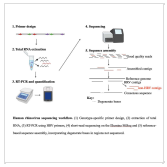

May 08, 2021

Whole-genome sequencing of two human rhinovirus A types - A15 and A101

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1 *Works for me* dx.doi.org/10.17504/protocols.io.bukxnuxn

HRV WGS

Martha Luka

ABSTRACT

Genome sequencing of pathogenic viruses has become an established tool for studying transmission, evolution and drug/vaccine design. Viral genome sequencing can either take up a targeted/enrichment approach or an agnostic/metagenomics approach. A standardised enrichment protocol for the whole-genome sequencing (WGS) of human rhinovirus (HRV) remains a challenge due to high viral genetic diversity. We present a type-specific WGS approach for two types - A15 and A101 using the Illumina Miseq.

Our protocol comprises of:-

Primer design

Polymerase Chain Reaction

Library preparation/ sequencing

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PROTOCOL CITATION

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KEYWORDS

null, human rhinovirus, whole-genome sequencing, MiSeq

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GUIDELINES

Quality control:

We used nuclease-free water (Qiagen, Germany) as a negative control in every batch to monitor potential cross-contamination. The negative controls were processed as samples (i.e. underwent extraction, reverse transcription, PCR and library preparation) and monitored for possible cross-contamination at different steps: gel electrophoresis, dsDNA quantification and analysis of sequence reads. We did not use a positive control as all samples were determined positive using real-time reverse-transcriptase PCR and confirmed by sequencing the VP4/2 region using the dideoxy technique. However, if available, positive sample controls with established reference sequences and comparable Ct values can check that the protocol is generating the intended results.

Below is a **Troubleshooting table** highlighting common challenges, their probable causes and proposed solutions

A	B	C
Problem	**Probable cause**	**Proposed solution(s)**
Geneious failed to generate a full scheme (primer pairs across entire genome)	Lack of suitable primers in local sequence	Manually scan the region for suitable primer(s)
Poor amplification/ faint bands in gel image	Sub-optimal PCR conditions	Double-check PCR conditions (volumes and thermocycling conditions)
Degraded RNA	Avoid freeze-thawing samples Target shorter amplicons	
No amplification/ no bands in gel image	Failed PCR	Modify PCR conditions e.g. annealing temperature
Unsuitable primers	Repeat primer design	
Amplification in negative control	Cross-contamination	Repeat experiment taking necessary precautions (e.g. disinfect surfaces, change pipet tips between all liquid transfers, good pipetting practice)
Non-specific bands in gel image	Non-specific amplification of host DNA	- Treat sample with DNAse - Centrifuge sample and use supernatant for extraction. - Use HRV reverse primers (in place of random hexamers) for reverse transcription
Insufficient reads	Error(s) in library preparation e.g. poor sample normalization	Troubleshoot library preparation (documented by manufacturer)

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MATERIALS TEXT

A
Clinical samples (nasopharyngeal swabs)
Qiagen Nuclease-Free Water
Eppendorf tubes
Type-specific primers
Q5® High-Fidelity 2X Master Mix
Casting block and plate
Agarose
Weighing balance & Weighing boat
1.5 kb ladder
QIAamp Viral RNA mini kit
SS III First-strand synthesis system
PCR plates
Primer pools 1-4
PCR plates
Electrophoresis tank
Combs, parafilm
0.5 % TBE
Pre- and post-PCR pipettes

DISCLAIMER:

BEFORE STARTING

Recommendations:
- To minimize the risk of cross-contamination, we recommend (i) single-tasking, (ii) frequent decontamination of surfaces with 1% (vol/vol) sodium hypochlorite solution or by UV exposure, (iii) physical separation of pre-and post-PCR areas and equipment (e.g. pipettes) and (iv) good pipetting practice.
- If processing several samples, create a master mix of the reagents to minimize inter-well variations due to pipetting errors

Primer design 4h

1 1. To retrieve publicly available (near) whole genomes, search GenBank using the search term: "Rhinovirus" AND {HRV type} AND 6000:8000 [SLEN]. 10m

1.1 Filter out any unsuitable genomes e.g. non-human. 10m

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1.2 Align the retrieved genomes. 10m

2 Design suitable primers using a suitable primer design tool. We used Geneious Prime 2019.2.1 (https://www.geneious.com), ensuring: 3h

a) A moderate GC content (40-60%)
b) Primer length of 18-27 bp, an annealing temperature between 55 °C and 65°C
c) Less than degenerate bases to minimize non-specific annealing.
d) No secondary structures (hairpins, self-dimers and primer dimers) or if present, kept below standard room temperature (18°C)
e) Primer pairs selected did not have a melting temperature (T_m) difference of more than 5 °C
f) Where possible, a GC clamp to improve PCR primer specificity.

RNA extraction 3h

3 Extract viral RNA from 140 µl of sample using the QIAamp viral RNA mini kit according to the manufacturer's recommendations, eluting in 60 µl of EB buffer 3h

Reverse transcription 2h 30m

4 Combine the following in a 1.5 ml tube: 20m

A	B
Component	**Volume**
RNA	5 µl
DEPC-treated water	3 µl
random primer (10 µM)	1 µl
10 mM dNTP mix	1 µl
Total Volume	10 µl

Mix thoroughly by pipetting up and down.

Note: One can also use the reverse HRV primers in place of random hexamers to generate consistently longer fragments.... ***see Troubleshooting table in Guidelines***

5 Place the sample in a thermal cycler and incubate the sample at 65°C for 5 minutes. 5m

Veriti 96-Well Thermal Cycler
Applied Biosystems **4375786**

6 Immediately transfer the tube to ice for at least 1 minute. 2m

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7 Assemble the first strand synthesis reaction on ice by adding the following components to the fragmented and primed RNA from step above 20m

A	B
Component	**Volume**
Fragmented and primed RNA (Step 4)	10 µl
10X RT buffer	2 µl
25 mM MgCl2	4 µl
0.1 M DTT	2 µl
RNaseOUT™ (40 U/µL)	1 µl
SuperScript® III RT (200 U/µL)	1 µl
Total volume	20 µl

Mix thoroughly by pipetting up and down several times.

8 Incubate the sample in a preheated thermal cycler with the heated lid set at ≥ 80°C as follows: 1h 5m

Step 1: 10 minutes at 25°C
Step 2: 50 minutes at 50°C
Step 3: 5 minutes at 85°C

Veriti 96-Well Thermal Cycler
Applied Biosystems **4375786**

9 (Optional) Briefly spin the plate. Add 1 µl of RNase H to each reaction. Incubate for 20 minutes at 37°C. 20m

Safe stop: Proceed directly to PCR or store at 4°C overnight

Preparing primer pools 1h 30m

10 30m

Note: To create the stock primer solution (100 µM), add the volume specified (of nuclease-free water) in the oligonucleotide QC report to the lyophilized primers.

Create a working concentration of 10 µM of each primer from the stock solution (100 µM) using nuclease free water. Alternatively, pool primers from the 100 µM solutions and dilute to 10 µM working solutions.

1h

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11

Note: Subsequent (overlapping) primer pairs should not be pooled into one reaction to avoid preferentially amplifying the shorter overlapping region.
Non-overlapping primer pairs with same annealing conditions can be pooled, with two primer pairs per reaction.
Pooling more primer pairs into one reaction reduced PCR success.

Label four 1.5 ml Eppendorf tubes (pool 1- pool 4).
Add equal volumes of 10 µM paired forward and reverse primers to either of the pools, e.g.

Primer pool 1 contains HRV-A101_amp1F, HRV-A101_amp1R, HRV-A101_amp3F and HRV-A101_amp3R.
Primer pool 2 contains HRV-A101_amp2F, HRV-A101_amp2R, HRV-A101_amp4F and HRV-A101_amp4R

Duplex PCR 4h

12 30m

Assemble the reaction on ice by adding the following to a reaction well

A	B	C
Component	**20 µl Reaction**	**Final Concentration**
Q5 High-Fidelity 2X Master Mix	10 µl	1X
Primer pool 10 µM (1-4)	2 µl	0.25 µM per primer
Template DNA	Variable	< 1,000 ng
Nuclease free water	Up to 20 µl	

Veriti 96-Well Thermal Cycler
Applied Biosystems **4375786**

Note: We recommend a primer concentration of below 0.5 µM per primer, though this can vary 0.1 – 1 µM per primer.

13 5m

Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Overlay the sample with mineral oil if using a PCR machine without a heated lid.

14 3h

Transfer PCR tubes to a PCR machine and begin thermocycling.

Note: The most suitable annealing temperature for individual primer pairs was sought by setting up a gradient PCR of annealing temperatures ±3°C from the estimated enzyme-specific annealing temperature.

Thermocycling conditions:

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A	B	C
STEP	**TEMP**	**TIME**
Initial Denaturation	98°C	30 seconds
35 Cycles	98°C	10 seconds
	Primer annealing temp.	30 seconds
	72°C	2 minutes
Final Extension	72°C	2 minutes
Hold	4°C	

Safe stop: You can store the PCR product at 4°C for up to one week or at -20°C for two months.

Gel electrophoresis

15 Preparing the gel

15.1 Using agarose powder and 0.5 % Tris Borate EDTA, prepare 1% Agarose Gel: It can effectively resolve small and large fragments.

15.2 Prepare the casting block; put in the comb(s) depending on the number of samples to be run.

15.3 Pour the gel into the casting plate and wait for 10-15 minutes for the gel to set and harden. When it hardens, remove the combs and put the gel into the electrophoresis tank containing 0.5 % TBE buffer. Ensure the gel is submerged in the buffer.

16 Load samples 15m

16.1 On a parafilm dispense 2µl of loading dye depending on the number of samples plus an extra for the ladder.

16.2 Pipette 4 µl of the ladder mix with the dye and load into the first well. Pipette 6 µl of each sample mix with the loading dye and load onto the well, do the same for all PCR products

17 Switch on the power pack set 90 Volts, 400 mA and 50- 60 minutes start the run. 1h

Note: Check that the terminals of the electrophoresis tank are connected correctly to avoid the products out into the buffer due to the wrong connections.

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18 Remove the gel after time lapses put in the gel doc machine; adjust the focus of the gel until a sharp focus is achieved. Capture the image 10m

Gel Doc XR+ Gel Documentation System
Gel Documentation System
Bio-rad Laboratories **1708195**

Quality Control
Check that the ladder has separated. If the ladder did not separate then the results are invalid.

Amplicon clean-up 2h

19 Purify PCR product using 1.0X Ampure beads according to manufacturer's instructions 2h

DNA quantification and pooling per sample 1h

20 Run Qubit according to manufacturer's recommendations. 1h

Qubit 4
Fluorometer
Invitrogen **Q33238**

21 Pool the amplicons per sample (samples with a dsDNA quantity of approximately 0.2ng/µl). 2h

Safe stop: You can store the PCR product at 4°C for up to one week or at -20°C for a couple of months.

Library preparation and sequencing 3d

22 The PCR products were tagmented, cleaned up and amplified. The libraries were then cleaned up, normalized and pooled as per the Nextera DNA Library Prep guidelines.

23 Sequencing was performed using the the Miseq Reagent kit v3 600 cycles with an output of 2 x 200 bp paired-end reads.

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MiSeq
Sequencer

illumina SY-410-1003

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