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# Multi-viral target Direct Detection and Nanopore Sequencing (DDNS)

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Alison Tedcastle[1], Erika Bujaki[1], Thomas Wilton[1], Javier Martin[1]

[1]MHRA

Alison Tedcastle
MHRA

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**We use this protocol and it's working**

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## Abstract

This protocol describes the target specific amplification for direct detection of multiple viral pathogens from wastewater extracted RNA. This enables sensitive identification of multiple different viral pathogens of interest for both surveillance and research purposes.
Targets include Enterovirus D68, Enterovirus 71, Rotavirus, Hepatitis A and E, Norovirus GI and GII and SARS-CoV-2.
Wastewater samples should be concentrated using an appropriate method and viral RNA extracted following the method; MagMAX Viral RNA Isolation Large volume extraction protocol using 1.2 mL of concentrate for each extraction (see Viral RNA extraction below). This method then utilises highly-sensitive nested PCR approaches where amplicons are subsequently pooled and purified ready for library preparation for Oxford Nanopore or Illumina sequencing. Barcoding the amplicons prior to nanopore sequencing allows multiplexing of many samples, reducing turn around time and cost per sample.

## Materials

- Nuclease-Free Water (Promega, Cat. No. P1193 or Merck, Cat. No. 3098)
- SuperScript™ III One-Step RT-PCR System with Platinum™ Taq High Fidelity DNA Polymerase (Invitrogen, Cat. No. 12574035)
- DreamTaq Hot Start PCR Master Mix (Thermo Scientific, Cat. No. K9011)
- Ethanol (96-100%)
- 0.2ml PCR tube strips
- 1.5 mL LoBind tubes (Eppendorf, Cat. No. 022431021)
- Gel electrophoresis agarose gel kit and buffers or Tapestation DNA kit
- Molecular weight marker (such as 1kb DNA Ladder, Promega, Cat. No. G571A)
- AMPure magnetic beads (Beckman Coulter, Cat. No. A63880)
- Qubit Broad Range dsDNA kit (Thermo Scientific, Cat. No. Q33853)

## Before start

### Viral RNA extraction

We recommend using the MagMAX Viral RNA Extraction method. Please follow the appropriate protocol depending on your starting material and manual or automated worklfow:

Purification of viral RNA from environmental samples using MagMAX Viral RNA Isolation Kit - https://www.protocols.io/view/large-volume-viral-rna-extraction-using-magmax-vir-81wgbzdr3gpk/v3

Using freshly extracted viral RNA is recommended whenever possible to facilitate successful amplification of long genome fragments.

Repeated freeze-thaw of samples or purified RNA can affect viral nucleic acid integrity, reducing amplification efficiency.

## Multi-viral RT-PCR

1 Wipe down lab bench surfaces and pipettes with 70% ethanol and an RNAse cleaning agent, such as RNAse Zap.

2 Prepare separate individual master mix preparations for each target using Superscript III One-Step RT-PCR System with Platinum Taq High Fidelity for each target (See Appendix 1). The reaction volumes are detailed in the tables below and should be prepared for the number of samples together with positive, negative controls plus 10% (for pipetting loss), where appropriate.

Note

Volume of nuclease free water can be reduced to allow larger volumes of RNA to be processed. **Each sample should be tested in duplicate.**

Note

PPMoV is used as an internal control for the monitoring of sample quality.

3

| | A | B |
|---|---|---|
| | **Target** | **Volume (µL)** |
| | **EV D68, EV71 or Hep E** | |
| | 2x Reaction Mix | 12.5 |
| | Anti-sense primer | 1 |
| | Enzyme Mix | 1 |
| | Water | 4.5 |
| | **Aliquot** | **19** |
| | | |
| | RNA | 5 |
| | **Total volume** | **24** |

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| | A | B |
|---|---|---|
| | **Target** | **Volume (µL)** |
| | **Rotavirus, Hep A, Norovirus or PMMoV** | |
| | 2x Reaction Mix | 12.5 |
| | Anti-sense primer | 1 |
| | Sense primer | 1 |
| | Enzyme Mix | 1 |
| | Water | 4.5 |
| | **Aliquot** | **20** |
| | | |
| | RNA | 5 |
| | **Total volume** | **25** |

5

| | A | B |
|---|---|---|
| | **Target** | **Volume (µL)** |
| | **SARS-CoV-2** | |
| | 2x Reaction Mix | 12.5 |
| | Anti-sense primer | 0.5 |
| | Sense primer | 0.5 |
| | Enzyme Mix | 1 |
| | Water | 5.5 |
| | **Aliquot** | **20** |
| | | |
| | RNA | 5 |
| | **Total volume** | **25** |

6 Vortex the master mix briefly and spin down to gather contents at the bottom of the tube. Aliquot the required volume of master mix as shown in the individual tables above (depending on reaction) into each pre-labelled PCR tube/strip.

7 Close the lids of the PCR tubes/strips and transfer the rack to the template addition room.

8 Add 5 µL of positive control or sample RNA to the appropriate reaction tubes. Add 5 µL of nuclease free water for the negative control.

9 Place PCR sample tubes in the Thermocycler and run the below conditions relevant to the target.

10

| | A | B |
|---|---|---|
| | **Profile: EV D68, EV71, Hep E** | |
| | 50°C for 30 min | |
| | Remove tubes, spin down sample and add 1µL of forward primer into each tube. | |
| | | |
| | 94°C for 2min | |
| | Cycles 42 | Step 1: 94°C for 15 secs |
| | | Step 2: 55°C for 30 secs |
| | | Step 3: 68°C for 1 min 30 secs |
| | 68°C for 5 min | |
| | 10°C hold | |

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| | A | B |
|---|---|---|
| | **Profile: Rotavirus, Hep A, Norovirus, PMMoV, SARS-CoV-2** | |
| | 50°C for 30 min | |

| | A | B |
|---|---|---|
| | 94°C for 2min | |
| | Cycles 42 | Step 1: 94°C for 15 secs |
| | | Step 2: 55°C for 30 secs |
| | | Step 3: 68°C for 1 min 30 secs |
| | 68°C for 5 min | |
| | 10°C hold | |

Note

At this point you can store the PCR products at -20°C. These can be stored indefinitely before continuing to step 12, however it is advised to continue the PCR protocol as soon as possible.

## Second round PCR

12 Wipe down lab bench surfaces and pipettes with 70% ethanol and an DNAse cleaning agent, such as DNAse Zap.

13 Prepare a separate master mix for each target using DreamTaq Hot Start PCR Master Mix (See Appendix 1). The reaction volumes are detailed in the tables below and should be prepared for the number of samples together with positive, negative controls plus 10% (for pipetting loss), where appropriate.

14

| | A | B |
|---|---|---|
| | **All targets** | **Volume (µL)** |
| | 2x Reaction Mix | 12.5 |
| | Forward primer | 1 |
| | Reverse primer | 1 |
| | Water | 8.5 |
| | First PCR product | 2 |
| | **Total volume** | **25** |

15 Vortex the master mix briefly and spin down to gather contents at the bottom of the tube. Aliquot 23 µL of the master mix into each PCR tube/strip.

16 Close the lids of the PCR tubes/strips and transfer the rack to the template addition room.

17 Add 2 µL of the first round PCR product to the appropriate reaction tubes. Add 2 µL of nuclease free water for the negative control.

18 Place PCR sample tubes in the Thermocycler and run the below conditions.

19 Run the following cycling conditions.

| | A | B |
|---|---|---|
| | **Profile: All targets** | |
| | 94°C for 2min | |
| | Cycles 40 | Step 1: 94°C for 15 secs |
| | | Step 2: 50°C for 30 secs |
| | | Step 3: 68°C for 1 min |
| | 68°C for 5 min | |
| | 10°C hold | |

20 Check all samples including positive and negative controls from the second round PCR on a 1% agarose gel. The expected band size for each target is in Appendix 1. Proceed to the next step with the confirmed positive reactions for purification and quantification of the DNA.

## Amplicon Pooling

21 Pool 10µL from each duplicate amplicon or 20µL from the only positive reaction (depending on successful amplification as noted in the gel) for each virus sample.

Note

We present a simplified approach here directed by electrophoresis results with purifying and
quantifying the pooled reactions. Alternatively, each amplicon can be purified and quantified individually for accurate equimolar pooling.

## Preparing input DNA

22 Allow AMPure beads to warm to room temperature then add an equal volume of beads to the individually pooled PCR samples and pipette gently to mix.
For example, for a 20µL pooled sample add 20µL of resuspended beads

23 Incubate at room temperature for 5 minutes.

24 Spin down and place on a magnetic rack until clear and colourless, then pipette off the supernatant.

25 Whilst on the magnet, wash the beads with 100µL of 80% Ethanol, remove and discard.

26 Repeat Step 25.

27 Spin down the tubes and place back on the magnet to remove any residual Ethanol. Allow to air dry for about 30 seconds.

28 Remove the tubes from the magnetic rack and resuspend the pellet in 20µL nuclease free water and incubate at room temperature for 2 minutes.

29 Pellet the beads on the magnet and remove 20µL (or as much as possible) of each sample into a clean tube.

30 Quantify the purified DNA with a Qubit Broad Range dsDNA kit.

31 In brief, create a master mix of 200µL (199µL buffer, 1µL Qubit reagent) for each sample + 2 standards + 10 %.

32 For the two standards, add 190µL master mix to 10µL of standard, and for samples add 198µL master mix to 2µL sample.

33 Vortex each sample or standard once they are added to the master mix and incubate for 2 minutes before
quantifying.

> Note
>
> Important - be sure to select the broad range kit on the Qubit

34 Record the concentration of DNA for each sample.

35 Transfer 200 fmol of DNA per sample into a clean 96-well plate and adjust volume to 12.5µL with nuclease free water. Mix gently by pipetting.
For example, 200 fmol of a 900bp fragment = ~110ng*

> Note
>
> Each amplicon is different in size; therefore, each will require different amounts of DNA to equal 200 fmol.

36 Once aliquoted, spin down briefly.

37 Continue to step 5 in the native barcoding protocol for direct detection nanopore sequencing. https://dx.doi.org/10.17504/protocols.io.e6nvwjko9lmk/v1

## Appendix 1: Primers (All primers should be at a working concentration of 10µM)

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| | A | B | C | D | E |
|---|---|---|---|---|---|
| | **Target** | **Primer name** | | **Sequence** | **Reference** |
| | EVD-68 (~900bp) | EVD-68 F0(1) | | YGCTAARTCAATYAATGCYAATGTYGG | |
| | | EVD-68 R0(1) | | CTTRCATCTTGCTATYTGRTGTYTTCC | |
| | | EVD-68 F1(2) | | CATGRRGCAGAGGCAGCYTACC | |
| | | EVD-68 R1(2) | | CAAARACYCCTCCGAAGCCTG | |
| | EV71 (~900bp) | EV71 F0(1) | | TCHGTYACCCTTGTRATACCRTGG | |
| | | EV71 R0(1) | | GARTTRCARTARTACACYCCTGTYTG | |
| | | EV71 F1(2) | | GGRGAYAGRGTGGCMGATG | |
| | | EV71 R1(2) | | GTCYTCCCADACDAGRTTYGC | |
| | Rotavirus (~900bp) | Beg9(1) | | GGCTTTAAAAGAGAGAATTTCCGTCTGG | 1, 2 |
| | | VP7R(1,2) | | AACTTGCCACCATTTTTTCC | |
| | | VP7F(2) | | ATGTATGGTATTGAATATACCAC | |
| | Hepatitis A (~550bp) | 2820F(1) | | YACAAGRAGAACAGGRAAYATTCAG | |
| | | 3788R(1) | | BACAGARTGATGRAAHCCAAACATC | |
| | | 2904F(2) | | GGAGAYAARACRGATTCHACWTTTGG | |
| | | 3469R(2) | | CATCYTTCATTTCYGTCCATTTYTCATC | |
| | Hepatitis E (450bp) | HE040_R(1) | | CCCTTRTCCTGCTGAGCRTTCTC | 3 |
| | | HE044_F(1) | | CAAGGHTGGCGYTCKGTTGAGAC | |

| | A | B | C | D | E |
|---|---|---|---|---|---|
| | | HE110.2_F(2) | HE110-2.1* | GYTCKGTTGAGACCTCYGGGGT | |
| | | | HE110-2.2* | GYTCKGTTGAGACCACGGGYGT | |
| | | | HE110-2.3* | GYTCKGTTGAGACCTCTGGTGT | |
| | | HE041_R(2) | | TTMACWGTCRGCTCGCCATTGGC | |
| | Norovirus GI (~300bp) | COG1F(1) | | CGYTGGATGCGNTTYCATGA | 4, 5 |
| | | G1-SKR(1,2) | | CCAACCCARCCATTRTACA | |
| | | G1SKF(2) | | CTGCCCGAATTYGTAAATGA | |
| | Norovirus GII (~300bp) | COG2F(1) | | CARGARBCNATGTTYAGRTGGATGAG | 4, 5 |
| | | G2-SKR(1,2) | | CCRCCNGCATRHCCRTTRTACAT | |
| | | G2SKF(2) | | CNTGGGAGGGCGATCGCAA | |
| | SARS-CoV-2 (~600bp) | Covid-501minus2F(1) | | GATCTCTGCTTTACTAATGTCTATGCAG | |
| | | Covid-501R0alt2(1) | | CCTGGTGTTATAACACTGACACCA | |
| | | Covid-501minus1FB(2) | | GAGGTGATGAAGTCAGACAAATYGC | |
| | | Covid-501R4alt(2) | | TCAAGAATSTCAAGTGTCTGTGGATC | |
| | PMMoV (~500bp) | CP/sF(1) | | ATGGCATACACAGTTACCAGT | 6 |
| | | CP/aR(1) | | TTAAGGAGTTGTAGCCCACGTA | |

(1) Primers used for Primary RT-PCR
(2) Primers used for Second round PCR
* Prepare primer mixes to a working concentration of 10µM:
1. HE110-2.1, HE110-2.2 and HE110-2.3

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