Construction of shotgun libraries from RNA virus assemblages

Alexander I. Culley, Curtis A. Suttle, and Grieg F. Steward

Abstract

The following protocol is used to construct shotgun libraries from RNA virus assemblages. This method is designed to detect all RNA viruses, regardless of their genome orientation, and therefore provides a broader assessment of RNA virus diversity compared to the single-gene–based method described in "A degenerate primer reverse transcriptionpolymerase chain reaction–based protocol to determine the diversity of picorna-like viruses."

Citation: Alexander I. Culley, Curtis A. Suttle, and Grieg F. Steward Construction of shotgun libraries from RNA virus assemblages. **protocols.io**
dx.doi.org/10.17504/protocols.io.dsi6cd
Published: 08 Dec 2015

Guidelines

Equipment—Thermocycler, heating block, incubator, gel electrophoresis unit, gel documentation system

Supplies—1.7 mL sterile, nucleic acid–free microcentrifuge tubes; 0.2 mL sterile, nucleic acid-free PCR tubes; sterile razor blades; QIAamp Viral RNA Mini kit (Qiagen); MinElute Gel Extraction kit (Qiagen); MinElute PCR Purification kit (Qiagen); MinElute Reaction Cleanup kit (Qiagen); PCRTerminator End Repair kit (Lucigen); CloneSmart HCKan Blunt Cloning kit with Ecloni Supreme cells (Lucigen); Turbo DNA-free Kit (Applied Biosystems); electroporation cuvettes

Solutions, reagents, and media—Superscript III Reverse Transcriptase and buffers (Invitrogen), 10 µM dNTP mix, RNase Out (Invitrogen), Klenow Fragment, 3′-5′ exo – (New England Biolabs); primers (Table 1): 0.5 × TBE (45 mM Tris-borate, 1 mM EDTA [pH 8.0]) electrophoresis buffer; nucleic acid-free, sterile water; Ampligold Taq Polymerase and buffers (Applied Biosystems); 0.02-filtered SM Buffer (100 mM NaCl, 8 mM MgSO4, 50 mM Tris pH 7.5)

Steps:

Sample collection, purification, and extraction—The ideal starting amount for this method is on the order of nanograms or greater of purified viral RNA, however the sensitivity of RTPCR suggests that sub-nanogram starting template will be successful. Because of how little RNA is present in a viral genome, the virus community in tens to thousands of liters of seawater must be isolated and concentrated before one can proceed with shotgun library construction. Other chapters in this book (Wommack et al. 2010, this volume; Steward and Culley 2010, this volume) discuss approaches to concentrating viral communities from seawater.

RP-SISPA will not discriminate between viral and non-viral nucleic acids, and it is therefore critical that only pure viral RNA is present as template. We have found that purification through two sequential

cesium chloride gradients (Protocol B in Lawrence and Steward 2010, this volume) is effective for removing contaminating cellular and exogenous nucleic acids prior to library construction.

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

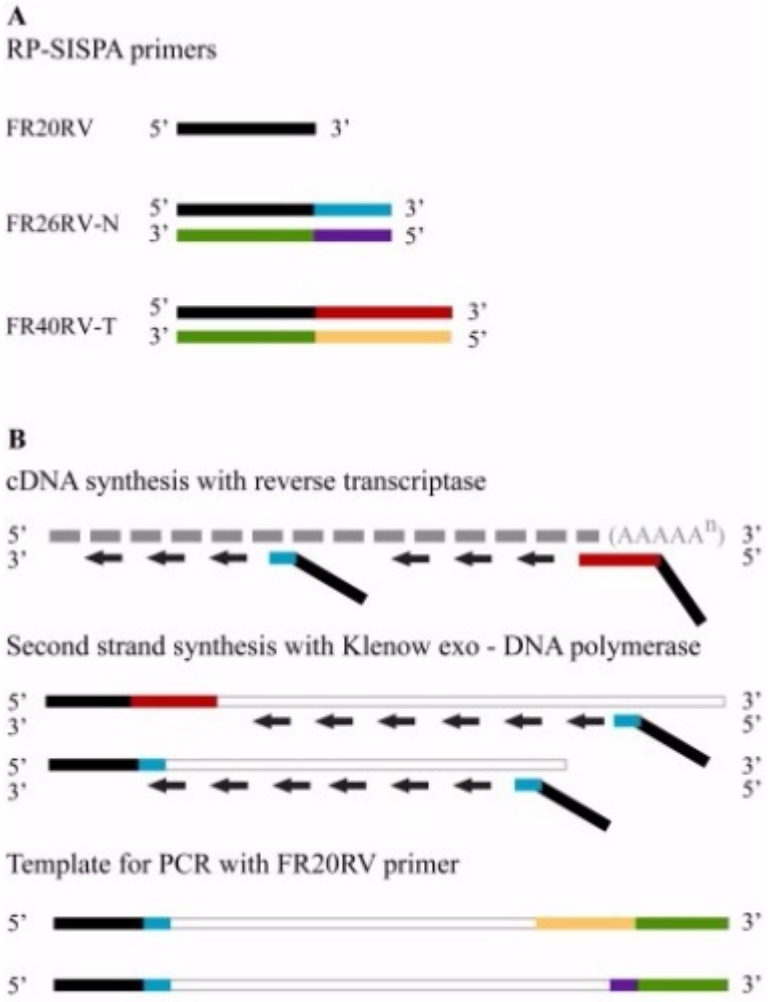

Figure 1. RP-SISPA Schematic. This figure is based on Djikeng et al. (2008). Figure 1A shows the three primers used in RP-SISPA. FR26RV-N is composed of the 20 bp 5′ primer sequence, FR20RV (black bar), and a random hexamer at the 3′ end (light blue bar). Primer FR40RV-T is a composite of the FR20RV primer (black bar) and a 20 bp poly T tail (red bar). FR20RV (black bar) is used in PCR. The reverse complement of FR26RV-N (green and orange bar) and FR40RV-T (green and yellow bar) are shown. In Figure 1B, cDNA is synthesized from viral RNA (gray segmented line) with reverse transcriptase primed with FR26RV-N and FR40RV-T. To synthesize the second strand, FR26RV-N is annealed to the newly synthesized cDNA strand where it primes Klenow exo – DNA polymerase "gap filling" activity, resulting in an FR20RV site on both 5′ and 3′ ends. The DNA templates, which represent random stretches of the initial viral RNA genomes, are then amplified with the FR20RV primer to generate more material for cloning and sequencing.

Protocol

Sample collection, purification, and extraction

Step 1.

Cesium chloride gradient is conducted to purify viruses from viral concentrate of seawater as described in others chapters of this book (Wommack et al. 2010, this volume; Steward and Culley 2010, this volume).

NOTES

Xu Zhong 08 Dec 2015
See guidelines

Sample collection, purification, and extraction

Step 2.
The relevant virus-containing gradient fractions are collected. The CsCl in the Virus-containing gradient fractions are removed by buffer exchange using a centrifugal ultrafiltration unit with a nominal molecular weight cutoff of 30 KD to 100 KD (Microcon, Millipore or Nanosep, Pall Life Sciences) as described in Steward and Culley (2010). Viruses are then recovered by eluting in 3 × 50 µL of 0.02-filtered SM buffer.

Sample collection, purification, and extraction

Step 3.
Each density fraction is then extracted with the QIAamp Viral RNA Mini kit (Qiagen) as directed by the manufacturer with the following exception (see annotation).

NOTES
Xu Zhong 09 Sep 2015
We do not add carrier RNA as suggested to the “AL” lysis buffer to avoid introducing non-target RNA to the sample. Note that there is no RNase treatment before extraction because exogenous RNA will pellet and is thus removed during the CsCl fractionation procedure. We recommend quantifying the RNA from each density fraction to identify in which fraction the concentration of viral RNA is highest. The template for RP-SISPA can be RNA extracted from mutiple pooled fractions or from a single fraction depending on your research objectives.

Enzymatic treatment

Step 4.
Remove contaminating DNA from RNA preparations with the Turbo DNAfree kit (Applied Biosystems) as described in the protocol provided with the kit.

NOTES
Xu Zhong 07 Oct 2015
See figure 1B in guidelines.

RP-SISPA: cDNA synthesis

Step 5.
In preparation for cDNA synthesis, 10 µL purified RNA viral template is mixed with a final dNTP concentration of 0.2 mM and 1 µM and 5 nM final concentrations of FR26RV-N and FR40RV-T primer, respectively.

NOTES
Xu Zhong 09 Sep 2015
See Table 1 for the sequence of each primer.
Xu Zhong 09 Sep 2015
FR40RV-T is added to take advantage of the fact that a majority of characterized RNA virus genomes have poly(A) tails. The addition of a poly(T) primer may increase the likelihood of the 3′ ends being sequenced.

RP-SISPA: cDNA synthesis

Step 6.
The reaction is heated to 65°C then cooled on ice to allow the primers to anneal.

RP-SISPA: cDNA synthesis

Step 7.
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 form RNAse activity.

RP-SISPA: cDNA synthesis

Step 8.

The complementary DNA strand is synthesized with 200 U of Superscript III (Invitrogen) reverse transcriptase.

NOTES

Xu Zhong 09 Sep 2015

The final reaction volume should be 20 µL.

RP-SISPA: cDNA synthesis

Step 9.

The reaction is incubated initially at 25°C for 10 min so that the hexamer 3′ end of primer FR26RV-N and the poly(T)$_{20}$ 3′ end of primer FR40RV-T remain annealed to the template while cDNA synthesis commences.

DURATION

00:10:00

RP-SISPA: cDNA synthesis

Step 10.

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

RP-SISPA: Second strand sythesis

Step 11.

After the hour-long incubation at 50°C, the first strand synthesis reaction is heated immediately to 94°C for 3 min and then rapidly cooled on ice.

DURATION

00:03:00

NOTES

Xu Zhong 09 Sep 2015

This step results in the reannealing of excess FR26RV-N primer to the nascent cDNA strand.

Xu Zhong 07 Oct 2015

In a simple and elegant step, the second strand synthesis reaction results in a ds cDNA template with a primer site added to both the 5′ and 3′ end (Figure 1B).

RP-SISPA: Second strand sythesis

Step 12.

A complementary second strand is subsequently synthesized at 37°C for 60 min with the addition of 2.5 U of Klenow Fragment, 3′-5′ exo – (New England Biolabs).

DURATION

01:00:00

RP-SISPA: Second strand sythesis

Step 13.

The Klenow reaction is terminated with a final incubation at 75°C for 10 min.

DURATION

00:10:00

PCR

Step 14.

One PCR reaction contains 5 µL of template taken directly from the second strand synthesis reaction, 40 pM of FR20RV primer (see Table 1), a final dNTP concentration of 0.2 mM, 1 × Gold buffer, 2.5 mM $MgCl_2$, and 2.5 U of Ampligold DNA polymerase (Applied Biosystems) in a final volume of 50 µL.

NOTES

Xu Zhong 09 Sep 2015

PCR is used to produce a sufficient quantity of DNA from the ds cDNA template from the second strand reaction for sequencing.

Xu Zhong 07 Oct 2015

See figure 1B in guidelines.

PCR

Step 15.

The reaction is incubated at 94°C for 10 min to fully denature the template and activate the hot start enzyme.

DURATION

00:10:00

NOTES

Xu Zhong 09 Sep 2015

We have found that a hot start is absolutely essential.

PCR

Step 16.

Followed by 35 cycles of denaturation at 94°C for 1 min.

DURATION

00:01:00

PCR

Step 17.

Annealing at 65°C for 1 min.

DURATION

00:01:00

PCR

Step 18.

Extension at 72°C for 2 min.

DURATION

00:02:00

PCR

Step 19.

A final extension for 13 min that permits the completion of complementary strand synthesis.

DURATION

00:13:00

PCR

Step 20.

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

NOTES

Xu Zhong 09 Sep 2015

This reduces the thickness of the gel, ultimately resulting in a more efficient recovery of target DNA from the excised band.

PCR

Step 21.

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

PCR

Step 22.

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

NOTES

Xu Zhong 09 Sep 2015

The size range excised should be based on what type of sequencing method is being used; for example, we target the 800-2000 bp size range for Sanger sequencing.

Xu Zhong 09 Sep 2015

When visualizing the gel, prolonged exposure to UV irradiation can damage the DNA and greatly reduce downstream cloning efficiency. An illuminator with blue light-emitting bulbs is ideal for gel visualization. If this is not available, take steps to reduce the exposure of the gel to ultraviolet irradiation during excision as much as possible. To mitigate the biases associated with high cycle number PCR, we recommend pooling the products from multiple PCR reactions using the same ds cDNA template.

PCR

Step 23.

If the sample is to be cloned for Sanger sequencing, we recommend eluting DNA from the column with three washes of 10 µL nuclease-free water in preparation for the PCRTerminator (Lucigen) end repair reaction.

PCR

Step 24.

For the cloning protocol, please refer to the "Cloning and sequencing" section of "A degenerate primer reverse transcriptionpolymerase chain reaction–based protocol to determine the diversity of picorna-like viruses".

NOTES

Xu Zhong 09 Sep 2015

The procedure from this point forward is the same. The material purified from the gel may instead be processed for pyrosequencing.