A degenerate primer reverse transcriptionpolymerase chain reaction protocol to determine the diversity of picorna-like viruses

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Abstract

The diversity of ribonucleic acid (RNA) viruses in the ocean and the ongoing isolation and characterization of RNA viruses that infect important primary producers suggests that RNA viruses are active members of the marine microbial assemblage. At this point, little is known about the composition, dynamics, and ecology of the RNA virioplankton. In this protocol, we describe a method to assess RNA virus diversity from seawater.

Citation: Alexander I. Culley, Curtis A. Suttle, and Grieg F. Steward A degenerate primer reverse transcriptionpolymerase chain reaction protocol to determine the diversity of picorna-like viruses. **protocols.io** dx.doi.org/10.17504/protocols.io.dse6bd

Published: 08 Dec 2015

Guidelines

This protocol is designed to detect picorna-like viruses from marine samples. It is based on a strategy first reported by Culley et al. (2003) and refined by Culley and Steward (2007). All picorna-like viruses have single-stranded positive-sense RNA genomes and are classified in the order Picornavirales (Le Gall et al. 2008). Picorna-like viruses are responsible for several significant human and animal diseases and infect a diversity of marine protists including a diatom (Nagasaki 2008; Lang et al. 2009). The procedure outlined below uses direct filtration of relatively small volumes of water and reverse transcriptionpolymerase chain reaction (RT-PCR) amplification of an RNAdependent RNA polymerase (RdRP) gene fragment using degenerate primer sets.

With this method, viral polymerase sequences were amplified in several distinct aquatic environments, including an estuarine urban canal (Ala Wai canal, Waikiki, HI, USA), a tropical bay (Kaneohe Bay, HI, USA), and a temperate bay (Monterey Bay, CA, USA). Amplification occurred in samples from the same site in different seasons and at different depths showing that RNA viruses are widespread and consistently present. Sequencing of the amplified gene fragments revealed novel sequences that are highly divergent from any known isolates.

Equipment—Peristaltic pump and pump heads, thermocycler, heating block, incubator, gel electrophoresis unit, electroporator, gel documentation system

Supplies—Sterile, 1.7 mL nucleic acid-free microcentrifuge tubes; 0.2 mL sterile, nucleic acid-free PCR tubes; 0.02 µm aluminum oxide filters (Anotop, Whatman); 10 mL sterile syringes; peristaltic pump tubing; sterile razor blades; MinElute Gel Extraction kit (Qiagen); MinElute PCR Purification kit (Qiagen); Masterpure Complete DNA and RNA Purification kit (Epicenter Biotechnologies); PCRTerminator End Repair kit (Lucigen); CloneSmart HCKan Blunt Cloning kit with Ecloni Supreme cells (Lucigen); Turbo DNA-free Kit (Applied Biosystems)

Solutions, reagents, and media—0.02-filtered, sterile, nucleic acid-free TE buffer (10 mM Tris, 1 mM EDTA; pH 8); 0.1 M dithiothreitol (DTT); Superscript III Reverse Transcriptase and buffers (Invitrogen); RNase Out (Invitrogen); RNase H (Invitrogen); Platinum Taq DNA polymerase and buffers (Invitrogen)

Primers in concentrations discussed as follows: 0.5 × TBE (45 mM Tris-borate, 1 mM EDTA; pH 8.0) electrophoresis buffer; nucleic acid–free, sterile water; 10 mM dNTP mix.

Table 1: RT and PCR primer details

Name	Sequence (5'-3')	°C annealing	~Product (bp)
Mpl.sc1F	TIGCIGGWGAYTWYARM	50	500
Mpl.sc1R	YTCCTTWTCRGSCATKGTA		
Mpl.sc2F	ITWGCIGGIGATTWCA	43	500
Mpl.sc2R	CKYTTCARRAAWTCAGCATC		
Mpl.sc3F	TIATIGMKGGIGAYTA	49	500
Mpl.sc3R	TTMARGAAIKMAGCATCTT		
Mpl.cdhF	GMIGGTGAYTAYAGCGCTTWYGAY	44	500
Mpl.cdhR	ATACCCAATGCCTYTTIARRAA		
RdRp1	GGRGAYTACASCIRWTTTGAT	50	450
RdRp2	MACCCAACKMCKCTTSARRAA		
SL1	CAGTCCAGTTACGCTGGAGTC	50	NA
SR2	GGTCAGGTATGATTTAAATGGTCAGT		
FR26RV-N	GCCGGAGCTCTGCAGATATCNNNNNN	NA	NA
FR40RV-T	GCCGGAGCTCTGCAGATATC(T)20	NA	NA
FR20RV	GCCGGAGCTCTGCAGATATC	65	NA

NA, not applicable

Protocol

Collection

Step 1.

Whole seawater (if intracellular viruses are to be included in the analysis) or 0.22-filtered seawater (if only the free virus community is targeted) is gently filtered (7 mmHg) through a 0.02 µm aluminum oxide filter (Anotop, Whatman).

Collection

Step 2.

Filtration should continue until the rate slows dramatically or drops to zero.

NOTES

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Note that it is important to measure the total volume of sample that passes through the filter.

Collection

Step 3.

Use pumped air to remove as much residual sample as possible.

Collection

Step 4.

Once dry, seal the filter inlets and outlets with parafilm.

Collection

Step 5.

Label the filter.

Collection

Step 6.

Flash-freeze the filter in liquid nitrogen.

Collection

Step 7.

Store it at –80°C until extraction.

Extraction

Step 8.

Total nucleic acids are extracted from the aluminum oxide filters using a Masterpure complete DNA and RNA Purification kit (Epicenter, Biotechnologies).

NOTES

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With a protocol slightly modified from the manufacturer's instructions. The detailed protocol of this method is presented elsewhere in this book (Steward and Culley 2010, this volume).

DNase Treatment

Step 9.

Remove contaminating DNA from RNA preparations with the Turbo DNA-free kit (Applied Biosystems) as described in the protocol provided with the kit.

NOTES

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To reduce the likelihood of mispriming and increase the sensitivity of the subsequent cDNA synthesis and PCR reactions.

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Although the manufacturers suggest that treated RNA template should not exceed 40% of the total cDNA synthesis reaction, using the reaction discussed below, we have used 55% template without inhibition.

cDNA synthesis

Step 10.

Each cDNA reaction contains 11 µL of extracted Turbo DNA-free–treated RNA template, 0.2 mM of each dNTP, and 100 ng of N6 primer in a total volume of 13 µL.

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We have synthesized complementary DNA (cDNA) using reverse transcriptase (Superscript III, Invitrogen) in reactions primed with random hexamers (N6) or the specific primers RdRp2, Mpl.sc1R, Mpl.sc2R, Mpl.sc3R, or Mpl.cdhR (details for these primers are listed in Table 1), and have found no detectable difference in sensitivity between the two approaches. Priming with random hexamers is preferable because the resultant cDNA can be used in a PCR reaction with any of the five primer sets listed in Table 1, eliminating the necessity for a different cDNA reaction for each primer set.

cDNA synthesis

Step 11.
Denaturation and annealing of the primers to the RNA template occurs by heating the sample to 65°C for 5 min and then cooling it on ice.

DURATION
00:05:00

cDNA synthesis

Step 12.
While still on ice, DTT (0.5 mM final conc.) is added to the reaction as an enzyme stabilization reagent with 40 U RNase OUT (Invitrogen) to protect the sample from RNAse activity.

cDNA synthesis

Step 13.
The complementary DNA strand is synthesized with 200 U Superscript III (Invitrogen) Reverse Transcriptase.

cDNA synthesis

Step 14.
The final reaction volume should be 20 µL.

cDNA synthesis

Step 15.
The reaction is incubated initially at 25°C for 5 min so that the relatively unstable hexamer primers remain annealed to the template while cDNA synthesis commences.

DURATION
00:05:00

cDNA synthesis

Step 16.
The temperature is then increased to 50°C, the temperature at which Superscript III's processivity is highest, for 60 min.

DURATION
01:00:00

cDNA synthesis

Step 17.
The activity of the enzyme is terminated by incubating the reaction at 85°C for 5 min.

DURATION
00:05:00

cDNA synthesis

Step 18.
After cDNA synthesis, add 1 µL (2 U) of RNase H (Invitrogen) to the reaction and incubate at 37°C for 20 min to digest the RNA template from the cDNA:RNA molecule.

DURATION
00:20:00

PCR

Step 19.
PCR can be performed with the RdRp, Mpl.sc1, Mpl.sc2, Mplsc3, and Mpl.cdh primer sets listed in Table 1 and the cDNA synthesized in the previous step.

PCR

Step 20.
In a 0.2 mL nuclease-free PCR tube, add reaction components to achieve final concentrations of 1× Platinum Taq buffer, 3 mM $MgCl_2$, 0.2 mM of each dNTP, 1 µM of each primer, and 1 unit of Platinum

Taq DNA polymerase (Invitrogen).

PCR

Step 21.

Incubate the reactions with the following thermal cycling conditions: 94°C for 75 s (this is necessary to activate the enzyme and ensures complete initial denaturation of template), followed by 35 cycles of denaturation at 94°C for 45 s, annealing at a primer-specific temperature (Table 1) for 45 s, and extension at 72°C for 45 s.

NOTES

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Note that the target product size is approximately 500 bp.

PCR

Step 22.

Complete the cycle with a single final extension step of 9 min 15 s at 72°C.

DURATION

00:09:15

PCR

Step 23.

Before gel separation, we purify and concentrate the PCR reactions with a MinElute PCR cleanup column (Qiagen) as described by the manufacturer.

NOTES

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A smaller volume of product allows one to load the product on a thinner gel, ultimately resulting in a more efficient recovery of target DNA from the excised band.

PCR

Step 24.

Purified PCR products are loaded onto a 1% agarose gel containing 1× SYBR safe stain (Invitrogen) and 0.5× TBE buffer.

PCR

Step 25.

Bands of DNA of the appropriate size (approximately 500 bp) are excised and purified with a MinElute Gel Extraction kit (Qiagen) according to the manufacturer's instructions.

PCR

Step 26.

We recommend eluting DNA from the column with three washes of 10 µL nuclease-free water in preparation for the end repair reaction.

Cloning and sequencing

Step 27.

In preparation for ligation, PCR products are polished and phosphorylated with the PCRTerminator End Repair kit (Lucigen) as described by the manufacturer.

NOTES

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Several commercial kits are available for the efficient cloning of PCR products. We use the CloneSmart Blunt Cloning kit from Lucigen. Some advantages of this cloning kit are the high efficiency of recombinants that eliminate the need for screening (e.g., XGAL/IPTG) and the prevention of transcription of inserts in the vector reduces cloning bias. However, the pSMART

vector requires blunt ends with 5′ phosphate groups (unlike TOPO-TA [Invitrogen] cloning kits for example). The additional step adds time, increases cost, and increases risk of sample loss, but we have found that it works well.

Cloning and sequencing

Step 28.

After a 15-min incubation at room temperature the reaction is purified and concentrated with a MinElute Reaction Cleanup column (Qiagen).

DURATION

00:15:00

Cloning and sequencing

Step 29.

The eluted PCR products are subsequently ligated into the pSMART-HCKan vector (Lucigen) according to the manufacturer.

NOTES

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In this reaction, we typically use the maximum amount of template (6.5 µL) and the maximum incubation time (2 h).

Cloning and sequencing

Step 30.

After terminating the ligation reaction by incubating for 15 min at 70°C, 2 µL of the ligation reaction is transformed into Ecloni 10G Supreme cells (Lucigen) via electroporation.

DURATION

00:15:00

NOTES

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We do not recommend using greater than 2 µL of the ligation mixture in the transformation reaction because the chances of arcing are greatly increased during electroporation.

Cloning and sequencing

Step 31.

Before initiating a large-scale sequencing effort, we recommend screening 10 to 20 colonies for inserts by PCR amplification with the primers SL1 and SR2 (Table 1) to assess the quality of the library.

Cloning and sequencing

Step 32.

Colony PCR products can be visualized on a gel and the products in the correct size range purified and sequenced.