MPGIT: A concentrated monophasic phenol-guanidinium isothiocyanate reagent for isolation of viral RNA

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Received 13 September 2013; revised 5 August 2014; accepted 9 October 2014

A method to prepare a concentrated monophasic phenol guanidinium isothiocyanate-based reagent for extraction of RNA from liquid samples is described. The reagent was comparable to a commercial reagent for extraction of genetic material of several RNA viruses as assessed by limit of detection of RNA by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). The study showed that the concentrated monophasic reagent could be used in place of a commercial reagent.

Keywords: Acid phenol, RNA isolation, RT-PCR, virus RNA

Introduction

The isolation of undegraded RNA is indispensable for gene expression analyses, including RT-PCR, Northern hybridization, in vitro transcription and translation, transcriptomics etc., but the ubiquitous and sturdy nature of RNases1,2 makes RNA isolation difficult. The most common method of RNA isolation, which uses acid phenol and guanidinium isothiocyanate (GITC) was developed more than two decades ago3. This method relies on the partition of RNA and DNA at acidic pH into aqueous and organic phases, respectively, and the strong chaotropic nature of guanidinium salts effectively denatures the proteins including the unusually stable RNases. Based on this principle, a number of concentrated monophasic reagents are commercially available for RNA isolation from liquid samples. These include TRIzol® LS Reagent (Invitrogen, USA) and TRI Reagent® LS (Sigma Aldrich, USA). However, these reagents have the following drawbacks: (a) limitation in the volume of the sample that can be used because large amount of the reagent is required, (b) increase in handling time as a consequence of the increase in the number of tubes used, and (c) both of these contributing to significant costs for diagnostic procedures in addition to the already expensive reagents. Further, the composition of these reagents is proprietary.

Liquid samples, such as, serum and cell culture supernatants, contain diluted virus particles and hence a concentrated reagent is required for the isolation of RNA from these samples. Here we describe a method to prepare a concentrated monophasic reagent suitable for the extraction of viral RNA from liquid samples and compared our reagent to a commercially available reagent. Different RNA viruses, such as, bluetongue virus (BTV) and rota virus (both containing double-stranded-segmented RNA genome), influenza virus A (single-stranded segmented), polio virus (single-stranded positive sense) and rabies virus (single-stranded negative sense), were used for RNA isolation in the present study. The results indicate that concentrated monophasic phenol-guanidinium isothiocyanate (MPGIT) can be used instead of commercially available reagents.

Materials and Methods

Cells and Viruses

Vero cells (CCL-81) and PK15 cells (American Type Culture Collection, USA) were grown in DMEM (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (Gibco®, USA) and penicillin-streptomycin (Invitrogen, USA). Culture supernatants of cells infected with influenza, rota and BT viruses were used for RNA isolation. For rabies and polio viruses, RNA was isolated from the virus bulk obtained from Bharat Biotech International Limited, Hyderabad.
Preparation of Concentrated Monophasic reagent, MPGIT

GITC (29.4 g) (Sigma-Aldrich, USA) was dissolved in 58 mL of water-saturated phenol (SRL, India). This was followed by addition of 147 mg of N-lauryl sarcosine (Sigma-Aldrich, USA), 2.65 mL of isomyl alcohol (S D Fine-Chemicals, India), 125 mg of 8-hydroxy quinoline (Qualigens, India), 610 µL of 2-mercaptoethanol (SRL, India) and 5 mL of 2 M acetate buffer, pH 4.5 (18.5 mL of 2 M sodium acetate and 31.5 mL of 2 M glacial acetic acid) in that order. Sterile diethyl pyrocarbonate (DEPC)-treated water was then added to make up the volume to 100 mL. The reagent was stored at 4°C.

RNA Isolation

To 250 µL of virus harvest or 10⁵ cells resuspended in 250 µL of phosphate-buffered saline (PBS, pH 7.4), 750 µL of TRI Reagent® LS (Sigma-Aldrich, USA) or MPGIT was added and the mixture was vortexed briefly and incubated at room temperature (RT) (~25°C) for 5 min. This was followed by the addition of 250 µL of chloroform, mixing by inverting several times and incubation at RT for 10 min. The organic and aqueous phases were separated by centrifugation at 16,100×G for 15 min at RT. The aqueous phase was harvested and equal volume of isopropanol was added. After incubation for 20 min at RT, RNA was pelleted by centrifugation at 16,100×G for 20 min at RT. The resultant pellet was washed with 70% ethanol, air dried and resuspended in 20 µL of RNase-free water (Sigma-Aldrich, USA). The extracted RNA was further used for RT-PCR.

Total cellular RNA was harvested from a 90-95% confluent (approx 7×10⁵ cells) T25 flask of Vero cells. Cells were harvested by trypsinization and then resuspended in 250 µL of PBS, followed by the addition of 750 µL of TRI Reagent® LS (Sigma-Aldrich, St. Louis, MO, USA) or MPGIT. The cell extracts were then collected in separate tubes and the RNA extraction procedure followed was the same as described above. Finally, the pellet was resuspended in 150 µL of RNase-free water containing 0.5% sodium dodecyl sulphate (SDS) and heated to 60°C for 10 min to allow complete solubilization of the RNA.

DNA Extraction

For testing potential inhibition of PCR, DNA was isolated from porcine kidney cell line PK15, which harbours porcine circovirus type 1 (PCV-1), and spiked with BTV RNA. DNA was isolated from 10⁵ PK15 cells by using phenol chloroform extraction as described earlier⁶. The DNA was resuspended in 200 µL of RNase-free water.

Reverse Transcription-PCR and PCR

RNA was serially diluted 10-fold to obtain dilutions up to 10⁶. Undiluted or diluted (5 µL) viral RNA and 2 µL of cellular RNA were used as templates for reverse transcription. cDNA was synthesized in a 20 µL reaction mixture containing MMLV buffer (Bioline, USA), 50 ng of random hexamers (Thermo Fisher Scientific, USA), 500 µM of each dNTP (Thermo Fisher Scientific, USA), 20 units each of BioScript™ (Bioline, USA) and RNaseOUT™ (Invitrogen, USA). The reaction was carried out at 25°C for 10 min, followed by 37°C for 60 min. The enzyme was then inactivated by heating at 70°C for 10 min.

The synthesized cDNA (5 µL) was used as a template for PCR. The reaction mixture contained 10 pmole each of the gene-specific forward and reverse primers, 200 µM of each dNTP (Thermo Fisher Scientific, USA), and 2.5 U of Taq DNA polymerase (Sigma-Aldrich, USA) in a 50 µL reaction volume. The PCR was carried out for 35 cycles using the cycling conditions mentioned in Table 1.

Each of PK15 DNA or BTV RNA (2 µL) were serially diluted 10-fold. Then 5 µL of each dilution was used as a template for PCR. Detection of PCV was performed using 10 pmole of PCV specific primers PCV3F and PCV4R (Table 1) along with 200 µM of each dNTP (Thermo Fisher Scientific, USA) and 2.5 U of Taq DNA polymerase (Sigma-Aldrich, USA) in a 50 µL reaction volume. Alternatively, PK15 DNA alone was serially diluted and 5 µL of sample at each dilution was used as a template directly or spiked with 5 µL of undiluted RNA and amplified using PCV primers.

Results and Discussion

The pervasive nature of RNases makes RNA highly susceptible to degradation during and after the extraction procedure. The use of effective denaturants in the extraction reagent protects the RNA from being degraded. RNA isolation was a cumbersome procedure until Chomczynski and Sacchi² described the single step method. A decade after the first description of acid GITC, a modified monophasic reagent, PIG-B (Phenol, Isomyl alcohol, Guaninitium isothiocyanate and Beta mercaptoehanol), was described⁵.
MPGIT is easy to make and is an inexpensive reagent. Further, MPGIT can be prepared by direct addition of GITC salt instead of GITC solution to the equilibrated phenol. Use of acetate buffer provides adequate buffering required at low pH. Salt in the form of sodium acetate facilitates efficient precipitation of RNA. We evaluated the utility of MPGIT by isolating viral RNA as well as cellular RNA. Agarose gel electrophoresis showed the expected banding pattern for BTV RNA (Fig. 1A) and distinct bands of 28S and 18S rRNA for Vero cell samples (Fig. 1B). This shows that the integrity of RNA was maintained throughout the extraction procedure as undegraded RNA was isolated from both BTV as well as Vero cells. The extracted cellular RNA was also analyzed by RT-PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. Specific amplification of a 452 bp product showed that RNA extracted using the MPGIT reagent was suitable for RT-PCR (Fig. 1C). These results indicate that MPGIT is as good as TRI Reagent® LS in obtaining quality RNA.

**Table 1—Primers, product size and the cycling conditions of PCR used in the study**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer name</th>
<th>Sequence</th>
<th>Product size (bp)</th>
<th>PCR condition</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Bluetongue virus (NS 3)</td>
<td>NS3F</td>
<td>GTAAAAAGTGTCGTGTC</td>
<td>822</td>
<td>55°C-30sec</td>
<td>8</td>
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<tr>
<td></td>
<td>NS3R</td>
<td>GTAAGTGTGAGCAGGC</td>
<td></td>
<td>72°C-1min</td>
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<tr>
<td>Rota virus (NSP 5)</td>
<td>ROSE11F1</td>
<td>CTCTCAGATTGACGTAAC</td>
<td>579</td>
<td>55°C-30sec</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>ROSE11R1</td>
<td>CGATCAATTGACGAC</td>
<td></td>
<td>72°C-45sec</td>
<td></td>
</tr>
<tr>
<td>Influenza A virus (M protein)</td>
<td>M30F2/08</td>
<td>ATGAGYCTTAAACCGGTCGA</td>
<td>244</td>
<td>55°C-30sec</td>
<td>10</td>
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<tr>
<td></td>
<td>M264R3/08</td>
<td>TGGACAAAANGCTATCGCTAG</td>
<td></td>
<td>72°C-30sec</td>
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</tr>
<tr>
<td>Polio virus type 2 (5’ non-coding region)</td>
<td>PVNCR5F</td>
<td>ATTGTCACCATCAAGCAGC</td>
<td>434</td>
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<td>11</td>
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<tr>
<td></td>
<td>PVNCR5R</td>
<td>CAAGCAGCTCTGTTCCTCCCG</td>
<td></td>
<td>72°C-40sec</td>
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<tr>
<td>Rabies virus (G protein)</td>
<td>RABF</td>
<td>GACCTTGGGTCTCCCAAGTGGGG</td>
<td>880</td>
<td>45°C-90sec</td>
<td>12</td>
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<tr>
<td></td>
<td>RABR</td>
<td>CAAAGGAGATTGAGATTGATGTC</td>
<td></td>
<td>72°C-1min</td>
<td></td>
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<tr>
<td>Porcine circovirus (replicase gene)</td>
<td>PCV3F</td>
<td>GAAAGTGAAGGGGGA</td>
<td>261</td>
<td>55°C-30sec</td>
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<td></td>
<td>PCV4R</td>
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<td></td>
<td>GAPDHR</td>
<td>TCCACCACCTGGTCTGTA</td>
<td></td>
<td>72°C-40sec</td>
<td></td>
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</table>

*In all cases, initial denaturation was carried out at 95°C for 5 min. This was followed by 35 cycles of denaturation at 95°C for 30 sec, annealing and extension. The annealing and extension temperatures for each primer are mentioned above. Final extension was done for 10 min at 72°C.

Fig.1 (A-C)—Comparison of MPGIT and TRI Reagent® LS for extraction of BTV and cellular RNA: (A) RNA extracted from BTV showing RNA bands corresponding to the various segments of the viral genome [Note that we have DNA ladder for size comparison because BTV is a double-stranded segmented RNA virus]; (B) RNA extracted from Vero cells showing distinct 28S and 18S rRNA bands; & (C) The extracted cellular RNA was tested for suitability in RT-PCR. [The RNA was reverse transcribed and amplified using GAPDH primers. Fragment of GAPDH gene amplified from RNA extracted using MPGIT (lane 1) and TRI Reagent® LS (lane 3) is shown.]
To further compare MPGIT with TRI Reagent® LS, the extracted viral RNA in each case was serially diluted up to $10^8$ and subjected to RT-PCR. The detection limit observed with different viruses was different, albeit slightly, between MPGIT and TRI Reagent® LS (Figs 2A-E). MPGIT worked better than TRI Reagent® LS for rotavirus, influenza virus and rabies virus, whereas the two reagents are comparable for bluetongue virus, and poliovirus.

To evaluate whether these differences were due to presence of inhibitors during either of the extraction procedures, BTV RNA was extracted using both the reagents and spiked into PK15 DNA and then serially diluted. A PCR was then set up to detect PCV1 DNA. The amplification observed with RNA spiked PK15 DNA was similar to that observed with PK15 DNA control (Fig. 3A). In another experiment, the same amount of RNA was spiked into serially diluted PK15 DNA so that fixed amount of inhibitor, if present, could be tested at various concentrations of DNA. Serially diluted PK15 DNA alone served as a control. The intensities of amplicons observed in each of the experiments were identical, indicating that there was no inhibition of PCR (Figs 3B & C).

We then performed experiments to test the efficiency of RNA extraction when the sample to reagent ratio (1:3) recommended for TRI Reagent® LS was altered. The results showed that 1:1 ratio of sample to reagent can be effectively used for RNA isolation, allowing the use of lesser reagent and more sample (data not shown).

The single step method described by Chomczynski and Sacchi® involved the preparation of guanidinium salt containing Solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sodium lauryl sarcosine, 100 mM 2-mercaptoethanol) for initial lysis, followed by extraction using phenol, chloroform and isoamyl alcohol. We have standardized the preparation of a concentrated monophasic reagent containing all the components so as to reduce handling at different stages and for efficient extraction of RNA from liquid samples.

We did not quantify the extracted RNA spectrophotometrically, but instead assessed quantity by a secondary assay determining the limit of detection via RT-PCR. The results with MPGIT were reproducibly comparable to that obtained using TRI Reagent® LS. It is important to note here that we used RNAs belonging to diverse families of viruses whose genomes are single or double stranded, positive or negative sense, and are linear or segmented. This reagent is routinely used in our laboratory for all viral RNA extractions, and the BTV RNA isolated using this reagent was recently used for next-generation sequencing®. Thus we conclude that the monophasic reagent MPGIT can be used for efficient RNA extraction as an alternative to TRI Reagent® LS. Since our interest involved only RNA isolation, the reagent was not tested for DNA or protein extraction.
The single step concentrated reagent MPGIT will serve as a cost effective alternative to the commercially available reagents. The reagent is relatively easy to prepare. In addition, the use of 8-hydroxyquinoline and 2-mercaptoethanol in the mixture prevents the oxidation of phenol, making the reagent stable at 4°C for at least a year without affecting the quality of extracted RNA (data not shown).

Acknowledgment
The PK15 cells used in the present study were provided as gift by Dr David Johnson, Oregon Health and Science University, USA. We thank Bharat Biotech International Limited for providing us the virus bulks. We also thank the other members of the laboratory for discussion and help, and Mr Fakeera for the artwork.

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