Available online at www.sciencedirect.com

SCIENCE DIRECT

Journal of Pharmaceutical and Biomedical Analysis 33 (2003) 819–823

JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS

www.elsevier.com/locate/jpba

Short communication

# RNA extraction for quantitative enterovirus RT-PCR: comparison of three methods

Bart Verheyden [a], Anne Thielemans [b], Bart Rombaut [a], Peter Kronenberger [b,*]

[a] Departement Microbiologie en Hygiëne, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels, Belgium
[b] Laboratorium voor Biotechnologie, Erasmushogeschool Brussel, Laarbeeklaan 121, B-1090 Brussels, Belgium

Received 7 March 2003; received in revised form 27 May 2003; accepted 27 May 2003

**Abstract**

Quantification of virus-like RNA sequences in biological fluids, like serum and cerebrospinal fluid, requires an RNA extraction method that is both reproducible and fast. Three RNA extraction methods were tested on enteroviruses: (1) the acid guanidine thiocyanate–phenol/chloroform (AGPC) method; (2) a method based on differential precipitation of the RNA and (3) a 'bind–wash–elute' system based on silica-gel membrane binding. The latter two methods yielded a comparable detection limit as measured by RT-PCR ELISA. The detection limit for the AGPC method was 10 times higher. The relative standard deviation for the bind–wash–elute method (3%) was superior to that of the other methods tested (both 20%) and provides a reliable and fast method to extract (viral) RNA from biological fluids for quantification by RT-PCR.
© 2003 Elsevier B.V. All rights reserved.

*Keywords:* RT-PCR ELISA; Quantitative PCR; Enterovirus PCR; RNA extraction

## 1. Introduction

An enterovirus specific quantitative RT-PCR with ELISA detection was previously optimized and validated [1,2]. The primers and probe used were enterovirus specific sequences located in a 148 nucleotide sequence of the 5′ untranslated region of the viral RNA, a region that is a highly conserved among all enteroviruses (details in Ref. [1]). Thirteen parameters of the RT-PCR, such as primer concentration or annealing temperature, were first tested in a three-level multifactorial analysis [1]. Optimized ELISA conditions were then defined by a two-level analysis of seven ELISA parameters (e.g. hybridization temperature or antibody concentration) [2]. However, the very first step of the quantification procedure, i.e. extraction of the viral RNA, is arguably one of the most critical steps that determine the reproducibility and the detection limit of the whole assay.

Automated or simplified RNA extraction methods for quantitative enterovirus RT-PCR from cerebrospinal fluid (CSF) have recently been evaluated (e.g. [3,4]). Despite the existence of

* Corresponding author. Tel.: +32-2479-1890; fax: +32-2479-7102.
*E-mail address:* peter.kronenberger@docent.ehb.be (P. Kronenberger).

0731-7085/03/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0731-7085(03)00312-1

many reports dealing with the quantitation of enterovirus genomes in CSF and other body fluids (e.g. [5]), a comparison of different manual enteroviral RNA extraction methods from biological fluids has, to our knowledge, not been performed yet. We therefore compared three methods for manual RNA extraction: (1) the acid guanidine thiocyanate–phenol/chloroform (AGPC) method of Chomczynski and Sacchi [6], (2) a method based on differential precipitation of the RNA ('PURESCRIPT®', Gentra systems) and (3) a 'bind–wash–elute' system based on silica-gel membrane binding ('QIAmp® viral RNA mini kit', Qiagen). Our goal was to quantify enterovirus-like sequences in biological fluids of patients with diseases of suspect enterovirus involvement, such as diabetes type 1 and multiple sclerosis.

## 2. Experimental

### 2.1. Materials and reagents

Products for the PURESCRIPT® and QIAmp® RNA extractions were provided by the manufacturers as a kit. Products for RNA extraction by the AGPC method were from Sigma Chemical Co., St. Louis, USA. All materials and reagents used for RT-PCR ELISA were as described by Lauwers et al. [2]. Briefly: the 5′-biotine labelled upstream primer 5′-CGGCCCCTGAATGCGGCTAAT-3′, the downstream primer 5′-TTGTCACCATAAGCAGCCA-3′ and the 3′ digoxigenin-labelled capture probe 5′-CCAAAGTAGTCGGTTCCGC-3′ (all purified by chromatography) were from Pharmacia Biotech (Uppsala, Sweden); AMV-RT and PCR reagents from Roche (Penzberg, Germany), and Amplitaq Gold DNA polymerase from Perkin–Elmer (Boston, MA). DNase and RNase free water (Sigma) was used throughout. The cycler was an Amplitron II from Thermolyne corporation (Dubuque, IA).

The reagents for the peroxidase-based ELISA were from Roche. The samples were quantified using a computer-controlled ELx800 microtiter plate reader from Bio-Tek Instruments (Winooski, VT).

### 2.2. Preparation of the test solutions

A 1 μg/μl (as determined by UV spectrophotometry and assuming an $A^{1\%}$ at 260 nm for virus being = 81.6) poliovirus stock solution (type 1, Mahoney, purified as described in Ref. [7]) was serially diluted to a dilution of $10^{-12}$. The dilutions were made in bovine calf serum as a source of 'biological fluid'. Starting from the $10^{-5}$ dilution, 50 μl of each dilution was then subjected to one of the three RNA extraction methods.

### 2.3. RNA extraction

The RNA 'AGPC' extraction method using guanidine thiocyanate, phenol and chloroform (all from Sigma) was as described [6]. RNA extraction using the 'PURESCRIPT®' kit ('body fluid' protocol) was performed according to the instructions of the manufacturer (Gentra Systems, Minneapolis), except that 50 μl sample was lysed using 250 μl cell lysis solution, instead of 100 μl using 500 μl). The RNA extraction using the 'QIAmp® Viral RNA mini kit' ('mini spin protocol') was also performed according to the manufacturers instructions (Qiagen N.V., Venlo, The Netherlands), except that 50 μl was lysed with 550 μl AVL buffer, instead of 140 μl in 560 μl. In all methods the viral RNA was suspended in a volume of 60 μl.

### 2.4. RT-PCR ELISA

The RT-PCR ELISA procedure was as described [2]. Briefly, the RNA samples were first heated at 65 °C for 5 min (and cooled to 4 °C for 5 min) to eliminate secondary structure. The RT buffer contained 25 U of RNasin from Promega (Madison, WI). Reverse transcription was for 1 h at 42 °C (2 U of AMV-RT per reaction). The reaction was stopped by heating to 95 °C (5 min), followed by cooling to 4 °C (5 min). The RT-mixture was then adjusted for PCR by adding PCR buffer and primers. $MgCl_2$ concentration was 1.5 mM. The final PCR volume was 40 μl. Hot-start PCR amplification included a 15 min incubation step at 95 °C, followed by 40 amplification cycles, each lasting for 30 s. The annealing

temperature was 60 °C. A final elongation step was programmed at 72 °C for 7 min. RT-PCR amplicons of 148 bp were visualized on agarose gels (data not shown).

For ELISA detection, 10 μl of the RT-PCR product was suspended in saline sodium citrate (SSC)+Tween-20, vortexed, and incubated for 30 min at 37 °C in streptavidin-coated microtiter plates. After washing with SSC, 0.1 M NaOH was added to the biotin-bound DNA (10 min). The wells were washed with SSC, and the 3′ digoxigenin-labelled DNA probe was added and incubated for 30 min at 37 °C. Unbound probe was removed by washing with a 2× SSC-solution containing 0.1% sodium dodecyl sulfate and 3 M urea, followed by washing with 2× SSC. The anti-digoxigenin-peroxidase conjugate (anti-DIG-POD) was then added and incubated for 30 min at 37 °C. Following 4 washes with PBS/0.05% Tween-20, the plates were developed using a freshly-made 2,2′-azino-di-[3-ethylbenzithiazoline-sulfonate] solution (ABTS), and measured at 405 nm.

## 3. Results and discussion

### 3.1. Detection limit and linearity

Using the three methods, RNA was extracted from virus dilutions made in calf serum (Section 2). Following RT-PCR with enterovirus-specific primers, the samples were subjected to an ELISA to quantify the amount of PCR product (see Section 2.4), and the optical density (O.D.) values were plotted. As can be seen from Fig. 1, the three methods yielded similar graphs. The dilution range $10^{-5}$ to $10^{-8}$ was linear and above background for the AGPC, PURESCRIPT® and QlAmp® methods. Linear regression of this part of the graph yielded slopes of 0.38, 0.44 and 0.33 and correlation coefficients of 0.97, 0.92 and 0.98, respectively.

Virus dilutions $10^{-11}$ to $10^{-12}$ can be considered to be negative controls, since they theoretically contain less than one virion per vial. However, these dilutions yielded O.D. values (0.198–0.593) that were higher than the no-template RT-PCR control (0.113) in which the sample RNA was substituted with DNase and RNase-free water, and which did not contain serum (Table 1). This difference was therefore due to the presence of serum in the dilutions. This effect of serum is well known, although not entirely understood.

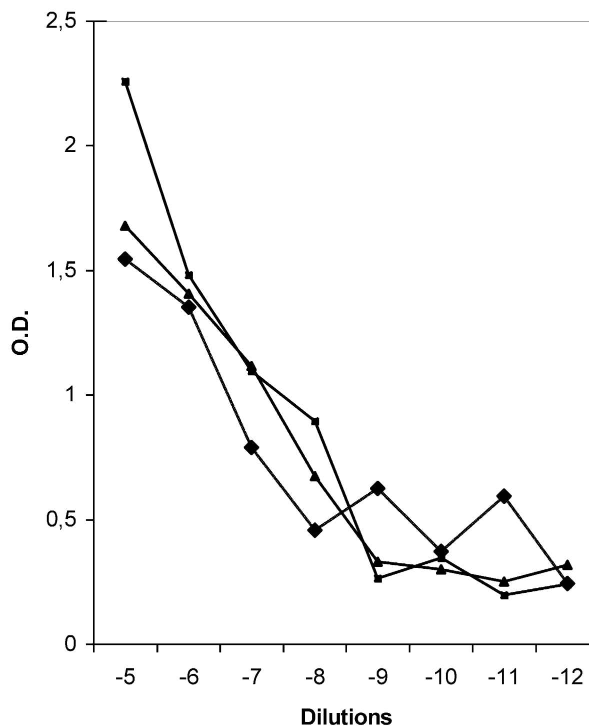


Fig. 1. O.D. values (*Y*-axis) as a function of poliovirus dilutions (*X*-axis) from a 1 μg/μl stock solution of purified virus. Virus dilutions were subjected to the three RNA extraction methods after which the RNA samples were RT-PCR amplified using enterovirus-specific primers. The amplicons were quantified using ELISA. (◆) AGPC, (■) PURESCRIPT®, (▲) QlAmp®.

Based on these data (shown in Fig. 1 and Table 1), the detection limit was situated between dilutions $10^{-8}$ and $10^{-9}$ for PURESCRIPT® and Qiagen kits (77–770 genome equivalents), whereas for the AGPC method the detection limit was 10-fold higher: between dilutions $10^{-7}$ and $10^{-8}$ (770–7700 genome equivalents).

### 3.2. Repeatability

To determine the intra-assay precision, a 0.5 μg/μl solution of purified poliovirus was diluted $10^{7}$

Table 1
O.D. values that were obtained for the three RNA extraction methods

| Dilution | Genome equivalents | O.D. values | | |
|---|---|---|---|---|
| | | AGPC | PURESCRIPT | QlAmp® |
| $10^5$ | $7.7 \times 10^5$ | 1.546 | 2.258 | 1.679 |
| $10^6$ | $7.7 \times 10^4$ | 1.352 | 1.482 | 1.407 |
| $10^7$ | $7.7 \times 10^3$ | 0.790 | 1.095 | 1.115 |
| $10^8$ | $7.7 \times 10^2$ | 0.457 | 0.896 | 0.674 |
| $10^9$ | $7.7 \times 10^1$ | 0.624 | 0.266 | 0.332 |
| $10^{10}$ | $7.7 \times 10^0$ | 0.374 | 0.348 | 0.302 |
| $10^{11}$ | $7.7 \times 10^{-1}$ | 0.593 | 0.198 | 0.253 |
| $10^{12}$ | $7.7 \times 10^{-2}$ | 0.245 | 0.242 | 0.319 |

The O.D. value of the no-template control (without serum) was 0.113, that of the ELISA negative control (without RT-PCR product) also 0.113.

times in bovine calf serum, and $4 \times 50$ μl aliquots were subjected to the three RNA extraction methods. The $10^7$ dilution was chosen because this dilution yielded optimal O.D. values around O.D. = 1.0 for the three methods (Fig. 1). For each method four dilutions without virus were also tested ('blank').

As shown in Table 2, the O.D. values obtained for the blanks were again higher than the no-template control and the ELISA negative control (as a result of the presence of serum used for the dilutions). The results were therefore re-interpreted by subtracting the mean of the four blank values for each method (0.396, 0.598 and 0.293; Table 3).

Based on the data from Table 3, the mean values of the three methods (AGPC, PURESCRIPT® and QlAmp®) were 0.267, 0.502 and 0.479, respectively. These data confirmed the higher yield of the PURESCRIPT® and QlAmp® methods (Fig. 1). The repeatability can be expressed as the standard deviation (S.D.), which was 0.053, 0.098 and 0.015, respectively, or as the relative standard deviation (R.S.D.) (to the mean), which was 20, 20 and 3%, respectively.

The data show that the highest yield was obtained with the PURESCRIPT® method, but that the precision of the QlAmp® method was superior. A R.S.D. of 3% indeed is far below the acceptable limit of 15% as described by the

Table 2
Repeatability of the three RNA extraction methods

| AGPC (mean = 0.663) | PURESCRIPT® (mean = 1.100) | QlAmp® (mean = 0.772) |
|---|---|---|
| 0.621 | 1.110 | 0.764 |
| 0.619 | 1.106 | 0.759 |
| 0.728 | 1.211 | 0.793 |
| 0.685 | 0.973 | 0.773 |
| Blanks (mean = 0.396) | Blanks (mean = 0.598) | Blanks (mean = 0.293) |
| 0.446 | 0.456 | 0.361 |
| 0.381 | 0.832 | 0.230 |
| 0.288 | 0.447 | 0.308 |
| 0.470 | 0.660 | 0.275 |

The O.D. values shown were obtained for a $10^7$ dilution of a 0.5 μg/μl poliovirus solution. The blanks were serum without virus. The O.D. value for the no template control (without serum) was 0.175, and for the ELISA negative control (without RT-PCR product): 0.112.

Table 3
Repeatability

| AGPC (mean = 0.267) | PURESCRIPT® (mean = 0.502) | QlAmp® (mean = 0.479) |
|---|---|---|
| 0.225 | 0.512 | 0.471 |
| 0.223 | 0.508 | 0.466 |
| 0.332 | 0.613 | 0.500 |
| 0.289 | 0.375 | 0.480 |
| S.D. = 0.053 R.S.D. = 20% | S.D. = 0.098 R.S.D. = 20% | S.D. = 0.015 R.S.D. = 3% |

The O.D. values from Table 2 were corrected for the mean of the blank values. The values of the S.D. or the R.S.D. to the mean are also shown for each method.

Biopharmaceutics Coordinating Committee of the US Food and Drug Administration [8]. The QIAmp® method, moreover, was much faster (on average 30 min) as compared to the other methods (PURESCRIPT®: 3.5 h, AGPC: 5–6 h). Taken together, RNA extraction using the QIAmp® Viral RNA kit is the method of choice for quantitative RT-PCR analysis of enterovirus-like sequences in CSF and serum from patients suffering from diseases that are possibly triggered by enteroviruses, such as multiple sclerosis or diabetes type 1.

## Acknowledgements

The help of Leen Espenhout and the technical assistance of Monique De Pelsmacker, Alfons De Rees and F. Van Der Kelen is appreciated. This work was supported by an OZR grant of the Vrije Universiteit Brussel, and by RaDO fund CBK/STI/009 of the Erasmushogeschool Brussel.

## References

[1] S. Lauwers, Y. Vander Heyden, B. Rombaut, J. Pharm. Biomed. Anal. 25 (2001) 131–142.
[2] S. Lauwers, Y. Vander Heyden, B. Rombaut, J. Pharm. Biomed. Anal. 29 (2002) 659–668.
[3] H.F. Rabenau, A.M. Clarici, G. Muhlbauer, A. Berger, A. Vince, S. Muller, E. Daghofer, B.I. Santner, E. Marth, H.H. Kessler, J. Clin. Virol. 25 (2002) 155–164.
[4] I. Casas, L. Powell, P.E. Klapper, G.M. Cleator, J. Virol. Methods 53 (1995) 25–36.
[5] W.A. Verstrepen, S. Kuhn, M.M. Kockx, M.E. Van De Vyvere, A.H. Mertens, J. Clin. Microbiol. 39 (2001) 4093–4096.
[6] P. Chomczynski, N. Sacchi, Anal. Biochem. 162 (1987) 156–159.
[7] B. Rombaut, R. Vrijsen, A. Boeyé, J. Gen. Virol. 66 (1985) 303–307.
[8] Bioanalytical Method Validation. Guidance for Industry, prepared by the Biopharmaceutics Coordinating Committee of the US Dept. of Health and Human Services Food and Drug Administration/Center for Drug Evaluation (CDER) in cooperation with the Center for Veterinary Medicine (CVM); 2001.