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Complete Hepatitis B Virus Sequencing using an ONT-Based Next-Generation Sequencing Protocol

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We are still developing and optimizing this protocol

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ABSTRACT

This protocol outlines the process of generating complete Hepatitis B virus (HBV) primers from DNA extracts for next-generation sequencing using Oxford Nanopore Technology (ONT). Specifically, we have designed pan-genotypic tiling primers to cover the entire HBV genome for sequencing. The hands-on time required for a batch of 48 samples is minimal, approximately 1 hour and 30 minutes. This protocol is straightforward and can be easily adapted in settings where the ONT protocol for SARS-CoV-2 has been implemented. We provide detailed instructions for PCR amplification using HBV-specific tiling primers, sample pooling, library construction using the Rapid Barcoding Kit (SQK-RBK110.96), quantification using Qubit, and subsequent sequencing on the GridION platform.

The Rapid barcoding protocol for up-to 96 samples "PCR tiling of SARS-CoV-2 virus with rapid barcoding (SQK-RBK110.96)" https://community.nanoporetech.com/protocols/pcr-tiling-of-sars-cov-2-virus-with-rapid-barcoding-sqk-rbk110/v/PCTR_9125_v110_revA_24Mar2021.

This protocol describes a modified version of the 1200bp amplicon "midnight" primer set for nCoV-2019 (SARS-CoV-2) amplification, utilizing the Nanopore Rapid kit for MinION. The modification was developed by Nikki Freed. https://dx.doi.org/10.17504/protocols.io.bwyppfvn and the original publication is found here : https://doi.org/10.1093/biomethods/bpaa014.

Primers were all designed using Primal Scheme: http://primal.zibraproject.org/, described here https://www.nature.com/articles/nprot.2017.066.

Primer sequences are here (and listed directly in the protocol) :

MATERIALS

QubitTM Assay TubesInvitrogen - Thermo Fisher Catalog #Q32856
Qubit dsDNA HS Assay kitThermo Fisher Scientific Catalog #Q32854
Q5 Hot Start High-Fidelity DNA Polymerase - 100 unitsNew England Biolabs Catalog #M0493S
DNA 1K / 12K / Hi Sensitivity Assay LabChipPerkin Elmer Catalog #760517
General PCR laboratory equipment and consumables Contributed by users (GridION, Qubit (Fluorometer), LabChip Fragment Analyzer)
Primers-specific for HBV according to Primal Scheme (Pool 1 -even numbers)
Artic Primers-specific for 2019-nCoV according to Primal Scheme (Pool 2 -odd numbers)
Rapid Barcoding Kit (RBK109)
Nuclease Free Water

Quantification of DNA using Qubit

1 Prepare the two standards calibrate the Qubit Fluorometer using Qubit dsDNA HS Assay kit Thermo Fisher Scientific (Qubit® dsDNA HS Reagent).

1.1 Label the tube lids. Do not label the side of the tube as this could interfere with the sample reading. **Use only thin-wall, clear, 0.5mL PCR tubes. Acceptable tubes include Qubit™ assay tubes (Cat. No. Q32856)**

1.2 For standards [STD]: Aliquot 190 µL of Qubit® dsDNA HS Reagent working solution to each 500 µL thin-walled polypropylene tubes .

For sample tubes: Aliquot 199 µL of Qubit® dsDNA HS Reagent working solution.

Note

The final volume in each tube must be 200 µL once sample/standard has been added.

1.3 Add 10 µL of STD 1 to the aliquoted 190 µL. 2m

Mix each tube vigorously by vortexing for 3–5 seconds without creating bubbles.

Incubate at room temperature for 00:02:00 prior to measuring the concentation using Qubit Fluorometer. **

Note

**** Do not delay (exceed) 00:02:00; the Qubit reagents are light sensitive.**

1.4 After calibrating the machine with STD 1 & 2 proceed to measuring DNA for samples. 2m

First, aliquot 199 µL of Qubit® dsDNA HS Reagent into a 500 µL thin-walled polypropylene tubes.

Add 1 µL of the specific sample to each well containing the pre-allocated 190 µL of sample.

Mix each tube vigorously by vortexing for 3–5 seconds without creating bubbles.

Incubate at room temperature for 00:02:00. Measure the concentation using Qubit Fluorometer.

Note

- If you are adding 1–2µL of sample, use a P-2 pipette for best results.

- Remember, do not delay (exceed) 00:02:00; the Qubit reagents are light sensitive.**

1.5 After calibrating the machine proceed to measuring DNA for samples. 6m

First, aliquot 199 µL of Qubit® dsDNA HS Reagent into a 500 µL thin-walled polypropylene tubes.

Add 1 µL of the specific sample to each well containing the pre-allocated 190 µL of sample.

Pulse vortex for 00:02:00 seconds to mix thoroughly without producing bubbles.

Incubate at room temperature for 00:02:00. Measure the concentation using Qubit Fluorometer.

Allow all tubes to incubate at room temperature for 2 minutes, then proceed to “Read standards and samples”.

** Do not delay (exceed) 00:02:00; the Qubit® dsDNA HS Reagent are light sensitive.**

Protocol

NAME

DNA quantification using the Qubit fluorometer

CREATED BY

Nikki Freed

PREVIEW

Primer design (Pan-genotypic/ universal tiling primers)

2 1. Download the HBV sequences of genotypes A-J were obtained from GenBank (n>8000) (https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/).

2. Design a consensus sequence based on 50% threshold per each genotype. Annotate the conserved and parsimony informative sites.

3. Use Primal scheme (https://primalscheme.com) to design the primers and the Amplicon-size was set at 1200.

4. Compare the variable regions and select the putatively universal primers.

5. To avoid any primer dropouts, add the other variable primers. These can be used to spike the master mix during PCR.

	A	B	C
	Primer Name	Sequence	Positions
	SC_1_LEFT	TTC CAC CAA GCT CTG CAA GATC	11 - 32
	SC_1_RIGHT	AGAGGAATATGATAAAACGCCGCA	384-407
	SC_2_LEFT	CATCATCATCAT CACCA CCTCC	325-346
	SC_2_RIGHT	AAAGCCCTACGAACCACTGAAC	692-713
	SC_3_LEFT	AAATACCTATGGGAGTGGGCCT	632-653
	SC_3_RIGHT	TTGTGTAAATGGAGCGGCAAAG	1 655-1 676
	SC_4_LEFT	AGAAAACTTCCTGTTAACAGACCTATTG	949-976
	SC_4_RIGHT	GGACGACAGAATTATCAGTCCCG	1 326-1 348
	SC_5_LEFT	TCCATACTGCGGAACTCCTAGC	1 265-1 286
	SC_5_RIGHT	TGTAAGACCTTGGGCAGGATTTG	1 632-1 654
	SC_6_LEFT	CTTCTCATCTGCCGGTCCGTGT	1 559-1580
	SC_6_RIGHT	AGA AGT CAG AAG GCA AAC GAGA	1 947-1 970
	SC_7_LEFT	GGCTTTGGGGCATGGACATT	1 890-1 909
	SC_7_RIGHT	ATCCACACTCCGAAAGAGACCA	2 256-2 277
	SC_8_LEFT	GACAACTATTGTGGTTTCATATTTCT	2 193-2 218
	SC_8_RIGHT	TTGTTGACACCTATTAATAATGTCCTCA	2 576-2 594
	SC_9_LEFT	TGGGCTTTATTCCTCTACTGTCCC	2 492-2 515
	SC_9_RIGHT	GGGAACAGAAAGATTCGTCCCC	2 889-2 910
	SC_10_LEFT	TTGCGGGTCACCATATTCTTGG	2 816-2 837
	SC_10_RIGHT	GGCCTGAGGATGACTGTCTCTT	3 189-3 210

Table1: Primers for Pool 1 & 2. Pool One are odd Numbers (SC_1, SC_3, ...) and Pool two are even Numbers (SC_2, SC_4,..)

Reconstitution of Primer Pools

3 To ensure proper primer dilution and pooling, follow these steps in a clean master-mix hood start by decontaminating of the working area (PCR hood/cabinet). 15m

1. Prior to use, decontaminate the master-mix hood using 10% bleach and 70% ethanol.

2. Sterilize the master-mix hood by exposing it to ultraviolet (UV) light for 00:15:00 .

3.1 Depending on the nature of the primers (lyophilised/or solution); if required, re-suspend lyophilised primers at a concentration of 100 µM each using nuclease-free water.

****Adhere to the primer reconstitution instructions provided by the supplier or manufacturer.****

3.2 **1.** To create a 100 µM stock of the primer pool for Pool 1, combine 5 µL of Pool 1 with 485 µL of nuclease-free water in a labeled 1.5 mL Eppendorf tube called "Pool 1 (100 µM)". This will yield a total volume of 490 µL, resulting in a 100 µM concentration of the primer pool stock.

2. Repeat the above procedure to create 100 M of primer pool for Pool 2.

Note

Primers should be diluted and pooled in the mastermix cabinet which should be cleaned with decontamination wipes and UV sterilised before for 00:15:00 and after use.

3.3 For each 100µM primer pool (1 & 2), dilute 1:10 in molecular grade water, to generate 10 µM primer stocks. Make several aliquotes for each primer pool in case of degradation or contamination.

Note

To achieve a final concentration of 0.015µM per primer in a 25 µL reaction, the following modifications can be made:

For pool 1, which consists of 5 primers:
- The required volume of the 10µM primer stock is calculated as follows:
(0.015µM) x (25µL) / (10µM) = 0.0375 µL
- Rounding up to 0.04µL, the volume of primer pool 1 to be added is adjusted to 1.1 µL.

For pool 2, which consists of 5 primers:
- Using the same calculation as above:
(0.015µM) x (25µL) / (10µM) = 0.0375 µL
- Rounding down to 0.03µL, the volume of primer pool 2 to be added is adjusted to 1.1 µL.

By rounding both volumes to 1.1 µL for both pools, they can be prepared in a similar fashion. If you are using a different primer pool scheme, adjust the volume accordingly.

Tiling Polymerase chain reaction (PCR)

4 To ensure contamination free master-mixes start by decontaminating all the working area in the clean room including workbench and the master mix hood. 15m

#*1. Decontaminate the master-mix hood using 10% bleach and 70% ethanol.

#*2. Sterilize the mastermix hood by exposing it to ultraviolet (UV) light for 00:15:00.

4.1 **Each sample requires two PCR reactions (1 for each primer pool, to be combined later).**

1. Arrange the PCR reactions for each sample in strip-tubes or plates according to the following instructions.

2. Mix the following components in a labeled 1.5ml eppendorf tube. Combine other reagents/components except the template as master-mix and divide into aliquots before adding DNA.

3. Mix gently by pipetting and briefly spin the tube to ensure the liquid collects at the

bottom.

	A	B	C
	Component	**PCR 1**	**PCR 2**
	Q5 2x Master Mix	12.5 µL	12.5 µL
	Primer pool 1 (10µM)	1.1 µL	-
	Primer pool 2 (10µM)	-	1.1 µL
	Nuclease-free water	8.9 µL	8.9 µL
	DNA template	2.5 µL	2.5 µL
	Total Volume	**25µL**	**25µL**

Table 2. PCR mastermix

4.2 In clean MasterMix cabinet:

1. Add 12.5 µL 5X Q5 Reaction Buffer to a labeled 1.5ml eppendorf tube.

2. Add 1.1 µL Primer Pool 1 or 2 (10µM) to the 1.5ml Eppendorf tube containing 12.55 µL 5X Q5 Reaction Buffer.

3. Add 8.9 µL of Nuclease free-water to the 1.5ml eppendorf mixture. The total volume should now be 25 µL

4. Aliquot the 22.5 µL of master-mix in labelled PCR strip tubes and transfer the master-mixes to the decontaminated**#*** extraction hood.

4.3 In the extraction and sample addition cabinet:

1. Add 2.5 µL of DNA template into the master-mixes, both pool 1 and 2. After adding; mix well by pipetting.

- Add 2.5 µL each DNA sample to a tube containing 22.5 µL Pool1.
- Add 2.5 µL each DNA sample to a tube containing 22.5 µL Pool 2.

2. Carefully mix the contents by pipetting in a gentle manner, and pulse centrifuge the tubes to collect the contents at the bottom of the tube.

Note

NB* : Maximum cation is required to avoid contamination.

Note: To prevent pre-PCR contamination the master-mix for each pool should be made up in the **master-mix** cabinet, which should be cleaned with decontamination wipes and UV sterilised for 00:15:00 before and after use and aliquoted into PCR strip-tubes/plate.

Store residual samples at -80 °C to maintain DNA integrity.

4.4

1. Incubate both PCR reactions in a thermocycler with the following settings:

	A	B	C
	Heat Activation	98°C	30 seconds
	Denaturation	98°C	15 seconds
	Annealing	65°C	5 minutes
	Repeat denaturation and annealing for a total of 25 cycles		
	Hold	4°C	∞

Tabe 3: Tiling PCR conditions

Expected result

Final concentrations of PCR products typically range from ~5 - 150ng/ul as measured by Qubit.

Pooling and PCR quantification

5

Label a 1.5 mL Eppendorf tube for each sample.

Transfer and merge all the components from the "Pool 1" and "Pool 2" PCR reactions of each biological sample into a single 1.5 mL Eppendorf tube, ensuring that all the contents are from sample. ** Avoid mixing samples**

Component	Volume
Pool 1 PCR reaction	10 µL
Pool 2 PCR reaction	10 µL
Total	20 µL

Note

It is crucial to exercise caution and follow proper laboratory practices when handling amplified PCR products. To ensure the integrity of your samples and prevent contamination, adhere to the following guidelines:

1. Designate a separate post-PCR workspace: Set up a dedicated area separate from where primers and master mixes are handled. Ideally, this space should be physically isolated or located in a different room to minimize the risk of contamination.
2. Equip the workspace appropriately: Outfit the post-PCR workspace with equipment solely dedicated to handling PCR products. This includes pipettes, centrifuges, and any other necessary instruments. Avoid using equipment that may have come into contact with primers or master mixes to prevent cross-contamination.
3. Take precautions during tube handling: When working with PCR tubes containing amplified products, open them only within the designated post-PCR workspace. Be mindful of not spilling or splashing the contents, as this could lead to contamination.
4. Use separate lab coats, gloves, and other personal protective equipment (PPE): Designate specific lab coats, gloves, and other PPE items for use in the post-PCR workspace. This prevents the transfer of contaminants between different areas of the laboratory.

By maintaining a separate workspace and adhering to these precautions, you can minimize the risk of contamination and ensure the reliability of your PCR results.

An alternative application for these amplicons is Oxford Nanopore Sequencing, specifically using Josh Quick's ligation-based protocol outlined in the CoV-2019 sequencing protocol v2. This protocol can be found at

CITATION

Josh Quick. nCoV-2019 sequencing protocol v2 (GunIt). protocols.io.
LINK
https://protocols.io/view/ncov-2019-sequencing-protocol-v2-bdp7i5rn

To quantify the DNA, it is recommended to use a Qubit or any other suitable method.

5.1 Nanodrop is not recommended for this purpose. However, if you do not have access to a Qubit or prefer to save time and costs, you may choose to omit this quantification step.

See go to step #1

Visualising the size of amplicons using the LabChip Fragme...

6 **"To add the lab chip preparation protocol"**

Rapid barcoding using the RBK110.96 kit for 96 samples

7 To accommodate multiple samples on the same flow cell, barcoding can be employed. Using the RBK110.96 kit, it is possible to run up to 96 samples simultaneously. Each sample's amplicons will be individually barcoded in the subsequent steps. It is strongly advised to refer to the current Oxford Nanopore Protocol for detailed instructions on these steps. As a tip, you can aliquot the Rapid barcodes into a PCR strip to facilitate multi-channeling. For comprehensive information, please consult the appropriate documentation.

CITATION

Nikki Freed, Olin Silander. SARS-CoV2 genome sequencing protocol (1200bp amplicon "midnight" primer set, using Nanopore Rapid kit). protocols.io.
LINK
https://protocols.io/view/sars-cov2-genome-sequencing-protocol-1200bp-amplic-bwyppfvn

8 Add 7.5 µL of each diluted PCR reaction from step 15 to the labeled PCR tube.
Set up the following reaction for each sample:

Component	Volume
DNA amplicons from step 15 (100ng total)	7.5 µL
Fragmentation Mix RB01-12 (one for each sample, included in kit)	2.5 µL
Total	10 µL

NB* Mix gently by flicking the tube, and spin down.

9 Incubate the reaction in a thermocycler with the following settings:

	A	B
	20°C	20 minutes
	65°C	10 seconds
	4°C	1 minute

TABLE 4:

Note: All PCR products now contain DNA barcodes that will be resolved during the sequencing process.

Rapid barocoding using the SLK RBK109

9.1 Pool all barcoded samples, noting the total volume.

9.2 Add an equal volume (1:1) of Ampure beads to the pooled sample tube and mix gently by either flicking or pipetting.

Protocol

NAME

Amplicon clean-up using SPRI beads for RAPID nanopore kit RBK004

CREATED BY

Nikki Freed

PREVIEW

9.3 Pulse centrifuge to collect all liquid at the bottom of the tube.

9.4 Incubate for 00:05:00 at Room temperature.

9.5 Place on magnetic rack and incubate for or until the beads have pelleted and the supernatant is completely clear.

9.6 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

9.7 Add 200 µL of freshly prepared 70% ethanol (at room temperature) to the pellet. Take caution to avoid touching the bead pellet. Remove the ethanol carefully and discard it.

9.8 Repeat **Step above.**

9.9 Centrifuge the tube briefly in pulses to ensure that all the liquid gathers at the bottom. Subsequently, using a P10 pipette, cautiously extract as much residual ethanol as possible from the tube.

9.10 Leave the tube lid open and incubate for 1 minute or until the pellet loses its shine.

NB*It is important to note that if the pellet dries completely, it may crack and become challenging to resuspend.

9.11 Resuspend pellet in 30 µLElution Buffer (EB), mix gently by either flicking or pipetting and incubate for 00:02:00.

9.12 Place on magnetic stand and transfer sample to a clean 1.5mL Eppendorf tube ensuring no beads are transferred into this tube.

9.13 Measure the concentration of samples using Qubit. (**See Section 1**).

MinION NGS sequencing

10 Prepare the flow-cells for sequencing. Prime the flow cell and add the priming fluid as recommend.

Start the sequencing run using MinKNOW latest version.

https://community.nanoporetech.com/protocols/pcr-tiling-of-sars-cov-2-virus-with-rapid-barcoding-sqk-rbk110/v/PCTR_9125_v110_revA_24Mar2021.