Review Article

Development of Next Generation Sequencing Protocols for dsRNA Viruses

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A B S T R A C T

Next generations sequencing (NGS) is a very important tool to identify pathogens in different forms i.e. viruses, bacteria etc. This helpful in decode life mysteries, detect pathogens and improve life qualities. Next generation sequencing/Massively parallel sequencing it allows millions of sequencing reactions to be carried out in parallel due to this accuracy and sequencing rate is high as compare to other sequencing methods. In this communication we tried to develop a protocol for dsRNA viruses for MIseq platform.

Introduction

Next-generation sequencing methodologies is now changing the way that we understand viruses, particularly in the areas of genome sequencing, evolution, ecology, discovery and transcriptomics. These technologies have opened further applications in virology including routine diagnostic work and new understanding of the interaction between viral and host transcriptomes. Although this opens an exciting era of viral exploration yet we expect new challenges to understand the role of newly discovered viral diversity in both disease and health (Radford *et al.,* 2012, Marston *et al.,* 2013).

In virology NGS methodology offers many benefits like no prior information or knowledge of virus genome is required

Schuster (2008). Furthermore, NGS is even useful in situation where low number of viral load is present. For execution the most commonly used platform is Illumina. We have recently developed a NGS protocol for dsRNA viruses using MiSeq illumina platform which is described in this report.

Protocols

Viral RNA isolation

The viral dsRNA will be extracted from various clinical samples tissue/cell culture isolates/blood/swabs by using different protocols e.g. using Trizol reagents (Attoui *et al.,* 2000) or using various RNA isolation kits available from various companies e.g., RNeasy plus universal mini kit (Qiagen). Samples after trizol extraction are treated either with DNase I (0.5 U/µl; TaKaRa Bio Inc., Otsu, Japan) or with S1 nuclease (27 U/µl; TaKaRa Bio Inc.) at 23°C for 15 min as per method described by Shimada *et al.,* (2015).

This extracted RNA will be further used as template for downstream reactions for sequencing. The steps are following:

cDNA synthesis

First Strand RT

(For low RNA concentrations samples such as swab, use double concentrations)

Mix I:

Random hexamer (50 µM):1µl

RNA: 2µl (50-300ng or cell culture) or 11µl

(serum, plasma, CSF)

Water: Up to 12µl total

5 min @ 75°C, then put on ice

Mix II

5X Buffer	4 µl
0.1M DTT	1 µl
25mM dNTPs	1 µl
RNaseOUT	1 µl
SuperScript III RT (200 units/µl)	1 µl

Add Mix II to Mix I
Total volume 20µl

Prepare Mix II beforehand or while sample incubates at 75°C; add Mix II right after Mix I is in the ice bath; mix briefly, quick spin, and incubate at 25°C for 10min, 50°C for 60min, 85°C for 5min

RNase H

Add RNase H = 1µl; Incubate at 37°C for 20 min, 75°C for 15 min

Second Strand synthesis (work inside clean PCR hood)

cDNA	21 µl
10X NEBuffer 2	10 µl
25mM dNTPs	2 µl
exo- Klenow fragment 5 unit/ul	4 µl
Random hexamers (600ng/ul)	1 µl
Nuclease-free water	62 µl

45 min @ 37°C
Quantify samples with Qubit 2.0, fluorometer. If the sample is less than 0.05 ng/µl, do not use it for library prep.).

Purification of cDNA samples using AM PureXP magnetic beads

1. Take 90µlof cDNA sample, to it add 162 µl beads, and mix for two minutes on VWR shaker. Incubate plates for 5 mins at room temperature.

2. Put this plate on magnetic stand for two minutes at room temperature. Discard the supernatant.
3. While plate on magnetic stand, wash the plates with 80% ethanol for 30 sec., without disturbing the beads. Discard ethanol and proceed to second wash step.
4. While plate on magnetic stand, wash the plates with 80% ethanol for 30 sec., without disturbing the beads.
5. Discard ethanol and incubate the plate at room temperature for 15 minutes. Make sure that ethanol is completely evaporated.
6. Now add 30 µl of elution buffer/NFW to plate and afterwards remove the plate form the magnetic stand. Seal the plate and keep it on shaker for 02 minutes.
7. Again put plate on magnetic stand, transfer whole of the (30 µl) elute to the PCR plate.

*Quantify samples with Qubit 2.0, fluorometer. If the sample is less than 0.05 ng/µl, do not use it for library prep.)

cDNA library preparation

Nextera XT DNA Library Prep Kit
(https://www.illumina.com/products/by-type/sequencing-kits/library-prep-kits/nextera-xt-dna.html)

Library preparation is a very important step in NGS based sequencing. This has 5 sub steps. First

Tagment Genomic DNA, Amplify the Libraries, Cleanup of this, normalize these libraries and finally pool it.

Tagment Genomic DNA

This step uses the Nexteratransposome to tagment gDNA, which is a process that fragments DNA and then tags the DNA with adapter sequences in a single step.

Procedure

1. Tagment DNA Buffer (TD) (10 µl) and normalized gDNA (5 µl) are added in new Hard-Shell skirted PCR plate. Add 5 µl Amplicon Tagment Mix (ATM) to each well. Pipette to mix.
2. Centrifuge at 280 × g at 20°C for 1 minute.
3. Place on the preprogrammed thermal cycler and run the tagmentation program (55°c for 5 minutes, Hold at 10°c). When the sample reaches 10°C, immediately proceed to step 5 because the transposome is still active.
4. Add 5 µlNeutralize Tagment Buffer (NT) to each well. Pipette to mix. Centrifuge at 280 × g at 20°C for 1 minute. Incubate at room temperature for 5 minutes.

Amplify Libraries

This step amplifies the tagmented DNA using a limited-cycle PCR program. PCR adds the Index 1 (i7), Index 2 (i5), and full adapter sequences to the tagmented DNA from the previous step. The index primers and Nextera PCR master mix are added directly to the 25 µl of tagmented gDNA from the previous step. The adapters and sequences are required for cluster formation. Use the full amount of recommended input DNA and the specified number of PCR cycles, which helps ensure high-quality sequencing results. When planning the index scheme for libraries, use the same Index 1 (i7) in each column of the PCR plate. This scheme allows use of a multichannel pipette to transfer indexes from the tubes to the plate.

Procedure

1. [24 libraries] Arrange the index primers in the TruSeq Index Plate Fixture as follows. Arrange Index 1 (i7) adapters in columns 1–6 of the TruSeq Index Plate Fixture. Arrange Index 2 (i5) adapter in rows A–D

of the TruSeq Index Plate Fixture.

2. [96 libraries] Arrange the index primers in the TruSeq Index Plate Fixture as follows. Arrange Index 1 (i7) adapters in columns 1–12 of the TruSeq Index Plate Fixture. Arrange Index 2 (i5) adapter in rows A–H of the TruSeq Index Plate Fixture.

3. Using a multichannel pipette, add 5 µl of each Index 1 (i7) adapter down each column. Replace the cap on each i7 adapter tube with a new orange cap.

4. Using a multichannel pipette, add 5 µl of each Index 2 (i5) adapter across each row. Replace the cap on each i5 adapter tube with a new white cap.

5. Add 15 µl NPM to each well containing index adapters. Pipette to mix.

6. Centrifuge at 280 × g at 20°C for 1 minute.

7. Place on the preprogrammed thermal cycler (72°c for 3 minutes, 95°c for 30 seconds, 12 cycles of: 95°c for 10 seconds, 55°c for 30 seconds, 72°c for 30 seconds, 72°c for 5 minutes, Hold at 10°c).

8. If you are stopping, seal the plate and store at 2°C to 8°C for up to 2 days. Alternatively, leave on the thermal cycler overnight.

Clean up libraries

This step uses AMPure XP beads to purify the library DNA and remove short library fragments.

Procedure

1. Centrifuge at 280 × g at 20°C for 1 minute.

2. Transfer 50 µl PCR product from each well of the PCR plate to corresponding wells of a new midi plate.

3. Add 30 µlAM Pure XP beads to each well. Shake at 1800 rpm for 2 minutes. Incubate at room temperature for 5

minutes.

4. Place on a magnetic stand and wait until the liquid is clear (~2 minutes). Remove and discard all supernatant from each well.

5. Wash two times as follows.

a Add 200 µl fresh 80% EtOH to each well.
b Incubate on the magnetic stand for 30 seconds.
c Remove and discard all supernatant from each well.

6. Using a 20 µl pipette, remove residual 80% EtOH from each well. Air-dry on the magnetic stand for 15 minutes.

7. Remove from the magnetic stand. Add 52.5 µl RSB to each well. Shake at 1800 rpm for 2 minutes. Incubate at room temperature for 2 minutes.

8. Place on a magnetic stand and wait until the liquid is clear (~2 minutes). Transfer 50 µl supernatant from the midi plate to a new Hard-Shell PCR plate. If you are stopping, seal the plate and store at -25°C to -15°C for up to 7 days. Quantify library with fragment analyzer and Qubit 2.0, fluorometer.

Normalization of libraries by denaturation of libraries

MiSeq Reagent Kit v3 = 4 nM library—Results in a 6–20 pM loading concentration.

MiSeq Reagent Kit v2 = **4 nM library**—Results in a 6–20 pM loading concentration.

nM library—Results in a 6–10 pM loading concentration.

Denature a 4 nMlibrary

1. Combine the following volumes in a microcentrifuge tube. 4 nM library (5 µl) and 0.2 N NaOH freshly prepared (5 µl),

Vortex briefly and then centrifuge at 280 × g for 1 minute.

2. Incubate for 5 minutes at room temperature.
3. Add 990 µl prechilled HT1 to the tube containing denatured library. The result is 1 ml of a 20 pM denatured library.

Denature a 2 nM library

1. Combine the following volumes in a microcentrifuge tube. 2 nM library (5 µl) and 0.2 N NaOH (5 µl), Vortex briefly

and then centrifuge at 280 × g for 1 minute.

2. Incubate for 5 minutes at room temperature.
3. Add 990 µl prechilled HT1 to the tube containing denatured library. The result is 1 ml of a 10 pM denatured library.

Dilute denatured 20 pM library

1. Dilute to the desired concentration using the following volumes.

Concentration	6 pM	8 pM	10 pM	12 pM	15 pM	20 pM
20 pM library	180 µl	240 µl	300 µl	360 µl	450 µl	600 µl
Prechilled HT1	420 µl	360 µl	300 µl	240 µl	150 µl	0 µl

2. Invert to mix and then pulse centrifuge.

Dilute PhiX to 4 nM

Combine the following volumes in a microcentrifuge tube.10 nMPhiX library (2µl), 10 mMTris-Cl, pH 8.5 with 0.1% Tween 20 (3 µl)

Denature PhiX Control

Combine the following volumes in a microcentrifuge tube.4 nMPhiX library (5 µl), 0.2 N NaOH (5 µl), Vortex briefly to mix. Centrifuge at 280 × g for 1 minute. Incubate at room temperature for 5 minutes.

Dilute denatured PhiX to 20 pM

Add prechilled HT1 to the denatured PhiX library. Denatured PhiX library (10 µl), Prechilled HT1 (990 µl). The result is 1 ml of a 20 pMPhiX library.

Combine library and PhiXcontrol as shown in below table. This put on ice until you are ready to load it onto the reagent cartridge.

	Most Libraries (1% Spike-In)	Low-Diversity Libraries (≥ 5% Spike-In)
Denatured and diluted PhiX	6 µl	30 µl
Denatured and diluted library (from protocol A or protocol B)	594 µl	570 µl

MiSeq workflow

MiSeq reagent kit microv2, MiSeq reagent kit v3

1. Prepare the prefilled reagent cartridge for use.
2. Load the library mix onto the reagent cartridge in the designated reservoir.
3. From the software interface, select Sequence to start the run setup steps. Wash and thoroughly dry the flow cell. Load the flow cell.
4. Load the PR2 bottle and make sure that the waste bottle is empty. Load the

reagent cartridge.
5. Review run parameters and pre-run check results. Select Start Run.
6. Monitor your run from the MCS interface or from another computer using Sequencing Analysis Viewer (SAV).
7. Perform a post-run wash.

Cluster generation

During cluster generation, single DNA molecules are bound to the surface of the flow cell, and then bridge-amplified to form clusters.

Sequencing

Following cluster generation, clusters are imaged using LED and filter combinations specific to each of the 4 fluorescently labelled dideoxynucleotides. After imaging of a tile is complete, the flow cell is moved into place to expose the next tile.

The process is repeated for each cycle of sequencing. Following image analysis, the software performs base calling, filtering, and quality scoring.

Analysis

When the run is complete, the MiSeq Reporter analysis software launches automatically to perform secondary analysis, which includes alignment and variant calling.

References

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