Library preparation

One-step RT-PCR protocol for amplicon-based viral genome sequencing

Introduction

The Zika virus disease continues to be a threat with periodic outbreaks in tropical and subtropical regions. Sequencing is a valuable tool for monitoring viral spread and identifying new mutations. Targeted amplification is a cost-effective approach in which PCR is used to generate amplicons to tile the entire viral genome prior to preparing the sequencing library. This approach generates enough coverage and depth to produce complete genome sequences even from samples that have partially degraded viral RNA genomes or low viral load.

Here we describe a multiplex one-step RT-PCR workflow for the preparation of next-generation sequencing (NGS) libraries from Zika virus RNA samples using two primer pools tiling the entire Zika virus genome, the Invitrogen™ SuperScript™ IV UniPrime One-Step RT-PCR System, and Invitrogen™ Collibri™ ES DNA Library Prep Kit for Illumina™ Systems. With its high specificity, high processivity, and a universal annealing feature, the SuperScript IV UniPrime One-Step RT-PCR System is an excellent choice for this application. It helps ensure high-level multiplexing capabilities in a simple one-step RT-PCR reaction.

Materials and methods

Samples

Quantitative genomic RNA isolated from the Zika virus strain PRVABC-59 (ATTC, Cat. No. ID VR-1843DQ) molecular standard was used in this study.

Primer pools

To successfully amplify and cover the whole Zika virus genome, published primer pool sequences generated by Primal Scheme software (primalscheme.com) were used [1]. All primers were divided into two primer pools: pool 1 (18 amplicons, 36 primers) and pool 2 (17 amplicons, 34 primers). To help ensure effective amplification in each pool, amplicons in each pool do not overlap with each other (example in Figure 2). Equal volumes of 100 µM primer stocks were combined into two pools following the recommendations for the Primal Scheme software, and later, the combined primer pool stocks were diluted 10-fold. The resulting concentrations of individual primers in the pool were 0.28 µM (pool 1) or 0.29 µM (pool 2).

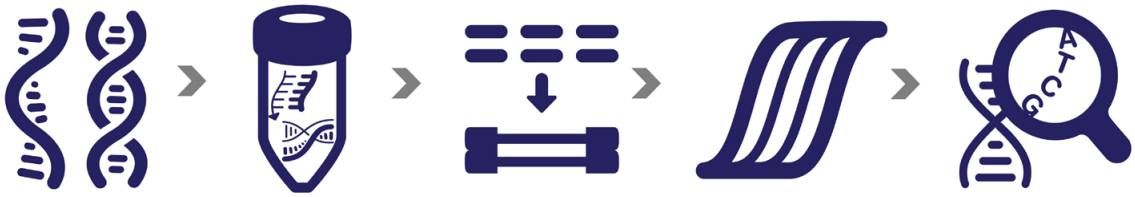

Figure 1. Next-generation sequencing (NGS) workflow for the analysis of viral RNA samples using a targeted one-step RT-PCR approach.

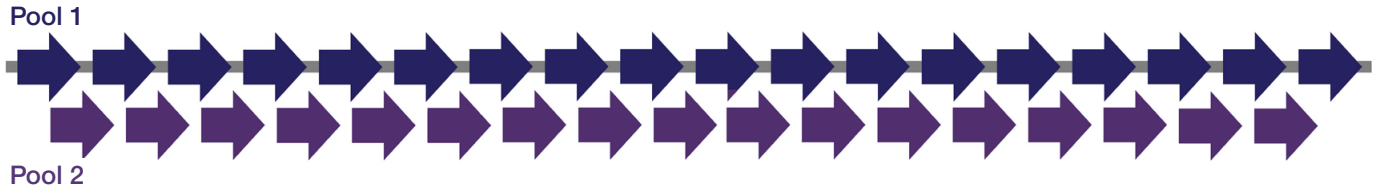

Figure 2. Arrangement of amplicons in primer pools.

One-step RT-PCR

A series of four dilutions of genomic Zika virus RNA (10–10,000 copies/reaction) were used for enrichment amplification using the SuperScript IV UniPrime One-Step RT-PCR System and the two previously made Zika virus genome primer pools. Two one-step RT-PCR reactions were performed in 12.5 µL (one per pool), following the reaction setup in Table 1. The optimized enrichment one-step RT-PCR program is provided in Table 2.

Table 1. Enrichment one-step RT-PCR reaction setup (two reactions per sample).

Reagent	Volume per 12.5 µL
2X SuperScript IV UniPrime RT-PCR Master Mix	6.25 µL
SuperScript IV RT Mix	0.5 µL
Primer pool (1 or 2)	1 µL (final concentration: ~0.02 µM per primer)*
RNA	2.5 µL
Water, nuclease-free	2.25 µL

* Final concentration per primer can vary between 0.015 µM and 0.03 µM depending on the target and size of the primer pool.

Table 2. Enrichment one-step RT-PCR program.

Step	Temperature	Time	Cycles
Reverse transcription	50°C	20 min	1
RT inactivation/initial denaturation	98°C	2 min	35**
Denaturation	98°C	15 sec	
Annealing/extension	60°C*	5 min	
–	4°C	Hold	–

* Annealing/extension temperature can vary from 60°C to 65°C depending on the target or thermal cycler.

** The number of amplification cycles can vary from 30 to 35 depending on the target and size of the primer pool.

Next, 12.5 µL of each enrichment one-step RT-PCR reaction was combined (25 µL total for each sample) and purified with magnetic beads using the Collibri ES DNA Library Prep Kit for Illumina Systems following the "Remove EDTA from reaction" protocol.

Library preparation

Purified amplicons were converted into sequencing-ready libraries by enzymatic fragmentation using the Collibri ES DNA Library Prep Kit for Illumina Systems. The optimized fragmentation reaction setup and program are provided in Tables 3 and 4. After completion of the fragmentation program, the dual-indexed adaptors were ligated according to the standard protocol and prepared libraries were purified using the magnetic beads.

Table 3. Fragmentation reaction setup.

Reagent	Volume per 50 µL
Purified DNA	20 µL
Elution buffer	15 µL
10X Fragmentation and dA-tailing buffer	5 µL
Fragmentation and dA-tailing enzyme mix	10 µL

Table 4. Fragmentation program.*

Step	Temperature	Time
Block pre-cooling	4°C	As required
Fragmentation	37°C	15 min
dA-tailing	65°C	10 min
–	4°C	Hold

* Incubate with lid heated to 80°C.

In this study libraries were not additionally amplified. If samples with low RNA concentrations are used, libraries can be amplified using the Invitrogen™ Collibri™ Library Amplification Master Mix and later purified using magnetic beads.

Average library size was measured on the Agilent™ 2100 Bioanalyzer™ instrument using the Agilent™ High Sensitivity DNA Kit. Library concentration was measured by qPCR using the Invitrogen™ Collibri™ Library Quantification Kit. Libraries were sequentially diluted to 1:10,000, and one of the dilutions was used to quantify 96 samples on two PCR plates. Further details on the library preparation process can be found in the Collibri ES DNA Library Prep Kit user manual (MAN0018545).

Sequencing and data analysis

The libraries were pooled and sequenced on an Illumina™ MiSeq™ instrument using the MiSeq™ Reagent Kit v2 Nano. Alignment with the reference genome NCBI KX377337.1 was performed using the BWA-MEM algorithm (bwa mem – 32 -r 1.0 -k 19 -M -N 6 -v 1) included in the Burrows–Wheeler Alignment Tool.

Results

Four serial dilutions of Zika virus genome RNA were sequenced (10–10,000 copies/reaction) using the optimized target enrichment workflow. The whole Zika virus genome (10 kb) was covered by 35 amplicons, which were amplified using the one-step RT-PCR method. The average percentage of aligned reads was 99.8%, providing 102.1x mean coverage of all samples (Figure 3, Table 5). No amplicons showed <20% sequencing depth, and the average of reads with ≥30x coverage was 95.4%, demonstrating high-quality results that can be used to sequence the Zika virus genome from various amounts of sample.

Table 5. Sequencing results for the whole Zika virus genome.

Sample concentration	Aligned reads	Mean coverage	≥30x coverage
10,000 copies/reaction	99.5%	143.8x	98.6%
1,000 copies/reaction	99.9%	114.7x	98.6%
100 copies/reaction	99.9%	116.9x	93.7%
10 copies/reaction	99.8%	33.1x	90.8%
Average	99.8%	102.1x	95.4%

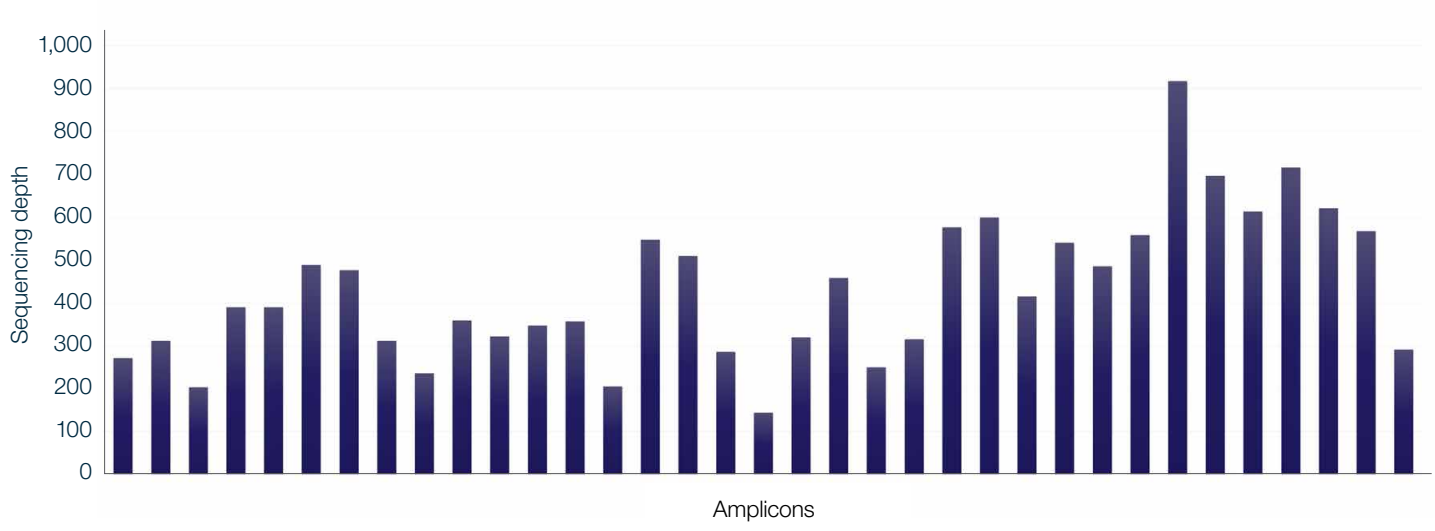

Figure 3. Average sequencing depth of samples containing 10–10,000 copies/reaction of Zika virus genomic RNA.

Conclusions

The amplicon enrichment–based library preparation approach using SuperScript IV UniPrime One-Step RT-PCR System and Collibri ES DNA Library Prep Kits for Illumina Systems provides sufficient sequencing coverage and depth for virus whole-genome sequencing, even at low copy numbers. The one-step RT-PCR amplification step simplifies the workflow by helping to save time and reducing pipetting steps, as well as minimizing possible errors and contamination.

Ordering information

Step	Product	Quantity	Cat. No.
Enrichment one-step RT-PCR	SuperScript IV UniPrime One-Step RT-PCR System, Colored	500 reactions	12597500
		100 reactions	12597100
		25 reactions	12597025
Enrichment one-step RT-PCR	SuperScript IV UniPrime One-Step RT-PCR System, Dye-free	500 reactions	12596500
		100 reactions	12596100
		25 reactions	12596025
Library generation	Collibri ES DNA Library Prep Kit for Illumina Systems	96 preps	A38607096W
Library amplification*	Collibri Library Amplification Master Mix	50 reactions	A38539050
Library quantification	Collibri Library Quantification Kit	500 reactions	A38524500

* Optional.

Reference

1. Quick J, Grubaugh ND, Pullan ST, et al. Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples. *Nat Protoc.* 2017;12(6):1261-1276. doi:10.1038/nprot.2017.066

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