.

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**

Do not touch the pellet while discarding the supernatant.

13.6 Resuspend the pellet with  13 μL of elution buffer(ONT) and incubate it for

 00:10:00 at  Room temperature

10m

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

Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at  Room temperature before mixing the reagents by vortexing, and pulse spin the tubes to collect liquid at the bottom of the tube.

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

It is very important not to introduce any air bubble.

19 Wait for 5 minutes.

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

It is very important not to introduce any air bubble.

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

Ensure each drop flows into the port before adding the next.

- 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

- 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.