

Simple and Rapid Purification of Infectious Laryngotracheitis Virus DNA from Infected Tissue

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Abstract: Little is known about the structure and physicochemical properties of the Infectious Laryngotracheitis Virus (ILTV) genome until recently a few years. To date, there is no protocol that is suitable for preparation of pure cell-associated herpesvirus DNA isolates from infected tissues or from tissue culture. The products of traditional methods are often found to be contaminated with host cellular DNA, especially for preparations of the large ILTV DNA genome. In addition, there is a need to develop methods for the isolation of highly purified viral DNA from cell culture or tissue samples to be used for high-throughput nucleotide sequencing. In this study, the isolation of ILTV from Chorioallantoic Membranes (CAM) was chosen as the model to test a method for purification of cell-associated herpesvirus DNA. The protocol was a combination of Triton X-100 lysis, nuclease treatment, nuclease denaturation, phenol-chloroform extraction and subsequent selective removal of small DNA fragments. The results showed that the cell background contamination was reduced significantly to a level that was suitable for 454 pyrosequencing and downstream applications such as cloning of viral DNA fragments or transfection of genomic viral DNA. This novel protocol, therefore has several advantages compared with existing methods.

Key words: Infectious laryngotracheitis virus, cell-associated herpesvirus, DNA isolation, infected tissue, treatment, China

INTRODUCTION

Avian Infectious Laryngotracheitis (ILT) is a respiratory disease caused by Herpesviridae Alphaherpesvirinae Gallid herpesvirus 1 (Bagust *et al.*, 2000). Natural infections of Infectious Laryngotracheitis Virus (ILTV) are limited to galliform birds and cause an acute respiratory disease which can cause significant mortality and loss of productivity in the poultry industry (Cover and Benton, 1958; Hughes *et al.*, 1991). For this virus, the morbidity is high but the mortality is lower than that found in the peracute form between 10 and 30%. Signs of infection include spasms of coughing and gasping with nasal and oral discharge and reduced egg production.

In addition, infected birds can become latent and virus can be re-excreted at a later date without clinical signs. Live attenuated ILT vaccines are available to protect against the disease but several reports have

implicated vaccine viruses as a factor in the possible recurrence and spread of the disease (Bagust and Johnson, 1995; Chin, 2009; Hughes *et al.*, 1991).

Little is known about the structure and physicochemical properties of the ILTV genome until recently a few years. This situation may be due to the difficulty in obtaining sufficient amounts of high quality ILTV virions of this cell-associated virus (Honda *et al.*, 1994; Honda, 1994; Taneno *et al.*, 1991, 1990). In previous studies, cell-free whole virus preparations have given poor yields, as the virus replicates poorly in Chicken Kidney (CK) cells to give relatively low titres. Hirt (1967) published the initial protocol for the isolation of polyoma virus DNA and subsequently, many researchers have used this method to obtain pure cell-free whole virus (Eizuru, 1984; Hirt, 1967; Pater *et al.*, 1976; Pignatti *et al.*, 1979; Rosenthal *et al.*, 1983; Walboomers and Schegget, 1976). However, these methods were found not to be applicable for ILTV which is a cell-associated virus. To

date, most molecular genetic studies on ILTV have used cell culture adapted strains as there is no ideal protocol that gives ILTV virions of sufficient high quality from samples isolated from Chorioallantoic Membrane (CAM). In addition, it is difficult to prepare sufficient amounts of pure viral DNA from clinical specimens and infected CAM.

Previous studies have used physical or chemical methods to release and purify cell-associated viruses from cells or tissue (Esposito, 1981; Hartley and Bowen, 1975; Wechsler *et al.*, 1985), however these protocols did not avoid cellular DNA contamination due to action of co-pelleting. Sinzger *et al.* (1999) developed a protocol to limit cellular DNA contamination by digestion of the cellular DNA into fragments <400 bp in length. These protocols have evolved to include a nuclease step either before or after centrifugation as it is difficult to removal cellular DNA by density gradient separation due to the similarity between virus and host-cell DNA densities (Muggeridge and Fraser, 1986; Plummer *et al.*, 1969; Sinzger *et al.*, 1999; Spiker *et al.*, 1983; Su, 2002). These low-molecular-weight DNA fragments are still present in the purified viral DNA and therefore may affect downstream applications. One group (Volkening and Spatz, 2009) improved this protocol by inclusion of an optimized Polyethylene Glycol (PEG) precipitation step to remove low-molecular-weight DNA fragments.

Samples produced using this protocol were of high quality viral DNA that was suitable for 454 pyrosequencing, a technology that does not rely on shotgun cloning of fragmented DNA. This method is perfect for specimens that have been produced by cell culture, however the experimental data showed this method was unfortunately not applicable for specimen isolated from infected tissues.

ILTV DNA replication, transcription and virus particle packaging takes place inside the cell nucleus (Guo, 1993; Prideaux, 1992), however no suitable cell line has been found for the growth of ILTV in tissue culture, although the LMH cell line can be used to grow ILTV but some adaption to this cell line would be required for field isolates, furthermore infectious virus is shed into the culture supernatant after a series of passages in primary cell culture. Therefore, the usual method for virus growth is to use chicken embryos or primary CK cells which means that a method for preparation of pure viral DNA from infected tissue is needed.

To this end, the aimed to develop an improved method for the preparation of pure viral DNA based on the established methods. This protocol aimed to

yield viral DNA of sufficient purity for downstream applications such as Restriction Fragment Length Analyses (RFLA), cloning of viral DNA fragments and transfection of genomic viral DNA. This protocol may be suitable for the isolation of other cell-associated alphaherpesviruses such as Pseudorabies Virus (PrV) (Caughman, 1985; Ihara *et al.*, 1983).

MATERIALS AND METHODS

Propagation and collection of the virus strain: The ILTV stain was isolated from commercial birds in Guangdong Province of China that had severe signs of disease which included conjunctivitis, gasping, coughing, sneezing, nasal discharge and expectoration of bloody mucus. Trachea, lungs and conjunctive tissues from these birds were collected and were homogenized in Phosphate Buffered Saline (PBS) pH 7.4 that contained 4000 U of freshly added penicillin-streptomycin stock solution and the homogenate was clarified by centrifugation at 2000 rpm for 5 min at 4°C. Clarified supernatants were inoculated via the CAM into five, 10 days old Specific-Pathogen-Free (SPF) embryonated chicken eggs (Guangdong Wen's Food Co., Ltd. China), a 200 µL aliquot was given per egg. CAMs were harvested and homogenized at 6 days post inoculation followed by four serial passages following the same procedure.

Preparation of rough nucleus: The infected CAMs were shredded by high-speed disintegration placed into 50 mL tubes and were freeze-thawed through three cycles at room temperature, next the samples were pelleted at 3000 g for 5 min at 4°C and the supernatant discarded. The pellet was resuspended in 20 mL of cold permeabilization buffer (320 mM sucrose, 5 mM MgCl₂, 10 mM Tris-HCl (pH 7.5) and 1% Triton X-100) and placed on ice for 10 min and then sonicated on ice with 2 bursts of 30 sec each. The samples were centrifuged at 300 g for 10 min at 4°C at which speed nucleus remained in the supernatant and the pellets were discarded, this step was repeated 3 times. The supernatants were then pelleted at 5000 rpm for 10 min at 4°C. The resulting pellet was washed 5 times with 40 mL PBS (pH 7.4) and pelleted at 5000 rpm for 10 min at 4°C to remove contaminating cellular fragments such as plasma membrane.

Elimination of contaminating cellular nucleic acid and mitochondria DNA: Three different protocols were compared to select the most appropriate method (Table 1). The details of the steps are as follows:

Table 1: Three protocols to release cellular DNA before digestion and stop reagent

Protocol	Cellular DNA release reagents and incubation time	Stop reagent
1	Cell mitochondria isolation solution, 2× nuclease buffer (2 h)	3 vol. 100% ethanol
2	Nuclei buffer, 2× nuclease buffer (30 min)	Final concentration of 1.2 mM EDTA
3	Cell mitochondria isolation solution, 2× nuclease buffer (2 h)	Final concentration of 1.2 mM EDTA

First protocol: The rough nucleus were resuspended in two volumes of cell mitochondrion isolation solution (Beyotime Institute of Biotechnology, China) to isolate host nucleolus and mitochondrial DNA. The samples were incubated at room temperature for 2 h before addition of two volumes of 2× nuclease buffer (40 mM PIPES (pH 7.0), 7% sucrose, 20 mM NaCl, 2 mM CaCl₂, 10 mM 2-mercaptoethanol and 200 μM PMSF) that contained 150 U of freshly added micrococcal nuclease (TaKaRa, 20U μL⁻¹, Dalian, China) and 10 μL of RNase A (TaKaRa, 100 mg mL⁻¹, Dalian, China). Cellular and unpackaged viral nucleic acids were degraded by incubation for 1 h at 37°C. The reaction was stopped by the addition of three volumes of 100% ethanol (-20°C), to give a final concentration of 75% ethanol and left for 10 min at 4°C. The same volume of 100% ethanol (-20°C) was added to denature micrococcal nuclease and cells left for 5 min at 4°C. The samples were pelleted at 10,000 rpm in a Eppendorf F34-6-38 rotor for 30 min at 4°C and the supernatants were removed completely. Next, 800 μL of digestion buffer (100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 25 mM Ethylenediaminetetraacetic Acid (EDTA) (pH 8.0), (1% SDS) that contained 15 μL of freshly added proteinase K (20 mg mL⁻¹) was then added to each sample and samples were mixed carefully to avoid shear of viral DNA and then incubated for 5 h at 55°C.

Second protocol: Viral DNA was purified using micrococcal nuclease digestion and polyethylene glycol precipitation as described by Volkening and Spatz (2009).

Third protocol: Viral DNA was prepared as described in protocol 1, except that the stop solution was 1.2 mM EDTA, instead of three volumes of 100% ethanol.

Extraction of total DNA: To remove proteins, 1 volume of saturated phenol (pH 8.0) was carefully mixed with the sample and the sample centrifuged for 3 min at a speed of 17,000 g. The aqueous phase was re-extracted 6 times with equal volumes of phenol/chloroform (50/50%, v/v) followed by 3 min centrifugation at 17,000 g. The redundant saturated phenol was removed by use of equal volume of chloroform and 3 min centrifugation at 17,000 g. A 0.5 volume of 7.5 mol L⁻¹ ammonium acetate and two volumes of 100% ethanol (-20°C) were added to the aqueous phase and the sample mixed gently by tapping, left for 10 min at -80°C and then precipitated by centrifugation at 17,000 g for 20 min at 4°C. The DNA pellet was washed with 300 μL of cold 70% ethanol, centrifuged for 10 min at 16,000 g and dried in an oven for 5 min at 65°C then resuspended in 200 μL Tris-EDTA buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) and the concentration of DNA was measured at 260 nm on a spectrophotometer. A 10 μL aliquot of the sample was

separated by agarose gel (0.6%) electrophoresis and stored at -20°C for downstream applications. This procedure was applied to the samples prepared from all 3 of the protocols described before.

Polyethylene glycol 8000 precipitation for selective removal of small DNA fragments: In the presence of 10 mM MgCl₂, the DNA solution was mixed with PEG at final concentration of 5%. After mixing, tubes were held at room temperature for 10 min before centrifugation (17,000 g for 10 min at room temperature). The supernatants were removed completely and the pellet was washed twice with 70% ethanol (17,000 g for 10 min at room temperature) and then resuspended in 50 μL TE (10 mM Tris-HCl (pH 7.5), 1 mM EDTA). The DNA solution after PEG precipitation was quantified at 260 nm in a spectrophotometer and detected by 0.6% gel electrophoresis and stored at -20°C for downstream applications. The list of components and their doses is shown in Table 2.

Detection of purified DNA: Researchers designed primers to amplify 1015 bp fragments of the chicken *β-actin* gene (based on Genbank Accession No. X00182) to detect contaminating host DNA. The primer sequences were A1 (5'-CAT CAC CAT TGG CAA TGA GAG G-3') and A2 (5'-GAT TCA TCG TAC TCC TGC TTG C-3'). In addition, researchers also designed primers to amplify 713 bp fragments of a chicken mitochondrion gene. The primer sequences were M1 (5'-CCA ACT ACC CAA CTA TCA ATA AAC A-3') and M2 (5'-TGA GTG GAA GAA GGC TCA GAA GAA-3').

Primers were designed to amplify 427 bp fragments of the virus Thymidine Kinase (*TK*) gene based on sequences published previously in the Genbank database (based on Genbank Accession No. EU360949) which included sequences for TK1 (5'-AAC TTG AAT GTC GGG AGG CG-3') and TK2 (5'-ACG TTG GAG GTA GGT GGT AG-3') DNA was synthesised using reaction conditions that were suitable for all three sets of primers as follows: one cycle of 94°C for 5 min as initial denaturation followed by 28 cycles of denaturation at 94°C for 40 sec, annealing reaction at 54°C for 40 sec and extension at 72°C for 40 sec and a final extension at 72°C for 10 min. The 25 μL PCR reactions contained 200 mM each of dATP, dCTP, dGTP and dTTP, 1 mM MgCl₂, 250 mM each of the primers, 1 μL Taq DNA polymerase (Takara, China), 5 μL of 10×Taq DNA polymerase buffer and 5 μL extracted DNA as

Table 2: Composition of sample used for the preparation for selective removal of small DNA fragments

Reagents and samples	Dose
15% PEG 8000 (w/w)	166.7 μL
1 M MgCl ₂	5 μL
Total DNA	40-288.3 μL
1× TE (10 mM Tris-HCl (pH 7.5), 1 mM EDTA)	Add to 500 μL

template. Finally, 10 μ L aliquots of the Polymerase Chain Reaction (PCR) products were separated by agarose gel (1%) electrophoresis and stained with SYBR Green I solution. At the same time, three sets of primers were used to check the rough total DNA and DNA solution purified previously using PEG 8000.

RESULTS AND DISCUSSION

Preparation of rough nucleus: ILTV DNA replication, transcription and virus particle packaging takes place inside host cell nucleus, therefore to obtain virus particles, it is necessary to purify these cell nucleus. Researchers showed that centrifugation at 300-3000 g was suitable for the preparation rough nucleus and these conditions gave an optimal balance of rough nucleus yield and purity.

Elimination of contaminating cellular nucleic acid and mitochondrial DNA: In order to reduce host DNA contamination in the preparations researchers developed a novel protocol and compared its efficiency with that of other published method. Nuclease treatment resulted in significant reduction of cellular DNA (Fig. 1 and 2).

Researchers obtain three different samples (Table 1). These samples were amplified to test the degree of host DNA contamination. All three protocols did not result in amplification of β -action after 28 reaction cycles. All three protocols resulted in the elimination of contamination with cellular nucleic acid (Fig. 1, lanes 7, 8 and 9). However, there was still abundant mitochondrial DNA present in samples isolated using the second protocol (Fig. 1 and

lane 5). Therefore, researchers concluded that the Volkening protocol (Volkening and Spatz, 2009) was not suitable for extraction of viral DNA from infected tissue and that the first and the third protocols could remove mitochondrial DNA contamination (Fig. 1, lanes 4 and 6). Primers TK1 and 2 amplified only the correct sized band (Fig. 1, lanes 1-3) after 28 reaction cycles despite PCR being a high sensitivity test method.

Stop reaction using micrococcal nuclease: To remove contaminating cellular DNA, the protocols initially included a nuclease digestion step before extraction of total DNA and EDTA was added at a final concentration of 1.2 mM to stop the reaction.

The experimental data showed this addition was not suitable to stop the reaction (Fig. 2, lanes 2 and 3). To find the ideal stop reagent, researchers designed two protocols (Table 1) using three different DNA samples as described before. A 5 μ L aliquot of the extraction product was separated by agarose gel (0.6%) electrophoresis and stained with SYBR Green I solution DNA (Fig. 2).

Polyethylene glycol 8000 precipitation for selective removal of small DNA fragments: Cellular and unpackaged viral nucleic acids were degraded in the micrococcal nuclease step before extraction of viral DNA (Fig. 2). Size-selective precipitation of DNA fragments with PEG is a long-established Dna Purification Method (Dunn and Blattner, 1987; Humphreys *et al.*, 1975; Just, 1983; Lis, 1980; Paithankar and Prasad, 1991; Sambrook *et al.*, 1989). Hartley and Bowen (1996) reported that a concentration of <5% PEG 8000 did not precipitate the 2652 bp fragments in the presence of 10 mM $MgCl_2$ and incubation of the PEG mixtures prior to centrifugation did not affect recovery of DNA.

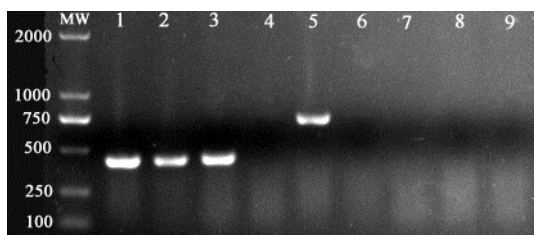


Fig. 1: Polymerase Chain Reaction (PCR) analysis of samples obtained using three different protocols. MW: DL2000 DNA Marker; lane 1, 4 and 7: Results of first protocol sample were subjected to PCR using primers TK1 and TK2; M1 and M2 and A1 and A2, respectively. Lane 2, 5 and 8: Results of second protocol sample were subjected to PCR using primers TK1 and TKA2; M1 and M2 and A1 and A2, respectively. Lane 3, 6 and 9: Results of third protocol sample were subjected to PCR using primer TK1 and TK2; M1 and M2 and A1 and A2, respectively

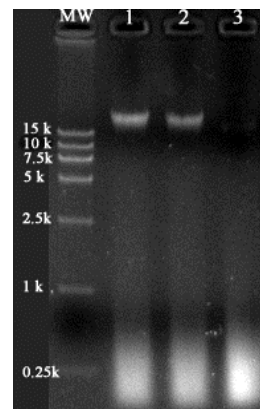


Fig. 2: The result of two different stop reagents. Lane 1: The results of first protocol sample; Lane 2: The results of second protocol sample; Lane 3: The results of third protocol sample. Molecular weight marker (lanes MW): The DL15000 DNA marker

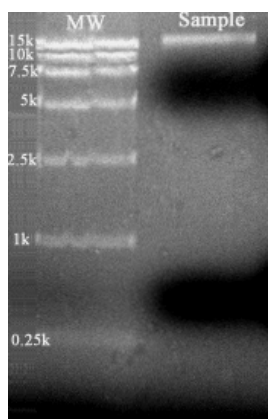


Fig. 3: The DNA used first protocol to prepare after Polyethylene Glycol (PEG) 8000 precipitation for removal of small DNA fragments. Molecular weight marker (lane MW) is the DL15000 DNA marker (Takara); Sample is purification of DNA

Researchers used the protocol described by James and Bowen (1996) to harvest the high MW DNA. As a result, researchers confirmed that it gave an optimal balance of yield and purity at a final concentration of 5% PEG plus 10 mM $MgCl_2$ (Fig. 3).

ILTV DNA replication, transcription and virus particle packaging takes place inside host cell nucleus, therefore to obtain virus particles, it is necessary to purify these cell nucleus. Theoretically, each cellular component can be concentrated easily by centrifugation because their sedimentation coefficient differs by more than two orders of magnitude (Wilson and Walker, 2006). In practice, this is difficult to do. One reason is that the CAM is a complicated structure and component tissue that is composed mainly of allantoic epithelial, stromal and villous epithelial cells (Coleman and Terepka, 1972; Gabrielli and Accili, 2010; Leeson and Leeson, 1963). As reported by Dunn and Fitzharris (1979), most superficial cells of the chorionic epithelium appear relatively electron lucent and contain euchromatic nuclei, prominently mitochondria, perinuclear golgi membranes and microfilaments. Another reason is that the mutual viscosity of components of the CAM mixture is very high after shredding by high-speed disintegration and therefore it is difficult to isolate each cellular component. In this study, researchers described a method suitable for preparation rough nucleus despite those difficulties that gives an optimal balance of yield and purity.

The similarity between viral and host-cell DNA densities have hampered the use of high-yield density gradient separation as contamination of preparations with host-cell DNA can occur: ILTV 1.704 g cm^{-3} compared with CK cell DNA 1.702 g cm^{-3} (Plummer *et al.*, 1969). In

addition, the protocols based on $CsCl_2$ gradients (Huang *et al.*, 1973) are more complicated, time consuming and may also be limited by the small amounts of viral DNA present in these cultures. Therefore, standard procedures for preparation of pure viral DNA from gradient fractions of virus particles are not applicable for these isolates but a solution could be to add nuclease before extraction of total DNA.

Existing protocols are suitable for isolation of virion DNA from cell culture but not applicable for these isolates from infectious tissue such as infected ILTV CAM. In order to reduce host DNA in the preparations, researchers developed a novel protocol of nuclease treatment that resulted in significant reduction of cellular DNA (Fig. 1 and 3).

The experimental data (Fig. 1 and 2) showed that the current method (second protocol) was not suitable for preparation of purified viral DNA from ILTV infected CAM as the samples still contained contaminating cellular components such as the mitochondria. Researchers concluded that the Volkening protocol (Volkening and Spatz, 2009) was not suitable for extraction viral DNA from infected tissue as did not take into account the contamination of mitochondrial DNA.

One potential reason may be the CAM has complicated structure. It has been reported that CAM cells contain large amounts mitochondria (Schmitz and Riesner, 2006). Another reason might be that the viscosity of the mixture CAM is very high after shredding by high-speed disintegration.

These preparations were still contaminated with cellular DNA and mitochondria due to the components co-pelleting together with the virus particle. Researchers speculated that the nuclei buffer cannot lyse rudimental mitochondria as it possesses a double layer plasma membrane structure. To solve this difficult problem, researchers used the first and the third protocol that preferably removed the mitochondrial DNA contamination as the mitochondria were digested by cell mitochondria isolation solution.

To remove contaminating cellular DNA, the protocols included a nuclease step whose action had to be stopped before extraction of total DNA. Sinzger *et al.* (1999) and Volkening and Spatz (2009) had reported methods that applied EDTA at a final concentration of 1.2 mM to stop that action. Lane 3 in Fig. 2 had no high-molecular weight DNA band which indicated that trace activated ribozyme had been digested completely. The results showed that EDTA did not inactivate the ribozyme activity. However, lane 2 in Fig. 2 distinctly showed a high-molecular-weight DNA fragment. When compared with the data shown

Table 3: Comparison of stop reagents

Items	EDTA	Ethanol
Incubation time	-	10 min
Need to calculate vol. of ribozyme in reactivity system	Yes	No
Need to calculate vol. of metallic ion in reactivity system	Yes	No
Need to search after vol. of stop reagent	Yes	No
Effect on protease K digestion	Yes	No

previously (Fig. 1 and lane 5), researchers concluded that the high-molecular-weight DNA band in lane 2 was mitochondrial DNA that was amplified in this sample. The nuclei buffer could not lyse the abundant mitochondria in this sample which resulted in the extraction of total DNA that contained a high yield of mitochondrial DNA.

As shown in Fig. 1 and 2, addition of three volumes of 100% ethanol stopped the micrococcal nuclease reaction. Researchers compared the ability of 100% ethanol and EDTA to stop the reaction (Table 3). EDTA was not the preferable reagent to stop micrococcal nuclease (Fig. 2 and Table 1) as the ability of EDTA to halt ribozyme activity depends on its ability to chelate metal ions and create water-soluble compounds. Its action depends on chelation of metals ion based on a 1:1 molar ratio and additional unused EDTA affects protease K digestion in downstream experiments. In contrast, the 100% ethanol is the preferred reagent to stop the reaction as no calibration of the quantity of micrococcal nuclease and metallic ions is needed (Fig. 2 and Table 3). Therefore, researchers concluded that the addition of ethanol to stop the reaction was more preferable than addition of EDTA.

Researchers assessed the PEG 8000 precipitation for selective removal of small DNA fragments. As a result, researchers confirmed that the Hartley protocol gave an optimal balance of ILTV DNA yield and purity at a final concentration of 5% PEG plus 10 mM MgCl₂ (Fig. 3).

CONCLUSION

Overall, the protocol described here can rapidly obtain high quality viral DNA that is suitable for use in advanced sequencers. The major advantage, however is that this protocol is suitable for preparation of pure viral DNA from cell-associated recent clinical specimens, infectious chicken embryos and isolates of material at a very early passage after primary CK cell infectious at which time infectious virus is not released into the culture supernatant.

What is more, the protocol is ideally suitable application for the other cell-associated viruses such as OvHV-2 (Ovine Herpesvirus-2) and DPV (Duck Plague Virus) and so on. It may, therefore help in the sequencing of complete virus genomes and determine changes in the virus sequences.

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