# Waiter, There's an Enteric Pathogen in My Shellfish Soup!

## Detection of Enteric Pathogenic Viruses in Shellfish by RT-PCR

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### Abstract

*Evidence linking the transmission of enteric viral (RNA viruses) disease to shellfish has been known for a long time. Described here is a simple and rapid method for the isolation and molecular detection of viral pathogens in shellfish using the Wizard® DNA Clean-Up System and the Access RT-PCR System.*

**This procedure allowed the detection of 20pfu of HAV per gram of hepato-pancreas tissue from shellfish samples spiked with HAV particles.**

### Introduction

Epidemiological evidence linking the transmission of enteric viral (RNA viruses) disease to the consumption of shellfish has been known since the mid 1950s. Scientists have described many methods for the detection of viral contaminants in shellfish using RT-PCR. However, these methods generally include numerous, often fastidious and time-consuming steps for virus release and RNA isolation from shellfish tissues.

We describe here a simplified procedure based on enzymatic liquefaction of shellfish digestive tissues with no mechanical homogenization step. Viral RNA was isolated directly from the shellfish tissue by a guanidine thiocyanate silica extraction method adapted for the use of a vacuum manifold system. RT-PCR assays were performed to detect genomic sequences of the predominant viral pathogens hepatitis A virus (HAV), Astrovirus and Norwalk-Like Viruses (NLV; from genogroups I or II).

We confirmed the specificity of the amplified products using hybridization with DIG-labeled probes (dot blot hybridization). This procedure allowed the detection of 20pfu of HAV per gram of hepato-pancreas tissue from shellfish samples spiked with HAV particles. In addition, up to 20 samples were tested within 24 hours.

### Shellfish Processing

Shellfish tissue was processed by incubation in an industrial protease, followed by dichloromethane solvent extraction as described by Legeay *et al.* (1).

### Viral mRNA Isolation

We used an RNA extraction procedure based on the method reported by Boom *et al.* (2). The method consists of the following steps: i) lysis of cell membranes and viral capsids in a lysis solution (5M guanidine thiocyanate, 0.02M EDTA and Triton® X-100 [1.3% w/v], in 0.1M Tris-HCl buffer [pH 6.4]) and binding of nucleic acids on a silica-based resin, ii) washing of the RNA-silica complex by centrifugation-resuspension steps with a guanidine thiocyanate-based solution (5M GTC in 0.1M Tris-HCl buffer [pH 6.4]), 70% ethanol and 80% acetone; iii) final elution in RNase-free water. This method was modified to allow the use of a vacuum system instead of the centrifugation-resuspension technique for the washing steps. With the Vac-Man® Laboratory Vacuum Manifold (Cat.# A7231), 20 simultaneous RNA extractions can be performed.

### RT-PCR Assays

We used Promega's single-tube Access RT-PCR System[a,b] (Cat.# A1250) for RT-PCR. For each virus-specific RT-PCR detection assay, 5µl of RNA sample was added to a 20µl reaction mix. The reaction mix contained: 1X AMV/*Tfl* buffer, 200µM each dNTP, 400nM each specific primer, 2.5 units each of AMV Reverse Transcriptase and *Tfl* DNA Polymerase. In addition, we optimized the concentration of $MgSO_4$ at 1mM for HAV and NLV II, 1.5mM for NLV I and

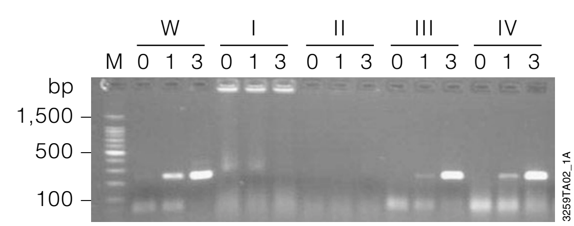


**Figure 1. Removal of RT-PCR inhibitors.** Four 5g mussel hepato-pancreas samples were digested with a protease. Dichloromethane extraction and RNA isolation procedures were then either applied or omitted before RT-PCR. Electrophoresis in 2% agarose of HAV-specific RT-PCR products obtained with 0, 10 and 1,000 ($10^3$) copies of HAV transcript in the presence of water (W), shellfish extract with no solvent extraction and no RNA isolation applied (I), shellfish extract with solvent extraction but no RNA isolation applied (II), shellfish extract with no solvent extraction but RNA isolation applied (III), shellfish extract with solvent extraction and RNA isolation applied (IV). Lane M, 100bp DNA Ladder (Cat.# G2101).

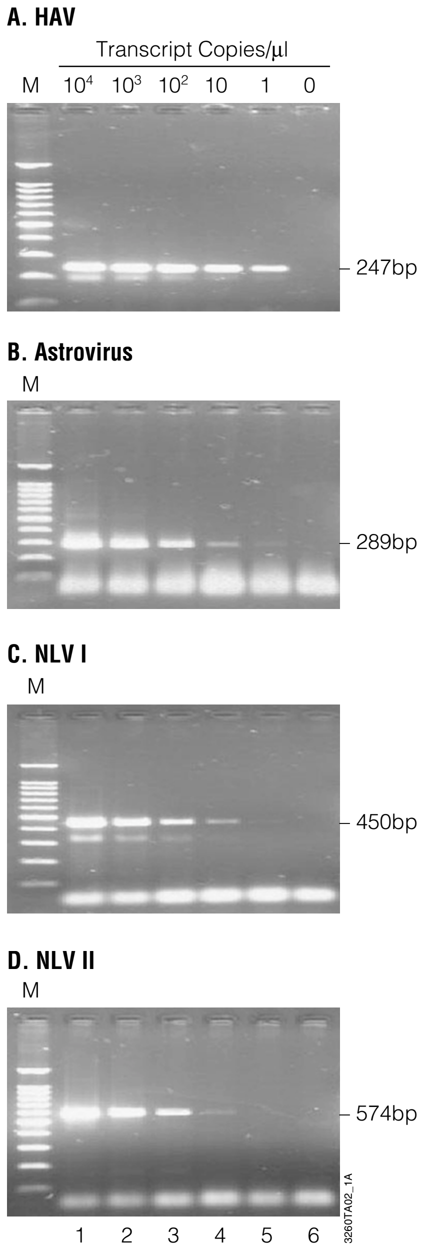


**Figure 2. Sensitivity of virus-specific RT-PCR assays.** Identical serial dilutions of HAV **(Panel A)**, Astrovirus **(Panel B)**, NLV I **(Panel C)** and NLV II **(Panel D)** transcripts were amplified using optimized virus-specific RT-PCR conditions. Five microliters of each RT-PCR product were then resolved on a 2% agarose gel. Lanes M, 100bp DNA Ladder (Cat.# G2101).

2.5mM for Astrovirus-specific detection assays. The RT-PCR assays were performed using a GeneAmp® PCR system 2400 (Perkin-Elmer), following uninterrupted thermal cycling programs consisting of 45 minutes at 48ºC, three minutes at 94ºC, 40 cycles of 30 seconds at 94ºC and 30 seconds at 55ºC (for HAV and NLV II RT-PCR assays) or 50ºC (for Astrovirus and NLV I RT-PCR assays), and a final elongation step of 20 minutes at 68ºC. The RT-PCR products, of 247bp, HAV; 289bp, Astrovirus; 450bp, NLV I and 574bp, NLV II, were separated by electrophoresis on a 2% agarose gel followed by ethidium bromide staining or detected with virus-specific probes using a dot blot hybridization assay. Positive (100 copies of virus-specific transcripts) and negative (water) controls were used with each RT-PCR assay.

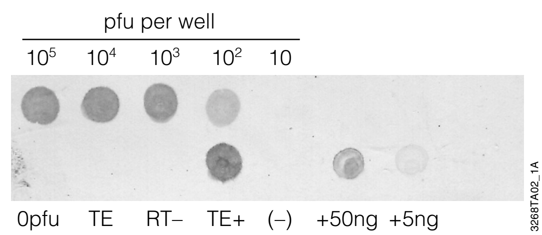


**Figure 3. Sensitivity of procedure.** Five grams of mussel hepato-pancreas samples were either spiked with serial dilutions, from $10^5$ to 10pfu, of HAV or noncontaminated (negative control) samples and processed. Results obtained from samples contaminated with $10^5$ to $10^1$pfu of HAV are shown in the top row. Controls, including a noncontaminated shellfish sample (0pfu), RNA isolation negative control (TE), RT-PCR assay negative control (RT–), RNA isolation positive controls (TE+), negative control (–) and positive controls (+50ng and +5ng) of hybridization, are shown in the bottom row.

## Results

In place of centrifugation and resuspension of RNA-silica complex for washing steps, we favored a convenient system using minicolumns fitted on a vacuum manifold system. Methods tested included several silica and silica-based resins, such as silica or diatomaceous earth (Sigma), prepared as described by Boom *et al.* (2), as well as ready-to-use resins, such as Wizard® DNA Clean-Up System (Cat.# A7280), SV Total RNA Isolation System (Cat.# Z3100), RNaid® system (QBIOgene, Inc.) or RNeasy® total RNA system (Qiagen, S.A.). Best results in regard to the compatibility with the Minicolumn-vacuum system and RNA yield recovery were obtained with the Wizard® DNA Clean-Up Resin. In addition, acetone, which was used initially as a washing solution, appeared to be too corrosive for minicolumns and was replaced by isopropanol.

Optimal conditions were obtained by pipetting 500µl of shellfish extract into a reaction tube containing 500µl of resin (Wizard® DNA Clean-Up Resin from Wizard® DNA Clean-Up System) and 1ml of lysis solution. The tube was vortexed and then placed on a rotating incubator for 20 minutes at room temperature. The mixture was transferred into a Wizard® Minicolumn (Cat.# A7211) fitted on the Vac-Man® Laboratory Vacuum Manifold (Cat.# A7231), where resin-bound RNA could be washed successively with 1ml of guanidine thiocyanate washing solution, 2ml of 70% ethanol and 1ml of 80% isopropanol. Residual isopropanol was removed from the column by centrifugation (12,000 x *g* for 2 minutes). RNA was then eluted in an RNase-free microcentrifuge tube by addition of 100µl of prewarmed RNase-free water and incubation at 80ºC for 10 minutes with a final spin at 12,000 x *g* for 2 minutes.

The optimized conditions led to the efficient elimination of RT-PCR inhibitors (Figure 1). A detection threshold of $10^2$ to $10^3$ copies in 100µl was obtained (data not shown), and thus the addition of $10^4$ copies (in 100µl) of RNA transcript in lysis solution was chosen as a positive control for monitoring potential interference on RT-PCR assays due to sample-specific inhibitors or extraction procedure failure.

Four commercial one-tube RT-PCR systems were tested. The Access RT-PCR System (Cat.# A1250) appeared to give the best results with regard to sensitivity in the presence of shellfish extracts (data not shown). Primers used for the detection of HAV and Astrovirus were derived from validated studies (3,4). Primers specific for NLV I and NLV II groups were designed as the result of multiple alignment of all sequences of NLV I and II coding for RNA polymerase from public data banks. Conditions were optimized for each virus-specific RT-PCR assay, allowing the detection of as little as 1–10 copies of transcript per microliter of aqueous solution on a 2% agarose gel (Figure 2) and the equivalent of 100pfu of HAV per 5g of hepatopancreas by dot blot hybridization (Figure 3).

## Discussion

The objective of this study was to develop a simple and rapid method for the molecular detection of viral pathogens in shellfish by RT-PCR. Many methods have already been described regarding shellfish tissue processing and isolation of viral RNA suitable for amplification (5–11). An industrial protease was used to liquefy the shellfish tissue, and a double extraction using dichloromethane appeared to be sufficient for clarification of the shellfish lysate. Removal of RT-PCR inhibitors was mainly achieved with the RNA isolation procedure (Figure 2).

The use of a vacuum manifold system, allowing rapid and simultaneous extraction of 20 samples, was particularly suitable for routine analysis perspectives. Similarly, the single-tube RT-PCR assay (Access RT-PCR System) is suitable for diagnostic purposes because of simplified manipulations and low risk of cross-contamination. Concerning sensitivity, as little as 1 copy of virus-specific transcript per microliter of aqueous solution (Figure 2) and the equivalent of $10^2$pfu of HAV per 5g of hepato-pancreas could be detected with this RT-PCR system (Figure 3).

Furthermore, given that i) 5g of hepatopancreas corresponds to 100–140g of whole animal and ii) viral contaminants are concentrated in the hepatopancreas (12,13), sensitivity of the procedure can be approximated at 0.8–1pfu of HAV per gram of whole animal. Such sensitivity is equivalent to that seen in studies using semi-nested RT-PCR or RT-PCR combined with hybridization (8,9,14). The main benefit of the dot blot hybridization assay, using DIG-labeled virus-specific probes and detection by a colorimetric reaction, was the confirmation of the specificity of RT-PCR products. Improvement in sensitivity was also observed compared with the gel electrophoresis technique. However, dot blot hybridization is time-consuming and the duration of the overall procedure can be shortened using only gel electrophoresis (24 hours for RT-PCR + dot blot [20 samples], 12 hours for RT-PCR + electrophoresis [20 samples]).

## Conclusion

We have developed a simple procedure for the detection of viral pathogens in shellfish that is suitable for routine diagnostic use. This procedure can be used for epidemiological studies for evaluation of the frequency of virus-specific nucleic acids in marketable shellfish or to determine viral pathogen circulation in shellfish collected either from producing areas or from natural environments. Specific detection of the predominant enteric viruses, epidemiologically linked to shellfish-associated viral diseases, i.e. HAV, Astrovirus and genogroups I and II of Norwalk-like viruses, were performed. Moreover, this procedure can easily be applied to the molecular detection of any other virus, such as emergent viruses or viral indicators, as well as other microbial pathogens, using appropriate PCR or RT-PCR conditions.

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## Protocols

- *Access RT-PCR System and Access RT-PCR Introductory System Technical Bulletin* #TB220, Promega Corporation. (www.promega.com/tbs/tb220/tb220.html)
- *Wizard® DNA Clean-Up System Technical Bulletin* #TB141, Promega Corporation. (www.promega.com/tbs/tb141/tb141.html)

**Product Bibliography: Access RT-PCR System**


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Additional resources available online at:
www.promega.com/amplification/

**Ordering Information**

| Product | Size | Cat.# | Price ($) |
|---|---|---|---|
| Access RT-PCR System[(a,b)]* | 500 reactions | A1280 | 1,425 |
| | 100 reactions | A1250 | 355 |
| Access RT-PCR Introductory System[(a,b)]* | 20 reactions | A1260 | 100 |
| Wizard® DNA Clean-Up System[(c)]* | 100 preps | A7280 | 100 |
| Vac-Man Laboratory Manifold | 20 samples | A7231 | 99 |

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(c)U.S. Pat. Nos. 5,658,548, 5,808,041, Australian Pat. No. 689815 and other patents pending.

**lab fact**

RT-PCR should be performed in the absence as well as presence of the reverse transcriptase, to assess DNA contamination in the template RNA. In addition, "no-template" negative control reactions should always be performed.

**Technical Questions?**
E-mail Promega Technical Services at:
*techserv@promega.com*