

Development, Technical Performance, and Clinical Evaluation of a NucliSens Basic Kit Application for Detection of Enterovirus RNA in Cerebrospinal Fluid

Christine C. Ginocchio,^{1,2*} Frank Zhang,¹ Amisha Malhotra,^{3†} Ryhana Manji,¹
Peter Sillekens,⁴ Helma Foolen,⁴ Marlieke Overdyk,⁴
and Margot Peeters⁴

North Shore-Long Island Jewish Health System Laboratories, Department of Molecular Diagnostics, Lake Success, New York,¹ North Shore University Hospital, Department of Laboratory Medicine,² and North Shore University Hospital, Department of Pediatrics,³ Manhasset, New York, and
bioMérieux, Boxtel, The Netherlands⁴

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The combination of nucleic acid sequence-based amplification and electrochemiluminescence detection was used to develop an internally controlled, highly sensitive and specific assay for the detection of enterovirus (EV) RNA in cerebrospinal fluid (CSF). The analytical performance of the assay was determined using both in vitro-transcribed EV RNAs and viral culture isolates. The sensitivity of the assay was 10 EV RNA copies per amplification reaction. The assay detected all enteroviral isolates tested with no cross-reactivity to 21 nonenteroviral species, including rhinovirus and parechovirus. The clinical performance of the assay was evaluated by testing 992 CSF specimens collected from adult and pediatric patients. NucliSens EV results from a subset of 327 CSF samples were compared to viral culture of nasopharyngeal specimens and rectal swabs ($n = 195$) and/or CSF ($n = 212$). Of the 212 CSF samples, 96 samples were positive by either the NucliSens EV assay (94/96; 97.9%) or culture (63/96; 65.6%), and 61/96 (63.5%) were positive by both methods. The inclusion of an EV-specific internal control monitored the entire process, including the efficiency of nucleic acid extraction, amplification, and detection. In total, only five blood-clotted CSF samples (0.5%) were inhibited. The NucliSens EV assay demonstrated superior sensitivity over viral culture ($P < 0.001$), excellent specificity, clear delineation of positive samples, and minimal amplification inhibition.

Enteroviruses (EV) are RNA viruses that are members of the *Picornaviridae* family (6). The nonpolio enteroviruses, including the coxsackieviruses, echoviruses, and numbered enteroviruses, are responsible for approximately 5 to 10 million symptomatic infections per year in the United States (14, 20). Enteroviruses cause a diverse array of illnesses, including respiratory, cardiac, and neurologic ones, in both adults and children. Children under 5 years of age appear to be the most susceptible to infection, due partly to a lack of acquired immunity and poor hygienic habits. In the neonate, enteroviruses can cause a sepsis-like picture (fever, irritability, failure to eat, poor responsiveness) or meningoencephalitis, which can be severe (14).

Enteroviral infections of the central nervous system will often mimic bacterial infection, and therefore, children with meningitis or febrile infants are hospitalized until a bacterial etiology is either confirmed or ruled out. Many enteroviruses can be grown in primate cell lines; however, results of viral culture can take 3 to 8 days, and therefore, the results are generally not rapid enough to affect either treatment options

or length of hospitalization (4, 6, 16, 21). In addition, viral culture has a sensitivity of approximately 65 to 75%, in part because of the inability to culture all enteroviral serotypes, including several coxsackievirus group A strains that require mouse inoculation for detection (6). To increase the sensitivity of enteroviral detection and reduce the turnaround time for results, molecular methods, including those using reverse transcriptase PCR (RT-PCR) (2, 7, 10, 13–15, 18, 19, 21, 23–25) and nucleic acid sequence-based amplification (NASBA) (5, 9, 10), have been developed for the diagnosis of enteroviral infections.

This study describes the development, analytical performance, and clinical evaluation of an internally controlled NucliSens basic kit assay (bioMérieux, Boxtel, The Netherlands) for the detection of EV from cerebrospinal fluid (CSF). The assay is a modification of a method first described by Fox et al. (5) and is based on NASBA and electrochemiluminescence (ECL) detection (3, 17). Total CSF nucleic acids and an EV-specific RNA internal control (IC) are coextracted according to the method of Boom (1). Single-tube coamplification of wild-type patient EV RNA (sense RNA) and an EV-specific IC is achieved through the coordinate activities of three enzymes (avian myeloblastosis virus reverse transcriptase, RNase H, and T7 RNA polymerase) and two DNA oligonucleotides specific for the 5'-nontranslated region (NTR) of the enterovirus genome. One oligonucleotide (P1) contains the T7 RNA polymerase promoter sequence, and the second oligonucleotide probe (P2) contains a generic ECL detection probe se-

* Corresponding author. Mailing address: North Shore Long Island Jewish Health System Laboratories, 10 Nevada Drive, Lake Success, NY 11042. Phone: (516) 719-1079. Fax: (516) 719-1254. E-mail: cginocch@nshs.edu.

† Present address: University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, New Brunswick, NJ 08903.

TABLE 1. Primers used for the construction of the enterovirus-specific external RNA standard and enterovirus-specific internal control

Primer	Sequence
Polio-1 EcoRI ^a	5'-GCGGAATTCTACCACCTCGGAATCTTCGA-3'
Polio-1 Csp45I ^b	5'-GCGTTCGAAATAAACACACTCAATGGAGCG-3'
Entero/PLRV-F ^c	5'-GCAAAGTATCATCCCTCCAGCCCAACCTCGGGGCA-3'
Entero/PLRV-R ^d	5'-CTGGAGGGATGATACTTTGCGATTCTTATGTAGC-3'
T7PCR ^e	5'-TAATACGACTCACTATAGGG-3'
Sp6PCR ^f	5'-GATTTAGGTGACACTATAG-3'

^a Upstream polio primer.

^b Downstream polio primer.

^c Downstream polio/PLRV primer.

^d Upstream polio/PLRV primer.

^e Upstream T7 PCR primer.

^f Downstream SP6 PCR primer.

^g Boldface denotes the EcoRI restriction site, and underlining denotes the Csp45I restriction site.

quence. The amplification results in the production of large amounts of single-stranded RNA that is antisense to the original target RNA and contains the ECL detection sequence. The single-stranded RNA product can then be readily detected using ECL after hybridization with a generic ruthenium (Ru²⁺) probe and a capture probe that is either EV or IC specific. Technical parameters analyzed in the study included assay sensitivity, specificity, IC validation, and the determination of positive/negative cutoff values. Results from clinical specimens were evaluated based upon clinical presentation and other laboratory data, including bacterial and viral culture.

(This study was presented in part at the 18th Annual Clinical Virology Symposium in 2002 and the 102nd General Meeting of the American Society for Microbiology in 2003.)

MATERIALS AND METHODS

Assay development. Construction and generation of the EV-specific external RNA standard and the EV internal RNA control were performed under good manufacturing practices conditions at bioMérieux, Boxtel, The Netherlands, using strict isolation precautions to prevent test sample contamination. Upon molecular cloning, a glycerol stock is produced from the transformant containing the plasmid, and portions of this glycerol stock are stored at -70°C as master bank stock and as master bank working stock. The transformant, stored under these conditions, is stable for years. During each large-scale production of the plasmid, genetic stability is checked by sequence analysis of a small part of the resulting plasmid DNA prior to the production of in vitro RNA. The external RNA standards and internal RNA controls were stored at -70°C until use.

Generation of an enterovirus-specific external RNA standard. For construction of pG30/POLIO-1 WT, nucleic acid was isolated from a cultured poliovirus type 1 vaccine strain using the silica/guanidine isothiocyanate-based extraction procedure as essentially described by Boom et al. (1). The extracted nucleic acid was used to amplify a 438-bp fragment of the 5'-noncoding region (5'-NCR) of the viral genome by RT-PCR. First-strand synthesis was performed using random hexamer primers and SuperScript II RT (Gibco BRL SuperScript preamplification system). After reverse transcription, RNA from the cDNA:RNA hybrids was degraded by RNase H incubation. The resulting first-strand cDNA was amplified directly by PCR using oligonucleotide primers POLIO-1 EcoRI and POLIO-1 Csp45I (Table 1). The amplified DNA fragment was digested with restriction enzymes EcoRI and Csp45I, whose restriction sites are located in the extensions of the oligonucleotide primers. Digested DNA was cloned into a modified pGEM vector (pG30) after removal of a 300-bp fragment from this plasmid by EcoRI/Csp45I digestion. This resulted in recombinant plasmid pG30/POLIO-1 WT. For large-scale plasmid production, the plasmid was transfected into MC1061 cells by electroporation. Cloning of the correct poliovirus type 1 5'-NCR fragment was confirmed by sequence analysis. The plasmid was linearized with BamHI, and in vitro RNA was generated using T7 RNA polymerase. EV-specific RNA was purified, and the concentration of in vitro-generated RNA solutions is based on spectrophotometry. An appropriate dilution of the RNA stock solution is prepared in water, and the optical density at 260 nm is measured. From the measured value for optical density at 260 nm, the RNA

concentration is calculated by making use of the dilution factor and the molar extinction coefficient of the in vitro RNA. This calculation results in a molar concentration. Using Avogadro's number (6.022×10^{23}), this molar concentration is transformed into copies per microliter. The molar extinction coefficient for a certain RNA is based on its base composition and standard molar extinction values for the nucleotides.

Generation of enterovirus-specific internal RNA control. For the construction of a plasmid from which IC RNA can be generated, a segment of 24 bp in the cloned poliovirus 5'-NCR wild-type sequence of plasmid pG30/POLIO-1 WT was replaced by a fragment of 20 bp derived from the genome of potato leaf roll virus (PLRV; EMBL accession number Y07496, positions 4003 to 4022). Two PCR fragments were generated using the plasmid DNA of pG30/POLIO-1 WT as a template and utilizing oligonucleotide primer T7 PCR in combination with Entero/PLRV-R and SP6 PCR in combination with Entero/PLRV-F (Table 1). The resulting PCR products were combined and again amplified using the outer oligonucleotide primers T7 PCR and SP6 PCR of the first round of PCRs. The amplified DNA fragment from this second PCR was digested with EcoRI and Csp45I and cloned into EcoRI/Csp45I-digested pG30 vector, revealing plasmid pG30/POLIO-1 SC1. Correct insertion of the PLRV fragment into the poliovirus 5'-NCR wild-type sequence was confirmed by sequence analysis. For large-scale plasmid production, recombinant plasmid pG30/POLIO-1 IC1 was transfected into MC1061 cells by electroporation. The plasmid was linearized with BamHI, and in vitro RNA was generated using T7 RNA polymerase. The final RNA product is 1,271 nucleotides (with the T7 start at position 621 and the BamHI site at position 1735). The primer binding sites are the same for the IC and for wild-type EV, permitting coamplification in a single-tube format. Differential detection of wild-type EV and IC is possible using two different biotinylated capture probes, one specific for the 23-bp enterovirus sequence and one specific for the 20-bp PLRV sequence (Table 2). IC RNA was purified and quantitated by spectrophotometry, as previously described.

NASBA amplification and detection of wild-type enterovirus and IC RNAs. (i) Nucleic acid isolation. One-hundred-microliter aliquots of enteroviral or non-enteroviral viral culture isolates or 200- μ l CSF samples, 100 μ l of base matrix (Boston Biomedica, Inc., West Bridgewater, MA), and 20 μ l IC RNA were added directly to 0.9-ml NucliSens lysis buffer tubes containing guanidine thiocyanate and Triton X-100 (bioMérieux, Durham, NC). Samples were lysed for 15 min at room temperature and then stored at -70°C until time of analysis. Total nucleic acids were isolated using the NucliSens basic kit isolation reagents (bioMérieux, Durham, NC) according to the manufacturer's instructions.

(ii) Nucleic acid amplification. Amplification was performed as previously described (9) using enterovirus-specific primers and probes (Table 2). The P1.3 (antisense) oligonucleotide primer includes the sequence encoding the T7 polymerase promoter that is required for the transcription-based NASBA process. The P2.2 (sense) oligonucleotide primer includes a sequence that is identical to the generic ECL detection probe supplied in the NucliSens basic kit. Amplification reactions were conducted at $41 \pm 0.5^\circ\text{C}$ in a circulating water bath for 150 min. The EV amplicon size with the generic ECL capture probe sequence is 248 nucleotides. The IC amplicon size with the generic ECL capture probe sequence is 244 nucleotides.

(iii) Detection of NASBA amplicons. Products of the amplification reaction were detected using capture oligonucleotide sequences (capture probes) specific for either the EV RNA or the IC RNA with a generic ruthenium (Ru²⁺)-labeled ECL probe (Table 2) as previously described (9). After incubation, the detection reactions were analyzed in a NucliSens reader (bioMérieux, Durham, NC).

TABLE 2. Enterovirus and IC primer and probe sequences^a

Primer or probe	Sequence
P1.3 ^b	5'- <u>AATTCTAATACGACTCACTATAGGG</u> CACCGGATGGCCAATCCA-3'
P2.2 ^c	5'-GATGCAAGGTCGCATATGAGGGTGTGAAGAGCCTATTGAG-3'
Wild-type EV-specific capture probe ^d	5'-biotin-CTCCGGCCCCTGAATGCGGCTAAT-3'
IC-specific capture probe ^e	5'-biotin-GCAAAGTATCATCCCTCCAG-3'
Generic ECL detection probe ^f	5'-ruthenium-labeled-GATGCAAGGTCGCATATGAG-3'

^a Located in the 5'-NCR of the enteroviral genome.

^b Underlining designates the overhang portion encoding the T7 RNA polymerase promoter. The primer is located at nucleotides 623 to 640 of V01150, poliovirus 1, strain Sabin 1.

^c Underlining denotes the generic ECL detection probe sequence. The primer is located at nucleotides 413 to 432 of V01150, poliovirus 1, strain Sabin 1.

^d The capture probe is located at nucleotides 446 to 469 of V01150, poliovirus 1, strain Sabin 1.

^e A potato leaf roll virus sequence is substituted for the enterovirus sequence located between nucleotides 446 and 469 of V01150, poliovirus 1, strain Sabin 1.

^f Supplied in the NucliSens basic kit.

Included in every run were two assay-negative (AN) controls, consisting of the ECL detection probe in combination with either the EV capture probe or the IC capture probe and 5 μ l of specimen diluent, instead of diluted amplification products. The purpose of the EV and IC AN controls was to determine the magnitude of the background ECL signal for each probe and for each run. In addition, an internal reference solution (IRS) was included in every run to allow standardization of the ECL values from run to run.

(iv) **Establishment of ECL positive/negative cutoff values.** To determine the positive/negative cutoff values for the NucliSens EV assay, 60 negative control specimens consisting of 100 μ l of base matrix and 200 μ l of CSF were added to 900 μ l of NucliSens lysis buffer, extracted, and amplified. The specimens were analyzed in a number of different runs, each run having its own value for the AN control and the IRS. Due to run-to-run variations observed for the IRS ECL values, all sample ECL values were normalized based on the mean value for all the IRS ECL values obtained for all runs included in this study. This normalization rules out any run-to-run variability in establishing the positive/negative ECL cutoff value. The positive/negative ECL cutoff value was reevaluated at the end of the entire study after consideration of the combined ECL results of the initial negative control studies, the sensitivity, specificity, and IC studies, and the ECL values obtained with the clinical samples tested in real time, for an overall total of 1,254 assay results.

(v) **Determination of assay specificity.** Serotype reactivity was tested using frozen clinical isolates obtained from the virology laboratories at Nassau University Medical Center (East Meadow, NY), North Shore University Hospital (Manhasset, NY), and the Department of Health Services (Berkeley, CA). In total, 16 enteroviral isolates, including poliovirus Sabin 2, coxsackievirus types A7, A8, A9, A10, A15, B1, B2, B3, B4, and B5, echovirus types 6, 9, 11, and 30, and enterovirus type 71, and 22 nonenteroviral viral isolates, including adenovirus types 7 and 21, parainfluenza virus types 1, 2, and 3, influenza virus types A and B, rubella virus, mumps, herpes simplex virus type 1 and type 2, varicella virus, respiratory syncytial virus, cytomegalovirus, parechovirus 1, and 7 isolates of rhinovirus, were used. All isolates with ECL signals greater than or equal to the positive cutoff value (650 ECL units) were scored as positive, as determined by clinical samples and control EV RNAs as described in the previous section. Isolates with ECL values in the range of 350 to <650 ECL units were scored as indeterminate, and isolates with ECL signals of <350 ECL units were scored as negative.

(vi) **Determination of assay sensitivity.** Several different studies were conducted to determine the analytical sensitivity of the assay and to determine whether the IC RNA would affect the sensitivity of wild-type EV detection. For each study replicate samples were tested on multiple runs. Nucleic acid extraction, amplification, and detection were performed as described above. The following samples were tested: (a) EV RNA control at input concentrations of 10, 50, 100, 500, and 5,000 copies (four tests per concentration) added directly to the NASBA amplification reaction mixture; (b) EV RNA control at input concentrations of 100 copies ($n = 18$), 200 copies ($n = 18$), and 300 copies ($n = 12$) added to NucliSens lysis buffer containing 100 μ l of base matrix and IC RNA at concentrations of either 2,000 copies ($n = 12$), 3,000 copies ($n = 12$), or 4,000 copies ($n = 7$); (c) EV RNA control at concentrations of 250, 500, 1,000, and 2,000 copies added to NucliSens lysis buffer containing 100 μ l of base matrix and IC RNA at a concentration of 4,000 copies (four tests per EV RNA concentration); (d) serial 10-fold dilutions of coxsackievirus B1 (range, $1 \times 10^{5.5}$ 50% tissue culture infective doses [TCID₅₀] to $1 \times 10^{1.5}$ TCID₅₀) and echovirus 30 (range, $1 \times 10^{6.5}$ TCID₅₀ to $1 \times 10^{1.5}$ TCID₅₀) made using NucliSens lysis buffer; (e) the strains included in the third European Union (EU) Concerted

Action for Quality Control (QCCA) proficiency panel for the molecular detection of enteroviruses (issued in 2001), including dilution series of coxsackievirus A9 and echovirus 11, three individual samples containing coxsackievirus B5, echovirus 6, and enterovirus 71, and two negative control samples. The lyophilized samples were reconstituted in 1 ml water, and nucleic acids were extracted from 100 μ l of the sample.

(vii) **Detection of amplification inhibition.** To determine the IC ECL cutoff value that would indicate amplification inhibition, the data obtained from two study sample groups were evaluated. The data included the range of IC ECL values and the mean IC ECL value obtained for the negative control samples tested in study iv and the results from 28 independent assay runs that detected EV RNA (100 copies per isolation) with IC RNA at a concentration of 2,000 copies per isolation. The IC ECL cutoff value for amplification inhibition was set at 50,000 IC ECL units, which was 2.6 times the minimum IC ECL value obtained for all the above samples. Samples positive for enterovirus can have IC ECL values above or below the IC ECL cutoff value. Enterovirus-negative samples with IC ECL values below the IC ECL cutoff value were considered inhibited and were reamplified from the original nucleic acid extraction. If inhibition was detected again and additional sample was available, the CSF was reextracted and reamplified. If inhibition was still detected with a negative EV ECL signal, the results were considered inhibited. This IC ECL cutoff value was reevaluated at the time of completion of the clinical studies and assessment of the IC ECL values for all negative clinical samples.

Clinical evaluation. (i) Clinical sample study 1. Initial clinical studies were performed using 144 CSF samples submitted to the North Shore University Hospital Clinical Virology Laboratory for routine viral culture. Samples were collected from pediatric patients admitted to the hospital with a diagnosis of sepsis and/or meningitis. Informed consent was obtained under an institutional review board-approved protocol. For 105 samples, routine CSF analysis including cell counting; protein and glucose measurements; and bacterial and viral cultures, including routine CSF, nasopharyngeal (NP), and rectal specimens, were performed according to standard laboratory procedures.

(ii) **Clinical sample study 2.** From July 2001 through December 2003, 848 CSF samples (submitted from eight North Shore-Long Island Jewish Health System hospitals, Long Island, NY) were collected by lumbar puncture in accordance with routine diagnostic protocols from adult and pediatric patients with clinical symptoms suggestive of aseptic meningitis. A refrigerated or frozen aliquot of each CSF was submitted for routine enterovirus detection by the NucliSens EV assay. A subset of the patient samples was also submitted for routine viral cultures, including CSF ($n = 117$), nasopharyngeal ($n = 102$), and rectal ($n = 38$) samples. For this subset, NASBA results were compared to viral culture results.

(iii) **Clinical sample processing.** CSF samples were stored at -70°C until tested. A 200- μ l aliquot of CSF was added to NucliSens lysis buffer containing 100 μ l of base matrix and 20 μ l of IC RNA (2,000 copies) and tested as described above. Remaining CSF was stored in cryovials at -70°C in case additional or repeat testing was necessary.

(iv) **Viral culture.** A single tube of primary rhesus monkey kidney (RhMK), human embryonic lung fibroblast (MRC-5) and human epidermoid tumor (A549) cells (all from both Diagnostic Hybrids, Athens, Ohio, and Viomed Laboratories, Minneapolis, Minn.) were inoculated with 0.1 to 0.2 ml of CSF, depending on the available volume. NP aspirates, NP washes, and NP swabs submitted in viral transport medium (M4; MicroTest, Inc., Lilburn, Ga.) were inoculated onto HEp-2, A549, MRC-5, and RhMK cells and/or R-Mix trays (Diagnostic Hybrids, Athens, Ohio). Rectal swabs were submitted in VTM and inoculated onto RhMK, A549, and MRC-5 cells. Culture tubes were incubated

at 37°C for 14 days and examined daily for cytopathic effect (CPE). R-Mix trays were screened daily for 7 days for enterovirus CPE. Tubes or R-Mix trays exhibiting characteristic enteroviral CPE were identified by using pan-enterovirus fluorescent antibody stains (Chemicon International, Temecula, Calif., and DakoCytomation, Real Carpinteria, Calif.) and/or EV monoclonal antibody pools (Chemicon International, Temecula, CA).

(v) **Resolution of discrepant results.** Resolution of discrepant NucliSens EV and CSF culture results was based upon all available laboratory results and clinical findings. CSF specimens positive by NucliSens EV and negative by culture were considered true positives if (a) either the NP or rectal culture was positive for the isolation of an enterovirus, (b) an additional CSF aliquot was extracted and retested positive or, if sufficient CSF volume was not available, a repeat of the same nucleic acid eluate was NASBA positive, or (c) the CSF was from a patient with a concordant clinical presentation and CSF parameters were consistent with aseptic meningitis (negative Gram stain and bacterial cultures, normal glucose, elevated protein, leukocytosis, negative herpesvirus or other viral PCR). A negative NucliSens EV sample was considered a false negative if (a) the CSF culture was positive or (b) the NP or rectal specimen was positive, and the CSF was from a patient with a concordant clinical presentation and CSF parameters were consistent with aseptic meningitis.

(vi) **Statistical analysis.** The clinical sensitivity and specificity of the NucliSens EV assay and viral culture results were calculated according to standard formulas. The McNemar test for paired sample nominal-scale-using pairs of data with a value for α of 0.05 was used to determine the statistical difference between the results obtained by the EV NASBA assay and EV culture. This test results in a *P* value of 5.12E-04, which should be reported as a *P* value of <0.001.

RESULTS

Establishment of ECL positive cutoff values. Initially, assay positive/negative ECL cutoff values were determined by testing samples consisting of base matrix and IC RNA added to normal CSF. The IRS and the AN controls were included with every test run. AN values varied from 77 ECL counts to 348 ECL counts. IRS values ranged from 27,631 ECL counts to 65,553 ECL counts. Due to the variations observed for the IRS, all EV ECL signals were normalized to the mean value for all the IRS values (ECL signal of 36,500 units). Raw EV ECL signals for the negative samples varied from 1 ECL count to 477 ECL counts. Upon normalization based on the corresponding IRS signal and subtraction of the AN value, corrected EV ECL signals varied from 0 (negative values, obtained for specimens for which the AN value exceeded the raw EV ECL signal, were set to 0) to 207 ECL counts. Based on these results the positive/negative EV ECL cutoff value was set at ≥ 650 ECL units, which was three times the highest normalized EV ECL signal for a negative sample. After evaluation of the clinical sample data (992 CSF samples), an indeterminate zone was established for values ranging from 350 to <650 EV ECL units. This range was chosen because two samples (0.2%) had initial EV ECL values that fell within this range, and repeat testing of the samples yielded one positive result and one repeat indeterminate result.

Evaluation of the IC. To monitor the fidelity of the NucliSens EV assay, an EV-specific IC RNA was developed and evaluated. The IC RNA was designed to monitor all steps of the assay, including nucleic acid isolation, amplification, and detection. Importantly, the IC RNA should indicate the presence of any amplification inhibitors in the sample but also not competitively inhibit the detection of any wild-type EV that would cause false-negative results. As shown in Table 3, all three IC RNA isolation input concentrations (2,000, 3,000, and 4,000 copies, which is equivalent to 200, 300 and 400 copies per amplification reaction) did not inhibit the detection of EV

TABLE 3. Assay sensitivity

No. of samples tested	EV iso input ^a	EV amp input ^b	EV ECL mean ^c	IC iso input ^d	IC amp input ^e	IC ECL mean
3	100	10	194,682	4,000	400	1,039,194
3	100	10	132,755	3,000	300	891,208
12	100	10	174,768	2,000	200	829,753
3	200	20	188,056	4,000	400	687,146
3	200	20	244,899	3,000	300	813,625
12	200	20	245,340	2,000	200	873,081
3	300	30	122,656	4,000	400	973,012
3	300	30	424,469	3,000	300	729,375
7	300	30	400,563	2,000	200	747,121

^a Total number of enterovirus RNA copies in 200 μ l of CSF per isolation (iso).

^b Total number of enterovirus RNA copies in 5 μ l of eluate used per amplification reaction (amp).

^c ECL mean RLU value for samples tested.

^d Total number of enterovirus IC RNA copies added to CSF extraction.

^e Total number of enterovirus IC RNA copies in 5 μ l of eluate used per amplification reaction.

RNA at the lowest number tested (100 copies per isolation; 10 copies per amplification reaction). IC RNA at a concentration of 2,000 copies per isolation gave a mean IC ECL signal of 845,461 ECL units. Therefore, 2,000 IC RNA copies per isolation was selected as the optimal input since it was the lowest value that gave positive results 100% of the time. The lower IC RNA copy number should also be a more sensitive detector of low levels of inhibition. Review of the data from 28 independent assay runs determined that EV RNA (100 copies per isolation) was always detected when the IC RNA (2,000 copies per isolation) was at least 19,000 ECL units. Therefore, to be conservative, the IC ECL threshold value for no amplification inhibition was set at 2.6 times the minimum value (equal to 50,000 IC ECL units). IC RNA aliquots, stored at -70°C , were highly stable and gave reproducible results for a minimum of 1 year. Each aliquot could be thawed and refrozen two times for a total of three uses per aliquot (data not shown).

Detection of amplification inhibition in clinical samples. In the presence of a positive EV ECL signal, the IC ECL may be positive, or it may be negative due to competition from the EV RNA. If the IC RNA was <50,000 ECL units and the EV ECL counts were <650 (negative result), the amplification was considered inhibited, and the sample was reamplified from the original nucleic acid extraction. If inhibition was detected again and additional sample was available, the CSF was reextracted and reamplified. If inhibition was still detected with a negative EV ECL signal, the results were considered inhibited. The mean IC ECL reading was 948,513 ECL units for the 992 CSF samples tested. In total, only 5 (0.50%) of the 992 CSF samples demonstrated amplification inhibition. All five samples consisted of <300 μ l of CSF containing copious amounts of clotted blood. Sufficient sample material was not available for repeat extraction, and all five samples were inhibited upon repeat testing from the same extraction.

Assay analytical sensitivity. The analytical sensitivity of the assay was evaluated in four sets of experiments using either in vitro-transcribed EV RNA or viral stock cultures of known TCID₅₀. Study I evaluated the assay sensitivity when EV RNA was added directly to the amplification reaction. All EV RNA samples at the concentrations tested (10 copies to 5,000 copies per amplification reaction) were detected (data not shown).

TABLE 4. Results of the third EU QCCA proficiency panel for the molecular detection of enteroviruses^b

Sample code	Enterovirus serotype	Virus titer (TCID ₅₀ /ml)	Expected result ^a	NucliSens EV result
EV-C03	Cox A9	3.6	Pos	Pos
EV-C02	Cox A9	0.36	Pos	Neg
EV-C01	Cox A9	0.036	Neg	Neg
EV-C04			Neg	Neg
EV-C09	Echo 11	25,000	Pos	Pos
EV-C11	Echo 11	250	Pos	Pos
EV-C06	Echo 11	25	Pos	Pos
EV-C10			Neg	Neg
EV-C07	Cox B5	320	Pos	Pos
EV-C05	Echo 6	20,000	Pos	Pos
Ev-C08	Entero 71	56	Pos	Pos

^a Based on results obtained by three reference laboratories.

^b Abbreviations: Cox, coxsackievirus; Echo, echovirus; Entero, enterovirus; Pos, positive; Neg, negative.

Studies II and III evaluated the sensitivity of the assay when various concentrations of EV RNA (ranging from 100 to 2,000 copies) and IC RNA (2,000, 3,000, and 4,000 copies) were added to the lysis buffer and coextracted. For both studies, all input concentrations of EV RNA were detected, demonstrating that the sensitivity of the assay was not affected by the extraction procedure nor the addition of IC RNA at all three concentrations (Table 3 and data not shown). The lowest concentration of EV RNA detected in the amplification reactions was 10 copies (Table 3). Serial dilutions of the viral stock cultures demonstrated a detection level of approximately 10¹ to 10^{1.5} TCID₅₀ (study IV) (data not shown). Results from the third EU QCCA proficiency panel are summarized in Table 4 and were concordant with the expected results, with the exception of two coxsackievirus type A9 samples (EV-CO1, EV-CO2) containing less than 1.0 TCID₅₀ in the original sample.

Assay specificity. To determine the specificity of the assay, stock isolates of 16 strains of enterovirus and 22 nonenteroviral isolates (Table 5) were tested with the assay. All enteroviral isolates gave EV ECL signals (range, 537,790 to 10,000,000

ECL units) greater than the positive cutoff value. All nonenteroviral isolates gave EV ECL signals below the positive cutoff value (range, 1 to 243 ECL units). The one isolate with the 243-ECL-unit signal was a rhinovirus. Six additional rhinovirus isolates were then tested, and all demonstrated ECL signals of 1. The NucliSens assay was 100% specific for the detection of enterovirus.

Clinical evaluation. In the original pilot study, 144 CSF samples were tested with the NucliSens EV assay. Overall, there were 74 positive samples (51.4%), 68 negative samples (47.2%), and two grossly bloody CSF samples (1.4%) that were identified as inhibited. Results from 105 of the patients were compared to CSF, nasopharyngeal, and/or rectal viral culture results and assessed in combination with other laboratory data and clinical findings. In comparison to CSF viral culture, the NucliSens EV assay detected 14 more EV-positive samples (29.4%) ($P < 0.001$). Ten of the 14 samples were EV culture positive from either an NP specimen or a rectal specimen. The remaining four NucliSens EV-positive samples were considered true positives based on repeat positive NucliSens EV results from a stored frozen aliquot, CSF cell counts, negative bacterial cultures, and clinical symptoms.

In the supplemental clinical evaluation, 848 CSFs were tested, 603 from children <18 years of age and 245 from adults ≥18 years of age for the presence of enterovirus. There were 246 (29.0%) CSF samples positive for the detection of enterovirus. The overall detection rates were 31.5% for children and 22.6% for adults. During the peak enterovirus seasons (July through October) in 2001, 2002, and 2003, the percentages of CSF-positive samples were 50.6%, 31.7%, and 33.8%, respectively, with a mean of 36.4%. The detection rates were 43.5% for children and 25.0% for adults. August and September had the highest average positivity rates of 45.3% and 47.0%, respectively. Throughout the rest of the year the overall mean positivity rate was 13.7% (adults, 10%; children, 12.5%).

NucliSens EV results ($n = 227$) were compared to a subset of the 848 CSF samples that had CSF viral culture ($n = 117$) and/or viral culture from nasopharyngeal and/or rectal speci-

TABLE 5. Assay specificity^a

Viral isolate	ECL value	Viral isolate	ECL value
Enterovirus 71.....	537,790	Adenovirus 3	1
Poliovirus Sabin 2.....	1,344,493	Adenovirus 7	1
Coxsackievirus A7.....	2,272,294	Adenovirus 21	1
Coxsackievirus A8.....	1,497,909	Parainfluenza virus 1	1
Coxsackievirus A9.....	1,182,959	Parainfluenza virus 2	1
Coxsackievirus A10.....	918,369	Parainfluenza virus 3	1
Coxsackievirus A15.....	1,603,073	Influenza A	1
Coxsackievirus B1.....	1,696,050	Influenza B	1
Coxsackievirus B2.....	1,374,858	Respiratory syncytial virus.....	1
Coxsackievirus B3.....	1,294,758	Rhinovirus 2513	243
Coxsackievirus B4.....	941,286	Rhinovirus 2514	1
Coxsackievirus B5.....	1,665,940	Rhinovirus 2520	1
Echovirus 6.....	309,335	Rhinovirus 2522	1
Echovirus 9.....	10,000,001	Rhinovirus 2523	1
Echovirus 30.....	901,437	Rhinovirus 2524	1
Echovirus 11.....	1,074,520	Rhinovirus 2525	1
Parechovirus 1	1	Measles.....	1
Herpes simplex 1	1	Mumps	1
Herpes simplex 2.....	1	Varicella	1

^a Samples with ECL values of ≥650 are considered positive.

mens ($n = 140$) performed as part of the routine diagnostic work-up. The overall correlation of NucliSens EV results with CSF culture results was 82.1%. From the 117 samples with both NucliSens EV and CSF culture results, there was a total of 32 EV CSF-positive samples. The NucliSens EV assay detected 30 (93.8%), and CSF viral culture detected 13 (40.6%) ($P < 0.001$). The two CSFs negative by NASBA and positive by culture required repassaging in viral culture for sufficient growth. Testing of the culture isolate by the NASBA confirmed the presence of an enterovirus. Two additional samples originally designated as culture positive stained poorly with the Chemicon Pan-enterovirus antibody stain, failed to stain with the enterovirus monoclonal pools and a supplemental direct stain (DakoCytomacron), and did not grow upon repassaging in culture. In addition, the culture supernatant was negative by the NucliSens EV assay. These two samples were then designated as true negatives. The remaining 19 NASBA-positive and culture-negative samples were retested from a new aliquot ($n = 16$) or, if insufficient volume remained, from the same eluate ($n = 3$). All 19 CSF samples were NucliSens EV positive by repeat testing. An additional 18 CSF samples that did not have CSF culture performed were positive by NucliSens EV and were classified as true positives because either rectal or nasopharyngeal specimens had tested culture positive for enterovirus.

For the two studies combined, the overall concordance between NucliSens EV and CSF culture was 88.2%. Of the 96 CSF EV-positive samples, NucliSens EV detected a significantly ($P < 0.001$) greater number of positive samples (97.9%; $n = 94$) than did viral culture (65.6%; $n = 63$).

DISCUSSION

This study describes the development, analytical performance, and clinical evaluation of an internally controlled NucliSens basic kit application for the detection of a wide variety of enteroviruses in CSF. This assay is a modification of the protocol first described by Fox et al. (5). The first modification was the substitution of a new consensus primer set that did not incorporate mixed bases. The purpose of the mixed bases in the original primer set was to compensate for the sequence divergence in the 5'-NTR of the enterovirus genome, thus expanding the scope of the enterovirus subtype detection. However, preliminary studies from our laboratory (unpublished data) indicated that the amplification was more efficient without the mixed bases, and the scope of enteroviral detection was comparable. Positive NucliSens EV results were obtained using viral stocks of a variety of echoviruses, enteroviruses, and coxsackievirus group A and B isolates, including the coxsackievirus group A strains that cannot be grown in routine viral culture (6). Although all strains of clinically relevant enteroviruses were not tested, additional studies by Fox et al. (5) demonstrated broad reactivity with additional serotypes, including those endemic in Europe and the United Kingdom (11, 12). Enterovirus strains included in an EU QCCA proficiency panel were all detected by NucliSens EV. Two samples containing very small amounts of coxsackievirus type A9 originally tested negative (Table 4), but when higher input levels were used, positive results could be obtained for these samples as well (results not shown). In addition, the significantly better

detection rate of enterovirus by NucliSens EV versus viral culture of local clinical samples demonstrated the ability to improve the detection of, at minimum, the predominant strains prevalent in our patient population. All other nonenteroviral isolates, including rhinovirus, did not give ECL signals above the positive cutoff value. One rhinovirus at a titer of approximately 10^6 TCID₅₀ gave a low-level ECL signal that was below the positive cutoff value. Based on this result and reports of cross-reactivity of rhinovirus with other enterovirus primer pairs (10, 18, 19), we tested several other clinical isolates. All six additional isolates were negative by our assay. A study by Landry et al., which compared the NucliSens EV assay with the Argene Biosoft (Varhilles, France) enterovirus consensus RT-PCR assay, identified two isolates of rhinovirus that were negative by the NucliSens EV assay but called positive by the Argene assay (10). Our laboratory currently uses the NucliSens EV assay to aid in the differentiation of rhinovirus from enterovirus culture isolates. The assay did not detect parechovirus 1 (formerly echovirus 22) (8), a result consistent with reports by other investigators using the NucliSens EV primer set (5, 9) or other RT-PCR assays that use primers designed from the same 5'-NTR sequence region (2, 7, 10, 13, 15, 18, 19, 23–25).

The second improvement to the assay was the inclusion of an EV IC that is added prior to the isolation of the sample nucleic acids, coextracted, and coamplified in the same tube with the wild-type EV RNA. Our studies demonstrated that the three IC concentrations tested all gave positive results 100% of the time and did not have any effect on the sensitivity of the EV RNA detection. The combination of an optimal input of IC RNA and a standardized IC ECL cutoff value should clearly indicate low-level amplification due to the presence of inhibition or a loss of RNA during the extraction process. Both of these factors are important for limiting the number of false-negative results and for improving the negative predictive value of the test. The ability to detect inhibition was demonstrated in five samples with significant amounts of clotted blood. Overall, the extraction and amplification methods were extremely robust since only 0.5% of the samples were inhibited.

The third modification to the test was the addition of base matrix to the sample lysis buffer. Preliminary studies (data not shown) demonstrated that the sensitivity of the assay was improved approximately 10-fold by the addition of the base matrix. Although the actual mechanism is not known, we can hypothesize that the base matrix may help stabilize the RNA after viral cell lysis and serve as an RNA carrier, similar to other nucleic acid extraction protocols that require the addition of tRNA to enhance the sensitivity of the nucleic acid isolation. The sensitivity of the NucliSens EV assay was 100 copies per sample input, which is equivalent to 10 copies per amplification reaction.

Clinical studies were performed to assess the validity of the assay and to aid in establishing positive/negative ECL cutoff values. After a review of all ECL data for positive and negative CSF patient samples, it was determined that a value of ≥ 650 ECL units identified all true positives that were confirmed either by culture or by testing another sample aliquot. This positive cutoff value was also reaffirmed in the studies by Landry et al. (9, 10). An indeterminate zone was established to

identify potential positive samples that may contain very low levels of EV RNA. Two CSF samples gave ECL values within this range and were retested. One sample retested as positive, and a nasopharyngeal culture was positive for enterovirus. The other sample retested as indeterminate, and the nasopharyngeal sample was negative by culture. Subsequent to this study we have identified a small number of additional samples that fell within the indeterminate range, were positive by repeat testing and were confirmed by culture, clearly indicating the need to retest the samples.

The NucliSens EV assay showed superior ability to detect enterovirus in CSF when compared to what was previously considered the "gold standard," i.e., viral culture. The assay detected 32.3% more positive CSF samples than viral culture did ($P < 0.001$). Our data were consistent with two studies by Landry et al. (9, 10) that compared this assay to viral culture. In the first study a variety of clinical samples (CSF, nasopharyngeal, throat, rectal or stool) were tested, and NASBA detected 96% of all the EV-positive samples compared to 78% for viral culture (9). The second study compared the NucliSens EV assay to the Argene Biosoft enterovirus consensus RT-PCR assay and viral culture (10). Overall, the NucliSens EV assay and the Argene RT-PCR assay had comparable clinical sensitivities (92.9% and 88.1%, respectively), and both were superior to viral culture, which had a sensitivity of 60.5%. We identified two samples that were initially suspected to be enterovirus culture positive based upon a low-level CPE and weak staining with the Chemicon Pan-enterovirus fluorescent antibody stain. However, the isolates were not confirmed by the EV monoclonal antibody pools or the DakoCytomacon EV direct stain, were not re-passaged in tissue culture, and were NucliSens EV negative when the viral culture cell lysates were tested. This highlights the difficulty in confirming and differentiating some true low-level enterovirus culture positives from nonspecific cross-reactivity found with the current commercially available EV fluorescent antibody reagents. We have found testing the viral culture lysates with the NucliSens EV assay to be an important tool for refereeing these difficult cultures (i.e., determining if the result was falsely positive due to nonspecific antibody staining). Two specimens that were NucliSens negative and culture positive had very low titers and took 5 to 7 days to demonstrate CPE in tissue culture. Our negative results may have been due to either levels of the virus in CSF below our detection threshold or loss of viral RNA due to improper handling, shipment, or storage conditions.

In summary, the NucliSens EV assay was very sensitive and demonstrated superior sensitivity over viral culture, excellent specificity, and a clear delineation of positive samples. The extraction method resulted in highly purified, concentrated total nucleic acids with minimal inhibitory substances. One extraction can be performed, and the eluate can be used for the detection of both RNA and DNA targets. In particular, this is extremely useful for low-volume pediatric samples and when multiple analytes are requested. In addition, the extraction procedure is applicable to many different sample types (22). Our laboratory has also validated this assay for use with nasopharyngeal specimens and stool samples and also as a method for culture confirmation (data not shown). The assay, including nucleic acid extraction, can be completed in approximately

6.5 h. Daily testing and the rapid turnaround time for results compared to routine viral culture has greatly impacted patient care in our institution by reducing hospitalization and the inappropriate use of antibiotics for viral infections. The cost of performing the assay was minimal compared to the savings in the treatment-associated costs (16, 21) and the emotional and traumatic impact of hospitalization for the children and parents.

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