Global Polio Laboratory Network

Guidance Paper 6

Poliovirus Inactivation

Document version (date)	Description of substantive revisions
Version 1 (March 2018)	
Version 2 (May 2019)	Addition of a section on Zymo RNA extraction and modified the next generation sequencing comments

Global Polio Laboratory Network

1. OVERVIEW

This document describes a protocol to be used for inactivation of type 2 poliovirus, which is subject to the Containment requirements described in WHO GAPIII. This document is directed toward Virus Isolation, ITD and Sequencing (VIIS) labs that are not polio-essential facilities (PEFs) and VIIS labs that are PEFs. It is expected to be used in conjunction with Guidance Paper 1 (GP1), which describes the safe handling and storage of type 2 poliovirus (PV2) in GPLN laboratories. In an attempt to develop more flexibility in handling poliovirus materials and to enable downstream extraction procedures such as the use of a microfuge without a sealed rotor or automated extraction instrument outside of a Biosafety Cabinet, methods were developed for the inactivation of poliovirus infectivity using heat treatment prior to the addition of lysis buffers available in commercial kits used for RNA extraction. This method ensures complete inactivation of high titer poliovirus isolates, while retaining the ability of extracted poliovirus RNA to be amplified in downstream molecular assays for VP1 Sanger sequencing.

2. SCOPE

Although this method is also compatible with poliovirus types 1 and 3, heating to 80°C prior to RNA extraction is recommended specifically for poliovirus type 2, which is currently subject to GAPIII requirements. Since types 1 and 3 polioviruses are not currently subject to GAPIII requirements, heat inactivation prior to RNA extraction is not recommended.

This document does not describe the inactivation of poliovirus using FTA cards, which is a validated method that has been used in the GPLN for more than 8 years. FTA methodology includes a 70°C / 4 minutes heat treatment prior to spotting isolates on the FTA cards, and this FTA methodology should be performed as previously described in written methods and instructional videos produced by the GLPN.

3. OBJECTIVES

The objectives of this document are the following;

1. To briefly describe a heat inactivation step to be performed prior to RNA extraction of type 2 poliovirus isolates.
2. To document RNA extraction methods that have been validated to completely inactivate poliovirus isolates.
3. To clarify the specific uses for heat inactivation in conjunction with RNA extraction prior to sequencing.

4. BACKGROUND

Nucleic acid extraction is central to molecular diagnosis and characterization of virtually all viral pathogens; however, many commercially available extraction kits fail to completely inactivate virus infectivity, posing a biosafety and biosecurity risk. This biosafety risk requires that the procedures must be carried out at higher containment level (e.g. such as BSL-3), limiting throughput and transferability of molecular diagnostic methods. In experiments designed to evaluate and validate the virucidal effect (or lack thereof) of common commercial nucleic acid extraction reagents, experimentation in a few GPLN labs found that lysis buffer in some popular commercial RNA extraction kits did not completely inactivate high-titer poliovirus isolates. Because poliovirus is not completely inactivated by the lysis buffer, virus materials

cannot be removed from a biosafety cabinet immediately after the lysis step in the standard RNA extraction protocol.

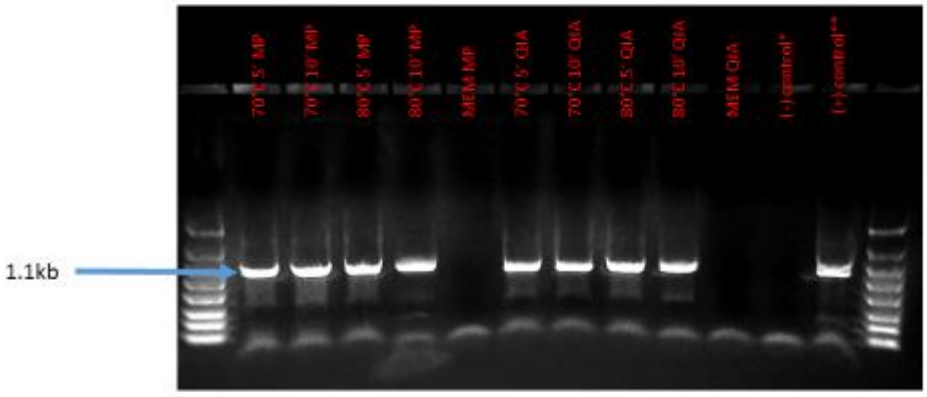
Therefore, the GPLN sought to develop and validate inactivation methods to improve safety and facilitate containment. Methods for rapid RNA extraction, including instrumentation that allows automation and standardization, were tested. Several GPLN laboratories have shown that the final extracted RNA prepared using select commercial technologies (Qiagen and MagnaPure) does not contain infectious poliovirus. In addition, the lysis buffer in the Zymo Quick-RNA Viral RNA kit has been shown to inactivate high titer poliovirus isolates. These validated methods can be used to prepare RNA that can be removed from GAPIII Containment laboratories for use in molecular assays, as stated in the Addendum to Annex 1 of GAP III (and in the Containment Advisory Group report from June 2017).

In an attempt to develop more flexibility in handling poliovirus materials and to enable downstream extraction procedures such as the use of a microfuge without a sealed rotor or automated extraction instrument outside of a Biosafety Cabinet, methods were developed for the inactivation of poliovirus infectivity using heat prior to the addition of lysis buffers available in commercial kits used for RNA extraction.

Heat inactivation methods were tested on select wild type 1 and 3 polioviruses and Sabin 1, 2, and 3 polioviruses. Temperatures from 70°C to 85°C for varying lengths of time were tested in a thermocycler with a heated lid. Heat treatment at 80°C for 10 minutes in a thermocycler with a heated lid was found to inactivate poliovirus infectivity completely, through testing for residual poliovirus infectivity using CCID50 and plaque assays. After extraction using either Qiagen viral RNA kits or MagNA Pure automated methodology, amplification of the VP1 coding region using Y7R/Q8 (VP1 gene region) or 247S/Q8 (Sabin 2) and the Qiagen One Step protocol following the WHO standard sequencing protocol was found to be robust. An example of the amplification results is included below.

RT-PCR of RNA Extracted from heat treated isolates using MagnaPure (MP) and Qiagen (QIA) Extraction Methods

DNA amplicon produced by generic primer pair Y7R and Q8



RT-PCR method using Qiagen One Step protocol followed as documented in PPLB-METHOD.032, WHO Poliovirus Diagnostic Sequence Protocol. No nuclease treatment was performed on the above samples

* Negative control (reagent blank)
**Positive control RNA (Sabin1 NIBSC)

5. AUDIENCE

The proposed audience for this document is the Head and the personnel of laboratory members of the Global Polio Laboratory Network.

6. REFERENCE DOCUMENTS

Additional information that may be useful to users of this document includes:

- *Polio Laboratory Manual.* [1]
- *The Global Action Plan to minimize poliovirus facility-associated risk after type specific eradication of wild polioviruses and sequential cessation of OPV use (GAP-III)* [2]
- *Guidance Paper 1 for safe handling and storage of type 2 poliovirus (PV2) in GPLN laboratories*
- *Laboratory Biosafety Manual* [3].
- *Qiagen QIAmp RNA extraction manual*
- *MagnaPure RNA extraction manual*
- *Zymo extraction manual*

[1] Polio Laboratory Manual and supplement at:
http://www.polioeradication.org/ResourceLibrary/GPLNpublications.aspx

[2] GAP-III. So far English and French are available at: http://www.polioeradication.org/Posteradication/Containment.aspx .

[3] Laboratory Biosafety Manual.
http://www.who.int/csr/delibepidemics/WHO_CDS_CSR_LYO_2004_11/en/

A. List of acronyms

FTA® Fast Technology for Analysis
GPEI Global Polio Eradication Initiative
GPLN Global Polio Laboratory Network
ITD Intratypic Differentiation
PEF Poliovirus Essential Facility (for storage and handling of PV2)
PV Poliovirus
RNA Ribonucleic Acid
rRTPCR real time Reverse Transcriptase Polymerase Chain Reaction
SL Sabin like
SOP Standard Operating Procedure
VP1 Viral protein 1 capsid protein
VI Viral Isolation
VII Viral Isolation and Intratypic Differentiation
VIIS Viral isolation Intratypic Differentiation and Sequencing
WHO World Health Organization

B. Materials:

- Poliovirus isolate of known virus titer
- Qiagen viral RNA extraction kit
- MagNA Pure Compact Nucleic Acid Isolation Kit 1
- Microcentrifuge tubes
- PCR tube strips (thin walled) with attached caps
- Mini centrifuge capable of spinning PCR tube strips (8 with attached caps)
- PCR thermocycler with heated lid
- Microcentrifuge
- MagNA Pure / Kingfisher compact instrument
- BSC Class II
- Lab coats
- Gloves
- Single channel pipettes
- Multi-channel pipettes (200ul and 1000ul)
- Pipet tips

C. Methods:

Heat treatment protocol*:*

Aliquot virus isolates in a type II BSC cabinet. 140ul aliquots of virus in thin wall PCR tubes should be placed in pre-heated PCR thermocycler at 80°C for 10 minutes in a thermocycler with

a heated lid. After heat treatment, the tubes may be spun down briefly in a microfuge (inside or outside of a biosafety cabinet) and cooled to room temperature.

RNA extraction– Aliquots designated for RNA extraction should be processed using Qiagen viral RNA kits, MagNA Pure compact instrument, or another validated RNA extraction method.

For Qiagen:

1. Prepare all the reagents contained in the Qiagen Qiamp viral RNA kit according to the manufacturer's instructions (AVL buffer, AW1 (wash buffer 1), AW2 (wash buffer 2))
2. Remove and set up enough QIAamp spin columns from the Qiagen kit (one for each FTA sample to be processed). Label with the specimen numbers.
3. Prepare and label RNAase free 1.5 ml microcentrifuge tubes for samples
4. Pipet 560 µl of prepared Buffer AVL containing carrier RNA into the labelled 1.5 ml microcentrifuge tubes.
5. In a biosafety cabinet, add 140 µl of the tissue culture fluid (TCF) for samples to the Buffer AVL–carrier RNA in the microcentrifuge tubes. Mix by pulse-vortexing for 15 seconds (s).
6. Incubate at room temperature (15–25°C) for 10 min.
7. Centrifuge the tube to remove drops from the inside of the lid.
8. Add 560 µl of ethanol (96–100 %) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the tube to remove drops from inside the lid.
9. Apply 630 µl of the solution from step 8 to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini column into a clean 2 ml collection tube, and discard the tube containing the filtrate.
10. Open the QIAamp Mini column, and repeat step 9.
11. Carefully open the QIAamp column and add 500 µl of Buffer AW1. Close the cap and centrifuge at 6000xg for one minute. Place the column into a new collection tube and discard the tube with the filtrate.
12. Carefully open the QIAamp column and add 500 µl of Buffer AW2. Close the cap and centrifuge the column at full speed (16,000xg) for 3 minutes. Place the column into a clean collection tube and centrifuge again at full speed for 1 minute.
13. Place the column into a corresponding labeled clean microfuge tube (from step 6) and discard the old collection tube. Add 50 µl of AVE buffer (Qiagen kit) to the column, close the cap and incubate at room temperature for 1 minute. Spin the column at 6000xg for 1 minute. Store the eluted RNA at -20° C.

For MagNA Pure:

1. For samples, set up one strip of tubes for each sample in the metal rack for the Kingfisher Magnetic bead processor in a Biosafety II MSC.
2. Load the plastic sheaths for the magnets into place on the Kingfisher Magnetic bead processor.
3. Prepare the Proteinase K by adding 6.7ml of elution buffer to the dry Proteinase K powder. Mix well and add 150µl of reconstituted Proteinase K to the first tube in each strip.
4. Working in a Biosafety II MSC, add 600 µl of the TCF for samples to the first tube of each set containing the Proteinase

5. Fill the other tubes as follows:

Tube

2 Wash Buffer I 700 µl
3 Wash Buffer II 300 µl
4 Wash Buffer III 700 µl
5 ElutionBuffer 50 µl

6. Add 600µl of Lysis buffer to tube 1, containing TCF and Proteinase K.
7. Mix the bottle of Magnetic Glass Beads thoroughly so that all the contents are evenly dispersed then add 225µl to tube 1.
8. Select the Q_RNA_ml_1 or MagNApure_Kit programme on the Kingfisher Magnetic bead processor, and start the machine.
9. After completion remove the metal rack from the machine and transfer the contents of tube 5 (nucleic acid + elution buffer) into a suitably labelled microtube and store at -20 °C.
10. Collect the remaining liquid from each tube into a falcon centrifuge tube and dispose of using an autoclavable bag

For Zymo RNA extraction:
Refer to the GPLN protocol for extraction of RNA using Zymo columns. The following materials are needed:

- β-mercaptoethanol (Sigma-Aldrich, M2650)
- Quick-RNA™ Viral Kit (Zymo Research, R1034/R1035):
- Viral RNA Buffer
- Viral Wash Buffer
- DNAse/RNAse-Free Water
- Zymo-Spin IC columns
- Collection Tubes (Optional: Extra Collection Tubes- Zymo Research, C1001)
- Ethanol (95-100%) (Decon Labs, V1101)
- Qβ virus process control (provided separately by CDC)

Limitations:
1. A PCR Thermocycler with a heated lid must be used in this protocol. Traditional dry heat blocks have limitations such as inefficient heat transfer and evaporation of the sample on the lids of the tubes; the method described here was not validated using a traditional heat block.
2. Testing to date has shown that 80°C heat inactivation prior to RNA extraction does not diminish the ability of the extracted RNA to be amplified in the VP1 region. More extensive VP1 amplification testing of samples in parallel, with and without 80°C heat inactivation, is ongoing. Likewise, the effect of 80°C heat inactivation on ITD has not been assessed, since ITD is normally performed on tissue culture isolates rather than extracted RNA. Effects are expected to be minimal because of the high sensitivity of ITD5.0 assays.
3. PCR amplification of the complete genome was found to be diminished somewhat after use of the 80°C heat inactivation method and subsequent RNA extraction; this could affect the efficiency of next generation sequencing. The use of a sequence-independent amplification technique for Illumina sequencing was not affected by the 80°C heat inactivation method. Temperatures ≥85°C resulted in reduced VP1 amplification in more than one laboratory.