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Unbiased deep sequencing of RNA viruses from clinical samples

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Abstract:	Here we outline a next-generation, RNA sequencing protocol that enables de novo assemblies and intra-host variant calls of viral genomes collected from clinical and biological sources. The method is unbiased and universal; it uses random primers for cDNA synthesis and requires no prior knowledge of the viral sequence content. Before library construction, selective RNase H-based digestion is used to deplete unwanted RNA—including poly(rA) carrier and ribosomal RNA—from the viral RNA sample. Selective depletion improves both the data quality and the number of unique reads in viral RNA sequencing libraries. Moreover, a transposase-based 'tagmentation' step is used in the protocol as it reduces the library construction from 3 days to about 4 hours. The protocol has enabled rapid deep sequencing of over 600 Lassa and Ebola virus samples—including collections from both blood and tissue isolates—and is broadly applicable to other microbial genomics studies.
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TITLE:
Unbiased deep sequencing of RNA viruses from clinical samples

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RNA viruses; Ebola virus; Lassa virus; intra-host variants; Lassa fever; poly(rA) carrier; rRNA; RNase H; RT-PCR

SHORT ABSTRACT:
This protocol describes a rapid and broadly applicable method for unbiased RNA-sequencing of viral samples from human clinical isolates.

LONG ABSTRACT:
Here we outline a next-generation RNA sequencing protocol that enables *de novo* assemblies and intra-host variant calls of viral genomes collected from clinical and biological sources. The method is unbiased and universal; it uses random primers for cDNA synthesis and requires no prior knowledge of the viral sequence content. Before library construction, selective RNase H-based digestion is used to deplete unwanted RNA—including poly(rA) carrier and ribosomal RNA—from the viral RNA sample. Selective depletion improves both the data quality and the number of unique reads in viral RNA sequencing libraries. Moreover, a transposase-based 'tagmentation' step is used in the protocol as it reduces overall library construction time. The protocol has enabled rapid deep sequencing of over 600 Lassa and Ebola virus samples—including collections from both blood and tissue isolates—and is broadly applicable to other microbial genomics studies.

INTRODUCTION:
Next generation sequencing of viruses from clinical sources can inform transmission and the epidemiology of infections, as well as help support novel diagnostic, vaccine and therapeutic development. cDNA synthesis using random primers has allowed the detection and assembly of genomes from divergent, co-infecting or even novel viruses[1,2]. As with other unbiased methods, unwanted contaminants occupy many sequencing reads and negatively impact sequencing results. Host and poly(rA) carrier RNA are contaminants present in many existing viral sample collections.

The protocol describes an efficient and cost-effective way of deep sequencing RNA virus genomes based on unbiased total RNA-seq. The method utilizes an RNase H selective depletion step[3] to remove unwanted host ribosomal and carrier RNA. Selective depletion enriches for viral content (Figure 1) and improves the overall quality of sequencing data (Figure 2) from clinical samples. Moreover, tagmentation is applied to the protocol as it significantly reduces library construction time. These methods have been used to rapidly generate large datasets of Ebola and Lassa virus genomes[2,4,5] and can be used to study a wide range of RNA viruses. Lastly, the approach is not limited to human samples; the utility of selective depletion was demonstrated on tissue samples collected from Lassa-infected rodents and non-human primate disease models[5,6].

[Place Figure 1 here]

[Place Figure 2 here]

The viral RNA-seq protocol details construction of libraries directly from extracted RNA collected from clinical and biological samples. To ensure personal safety, all viral serum, plasma and tissue samples should be inactivated in appropriate buffers prior to RNA extraction. In some inactivation and extraction kits, carrier poly(rA) RNA is included; this will be removed during the initial RNase H selective depletion step. Based on complete recovery, the expected concentration of carrier RNA is 100 ng/µL. In the protocol, 110 ng/µL oligo dT RNA (1.1x carrier concentration) is used for depletion. If poly(rA) carrier is not present in the sample, then oligo(dT) should not be added prior to depletion.

The following protocol is designed for 24 reactions in PCR plate format (up to 250 µL volume). An earlier version of this protocol was reported in Matranga, *et al.*[6].

PROTOCOL:

Ethics statement: Lassa fever patients were recruited for this study using protocols approved by human subjects committees at Tulane University, Harvard University, Broad Institute, Irrua Specialist Teaching Hospital (ISTH), Kenema Government Hospital (KGH), Oyo State Ministry of Health, Ibadan, Nigeria and Sierra Leone Ministry of Health. All patients were treated with a similar standard of care and were offered the drug Ribavirin, whether or not they decided to participate in the study. For Lassa fever (LF) patients, treatment with Ribavirin followed the currently recommended guidelines and was generally offered as soon as LF was strongly suspected.

Due to the severe outbreak for Ebola Virus Disease (EVD), patients could not be consented through our standard protocols. Instead use of clinical excess samples from EVD patients was evaluated and approved by Institutional Review Boards in Sierra Leone and at Harvard University. The Office of the Sierra Leone Ethics and Scientific Review Committee, the Sierra Leone Ministry of Health and Sanitation, and the Harvard Committee on the Use of Human Subjects have granted a waiver of consent to sequence and make publically available viral sequences obtained from patient and contact samples collected during the Ebola outbreak in Sierra Leone. These bodies also granted use of clinical and epidemiological data for de-identified samples collected from all suspected EVD patients receiving care during the outbreak response. The Sierra Leone Ministry of Health and Sanitation also approved shipments of non-infectious non-biological samples from Sierra Leone to the Broad Institute and Harvard University for genomic studies of outbreak samples.

1. DNase-treatment of sample RNA (up to 55 µL extracted total RNA, ~4 hr)

1.1) Set up the DNase reaction in a 96-well PCR plate on ice in a biosafety cabinet as described in Table 1, Step 1.1 (total volume, 70 µL/well). Note: A master mix can be prepared.

1.2) Vortex gently and thoroughly, then centrifuge at 280 x g at room temperature for 1 min. Incubate at 37°C for 30 min.

1.3) Cleanup using RNA Solid Phase Reversible Immobilization (SPRI) beads.

1.3.1) Warm RNA beads to room temperature for 30 min.

1.3.2) Gently shake RNA beads bottle to resuspend any magnetic particles that may have settled. Add 1.8x volume (126 µL) of RNA beads to DNase-treated RNA (70 µL), mix by pipette 10 times and incubate for 5 min at room temperature (total volume in well, 196 µL).

1.3.3) Place mixture on the magnetic station. Wait for the solution to clear (5-10 min).

1.3.4) Remove cleared solution while on the station by pipette and discard. While on station, wash beads by covering pellet with 70% ethanol and incubate for 1 min. Remove ethanol with pipette and discard. Repeat for a total of two washes.

Note: Using precisely 70% freshly prepared ethanol is critical, as a higher percentage will result in inefficient washing of smaller-sized molecules, whereas < 70% ethanol could cause loss of sample[7].

1.3.5) Keep plate on the station and leave open to air-dry. Note: Be sure to allow the beads to dry completely until beads begin to crack.

1.3.6) Add 55 µL of nuclease-free water to the plate to elute RNA. Remove plate from the station to mix the beads and water by pipetting thoroughly. Note: Alternatively, use less water (≤ 10 µL) in order to concentrate the total RNA.

1.3.7) Place plate back on the station. Wait until solution clears to transfer by pipette to new screw-cap tube for long-term storage (-80˚C). Place 5 µL RNA in new 96-well PCR plate for depletion (step. 2.4).

1.3.8) Optional: Save and dilute 1 µL in 19 µL water (1:20) for qRT-PCR of rRNA (e.g. 18S, 28S rRNA) (Table 2) and viral markers[5].

2. Selective depletion of ribosomal and carrier RNA from viral RNA sample (~4 hr)

2.1) Make 5x hybridization and 10x RNase H reaction buffers, and nuclease-free water with linear acrylamide carrier as described in Table 1.

2.2) Set up hybridization reaction by combining RNA with rRNA depletion oligos (Table 3) and oligo(dT) on ice in a 96-well PCR plate as described in Table 1.

Note: A master mix can be prepared. 50 femtograms (fg) of a unique synthetic RNA (ERCCs[8]) can be added for tracking both the viral sequencing process and potential index read cross-contamination.

2.2.1) Vortex gently and thoroughly, then centrifuge at 280 x g at room temperature for 1 min. Incubate at 95 °C for 2 min, slow ramping to 45 °C at -0.1 °C per sec. Pause the thermocycler at 45 °C.

2.3) Set up RNase H reaction mix on ice as described in Table 1, then preheat at 45 °C for 2 min. Note: A master mix can be prepared.

2.3.1) Add the pre-heated RNase H mix to the hybridization reaction in plate while keeping the plate in the thermocycler at 45°C.

2.3.2) Mix well by gentle pipetting 6-8 times. Incubate at 45 °C for another 30 min. Place on ice.

2.4) Set up the DNase reaction mix on ice as described in Table 1. Note: A master mix can be prepared.

2.4.1) Add to the RNase H reaction in plate, vortex gently and thoroughly, then centrifuge at 280 x g at room temperature for 1 min. Incubate at 37 °C for 30 min.

2.4.2) Stop DNase reaction by adding 5 µL 0.5M EDTA. Vortex gently and thoroughly, then centrifuge at 280 x g at room temperature for 1 min.

2.5) Cleanup using RNA beads (see step 1.3) using 1.8x volume (144 µL) beads. Elute in 11 µL of nuclease-free water. Note: For safe cold storage, store depleted RNA sample at -80 ˚C overnight.

3. cDNA synthesis (~6 hr)

3.1) Mix rRNA/carrier-depleted RNA with random primers on ice in a 96-well PCR plate as described in Table 1, vortex gently and thoroughly, then centrifuge at 280 x g at room temperature for 1 min.

3.1.1) Heat the mixture to 70 °C for 10 min in a thermocycler. Immediately after heat denaturation, place the RNA on ice for 1–5 min. Do not allow the RNA to stand (even on ice) for longer than 5 min prior to the first-strand reaction.

3.2) Set up first-strand synthesis reaction mix on ice as described in Table 1. Note: A master-mix may be prepared.

3.2.1) Add to RNA/random primer mix in plate, vortex gently and thoroughly, then centrifuge at 280 x g at room temperature for 1 min. Incubate at 22-25 °C for 10 min.

3.2.2) Incubate at 55°C in an air incubator for 60 min. Place the plate on ice to terminate the reaction. Note: The use of an air incubator is recommended to create gradual warming of the first-strand reaction during which the primers anneal and the first strand begins to elongate.

3.3) Set up second-strand synthesis reaction mix on ice as described in Table 1. Note: A master-mix may be prepared.

3.3.1) Add to first-strand synthesis reaction in plate, vortex gently and thoroughly, then centrifuge at 280 x g at room temperature for 1 min. Incubate for 2 hr at 16 °C (keep lid at 25 °C). Do not allow the temperature to rise above 16°C.

3.3.2) Place the plate on ice, then inactivate reaction by adding 5 µL of 0.5M EDTA, mix gently and thoroughly, then centrifuge at 280 x g at room temperature for 1 min.

3.4) Cleanup with DNA beads (see step 1.3 for protocol) using 1.8x volume (153 µL) of beads. Elute in 9 µL of elution buffer (EB). Save 1 µL for quantification. Use 1 ng of cDNA for subsequent steps. If cDNA concentration is too low to detect, use 4 µL of cDNA for tagmentation (see step 4.1).

3.5) For safe cold storage, store double-stranded cDNA at 4 °C overnight or -20 °C for long-term storage.

4. Library preparation—DNA library construction (~4 hr)

4.1) Transfer 4 µL of cDNA to 96 well plate and save remaining cDNA for second attempt if needed.

4.2) Set up the tagmentation reaction on ice as described in Table 1.

Note: A master-mix may be prepared. To reduce background and overall cost, the total volume of the tagmentation reaction is reduced from 20 to 10 µL. As cDNA is the limiting factor, the amount of ATM (*i.e.* transposome) used in the reaction is also reduced to decrease the number of integration sites.

4.2.1) Add tagmentation mix to cDNA in the plate, vortex gently and thoroughly and centrifuge at 280 x g (at room temperature) for 1 min. Incubate at 55 °C for 5 min, hold at 10 °C.

4.2.2) Once at 10 °C, immediately add 2.5 µL of Neutralize Tagment Buffer (NT) to end reaction. Mix by pipetting up and down, then centrifuge at 280 x g (at room temperature) for 1 min.

4.2.3) Incubate at room temperature for 5 min.

4.3) Set up PCR amplification reaction on ice as described in Table 1.

4.3.1) Vortex gently and thoroughly, then centrifuge at 280 x g at room temperature for 1 min.

4.3.2) Perform PCR on thermocycler using the conditions described in Table 1.

Note: 12 cycles of PCR are suggested for 1 ng of tagmented cDNA; however, viral clinical samples often have undetectable amounts of cDNA. For low amounts of cDNA (<1 ng), use up to 18 cycles of PCR to create enough library for sequencing.

4.4) Library preparation—cleanup and pooling for sequencing

4.4.1) Bring sample up to 50 µL with EB.

4.4.2) Cleanup with DNA beads (see step 1.3 for protocol) using 0.6x volume (30 µL) beads. Elute in 15 µL EB.

4.4.3) Determine concentration of library (Figure 3) by conducting region analysis (150 to 1000 bp) using bioanalyzer software[9], excluding primer dimers (~120 bp) from region analysis. Note: Alternatively, qPCR can be used to quantify libraries [10].

[Place Figure 3 here]

4.4.4) Pool libraries at the lowest molar concentration of 1 nM or greater. If library is below 1 nM, add a small volume of library to pool (~1x volume of other libraries) to capture sequence information from these libraries.

4.4.5) Cleanup pool with 0.7x DNA beads as outlined above (see step 2). Elute in 15 µL EB. Note: Volume of beads will depend on the final volume of the pool.

4.4.6) Analyze pool[9]. Determine molar concentration by conducting region analysis (150 to 1000 bp)[9]. Note: Alternatively, qPCR can be used to quantify library pool [10].

4.4.7) Load sequencer at 10 pM library concentration to generate 101 bp, paired-end reads with dual barcode reads[11].

REPRESENTATIVE RESULTS:

The described protocol enables the generation of high quality sequencing reads from low-input viral RNA samples while enriching for unique viral content. As shown in Figure 1, the protocol enriched unique Lassa virus content at least five-fold in all samples (compared to non-depleted controls) with at least one million copies of 18S rRNA (~100 pg total RNA). Likewise, sequencing success also correlated with the amount of virus within a given sample. Using qRT-PCR as a surrogate for viral quantity, samples that contained ~1,000 or more viral genome copies most often created full assemblies (data not shown). Moreover, depletion of poly(rA) carrier reduces homopolymer sequences of A and T in libraries, resulting in cleaner preparations and ensuring better quality sequencing reads (Figure 2). Final libraries from low input viral clinical samples often have a broad fragment length from 150 to 1000 bp (Figure 3).

After sequencing, to reduce sample misidentification and crosstalk between libraries within a pool[12], only index reads with a base quality score of 25 (q25) and zero mismatches are kept during the demultiplexing process. Viral genomes are assembled using a bioinformatics pipeline specific for divergent viruses[2,4-6]. These tools are available at https://github.com/broadinstitute/viral-ngs or through commercial cloud platforms[4].

Figure Legends:

Figure 1. Total RNA content reflects enrichment of Lassa virus content using selective depletion. Starting overall content (RNA input) and enrichment of unique Lassa virus (LASV) reads (Library content) upon rRNA depletion from nine different clinical isolates. This figure has been modified from [6].

Figure 2. Higher quality sequencing after carrier RNA depletion. Median base qualities per sequencing cycle of poly(rA)-contaminated Lassa virus libraries (red) and control (no carrier observed in library, black) from QC report [13]. Both read 1 and read 2 of paired end reads are merged in the library BAM file and the quality scores are shown at each base. This figure has been modified from [6].

Figure 3. Libraries constructed from Ebola virus clinical samples. Gel image of 4 representative Ebola virus (EBOV) libraries. Regions of library and primer dimers are shown.

Table 1: Reaction set-up and buffers. Step-by-step tables with contents of all buffers and reaction mixes.

Table 2: qRT-PCR primers sequences. Primers used for measuring host (18S rRNA) and viral (Ebola and Lassa) content. 'KGH' is Kenema Government Hospital in Sierra Leone, where the Ebola primers were tested [2]. 'Kulesh' is the investigator who designed the primer set [14].

Table 3: Ribosomal RNA (rRNA) depletion oligos. 195 50-nt long sequences complementary to human rRNA for selective depletion step[6].

DISCUSSION:

The outlined approach enables robust, universal, rapid sequencing and was used for sequencing Ebola virus during the 2014 outbreak[2,4]. By coupling selective depletion and cDNA synthesis with tagmentation library construction, the overall process time was reduced by ~2 days from previous adapter ligation methods. More recently, this protocol was employed by international collaborators and others with great success[15,16] and will be deployed to labs in West Africa to support local genomics-based research studies and diagnostics[17].

The protocol described here uses random primers to prepare cDNA for viral RNA-seq libraries. Unlike previous viral RNA-seq approaches, it requires no a priori knowledge of sequence data or elaborate and time-consuming primer design for a specific virus or clade. The method can be applied to any viral RNA sample. For example, it was used to generate viral content from both

Ebola and Lassa samples[6]. The protocol may also be used for host transcriptomic, metagenomic and pathogen discovery sequencing projects [1].

A critical step of the protocol is targeted RNase H digestion, a high-throughput, low cost method for removing unwanted carrier and host RNA from viral samples. The selective depletion step of the protocol uses many components and requires skill and accuracy. Extra time and care should be taken during the initial setup.

As most clinical serum and plasma samples often have very little nucleic acid material, contamination and sample loss are common. To avoid these issues, special care should be taken when using this protocol. First, RNA is highly susceptible to degradation; therefore all areas should be clean and free of nucleases. Second, to identify samples suitable for use in this protocol, qRT-PCR assays for both host RNA and virus should be used for quantification[5,6]. When comparing input amounts with sequencing results from the protocol, sequencing success (*i.e.* generation of sufficient data for full viral assembly) correlated with samples that contained at least 100 pg total RNA and 1,000 copies of virus. Third, exposure to environmental sources of nucleic acids should be avoided. The protocol outlined here is done in a biosafety cabinet for safety precautions and for limiting environmental contaminants. Moreover, our group and others have noticed that commercial enzymes may be another source of contaminating bacterial nucleic acids in low input samples[6,18]. Use of a clean workspace (e.g. PCR hood, biosafety cabinet) and negative controls (e.g. water or buffer) will help alleviate and track contamination respectively. For samples with <100 pg of total RNA, only poly(rA) carrier RNA, not rRNA, should be depleted to ensure high quality sequencing results while limiting loss of material. For very low input samples, cDNA-amplification methods may be more suitable[19], although poly(rA) carrier should be removed prior to the cDNA synthesis.

The depletion of host rRNA enriches for viral content in sequencing libraries and is applicable to different sample collections including serum or plasma, and multiple types of tissues from rodents and non-human primates[5,6]. In non-human organisms, reads aligning to 28S rRNA remained after depletion, suggesting 28S rRNA is less conserved between humans and other species[6,20]. When using this method with non-human isolates, it may be necessary to supplement with DNA oligos complementary to the divergent rRNA sequences of the specific host [3,21].

Since the protocol is unbiased, viral reads may represent only a small fraction of total library content. Although rRNA is the most abundant species of host RNA and only a small percentage of rRNA reads (<1%) are found after selective depletion, all other host RNA (*e.g.* mRNA) will remain after depletion and may account for many sequencing reads from the sample. Therefore “oversampling” (i.e. oversequencing) individual libraries is required in order to have enough coverage for viral assembly and variant calls. For our studies, we attempt to sequence ~20 million reads per sample to have enough depth for analysis of viral genomic and associated variants as well as metagenomic content[2,5]. For metagenomic and pathogen discovery studies, it is important to note that contaminating host DNA is removed by DNase digestion. Therefore

viruses and other pathogens that contain DNA genomes may be lost during the process, however RNA intermediates may still be sequenced.

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DISCLOSURES:
The authors have no competing financial interests.

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Figure 1

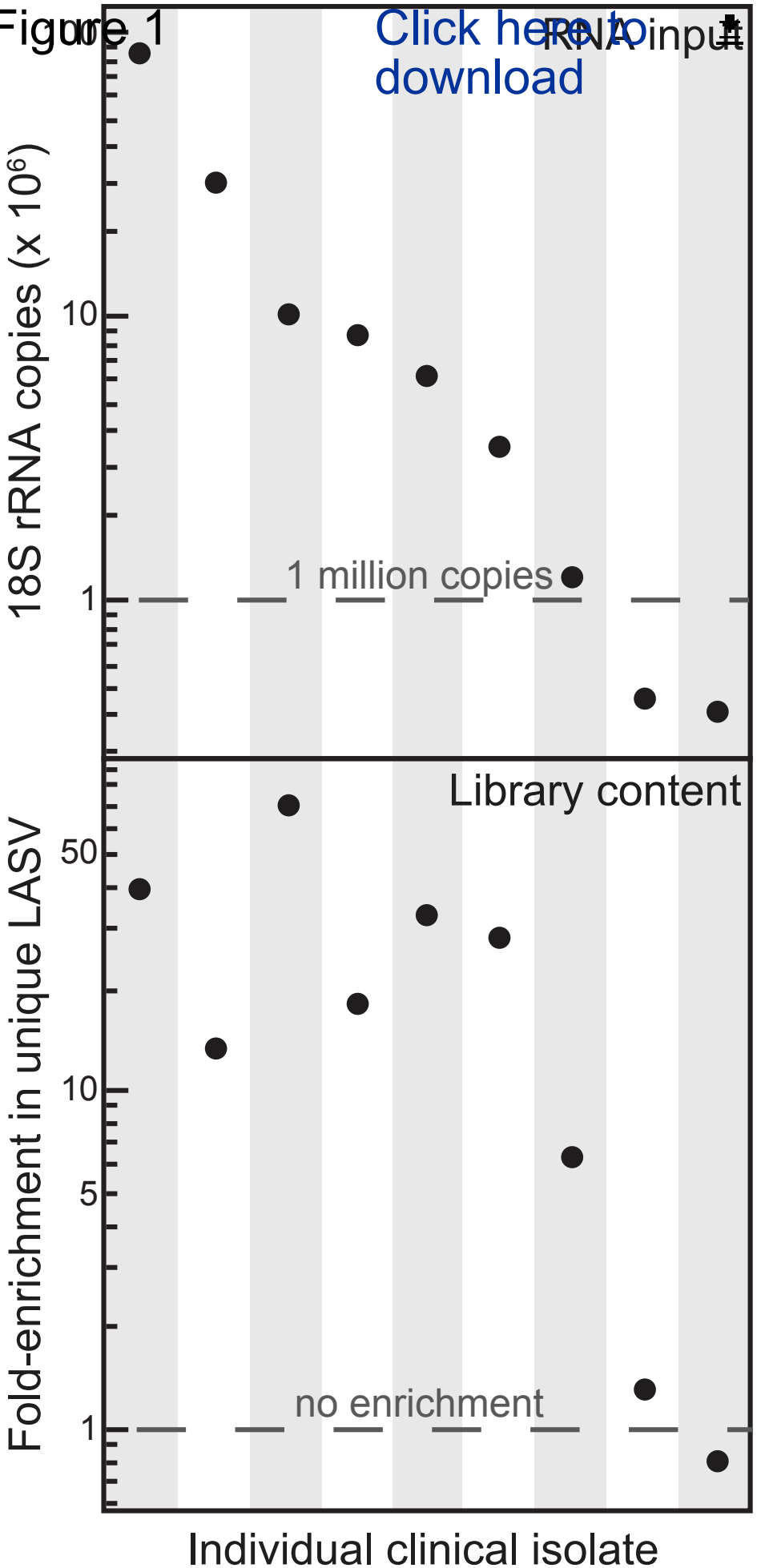

Figure 2

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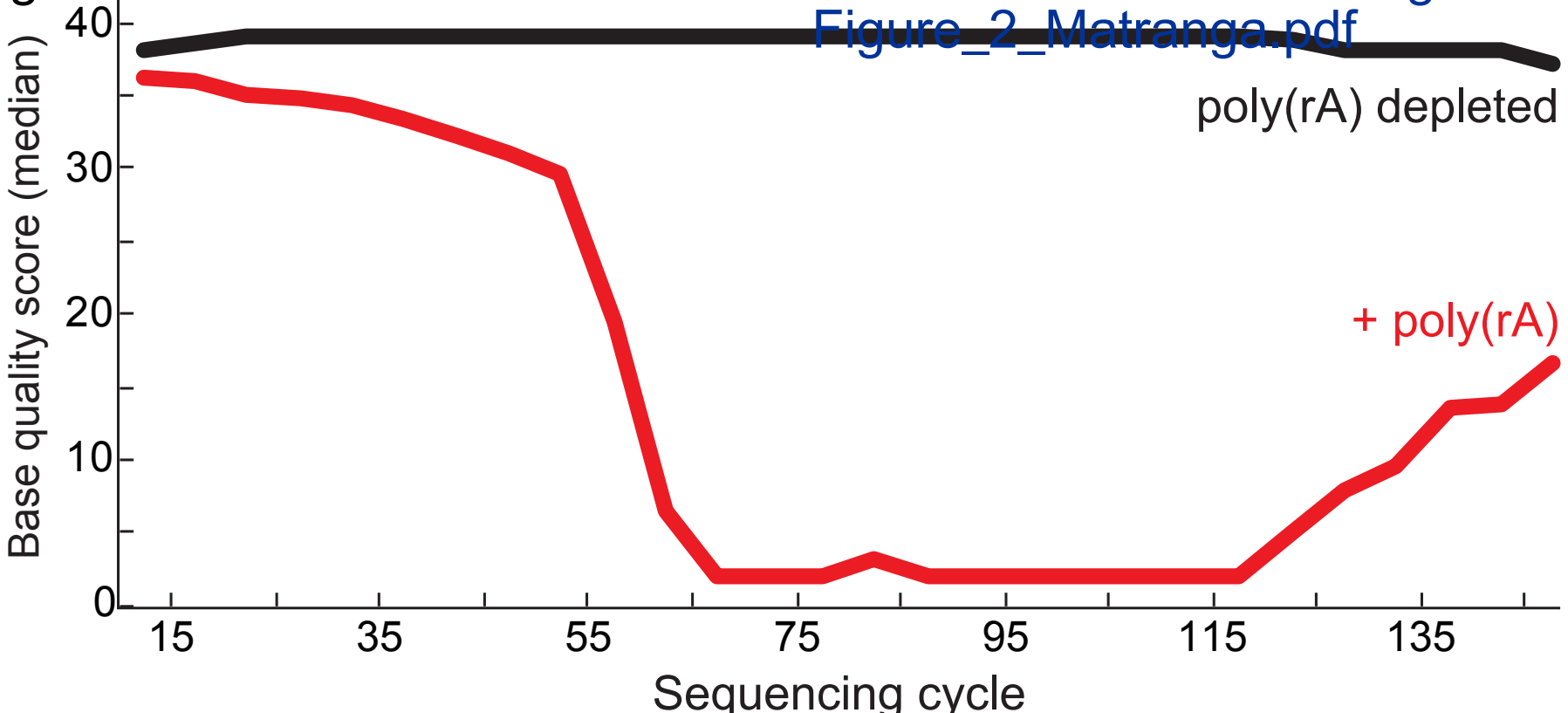

Figure 3

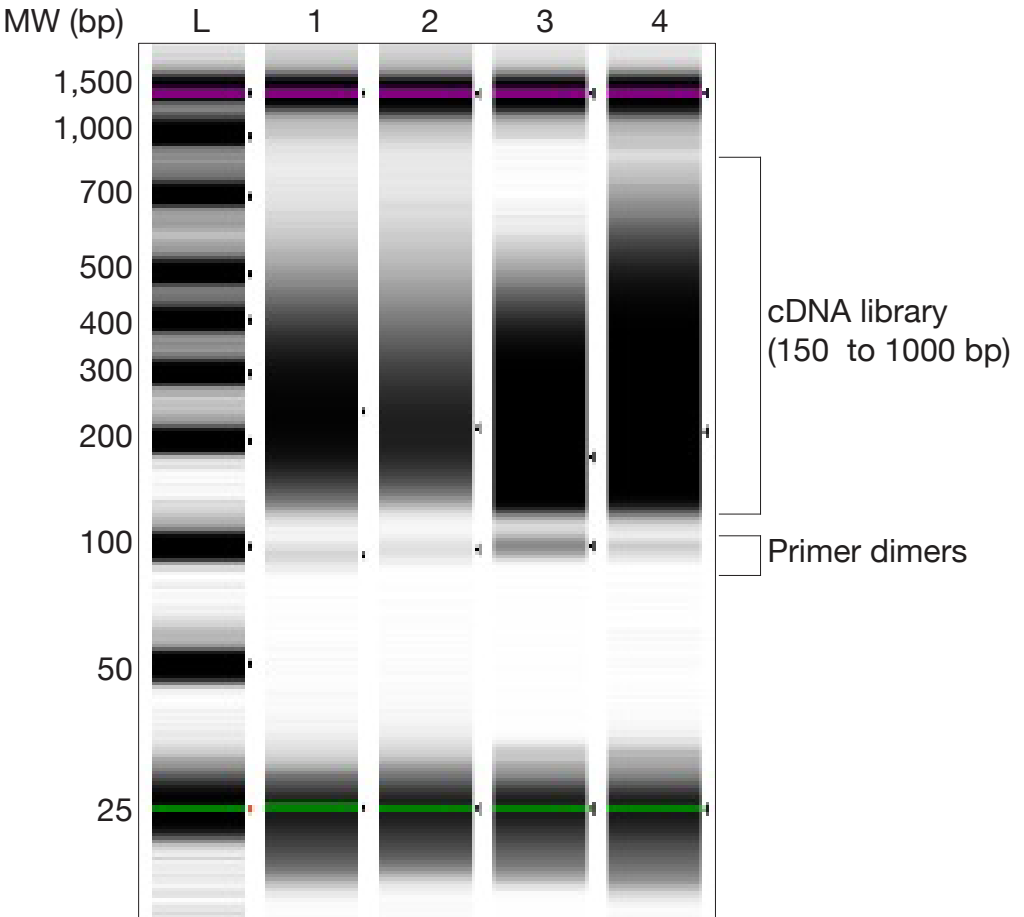

Table 1

Step 1.1: DNase reaction

Reagent	Volume per reaction (µL)
10x DNase buffer	7
Nuclease-free water	6
Extracted viral RNA	55
DNase (2 U/µL)	2
Total volume	70

Step 2.1: 5x Hybridization buffer

Reagent	Volume for 1 mL (µL)
5M NaCl	200
1M Tris-HCl (pH 7.4)	500
Nuclease-free water	300
Total volume	1000

Step 2.1: 10x RNase H reaction buffer

Reagent	Volume for 1 mL (µL)
5M NaCl	200
1M Tris-HCl (pH 7.5)	500
1M $MgCl_2$	200
Nuclease-free water	500
Total volume	*1000*

Step 2.1: Water with linear acrylamide

Reagent	Volume for 1 mL buffer (µL)
Nuclease-free water	992
Linear acrylamide (5 mg/mL)	8
Total volume	*1000*

Step 2.2: Hybridization reaction for selective depletion

Reagent	Volume per reaction (µL)
5x Hybridization Buffer	2
rRNA-depletion oligo mix (100 µM)	1.22
Oligo(d)T (550 ng/µL)	1
DNase-treated total RNA	up to 5
Spike-in RNA (This is optional)	0.5
Water (with linear acrylamide)	bring up to 10 total
Total volume	*10*

Step 2.3: RNase H reaction for selective depletion

Reagent	Volume per reaction (µL)
10x RNase H Reaction Buffer	2
Water (with linear acrylamide)	5
Thermostable RNase H (5U/µL)	3
Total volume	*10*

Step 2.4: DNase reaction post selective depletion

Reagent	Volume per reaction (µL)
10x DNase Buffer	7.5
Water (with linear acrylamide)	44.5
RNase inhibitor (20 U/µL)	1
RNase-free DNase I (2.72 U/µL)	2
Total volume (with RNase H reaction)	*75*

Step 3.1: cDNA synthesis, random primer hybridization

Reagent	Volume per reaction (µL)
rRNA/carrier-depleted RNA	10
3 µg random primer	1
Total volume	*11*

Step 3.2: First strand cDNA synthesis reaction

Reagent	Volume (µL)
5X First-Strand Reaction Buffer	4
0.1 M DTT	2
10 mM dNTP mix	1
RNase inhibitor (20 U/µL)	1
Reverse transcriptase (add last)	1
Total volume (with RNA above)	*20*

Step 3.3: Second strand cDNA synthesis reaction

Reagent	Volume (µL)
RNase-free water	43
10X Second-Strand Reaction Buffer	8
10 mM dNTP mix	3
E. coli DNA Ligase (10 U/µL)	1
E. coli DNA Polymerase I (10 U/µL)	4
E. coli RNase H (2 U/µL)	1
Total volume (with 1 st strand reaction)	*80*

Step 4.2: Tagmentation reaction

Reagent	Volume (µL)
Amplicon Tagment Mix (ATM)	1
Tagment DNA Buffer (TD)	5
Total volume (with cDNA)	*10*

Step 4.3: Library PCR reaction

Reagent	Volume (µL)
PCR Master Mix (NPM)	7.5
Index 1 primer (i7)	2.5
Index 2 primer (i5)	2.5
Total volume (with tagmented cDNA)	*25*

Step 4.3.2: Library PCR conditions

72°C, 3 min
95°C, 30 sec
up to 18 cycles-10 sec at 95°C, 30 sec at 55°C, 30 sec at 72°C
72°C, 5 min
10°C, forever

Primers

Click here to download Table Table 2.xlsx

Oligo Name	Sequence (5' to 3')
Ebola KGH FW	GTCGTTCCAACAATCGAGCG
Ebola KGH RV	CGTCCCGTAGCTTTRGCCAT
Ebola KULESH FW	TCTGACATGGATTACCACAAGATC
Ebola KULESH RV	GGATGACTCTTTGCCGAACAATC
Lassa SL FW	GTA AGC CCA GCD GYA AAB CC
Lassa SL RV	AAG CCA CAG AAA RCT GGS AGC A
Lassa Nigeria FW	CCC AAG CYC THC CYA CAA T
Lassa Nigeria RV	AAC CCT TAT GAG AAY ATA CTB TAY AA
18S rRNA FW	TCCTTTAACGAGGATCCATTGG
18S rRNA RV	CGAGCTTTTTAACTGCAGCAACT

Ribosomal rRNA oligos

Click here to download Table Table 3.xlsx

Oligo Name	Sequence (5' to 3')
AG9327_18_1	TAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTAC
AG9328_18_2	TTCCTCTAGATAGTCAAGTTCGACCGTCTTCTCAGCGCTCCGCCAGGGCC
AG9329_18_3	GTGGGCCGACCCCGGCGGGGCCGATCCGAGGGCCTCACTAAACCATCCAA
AG9330_18_4	TCGGTAGTAGCGACGGGCGGTGTGTACAAAGGGCAGGGACTTAATCAACG
AG9331_18_5	CAAGCTTATGACCCGCACTTACTCGGGAATTCCCTCGTTCATGGGGAATA
AG9332_18_6	ATTGCAATCCCCGATCCCCATCACGAATGGGGTTCAACGGGTTACCCGCG
AG9333_18_7	CCTGCCGGCGTAGGGTAGGCACACGCTGAGCCAGTCAGTGTAGCGCGCGT
AG9334_18_8	GCAGCCCCGGACATCTAAGGGCATCACAGACCTGTTATTGCTCAATCTCG
AG9335_18_9	GGTGGCTGAACGCCACTTGTCCCTCTAAGAAGTTGGGGGACGCCGACCGC
AG9336_18_10	TCGGGGGTCGCGTAACTAGTTAGCATGCCAGAGTCTCGTTCGTTATCGGA
AG9337_18_11	ATTAACCAGACAAATCGCTCCACCAACTAAGAACGGCCATGCACCACCAC
AG9338_18_12	CCACGGAATCGAGAAAGAGCTATCAATCTGTCAATCCTGTCCGTGTCCGG
AG9339_18_13	GCCGGGTGAGGTTTCCCGTGTTGAGTCAAATTAAGCCGCAGGCTCCACTC
AG9340_18_14	CTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGCTTTGCAACCATA
AG9341_18_15	CTCCCCCCGGAACCCAAAGACTTTGGTTTCCCGGAAGCTGCCCGGCGGGT
AG9342_18_16	CATGGGAATAACGCCGCCGCATCGCCGGTCGGCATCGTTTATGGTCGGAA
AG9343_18_17	CTACGACGGTATCTGATCGTCTTCGAACCTCCGACTTTCGTTCTTGATTA
AG9344_18_18	ATGAAAACATTCTTGGCAAATGCTTTCGCTCTGGTCCGTCTTGCGCCGGT
AG9345_18_19	CCAAGAATTTCACCTCTAGCGGCGCAATACGAATGCCCCCGGCCGTCCCT
AG9346_18_20	CTTAATCATGGCCTCAGTTCCGAAAACCAACAAAATAGAACCGCGGTCCT
AG9347_18_21	ATTCCATTATTCCTAGCTGCGGTATCCAGGCGGCTCGGGCCTGCTTTGAA
AG9348_18_22	CACTCTAATTTTTTCAAAGTAAACGCTTCGGGCCCCGCGGGACACTCAGC
AG9349_18_23	TAAGAGCATCGAGGGGGCGCCGAGAGGCAAGGGGCGGGGACGGGCGGTGG
AG9350_18_24	CTCGCCTCGCGGCGGACCGCCCGCCCGCTCCCAAGATCCAACTACGAGCT
AG9351_18_25	TTTTAACTGCAGCAACTTTAATATACGCTATTGGAGCTGGAATTACCGCG
AG9352_18_26	GCTGCTGGCACCAGACTTGCCCTCCAATGGATCCTCGTTAAAGGATTTAA
AG9353_18_27	AGTGGACTCATTCCAATTACAGGGCCTCGAAAGAGTCCTGTATTGTTATT
AG9354_18_28	TTTCGTCACTACCTCCCCGGGTCGGGAGTGGGTAATTTGCGCGCCTGCTG
AG9355_18_29	CCTTCCTTGGATGTGGTAGCCGTTTCTCAGGCTCCCTCTCCGGAATCGAA
AG9356_18_30	CCCTGATTCCCCGTCACCCGTGGTCACCATGGTAGGCACGGCGACTACCA
AG9357_18_31	TCGAAAGTTGATAGGGCAGACGTTCGAATGGGTCGTCGCCGCCACGGG
AG9358_18_32	GCGTGCGATCGGCCCGAGGTTATCTAGAGTCACCAAAGCCGCCGGCGCCC
AG9359_18_33	GCCCCCCGGCCGGGGCCGGAGAGGGGCTGACCGGGTTGGTTTTGATCTGA
AG9360_18_34	TAAATGCACGCATCCCCCCCGCGAAGGGGGTCAGCGCCCGTCGGCATGTA
AG9361_18_35	TTAGCTCTAGAATTACCACAGTTATCCAAGTAGGAGAGGAGCGAGCGACC
AG9362_18_36	AAAGGAACCATAACTGATTTAATGAGCCATTCGCAGTTTCACTGTACCGG
AG9363_18_37	CCGTGCGTACTTAGACATGCATGGCTTAATCTTTGAGACAAGCATATGCT
AG9364_18_38	TGGCTTAATCTTTGAGACAAGCATATGCTACTGGCAGGATCAACCAGGTA
AG9365_28_1	GACAAACCCTTGTGTCGAGGGCTGACTTTCAATAGATCGCAGCGAGGGAG
AG9366_28_2	CTGCTCTGCTACGTACGAAACCCCGACCCAGAAGCAGGTCGTCTACGAAT

AG9367_28_3	GGTTTAGCGCCAGGTTCCCCACGAACGTGCGGTGCGTGACGGGCGAGGG
AG9368_28_4	GCGGCCGCCTTTCCGGCCGCGCCCCGTTTCCCAGGACGAAGGGCACTCCG
AG9369_28_5	CACCGGACCCCGGTCCCGGCGCGCGGCGGGGCACGCGCCCTCCCGCGGCG
AG9370_28_6	GGGCGCGTGGAGGGGIGGGCGGCCCGCCGGCGGGGACAGGCGGGGGACCG
AG9371_28_7	GCTATCCGAGGCCAACCGAGGCTCCGCGGCGCTGCCGTATCGTTCGCCTG
AG9372_28_8	GGCGGGATTCTGACTTAGAGGCGTTCAGTCATAATCCCACAGATGGTAGC
AG9373_28_9	TTCGCCCCATTGGCTCCTCAGCCAAGCACATACACCAAATGTCTGAACCT
AG9374_28_10	GCGGTTCCTCTCGTACTGAGCAGGATTACCATGGCAACAACACATCATCA
AG9375_28_11	GTAGGGTAAAACTAACCTGTCTCACGACGGTCTAAACCCAGCTCACGTTC
AG9376_28_12	CCTATTAGTGGGTGAACAATCCAACGCTTGGCGAATTCTGCTTCACAATG
AG9377_28_13	ATAGGAAGAGCCGACATCGAAGGATCAAAAAGCGACGTCGCTATGAACGC
AG9378_28_14	TTGGCCGCCACAAGCCAGTTATCCCTGTGGTAACTTTTCTGACACCTCCT
AG9379_28_15	GCTTAAAACCCAAAAGGTCAGAAGGATCGTGAGGCCCCGCTTTCACGGTC
AG9380_28_16	TGTATTCGTACTGAAAATCAAGATCAAGCGAGCTTTTGCCCTTCTGCTCC
AG9381_28_17	ACGGGAGGTTTCTGTCCTCCCTGAGCTCGCCTTAGGACACCTGCGTTACC
AG9382_28_18	GTTTGACAGGTGTACCGCCCCAGTCAAACTCCCCACCTGGCACTGTCCCC
AG9383_28_19	GGAGCGGGTCGCGCCCGGCCGGGCGGGCGCTTGGCGCCAGAAGCGAGAGC
AG9384_28_20	CCCTCGGGCTCGCCCCCCCGCCTCACCGGGTCAGTGAAAAAACGATCAGA
AG9385_28_21	GTAGTGGTATTTCACCGGCGGCCCGCAGGGCCGCGGACCCCGCCCCGGGC
AG9386_28_22	CCCTCGCGGGGACACCGGGIGGGCGCCGGGGGCCTCCCACTTATTCTACA
AG9387_28_23	CCTCTCATGTCTCTTCACCGTGCCAGACTAGAGTCAAGCTCAACAGGGTC
AG9388_28_24	TTCTTTCCCCGCTGATTCCGCCAAGCCCGTTCCCTTGGCTGTGGTTTCGC
AG9389_28_25	TGGATAGTAGGTAGGGACAGTGGGAATCTCGTTCATCCATTCATGCGCGT
AG9390_28_26	CACTAATTAGATGACGAGGCATTTGGCTACCTTAAGAGAGTCATAGTTAC
AG9391_28_27	TCCCGCCGTTTACCCGCGCTTCATTGAATTTCTTCACTTTGACATTCAGA
AG9392_28_28	GCACTGGGCAGAAATCACATCGCGTCAACACCCGCCGCGGGCCTTCGCGA
AG9393_28_29	TGCTTTGTTTTAATTAAACAGTCGGATTCCCCTGGTCCGCACCAGTTCTA
AG9394_28_30	AGTCGGCTGCTAGGCGCCGGCCGAGGCGAGGCGCGCGCGGAACCGCGGCC
AG9395_28_31	CCGGGGGCGGACCCGGCGGGIGGGACCGGCCCGCGGCCCCTCCGCCGCCT
AG9396_28_32	GCCGCCGCCGCCGCCGCGCGCCGAGGAGGAGGGGGGAACGGGGGGCGGAC
AG9397_28_33	GGGCCGGGIGGGTAGGGCGGGGGGACGAACCGCCCCGCCCCGCCGCCCG
AG9398_28_34	CCGACCGCCGCCGCCCGACCGCTCCCGCCCCCAGCGGACGCGCGCGCGAC
AG9399_28_35	CGAGACGTGGGGTGGGGGTGGGGGGCGCGCCGCGCCGCCGCCGGGCTCCC
AG9400_28_36	CGGGGGCGGCCGCGACGCCCGCCGCAGCTGGGGCGATCCACGGGAAGGGC
AG9401_28_37	CCGGCTCGCGTCCAGAGTCCGCGCCGCCGCCGGCCCCCCGGGTCCCCGGG
AG9402_28_38	GCCCCCCTCGCGGGGACCTGCCCCCGCCGGCCGCCCCGGCGGCCGCCGCG
AG9403_28_39	CGGCCCCTGCCGCCCCGACCCTTCTCCCCCCGCCGCGCCCCCACGCGGCG
AG9404_28_40	CTCCCCCGGGGAGGGGGGAGGACGGGGAGCGGGGGAGAGAGAGAGAGAGA
AG9405_28_41	GGGCGCGGGGTGGGGAGGGAGCGAGCGGCGCGCGCGGGTGGGGCGGGGGA
AG9406_28_42	GGGCCGCGAGGGGGGTGCCCCGGGCGTGGGGIGGGCGCGCGCCTCGTCCA
AG9407_28_43	GCCGCGGCGCGCGCCCAGCCCCGCTTCGCGCCCCAGCCCGACCGACCCAG

AG9408_28_44	CCCTTAGAGCCAATCCTTATCCCGAAGTTACGGATCCGGCTTGCCGACTT
AG9409_28_45	CCCTTACCTACATTGTTCCAACATGCCAGAGGCTGTTCACCTTGGAGACC
AG9410_28_46	TGCTGCGGATATGGGTACGGCCCGGCGCGAGATTTACACCCTCTCCCCCG
AG9411_28_47	GATTTTCAAGGGCCAGCGAGAGCTCACCGGACGCCGCCGGAACCGCGACG
AG9412_28_48	CTTTCCAAGGCACGGGCCCCTCTCTCGGGGCGAACCCATTCCAGGGCGCC
AG9413_28_49	CTGCCCTTCACAAAGAAAAGAGAACTCTCCCCGGGGCTCCCGCCGGCTTC
AG9414_28_50	TCCGGGATCGGTCGCGTTACCGCACTGGACGCCTCGCGGCGCCCATCTCC
AG9415_28_51	GCCACTCCGGATTCGGGGATCTGAACCCGACTCCCTTTCGATCGGCCGAG
AG9416_28_52	GGCAACGGAGGCCATCGCCCGTCCCTTCGGAACGGCGCTCGCCCATCTCT
AG9417_28_53	CAGGACCGACTGACCCATGTTCAACTGCTGTTCACATGGAACCCTTCTCC
AG9418_28_54	ACTTCGGCCTTCAAAGTTCTCGTTTGAATATTTGCTACTACCACCAAGAT
AG9419_28_55	CTGCACCTGCGGCGGCTCCACCCGGGCCCGCGCCCTAGGCTTCAAGGCTC
AG9420_28_56	ACCGCAGCGGCCCTCCTACTCGTCGCGGCGTAGCGTCCGCGGGGCTCCGG
AG9421_28_57	GGGCGGGGAGCGGGGCGTGGGCGGGAGGAGGGGAGGAGGCGTGGG
AG9422_28_58	GGGCGGGGGAAGGACCCCACACCCCCGCCGCCGCCGCCGCCGCCCTC
AG9423_28_59	CGACGCACACCACACGCGCGCGCGCGCGCCGCCCCCGCCGCTCCCGTC
AG9424_28_60	CACTCTCGACTGCCGGCGACGGCCGGGTATGGGCCCGACGCTCCAGCGCC
AG9425_28_61	ATCCATTTTCAGGGCTAGTTGATTCGGCAGGTGAGTTGTTACACACTCCT
AG9426_28_62	TAGCGGATTCCGACTTCCATGGCCACCGTCCTGCTGTCTATATCAACCAA
AG9427_28_63	CACCTTTTCTGGGGTCTGATGAGCGTCGGCATCGGGCGCCTTAACCCGGC
AG9428_28_64	GTTCGGTTCATCCCGCAGCGCCAGTTCTGCTTACCAAAAGTGGCCCACTA
AG9429_28_65	GGCACTCGCATTCCACGCCCGGCTCCACGCCAGCGAGCCGGGCTTCTTAC
AG9430_28_66	CCATTTAAAGTTTGAGAATAGGTTGAGATCGTTTCGGCCCCAAGACCTCT
AG9431_28_67	AATCATTCGCTTTACCGGATAAAACTGCGTGGCGGGGGTGCGTCGGGTCT
AG9432_28_68	GCGAGAGCGCCAGCTATCCTGAGGGAAACTTCGGAGGGAACCAGCTACTA
AG9433_28_69	GATGGTTCGATTAGTCTTTCGCCCCTATACCCAGGTCGGACGACCGATTT
AG9434_28_70	GCACGTCAGGACCGCTACGGACCTCCACCAGAGTTTCCTCTGGCTTCGCC
AG9435_28_71	CTGCCCAGGCATAGTTCACCATCTTTCGGGTCCTAACACGTGCGCTCGTG
AG9436_28_72	CTCCACCTCCCCGGCGCGGCGGGCGAGACGGGCCGGTGGTGCGCCCTCGG
AG9437_28_73	CGGACTGGAGAGGCCTCGGGATCCCACCTCGGCCGGCGAGCGCGCCGGCC
AG9438_28_74	TTCACCTTCATTGCGCCACGGCGGCTTTCGTGCGAGCCCCCGACTCGCGC
AG9439_28_75	ACGTGTTAGACTCCTTGGTCCGTGTTTCAAGACGGGTCGGGTGGGTAGCC
AG9440_28_76	GACGTCGCCGCCGACCCCGTGCGCTCGCTCCGCCGTCCCCCTCTTCGGG
AG9441_28_77	GACGCGCGCGTGGCCCCGAGAGAACCTCCCCCGGGCCCGACGGCGCGACC
AG9442_28_78	CGCCCGGGGCGCACTGGGGACAGTCCGCCCCGCCCCCCGACCCGCGCGCG
AG9443_28_79	GCACCCCCCCCGTCGCCGGGGCGGGGGCGCGGGGAGGAGGGGTGGGAGAG
AG9444_28_80	CGGTCGCGCCGTGGGAGGGGTGGCCCGGCCCCCCCACGAGGAGACGCCGG
AG9445_28_81	CGCGCCCCCGCGGGGGAGACCCCCCTCGCGGGGGATTCCCCGCGGGGGTG
AG9446_28_82	GGCGCCGGGAGGGGGGAGAGCGCGGCGACGGGTCTCGCTCCCTCGGCCCC
AG9447_28_83	GGGATTCGGCGAGTGCTGCTGCCGGGGGGGCTGTAACACTCGGGGIGGGT
AG9448_28_84	TTCGGTCCCGCCGCCCCCGCCGCCGCCGCCACCGCCGCCGCCGCCGCCGC

AG9449_28_85	CCCGACCCGCGCGCCCTCCCGAGGGAGGACGCGGGGCCGGGGGGCGGAGA
AG9450_28_86	CGGGGGAGGAGGAGGACGGACGGACGGACGGGGCCCCCCGAGCCACCTTC
AG9451_28_87	CCCGCCGGGCCTTCCCAGCCGTCCCGGAGCCGGTCGCGGCGCACCGCCGC
AG9452_28_88	GGTGGAAATGCGCCCGGCGGCGGCCGGTCGCCGGTCGGGGGACGGTCCCC
AG9453_28_89	CGCCGACCCCACCCCCGGCCCCGCCCGCCCACCCCCGCACCCGCCGGAGC
AG9454_28_90	CCGCCCCCTCCGGGGAGGAGGAGGAGGGGCGGCGGGGGAAGGGAGGGCGG
AG9455_28_91	GTGGAGGGGTCGGGAGGAACGGGGGGCGGGAAAGATCCGCCGGGCCGCCG
AG9456_28_92	ACACGGCCGGACCCGCCGCCGGGTTGAATCCTCCGGGCGGACTGCGCGGA
AG9457_28_93	CCCCACCCGTTTACCTCTTAACGGTTTCACGCCCTCTTGAACTCTCTCTT
AG9458_28_94	CAAAGTTCTTTTCAACTTTCCCTTACGGTACTTGTTGACTATCGGTCTCG
AG9459_28_95	TGCCGGTATTTAGCCTTAGATGGAGTTTACCACCCGCTTTGGGCTGCATT
AG9460_28_96	CCCAAGCAACCCGACTCCGGGAAGACCCGGGCGCGCGCCGGCCGCTACCG
AG9461_28_97	GCCTCACACCGTCCACGGGCTGGGCCTCGATCAGAAGGACTTGGGCCCCC
AG9462_28_98	CACGAGCGGCGCCGGGGAGCGGGTCTTCCGTACGCCACATGTCCCGCGCC
AG9463_28_99	CCGCGGGGCGGGGATTCGGCGCTGGGCTCTTCCCTGTTCACTCGCCGTTA
AG9464_28_100	CTGAGGGAATCCTGGTTAGTTTCTTTTCCTCCGCTGACTAATATGCTTAA
AG9465_28_101	GACTAATATGCTTAAATTCAGCGGGTCGCCACGTCTGATCTGAGGTCGCG
AG9466_5.8_1	AAGCGACGCTCAGACAGGCGTAGCCCCGGGAGGAACCCGGGGCCGCAAGT
AG9467_5.8_2	GCGTTCGAAGTGTCGATGATCAATGTGTCCTGCAATTCACATTAATTCTC
AG9468_5.8_3	GCAGCTAGCTGCGTTCTTCATCGACGCACGAGCCGAGTGATCCACCGCTA
AG9469_16_1	AAACCCTGTTCTTGGGTGGGTGTGGGTATAATACTAAGTTGAGATGATAT
AG9470_16_2	CATTTACGGGGGAAGGCGCTTTGTGAAGTAGGCCTTATTTCTCTTGTCCT
AG9471_16_3	TTCGTACAGGGAGGAATTTGAANGTAGATAGAAACCGACCTGGATTACTC
AG9472_16_4	CGGTCTGAACTCAGATCACGTAGGACTTTAATCGTTGAACAAACGAACCT
AG9473_16_5	TTAATAGCGGCTGCACCATCGGGATGTCCTGATCCAACATCGAGGTCGTA
AG9474_16_6	AACCCTATTGTTGATATGGACTCTAGAATAGGATTGCGCTGTTATCCCTA
AG9475_16_7	GGGTAACTTGTTCCGTTGGTCAAGTTATTGGATCAATTGAGTATAGTAGT
AG9476_16_8	TCGCTTTGACTGGTGAAGTCTTAGCATGTACTGCTCGGAGGTTGGGTTCT
AG9477_16_9	GCTCCGAGGTCGCCCCAACCGAAATTTTTAATGCAGGTTTGGTAGTTTAG
AG9478_16_10	GACCTGTGGGTTTGTTAGGTACTGTTTGCATTAATAAATTAAAGCTCCAT
AG9479_16_11	AGGGTCTTCTCGTCTTGCTGTGTTATGCCCGCCTCTTCACGGGCAGGTCA
AG9480_16_12	ATTTCACTGGTTAAAAGTAAGAGACAGCTGAACCCTCGTGGAGCCATTCA
AG9481_16_13	TACAGGTCCCTATTTAAGGAACAAGTGATTATGCTACCTTTGCACGGTTA
AG9482_16_14	GGGTACCGCGGCCGTTAAACATGTGTCACTGGGCAGGCGGTGCCTCTAAT
AG9483_16_15	ACTGGTGATGCTAGAGGTGATGTTTTTGGTAAACAGGCGGGGTAAGATTT
AG9484_16_16	GCCGAGTTCCTTTTACTTTTTTTAACCTTTCCTTATGAGCATGCCTGTGT
AG9485_16_17	TGGGTTGACAGTGAGGGTAATAATGACTTGTTGGTTGATTGTAGATATTG
AG9486_16_18	GGCTGTTAATTGTCAGTTCAGTGTTTTAATCTGACGCAGGCTTATGCGGA
AG9487_16_19	GGAGAATGTTTTCATGTTACTTATACTAACATTAGTTCTTCTATAGGGTG
AG9488_16_20	ATAGATTGGTCCAATTGGGTGTGAGGAGTTCAGTTATATGTTTGGGATTT
AG9489_16_21	TTTAGGTAGTGGGTGTTGAGCTTGAACGCTTTCTTAATTGGTGGCTGCTT

AG9490_16_22	TTAGGCCTACTATGGGTGTTAAATTTTTTACTCTCTCTACAAGGTTTTTT
AG9491_16_23	CCTAGTGTCCAAAGAGCTGTTCCTCTTTGGACTAACAGTTAAATTTACAA
AG9492_16_24	GGGATTTAGAGGGTTCTGTGGGCAAATTTAAAGTTGAACTAAGATTCTA
AG9493_16_25	TCTTGGACAACCAGCTATCACCAGGCTCGGTAGGTTTGTCGCCTCTACCT
AG9494_16_26	ATAAATCTTCCCACTATTTTGCTACATAGACGGGTGTGCTCTTTTAGCTG
AG9495_16_27	TTCTTAGGTAGCTCGTCTGGTTTCGGGGGTCTTAGCTTTGGCTCTCCTTG
AG9496_16_28	CAAAGTTATTTCTAGTTAATTCATTATGCAGAAGGTATAGGGGTTAGTCC
AG9497_16_29	TTGCTATATTATGCTTGGTTATAATTTTTCATCTTTCCCTTGCGGTACTA
AG9498_16_30	TATCTATTGCGCCAGGTTTCAATTTCTATCGCCTATACTTTATTTGGGTA
AG9499_16_31	AATGGTTTGGCTAAGGTTGTCTGGTAGTAAGGTGGAGTGGGTTTGGGGCT
AG9500_12_1	GTTCGTCCAAGTGCACTTTCCAGTACACTTACCATGTTACGACTTGTCTC
AG9501_12_2	CTCTATATAAATGCGTAGGGGTTTTAGTTAAATGTCCTTTGAAGTATACT
AG9502_12_3	TGAGGAGGGTGACGGGCGGTGTGTACGCGCTTCAGGGCCCTGTTCAACTA
AG9503_12_4	AGCACTCTACTCTTAGTTTACTGCTAAATCCACCTTCGACCCTTAAGTTT
AG9504_12_5	CATAAGGGCTATCGTAGTTTTCTGGGGTAGAAAATGTAGCCCATTTCTTG
AG9505_12_6	CCACCTCATGGGCTACACCTTGACCTAACGTCTTTACGTGGGTACTTGCG
AG9506_12_7	CTTACTTTGTAGCCTTCATCAGGGTTTGCTGAAGATGGCGGTATATAGGC
AG9507_12_8	TGAGCAAGAGGTGGTGAGGTTGATCGGGGTTTATCGATTACAGAACAGGC
AG9508_12_9	TCCTCTAGAGGGATATGAAGCACCGCCAGGTCCTTTGAGTTTTAAGCTGT
AG9509_12_10	GGCTCGTAGTGTTCTGGCGAGCAGTTTTGTTGATTTAACTGTTGAGGTTT
AG9510_12_11	AGGGCTAAGCATAGTGGGGTATCTAATCCCAGTTTGGGTCTTAGCTATTG
AG9511_12_12	TGTGTTCAGATATGTTAAAGCCACTTTCGTAGTCTATTTTGTGTCAACTG
AG9512_12_13	GAGTTTTTTACAACTCAGGTGAGTTTTAGCTTTATTGGGGAGGGGGTGAT
AG9513_12_14	CTAAAACACTCTTTACGCCGGCTTCTATTGACTTGGGTTAATCGTGTGAC
AG9514_12_15	CGCGGTGGCTGGCACGAAATTGACCAACCCTGGGGTTAGTATAGCTTAGT
AG9515_12_16	TAAACTTTCGTTTATTGCTAAAGGTTAATCACTGCTGTTTCCCGTGGG
AG9516_12_17	TGTGGCTAGGCTAAGCGTTTTGAGCTGCATTGCTGCGTGCTTGATGCTTG
AG9517_12_18	TTCCTTTTGATCGTGGTGATTTAGAGGGTGAACTCACTGGAACGGGGATG
AG9518_12_19	CTTGCATGTGTAATCTTACTAAGAGCTAATAGAAAGGCTAGGACCAAACC
AG9519_5_1	AAAGCCTACAGCACCCGGTATTCCCAGGCGGTCTCCCATCCAAGTACTAA
AG9520_5_2	CCAGGCCCGACCCTGCTTAGCTTCCGAGATCAGACGAGATCGGGCGCGTT
AG9521_5_3	TTCCGAGATCAGACGAGATCGGGCGCGTTCAGGGTGGTATGGCCGTAGAC

Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment
JoVE_Materials_Matranga.xlsx

Name of Reagent/ Equipment	Company	Catalog Number
96-Well PCR Plates	VWR	47743-953
Strips of Eight Caps	VWR	47745-512
Nuclease-free water	Ambion	AM9937
TURBO DNase	Ambion	AM2238
PCR cycler		
Agencourt RNAClean XP SPRI beads	Beckman Coulter Genomics	A63987
Real Time qPCR system		
DynaMag-96 Side Skirted Magnet	Invitrogen	12027
70% Ethanol		
qRT-PCR primers	IDT DNA	
5M NaCl	Ambion	AM9760G
1M Tris-HCl pH 7.4	Sigma	T2663-1L
1M Tris-HCl pH 7.5	Invitrogen	15567-027
1M $MgCl_2$	Ambion	AM9530G
Linear acrylamide	Ambion	AM9520
DNA oligos covering entire rRNA region	IDT DNA	
Oligo (dT)	IDT DNA	
Hybridase Thermostable RNase H	Epicentre	H39100
RNase-free DNase Kit	Qiagen	79254
SUPERase-In RNase Inhibitor	Ambion	AM2694
Random Primers	Invitrogen	48190-011

10 mM dNTP mix	New England Biolabs	N0447L
SuperScript III Reverse Transcriptase	Invitrogen	18080-093
Air Incubator		
NEBNext Second Strand Synthesis (dNTP-free) Reaction Buf	New England Biolabs	B6117S
E. coli DNA Ligase	New England Biolabs	M0205L
E. coli DNA Polymerase I	New England Biolabs	M0209L
E. coli RNase H	New England Biolabs	M0297L
0.5M EDTA	Ambion	AM9261
Agencourt AMPure XP SPRI beads	Beckman Coulter Genomics	A63881
Elution Buffer	Qiagen	
Quant-iT dsDNA HS Assay Kit	Invitrogen	Q32854
Qubit fluorometer	Invitrogen	Q32857
Nextera XT DNA Sample Prep Kit	Illumina	FC-131-1096
Nextera XT DNA Index Kit	Illumina	FC-131-1001
Tapestation 2200	Agilent	G2965AA
High Sensitivity D1000 reagents	Agilent	5067-5585
High Sensitivity D1000 ScreenTape	Agilent	5067-5584
BioAnalyzer 2100	Agilent	G2939AA
High Sensitivity DNA reagents	Agilent	5067-4626
Library Quantification Complete kit (Universal)	Kapa Biosystems	KK4824

Comments/Description

50 ml bottle

post RNA extraction step, 2 U/µL, buffer included

any PCR cyclers

beads for RNA cleanup

any system

prepare fresh

see Table 2

see Table 3, order lab-ready at 100 µM

40 nt long, desalted

post selective depletion step

mostly hexamers

with first-strand buffer, DTT

any air incubator cyclers

10x

10 U/µl

10 U/µl

2 U/µl

beads for DNA cleanup

10 mM Tris HCl, pH 8.5

alternative to tapestation, bioanalyzer for library quantification

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Title of Article: Unbiased deep sequencing of RNA viruses from clinical samples

Author(s): C.B. Matranga; A. Gladden-Young; J. Qu; D. Nosenkeman; J.Z. Levin; P.C. Sabeti

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• *Your manuscript has been modified by your editor, please maintain the current formatting throughout the manuscript.* ***Please use the updated manuscript located in your Editorial Manager account (under "File Inventory") (for all subsequent revisions****. Updated manuscript is also attached to this email; Please track the changes in your word processor (e.g., Microsoft Word) or change the text color to identify all of the manuscript edits.*

Thank you for your edits. We have used the updated manuscript and tracked changes in the revised manuscript by changing text color to blue ('54117_R3_120715_cbm').

• *Discussion: Please elaborate on the future applications of this technique, and troubleshooting. *The second last paragraph of the Introduction ("Since most clinical.....these contaminants") discusses the critical steps within the protocol and briefly discusses troubleshooting. Please move this paragraph to the Discussion section.*

We moved the referenced paragraph with minor modifications to the Discussion section in the revised manuscript.

• *Step 2.5: Please rewrite the Note for clarity (especially, stopping point). Does stopping point indicate that the protocol step can be stopped at this point?; 3.5: It is not clear what "Stopping point" means. Please briefly explain stopping point as a "Note". Does stopping point indicate that the protocol step can be stopped at this point?*

In the revised manuscript, we replaced this statement with "Safe cold storage step".

• *Representative Results should refer to all of the results figures. Please briefly discuss Figure 3 in the results section in paragraph form.*

In the revised manuscript, we add a sentence describing Figure 3 in the Representative Results.

• *In Table 2, are the "KGH" and "KULESH" indicators identifiers for the hospital/location at which samples were collected? In your rebuttal letter, you state that "KGH is Kenema Government Hospital in Sierra Leone, where the Ebola primers were tested. Kulesh is the investigator who designed the primer set". Please provide these details in the Table legend. Please define all abbreviations.*

In the revised manuscript, we provided this information and a reference for each.