# In Pursuit of a Universal Extraction Method to Isolate Nucleic Acids from Neurotropic Pathogens

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## Abstract

**Background**: Rapid and sensitive diagnosis is critical to minimize morbidity and mortality for infectious meningitis (ME) and encephalitis (EN). The testing of cerebrospinal fluid with traditional methods, Gram stain and culture, has limited sensitivity, especially in patients treated with antibiotics. Molecular diagnostics are less affected by antimicrobial therapy and increasingly used for ME/EN diagnosis; however, performance depends on efficient extraction methods for isolation of nucleic acids (NAs). This study aimed to identify a universal method for NA extraction from viral and bacterial ME/EN pathogens.

**Methods**: Ten magnetic bead-based extraction methods (six in-house and four commercial kits) were evaluated on the KingFisher™ Flex (Thermo Scientific, Waltham, MA) using serial dilutions (1E5-1E1 units/mL) of the following: herpes simplex virus 1 and 2 (HSV-1, HSV-2), cytomegalovirus (CMV), Epstein-Barr virus (EBV), human herpesvirus 6B (HHV-6B), varicella-zoster virus (VZV), enteroviruses A71 and D68 (EV71, EV68), echovirus 9 (E9), human parechovirus (HPeV), West Nile virus (WNV), *Mycoplasma pneumoniae* (MPN), *Neisseria meningitidis* (NMG), and *Streptococcus pneumoniae* (SPN). Extraction efficiencies were evaluated by measuring NA yield and impurities on a NanoDrop™ (Thermo Scientific) with subsequent analysis by quantitative PCR (qPCR) or Target Enriched Multiplex PCR (TEM-PCR™).

**Results**: Measurable NA yield was detected in all samples for nine methods. One method showed no detectable NA yield but produced the lowest Cq values for all viral DNA targets. Impurities in eluates based on aberrant 260/230 ratios were detected in nine methods with no effect on qPCR detection of viral targets. While NA yield for bacteria was similar across nine methods, extraction was impeded for NMG at 1E2 CFU/mL in four methods. The extraction efficiency for viruses was target-specific across all ten methods with the lowest detectable concentration for viral RNA targets (E9, EV68, HPeV, and WNV) at 1E1 PFU/mL and viral DNA targets (CMV, EBV, HSV-1, HSV-2, HHV-6B, and VZV) from 1E4 to 1E1 units/mL.

**Conclusion**: Target-specific differences and residual impurities from extraction processes may adversely influence the sensitivity of detection for select bacterial and viral targets. We identified at least three methods for efficient and simultaneous extraction of NA from bacterial and viral targets. A universal extraction method for viral and bacterial NA provides an attractive and practical approach for limited sample sources, such as CSF, in molecular diagnostic assays.

## Introduction

- Rapid and accurate detection of neurotropic pathogens is critical for positive patient outcomes.
- Up to 10% of patients with encephalitis have normal cerebrospinal fluid (CSF) findings,[1] and viral etiologies are not easily identified with conventional culture diagnostics.
- Traditional bacterial meningitis diagnostics have low sensitivity, identifying only 25% or 60% of patients by Gram stain when bacterial load is ≤$10^3$ CFU/mL or $10^3$-$10^5$ CFU/mL, respectively.
- Molecular diagnostics, such as PCR, provide ≥94% sensitivity and specificity for bacterial meningitis diagnosis[2] and ≥95% for herpes simplex encephalitis.[1]
- The performance of molecular assays for neurotropic pathogen detection relies on an efficient NA extraction from CSF which may contain inhibitors and is usually limited in supply.
- This study aims to find a universal extraction method to isolate NA from neurotropic bacteria and viruses with minimal sample input.

## Materials & Methods

**Nucleic Acid Extraction.** Six extraction protocols were developed using components from MO BIO Laboratories (Carlsbad, CA), Omega Bio-tek (Norcross, GA) , and in-house reagents. Four additional extraction kits were provided by external vendors (Table 1). Fourteen organisms were serially diluted in Amies medium to clinically relevant concentrations (Table 2) using titered Zeptometrix (Buffalo, NY), BEI Resources, or International Reagent Resource (IRR, Manassas, VA) stock for *Mycoplasma pneumoniae* and viruses. McFarland standards were prepared for *Neisseria meningitidis* and *Streptococcus pneumoniae*. Nucleic acid isolation was performed on the KingFisher™ Flex platform (Thermo Scientific, Waltham, MA) per manufacturer or in-house protocol.

**Table 1.** Extraction methods evaluated for NA isolation from neurotropic pathogens.

| Extraction (Ext) | Method | Reagent/Kit Vendors | Sample Vol. (µL) |
|---|---|---|---|
| 1 | Viral Respiratory Panel Extraction | MO BIO/In-house | 200 |
| 2 | MagBind® Optimized Extraction | Omega/In-house | 200 |
| 3 | MagBind® Optimized Extraction – Modification 1 | Omega/In-house | 200 |
| 4 | MagBind® Optimized Extraction – Modification 2 | Omega/In-house | 200 |
| 5 | MagBind® Optimized Extraction – Modification 3 | Omega/In-house | 200 |
| 6 | XP2 Bacterial Extraction | MO BIO/Omega/In-house | 275 |
| 7 | MagListo™ 5M Viral DNA/RNA Extraction Kit | Bioneer | 250 |
| 8 | MagJET Viral DNA and RNA Kit | Thermo Scientific™ | 200 |
| 9 | MagMAX™ Pathogen DNA/RNA Kit | Applied Biosystems™ | 200 |
| 10 | KingFisher™ Pure Viral NA Kit | Thermo Scientific™ | 200 |

**NA Quantitation and Integrity.** Extracted NA were measured using the NanoDrop™ 1000 (Thermo Scientific). A subset of eluates [HSV-1 (1.0E5 PFU/mL): Ext 1 and Ext 6; EV68 (1.0E3 PFU/mL): Ext 1 and Ext 6] were submitted to the Genomic Services Laboratory (HudsonAlpha Institute for Biotechnology, Huntsville, AL) for analysis using the Agilent BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA).

## Materials & Methods *(continued)*

**Assay Design.** Using the National Center for Biotechnology Information database, assays were designed in-house to amplify gene targets for the organisms listed in Table 2.

**Table 2.** Target organisms and concentrations used in evaluation

| Organism | Abbreviation | Concentrations | Units |
|---|---|---|---|
| Cytomegalovirus | CMV | 1.0E4-1.0E2 | PFU/mL |
| Epstein-Barr virus | EBV | 2.2E3-2.2E1 | copies/mL |
| Echovirus 9 | E9 | 1.0E3-1.0E1 | PFU/mL |
| Enterovirus A71 | EV71 | 1.0E3-1.0E1 | PFU/mL |
| Enterovirus D68 | EV68 | 1.0E3-1.0E1 | PFU/mL |
| Herpes simplex virus 1 | HSV-1 | 1.0E5-1.0E3 | PFU/mL |
| Herpes simplex virus 2 | HSV-2 | 1.0E4-1.0E2 | PFU/mL |
| Human herpesvirus 6B | HHV-6B | 2.2E3-2.2E1 | copies/mL |
| Human parechovirus | HPeV | 1.0E3-1.0E1 | PFU/mL |
| Varicella-zoster virus | VZV | 1.0E3-1.0E1 | PFU/mL |
| West Nile virus | WNV | 1.0E3-1.0E1 | PFU/mL |
| *Mycoplasma pneumoniae* | MPN | 1.0E3-1.0E1 | CFU/mL |
| *Neisseria meningitidis* | NMG | 1.0E3-1.0E1 | CFU/mL |
| *Streptococcus pneumoniae* | SPN | 5.0E4-5.0E2 | CFU/mL |

**TEM-PCR™ Amplification and Detection.** Extracted bacterial DNA samples were amplified using TEM-PCR™ (Figure 1). Target-specific biotinylated amplicons were hybridized to complimentary detection sequences that were covalently coupled to a custom glass microarray (Microarrays, Inc, Huntsville, AL). Streptavidin-phycoerythrin was added for detection of hybridized biotinylated amplicons. Fluorescence, indicating the amplicon detection sequence was hybridized to the complementary sequence on the microarray, was measured using a Fluorescence Array Imaging Reader (FLAIR, Sensovation AG, Radolfzell, Germany).

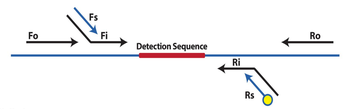


**Figure 1. TEM-PCR™ Scheme.**
Low concentration nested gene-specific primers are (Fo – forward out; Fi – forward in; Ri – reverse in; and Ro – reverse out) designed to enrich the targets during the initial PCR cycles. Later in the procedure, a pair of universal SuperPrimers (Fs and Rs) is used to amplify all targets. The Rs primer is labeled with biotin for subsequent detection.

**qPCR Amplification and Detection.** Extracted NAs were amplified and detected in triplicate using in-house developed TaqMan® Gene Expression assays on the QuantStudio 12K Flex (Thermo Scientific). For DNA viruses and bacteria, 10 µL reactions were prepared with 5 µL TaqMan® Gene Expression Master Mix (Thermo Scientific), 1.5 µL primer/probe mix, and 3.5 µL of template DNA. For RNA viruses, 10 µL reactions were prepared with 2.5 µL TaqPath™ 1-Step Multiplex Master Mix (Thermo Scientific), 1.5 µL primer/probe mix, 2.9 µL nuclease free water, and 3.1 µL of template RNA. Cycling protocols were developed in-house with a proprietary preamplification step.

## Results

### NA Quality Analysis

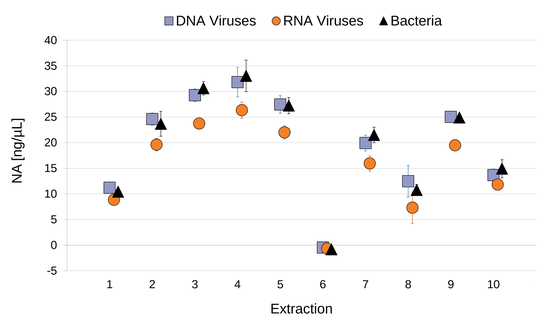


**Figure 2. NA yield varies across extraction methods but not by extracted concentration.** Six DNA viruses, five RNA viruses, and three bacteria were serially diluted to three clinically relevant concentrations. Nucleic acids were isolated using ten extraction methods. Eluates were measured on the NanoDrop™ 1000. Markers indicate average concentration of all measurements at three concentrations and error bars indicate standard deviation of all measurements (DNA viruses, n=36; RNA viruses, n=30; bacteria, n=18).

## Results *(continued)*

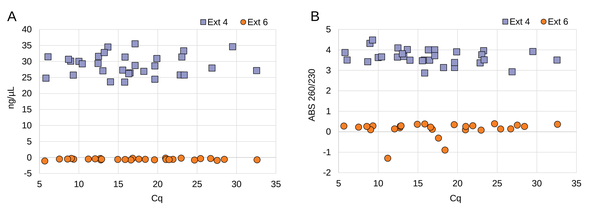


**Figure 3. Cq values do not correlate to NA yield or purity.** Extraction methods producing the (A) highest and lowest average NA yields and (B) highest and lowest 260/230 absorbance (ABS) measurements. Markers indicate individual eluate measurements for each virus at three concentrations.

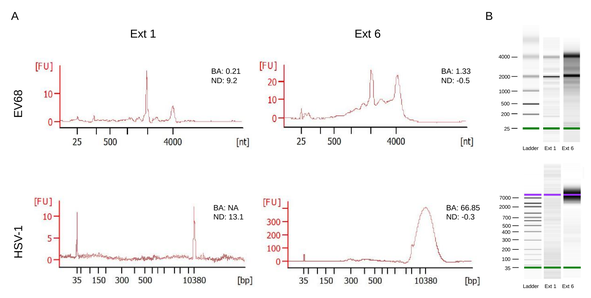


**Figure 4: Different extraction methods show varying levels of NA degradation and sample concentration.** NA yield for EV68 and HSV-1 eluates from Extractions 1 and 6 was measured on the NanoDrop™ 1000 (ND) and analyzed with the Agilent BioAnalyzer 2100 (BA). (A) Electropherograms with measured NA concentration [ng/µL] (inset) and (B) gel electrophoresis from quality analysis represent Extractions 1 and 6 for EV68 and HSV-1.

### TEM-PCR™ Results

**Table 4**. TEM-PCR™ detection of bacterial target organisms [CFU/mL] across all extraction methods.

| Organism | Conc. | Extraction | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *M. pneumoniae* | 1.0E3 | X | X | X | X | X | X | X | X | X | X |
| | 1.0E2 | X | X | X | X | X | X | X | X | X | X |
| | 1.0E1 | | | X | | | X | | | | |
| *N. meningitidis* | 1.0E3 | X | X | X | X | X | X | X | X | X | X |
| | 1.0E2 | X | | X | X | | X | | | X | X |
| | 1.0E1 | | X | | | | | | | | |
| *S. pneumoniae* | 5.0E4 | X | X | X | X | X | X | X | X | X | X |
| | 5.0E3 | X | X | X | X | X | X | X | X | X | X |
| | 5.0E2 | X | X | | X | X | X | | X | X | X |

X, Detected

### qPCR Results

**Table 5**. qPCR detection of viral targets [PFU/mL] and bacterial targets [CFU/mL] across all extraction methods at the lowest detectable target concentration. Best performing methods are bolded based on following selection criteria: method produced Cq value ≤ 1 Cq higher than lowest Cq and SD ≤ 1.5. If Cq and/or SD did not fall within this criteria, data are grayed out.

| Organism | Conc. | Extraction | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | | 2 | | 3 | | 4 | | 5 | | 6 | | 7 | | 8 | | 9 | | 10 | |
| | | Avg Cq | SD | Avg Cq | SD | Avg Cq | SD | Avg Cq | SD | Avg Cq | SD | Avg Cq | SD | Avg Cq | SD | Avg Cq | SD | Avg Cq | SD | Avg Cq | SD |
| CMV | 1.0E2 | 13.6 | 0.11 | **12.2** | **0.09** | 13.8 | 0.05 | **12.5** | **0.03** | **12.1** | **0.11** | **12.7** | **0.06** | 15.2 | 0.03 | 14.9 | 0.02 | 14.3 | 0.03 | 14.3 | 0.08 |
| EBV | 2.2E2* | **22.8** | **0.7** | 23.4 | NA | **23.4** | **0.18** | **23.3** | **0.2** | 23.5 | 1.56 | **23.0** | **1.02** | NA | NA | 38.7 | NA | 24.1 | NA | 26.8 | 6.91 |
| HSV-1 | 1.0E3 | **16.6** | **0.21** | **17.5** | **0.38** | 18.1 | 0.22 | **17.1** | **0.14** | 18.1 | 0.18 | **16.6** | **0.04** | 19.2 | 0.18 | 18.0 | 0.14 | 17.9 | 0.19 | 17.9 | 0.11 |
| HSV-2 | 1.0E2 | 17.4 | 0.10 | 16.0 | 0.06 | 16.2 | 0.21 | **15.9** | **0.10** | 16.1 | 0.12 | **14.9** | **0.06** | 18.7 | 0.28 | 16.4 | 0.20 | 16.5 | 0.26 | 16.2 | 0.07 |
| HHV-6B | 2.2E2* | 29.2 | 1.35 | 29.0 | 5.24 | 27.8 | 1.60 | 29.5 | 5.91 | 33.1 | 5.57 | **25.5** | **0.90** | 31.9 | 5.29 | 29.4 | 1.29 | 30.8 | 8.14 | 27.5 | 1.26 |
| VZV | 1.0E1 | 13.5 | 0.03 | 13.3 | 0.05 | 15.2 | 0.08 | 13.2 | 0.07 | 14.9 | 0.13 | **12.1** | **0.15** | 16.5 | 0.33 | 14.7 | 0.07 | 14.4 | 0.11 | 14.6 | 0.08 |
| E9 | 1.0E1 | 18.3 | 0.33 | 18.0 | 0.44 | **17.5** | **0.13** | 18.2 | 0.18 | **17.6** | **0.2** | 18.4 | 0.13 | 20.1 | 0.3 | 18.0 | 0.28 | **16.9** | **0.12** | **17.6** | **0.26** |
| EV71 | 1.0E1 | **30.6** | **0.35** | 31.1 | 3.03 | 31.1 | 2.08 | 32.6 | 0.29 | 31.1 | 2.66 | 32.6 | 1.78 | 33.3 | 0.5 | 32.6 | 0.8 | **29.7** | **1.37** | 29.5 | 2.06 |
| EV68 | 1.0E1 | **21.5** | **0.36** | **21.9** | **0.84** | **22.3** | **1.09** | 22.9 | 1.05 | **22.5** | **0.55** | 28.5 | 3.52 | 29.1 | 4.58 | 22.8 | 0.65 | **21.7** | **0.48** | 23.5 | 0.83 |
| HPeV | 1.0E1 | **20.7** | **0.12** | **20.4** | **0.32** | 21.7 | 1.28 | 19.6 | 2.62 | **19.8** | **0.80** | 21.5 | NA | **20.7** | **1.37** | 22.0 | 1.25 | 21.4 | 1.59 | 18.8 | 2.63 |
| WNV | 1.0E1 | **14.2** | **0.15** | 16.2 | 0.01 | 15.9 | 0.13 | 16.3 | 0.31 | **14.1** | **0.11** | Not Performed* | | 18.5 | 0.17 | 16.0 | 0.07 | **15.1** | **0.22** | 15.9 | 0.14 |
| *M. pneumoniae* | 1.0E2 | 27.82 | 1.83 | 25.63 | 1.62 | 24.32 | 0.56 | 21.35 | 0.52 | 22.74 | 0.25 | **20.05** | **0.13** | 26.85 | 2.8 | 21.11 | 0.06 | 24.48 | 0.5 | 24.21 | 0.13 |
| *N. meningitidis* | 1.0E2 | 22.47 | 0.77 | 22.91 | 0.40 | 23.86 | 0.89 | 22.76 | 0.39 | NA | NA | **21.23** | **0.32** | 23.41 | 0.26 | 22.61 | 0.03 | 22.72 | 0.86 | NA | NA |
| *S. pneumoniae* | 5.0E2 | 32.61 | 0.53 | 33.33 | 0.84 | 32.97 | 0.15 | 34.08 | 0.56 | 33.62 | 1.22 | **31.37** | **0.48** | NA | NA | 33.85 | 0.66 | 33.13 | 0.22 | NA | NA |

*EBV and HHV-6B concentrations reported as copies/mL. An incubation step for Extraction 6 could not performed for WNV due to laboratory restrictions.

## Results *(continued)*

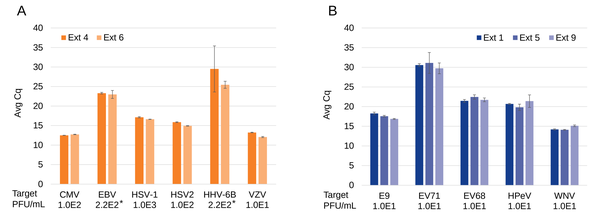


**Figure 5**. Top performing extraction methods for (A) DNA viruses and (B) RNA viruses based on average Cq and standard deviation of triplicate reactions. *EBV and HHV-6B concentrations reported as copies/mL.

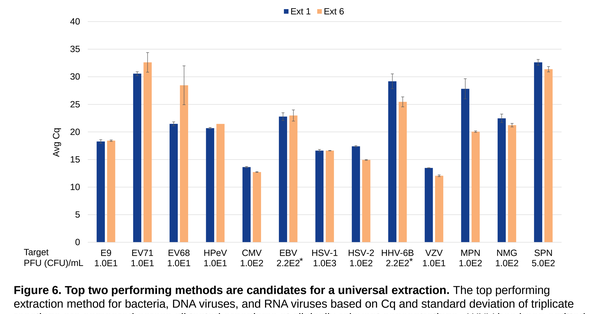


**Figure 6. Top two performing methods are candidates for a universal extraction.** The top performing extraction method for bacteria, DNA viruses, and RNA viruses based on Cq and standard deviation of triplicate reactions are compared across all tested organisms at clinically relevant concentrations. WNV has been omitted from the figure due to Extraction 6 not being performed for this target. *EBV and HHV-6B concentrations reported as copies/mL.

## Discussion

- The neurotropic pathogens evaluated in this study require a robust NA extraction method to include lysis of bacteria and viruses for DNA isolation without compromising RNA integrity from viral targets.
- Cq values did not correlate to NA yield or 260/230 absorbance measurements produced by the Agilent BioAnalyzer 2100 or NanoDrop™ 1000. This could be due to mammalian gDNA carryover from viral culture supernatant, NA input being lower than the NanoDrop™ 1000 sensitivity, or residual reagent carryover with absorbance at 260 nm.
- The NanoDrop™ 1000 and Agilent BioAnalyzer 2100 produced discrepant NA yield measurements. Data suggest that the Agilent BioAnalyzer 2100 results were more accurate displaying very low sample concentrations.
- Viral RNA isolation was most efficient with Extractions 1, 5, and 9; whereas, the most effective NA extraction for DNA viruses and bacteria was achieved with Extractions 4 and 6.
- Extraction 1 or 6 was suitable for NA isolation from all tested organisms.

## Conclusions

- Overall NA extraction efficiency varies between microorganisms.
- Standard measurements and methods, such as NA yield and quality analysis, are not sufficient for selecting the most efficient extraction method.
- A functional test, such as qPCR, is the best method to asses NA extraction efficiency.
- A single, universal extraction method can be utilized for the isolation of NA from neurotropic pathogens at clinically-relevant concentrations.

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**References**

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[2]Allan R. Tunkel, Barry J. Hartman, Sheldon L. Kaplan, Bruce A. Kaufman, Karen L. Roos, W. Michael Scheld, Richard J. Whitley; Practice Guidelines for the Management of Bacterial Meningitis. *Clin Infect Dis* 2004; 39 (9): 1267-1284. doi: 10.1086/425368


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