

Chapter 11

Partial Purification of IBV and Subsequent Isolation of Viral RNA for Next-Generation Sequencing

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Abstract

RNA viruses are known for a high mutation rate and rapid genomic evolution. As such an RNA virus population does not consist of a single genotype but is rather a collection of individual viruses with closely related genotypes—a quasispecies, which can be analyzed by next-generation sequencing (NGS). This diversity of genotypes provides a mechanism in which a virus population can evolve and adapt to a changing environment. Sample preparation is vital for successful sequencing. The following protocol describes the process of generating a high-quality RNA preparation from IBV grown in embryonated eggs and then partially purified and concentrated through a 30 % sucrose cushion for NGS.

Key words Quasispecies, Next-generation sequencing, RNA, Infectious bronchitis virus (IBV)

1 Introduction

RNA viruses are known for a high mutation rate and rapid genomic evolution. As such an RNA virus population does not consist of a single genotype but is rather a collection of individual viruses with closely related genotypes—a quasispecies [1]. This diversity of genotypes provides a mechanism in which a virus population can evolve and adapt to a changing environment.

It is becoming increasingly important to understand virus population dynamics and the evolution of quasispecies. Standard RT-PCR and sequencing assembly methods in which genomic sequences are generated from the consensus of all aligned reads are not sufficient. To understand the depth of diversity in the population, next-generation sequencing (NGS), commonly referred to as deep sequencing is used. This process involves the parallel sequencing of genomic fragments, generating, depending on the protocol and sample preparation, thousands to millions of short sequencing reads in a single run which ultimately allows for greater coverage at each individual nucleotide.

Recent research has utilized NGS to model the mutational dynamics of bovine coronavirus during adaptation to new host environments, revealing the presence of two distinct circulating genotypes that altered in frequency depending on the host cell type [2]. Cotten et al. [3] studied an outbreak of MERS coronavirus (MERS-CoV) using a combination of NGS from clinical samples and phylogenetic analysis to map transmission of the virus within a hospital, providing information about the emergence and evolution of MERS-CoV. A further study incorporating greater numbers of clinical samples determined the evolutionary rate of MERS-CoV and identified regions of the genome that are under positive selection pressure [4].

Sample preparation is key to successful deep sequencing. It is important to enrich the RNA of interest and then to generate a high-quality preparation which is DNA free. The following protocol describes the process of generating a high-quality RNA preparation from IBV grown in embryonated eggs, and then partially purified and concentrated through a 30 % sucrose cushion, which was suitable for use in 454 sequencing requiring a minimum quantity of 200 ng in 19 μ l. The growth of IBV in embryonated eggs has been described previously and is not discussed in this protocol (*see* Chapter 7 for further information). It is important to start this protocol with allantoic fluid containing IBV of high titer with 10^6 – 10^7 pfu/ml preferable. The fluid must also be free of membrane or other solid masses and ideally be free from blood. If the IBV in question causes hemorrhaging of the blood vessels, it is important to harvest the allantoic fluid before this happens.

2 Materials

2.1 *Partial Purification of IBV*

1. 50 ml Falcon tubes.
2. 30 % sucrose (w/v) in PBS adjusted to pH 7.2 with HCl, filtered through 0.22 μ m.
3. Refrigerated benchtop centrifuge.
4. SureSpin 630 rotor and Sorvall OTD65B ultracentrifuge or equivalent.
5. Beckman ultra-clear (25 \times 89 mm) ultracentrifuge tubes or equivalent.

2.2 *RNA Extraction*

1. TRIzol reagent.
2. 75 % ethanol.
3. Isopropanol.
4. Chloroform.

3 Methods

3.1 *Partial Purification of IBV*

1. Place 25 ml of IBV-infected allantoic fluid into a 50 ml Falcon tube and centrifuge for 10 min, $1,150\times g$, $4\text{ }^{\circ}\text{C}$ in a benchtop centrifuge (*see Note 1*).
2. Take the supernatant and layer on top of 10 ml 30 % sucrose in an ultracentrifuge tube (*see Note 2*). Balance the tubes carefully.
3. Centrifuge for 4 h, $102,400\times g$, $4\text{ }^{\circ}\text{C}$ in an ultracentrifuge.
4. Remove the supernatant in layers, careful not to disturb the pellet (*see Note 3*).
5. Wipe the sides of the tube with tissue and proceed directly to the next stage, RNA extraction.

3.2 *RNA Extraction*

1. Add 1 ml TRIzol reagent directly to the virus pellet from **step 5**, Subheading **3.1**. Carefully pipette up and down to mix (*see Note 4*).
2. Incubate for 5 min at room temperature (*see Note 5*).
3. Add 200 μl chloroform and mix by shaking for 15 s.
4. Incubate for 3 min at room temperature.
5. Centrifuge in a benchtop centrifuge for 15 min, $4\text{ }^{\circ}\text{C}$, $12,075\times g$.
6. Carefully take the aqueous top layer, which should be clear, and place in a clean 1.5 ml tube (*see Note 6*).
7. Add 0.5 ml isopropanol and incubate for 10 min at room temperature (*see Note 7*).
8. Centrifuge for 10 min at $2,100\times g$, $4\text{ }^{\circ}\text{C}$ in a benchtop centrifuge.
9. Carefully remove the supernatant without disturbing the pellet (*see Note 8*).
10. Add 0.75 ml 75 % ethanol to the pellet and mix by pipetting.
11. Centrifuge for 10 min, $12,075\times g$, $4\text{ }^{\circ}\text{C}$ in a benchtop centrifuge.
12. Remove the supernatant, very carefully, and wipe the sides of the tube with tissue (*see Note 9*).
13. Air-dry the pellet for 5–10 min (*see Note 10*).
14. Resuspend the pellet in 25 μl RNAase-free sterile water (*see Note 11*) and store at either -20 or $-80\text{ }^{\circ}\text{C}$.

4 Notes

1. This first spin is to remove any solid matter from the IBV infected allantoic fluid.
2. It is important that the layers do not mix.

3. The pellet will be difficult to see and will have a speckled appearance. The pellet should not be colored.
4. Make sure the pellet is completely resuspended in the TRIzol reagent.
5. This incubation step allows the TRIzol reagent to break down the virus particles.
6. It is important to take the top aqueous layer only. The interface or organic layer, which will have a cloudy white appearance, contains DNA and protein. It is therefore best to be cautious when taking the top layer and is preferable to leave a little bit behind rather than risk contaminating the RNA sample.
7. When precipitating RNA from small sample quantities RNase-free glycogen can be added at this stage. The glycogen will act as a carrier to the aqueous phase but will be co-precipitated with the RNA. It is important therefore to consider if this will have implications to downstream applications.
8. It is highly unlikely that a pellet will be visible; a little bit of faith is required at this stage. It helps to position the tubes in the centrifuge so that the general location of the pellet can be estimated. It is prudent to keep the supernatant, and store on ice, until you are sure the RNA extraction has been successful.
9. Keep the supernatant, and store on ice, until you are sure the RNA extraction has been successful.
10. Do not allow the RNA to dry completely as the pellet can lose solubility.
11. Make sure the pellet is completely dissolved (partially dissolved RNA samples have an A260/280 ratio <1.6) and then assess the quantity and quality using a NanoDrop or RiboGreen assay.

References

1. Domingo E, Sheldon J, Perales C (2012) Viral quasi species evolution. *Microbiol Mol Biol Rev* 76:159
2. Borucki MK, Allen JE, Chen-Harris H et al (2013) The role of viral population diversity in adaptation of bovine coronavirus to new host environments. *PLoS One* 8: e52752
3. Cotten M, Lam TT, Watson SJ et al (2013) Full-genome deep sequencing and phylogenetic analysis of novel human betacoronavirus. *Emerg Infect Dis* 19:736B–742B
4. Cotten M, Watson S, Zumla A et al (2014) Spread, circulation, and evolution of the Middle East respiratory syndrome coronavirus. *MBio* 5: pii: e01062–13. doi:10.1128/mBio.01062–13