

# Development and evaluation of nucleic acid sequence based amplification (NASBA) for diagnosis of enterovirus infections using the NucliSens<sup>®</sup> Basic Kit

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## Abstract

**Background:** Molecular methods based on RNA amplification are needed for sensitive detection of enteroviruses in clinical samples. Many ‘in house’ methods based on reverse-transcribed PCR (RT-PCR) could be difficult to use in the routine diagnostic laboratory since they tend to be time-consuming, use reagents from many different suppliers and include non-routine procedures. **Objectives:** The aim of this study was to develop and evaluate methods based on nucleic acid sequence based amplification (NASBA) for detection of enterovirus sequences. **Study design:** ‘In house’ prepared and commercially available reagents were utilised to develop enterovirus-specific NASBA assays. Optimised methods were evaluated using clinical samples (cerebrospinal fluid, respiratory and stool samples), titred virus controls and in vitro produced synthetic RNA. Results for NASBA were compared with RT-PCR and virus culture. **Results:** Kit-based reagents gave an equivalent sensitivity to the more laborious ‘in house’ molecular assays (NASBA and RT-PCR) on clinical material and controls. All molecular methods picked up enterovirus positive clinical samples that were not identified by culture. End point detection sensitivity for the NASBA assay based on the NucliSens<sup>®</sup> Basic Kit was  $\leq 1$  tissue culture infective dose 50% of a range of enteroviruses or  $< 100$  copies RNA input. The assay was specific for enteroviruses and did not pick up high titre rhinovirus preparations. Enterovirus Basic Kit NASBA results for clinical samples were easily obtained within a single working day. **Conclusions:** NASBA is a suitable alternative to RT-PCR for sensitive amplification and detection of enterovirus sequences in a range of clinical

**Abbreviations:** BSA, bovine serum albumin; CMV, cytomegalovirus; CSF, cerebrospinal fluid; DIG, digoxigenin; ECL, electrochemiluminescence; ELGA, enzyme linked gel assay; HRP, horse radish peroxidase; KCl, potassium chloride; mRNA, messenger ribonucleic acid; NaCl, sodium chloride; NaOH, sodium hydroxide; NASBA, nucleic acid sequence based amplification; NTR, non-translated region; OD, optical density; PCR, polymerase chain reaction; RS, reference solution; RT, reverse transcriptase; RT-PCR, reverse transcribed polymerase chain reaction; TBS, tris-buffered saline; TCID<sub>50</sub>, tissue culture infective dose 50%.

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specimens. The use of kit-based reagents will enable a wide range of laboratories to undertake molecular-based diagnostic procedures for RNA viruses and provide results within a time frame relevant to patient management. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** Enterovirus; Molecular diagnosis; RT-PCR; NASBA; NucliSens® Basic Kit

## 1. Introduction

Enteroviruses are important pathogens with a wide range of clinical manifestations. Non-polio enteroviruses account for most hospital admissions for viral meningitis. Enteroviruses can also cause severe neonatal infection (large hospital outbreaks have been described) pyrexias, rashes and respiratory symptoms (Melnick, 1996). The association of enteroviruses with diabetes and chronic fatigue syndrome has also been widely reported and warrants further study (Yousef et al., 1988; Clements et al., 1995a,b; Galbraith et al., 1995; Vedhara et al., 1997). Isolation methods have been widely used for diagnosis of enterovirus infection but many of the viruses in this diverse group do not grow well in tissue culture (Grandien et al., 1995). There has been much discussion about the safety and ethics of using wild caught primary monkey cells for virus isolation. Many laboratories still rely on primary cells for isolation directly from clinical material despite the availability of continuous cell lines and, as such cells become less widely available, culture for a wide range of enterovirus types will become difficult in some centres.

Sensitive molecular-based amplification methods are required to detect enterovirus nucleic acid in cerebrospinal fluid (CSF). Many procedures based on reverse-transcribed polymerase chain reaction (RT-PCR) have been described (Rotbart, 1990; Zoll et al., 1992; Glimåker et al., 1993; Nicholson et al., 1994; Abzug et al., 1995; Clements et al., 1995b). Although these assays have been useful for diagnosis of enterovirus infections, laboratories often find 'in house' RT-PCR time consuming and difficult to control. Many protocols do not fit easily into a diagnostic laboratory set-up and utilise complicated mixes of reagents from a variety of sources.

NASBA is an isothermal, transcription-based amplification method first described by Guatelli et al. (1990) which has some advantages over RT-PCR for diagnostic detection of RNA targets. The method has been reviewed recently along with other isothermal RNA amplification formats (Chan and Fox, 1999). The aim of this study was to develop and evaluate methods based on NASBA for diagnosis of enterovirus infections as an alternative to RT-PCR. The availability of reagents for extraction, amplification and probe-specific detection of NASBA products allowed comparison of 'in house' and simple to use kit-based reagents for undertaking NASBA in a diagnostic setting.

## 2. Materials and methods

### 2.1. Samples and controls

During the course of this study a range of different enteroviruses and rhinovirus cultures, spiked into negative stool, respiratory sample or CSF were utilised ( $n = 30$ ). Cultures included preparations of polioviruses 1, 2, 3, enterovirus 70, echoviruses 4, 6, 11, 30, coxsackievirus B1, 2, 3, 4, 5, coxsackievirus A9 and rhinoviruses 1B, 2, 7, 9, 14, 16, 41, 58 and 70. Enteroviruses and rhinoviruses were propagated and typed using well-validated methods (Johnston et al., 1995; Grandien et al., 1995). Titration of virus stocks and calculation of tissue culture infective dose 50 (TCID<sub>50</sub>) used the method of Reed and Muench (1938).

Local ethical approval guidelines were followed for use of clinical material and access to diagnostic results. Clinical samples analysed included stool sample suspensions ( $n = 100$ ), CSF samples submitted for investigation of possible viral central nervous system disease ( $n = 200$ ) and respira-

tory samples taken during acute upper and lower respiratory tract infection ( $n = 80$ ). Enterovirus positive and negative material sent as quality control specimens ( $n = 130$ ) were also analysed. Culture results were available for all stool and CSF samples and the majority of respiratory samples ( $n = 50$ ). RT-PCR results were available for all stool and respiratory samples and for those CSF samples which gave a positive NASBA or culture result.

## 2.2. Summary of molecular methods

Three different assay approaches incorporating different detection systems were utilised during the course of this study to evaluate NASBA for detection of enterovirus RNA. The various methodological approaches utilised in this study are summarised in Fig. 1.

## 2.3. Design of oligonucleotide primers and probes

Suitable primers and probes were designed in the 5' non-translated region (5'NTR) based on alignment of representative enterovirus sequences. Mixed bases were incorporated as appropriate. Using this procedure all enterovirus types for

which sequences were available would be expected to be amplified and detected without cross-reaction with human rhinoviruses. The parechoviruses 1 and 2 would not be amplified. Alignment was undertaken using DNAsis software (Hitachi Software Engineering Co Ltd, San Francisco, CA) on sequences taken from the GenBank database. Target-specific regions of primers and probes were analysed using BIO-OLIGO software (BioGene Ltd, Cambridge, UK) to avoid problems of primer and probe secondary structure and were screened using the BLAST programme (Altschul et al., 1990) to ensure that there would be no likely cross-reaction with non-enterovirus targets. Oligonucleotide primers and labelled probes were synthesised and HPLC purified (Oswel DNA Services, Southampton, UK) before use in RT-PCR or NASBA.

## 2.4. Extraction of nucleic acid

Extraction of RNA from cultured material, spiked controls and clinical samples was undertaken according to the method first described by Boom et al. (1990) with a pre-extraction step for stool samples and some respiratory specimens. Stool suspensions were freeze-thawed in Minimal Eagles Medium with added antibiotics and bench

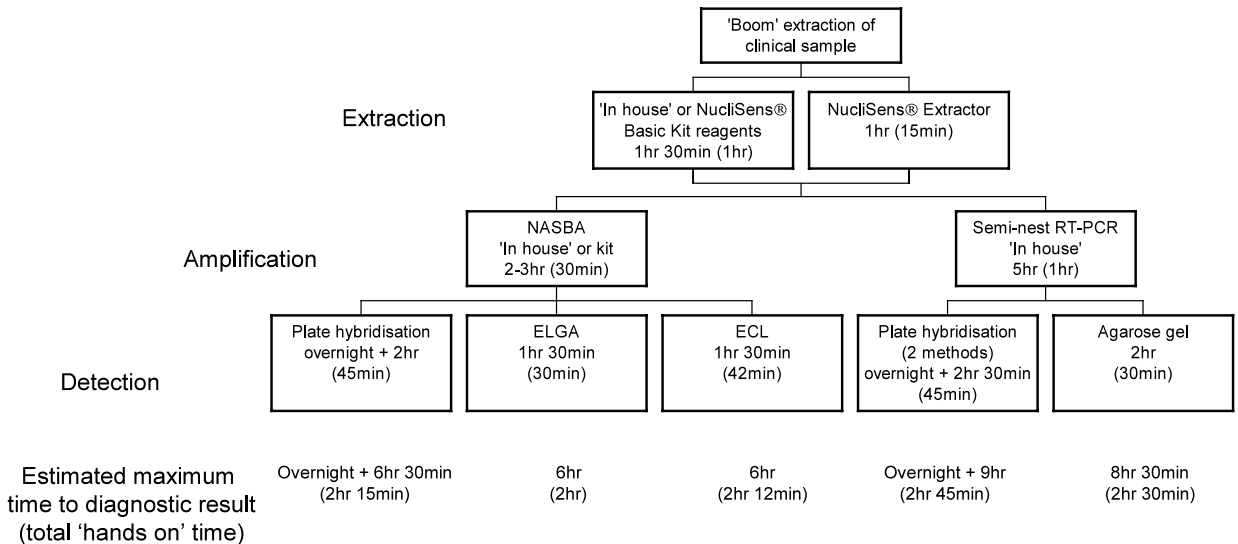


Fig. 1. Estimated time for each step is indicated with 'hands-on' time in brackets. Estimates are based on handling of 20 samples and controls. ELGA, enzyme linked gel assay, ECL, electrochemiluminescence.

centrifuge clarified prior to addition of silica in a similar way to that described previously (van der Hoek et al., 1995). For high volume, dilute clinical material (e.g. pernasal swabs taken into culture medium or nasopharyngeal aspirates) a modification of the silica-extraction procedure utilising a concentration step was included, as described previously (Samuelson et al., 1998; Notermans et al., 2000). This concentration allowed up to 2 ml of diluted sample to be extracted and eluted into a 50 µl volume. Extraction of material for use in RT-PCR and NASBA was undertaken manually (using 'in house' prepared reagents), using the extraction module of the NucliSens<sup>®</sup> Basic Kit (Organon Teknika) and or using the NucliSens<sup>®</sup> Extractor (Organon Teknika) (Fig. 1).

### 2.5. RT-PCR for amplification of enterovirus RNA

This was undertaken using a semi-nested procedure according to a previously published method (Glimåker et al., 1993) with only minor modifications. The primers consisted of one upstream primer (BJent1) and one downstream primer (Rot2) for the RT step and first round of PCR. For the second round of PCR a nested downstream primer (BJent2) was used with BJent1 (Glimåker et al., 1993). In one format of the PCR the primer BJent2 was labelled at the 5' end with biotin which enabled capture of the PCR products to streptavidin coated plates (see below). All thermocycling was undertaken in a TC1 PCR machine (Applied Biosystems, Cheshire, UK.)

### 2.6. Detection of RT-PCR products by gel electrophoresis

The amplified cDNA product was subjected to standard agarose gel electrophoresis (2% w/v agarose) in the presence of ethidium bromide (final concentration in gel and buffer 0.5 µg/ml) and visualised by UV illumination. A visible band of approximately 120 bp was considered to be a positive result.

### 2.7. Detection of RT-PCR products by plate hybridisation

One amplification procedure utilised a biotinylated version of primer BJent2 (Glimåker et al., 1993) for capture of product and detection using an 'in house' method. After denaturation of products from the second round of PCR, biotinylated negative sense sequences were captured on to a solid phase. Detection of captured products was by using a digoxigenin-labelled probe (Rvdet, Samuelson et al., 1998). The optimum dilution of digoxigenin-labelled probe was pre-determined (1 in 100 dilution of purified probe). For denaturation of the PCR products 40 µl of 0.1 M NaOH was mixed with 5 µl of the products and left at room temperature for 10 min followed by the addition of 500 µl Tris-buffered saline (TBS, 100 mM Tris, 150 mM NaCl, pH 7.5) with 0.1% w/v BSA (Wilfred Smith, Edgware, UK). To each well in a streptavidin coated plate (Boehringer–Mannheim Diagnostics and Biochemicals Ltd, Lewes, UK) 100 µl of the denaturated products in TBS was added. A control well consisted of TBS/0.1% BSA only. The plate was incubated overnight at 4 °C. The next day the wells were washed four times with TBS containing 0.1% Tween 20 (TBST). The method for detection of bound product was then as described previously (Samuelson et al., 1998; Darke et al., 1998). The cut-off was set at approximately 0.3 (three times the mean value of OD for negative controls plus three standard deviations (S.D.)).

A second plate hybridisation procedure used a 5' biotin-labelled capture probe (EnterBio1: 5'biotin GYAASTCYGYRGCGGAACCGA3') with detection of amplified products using a commercially-available PCR detection kit according to the manufacturers' instructions (DiaSorin Ltd, Wokingham, UK). In brief, streptavidin coated microwell plates were coated overnight at 4 °C with the 5' biotin-labelled capture probe. The wells were then washed. Single-round PCR products were denatured at 94 °C for 15 min and immediately placed on ice to prevent reannealing of strands. Twenty microlitres of each product were added to a well containing hybridisation buffer and incubated at 50 °C for 1 h. Microwell

Table 1  
Summary of primers and probes used for 'in house' and Basic Kit enterovirus NASBA

Name	Sequence 5'–3'	End label/assay format	Oligonucleotide location <sup>a</sup>
Entero2	GGTGYGAAGAGYCTAYTGAG	no label/'in house' and kit amplification with enterovirus specific probes	412–431
EnteroBK2	GGTGYGAAGAGYCTAYTGAG	5'ECL tail <sup>b</sup> /Basic Kit amplification and ECL detection	412–431
EnteroBIO2	CTCCGGCCCTGAATGCGGCTAAT	5' biotin/'in house' and Basic Kit detection	445–468
EnteroDIG2	TACTTTGGGTGTCCGTGTTTC	5' digoxigenin/'in house' plate detection	542–562
EnteroECL	TACTTTGGGTGTCCGTGTTTC	5' ruthenium/enterovirus-specific ECL detection	542–562
EnteroHRP	ACCGACTACTTTGGGTGWCCGTG	5' HRP/ELGA detection	536–558
Entero1	CACYGGWTGGCCAATCCAA	5' T7 tail <sup>c</sup> /'in house' and Basic Kit detection	639–621

Y = C or T, W = A or T, R = A or G; ECL, electrochemiluminescence; DIG, digoxigenin; HRP, horseradish peroxidase; ELGA, enzyme-linked gel assay.

<sup>a</sup> Refers to GenBank Accession No. NC 002058.

<sup>b</sup> ECL tail sequence (Basic Kit) 5' GATGCAAGGTCGCATATGAG.

<sup>c</sup> T7 sequence 5' AATTCTAATACGACTCACTATAGGG.

plates were washed and anti-double-stranded, protein A conjugated to horseradish peroxidase and a chromogenic substrate were added successively. The reaction was stopped, absorbance read at 450 nm and the cut-off value set at approximately 0.16 (calculated according to the manufacturers' instructions).

### 2.8. Primer and probe design for NASBA

Primers and capture probe were designed to include a T7 RNA polymerase promoter on primer 1 and a common tail sequence on primer 2 for detection of amplified products using a single ruthenium-labelled probe provided in the NucliSens<sup>®</sup> Basic Kit. In this study an enterovirus-specific ruthenium-labelled probe was also utilised to validate the Basic Kit format. Selection of primers and probes for the 'in house' and Basic Kit systems was a compromise and had to take into account the limited areas of conservation between different enteroviruses. Primers and probes for 'in house' and Basic Kit NASBA are given in Table 1.

### 2.9. Set up of NASBA using 'in house' reagents

This was undertaken according to the method described previously (Samuelson et al., 1998; Darke et al., 1998) using primers Entero1 and Entero2 (Table 1). Amplification was for 90–150 min (150 min in the final protocol) at 41 °C and the reaction was stopped by cooling on ice. The amplified products were either analysed immediately or stored at –80 °C for later study.

### 2.10. Set up of enterovirus NASBA using Basic Kit reagents

The set up of NASBA using the NucliSens<sup>®</sup> Basic Kit was much more straightforward than when using 'in house' reagents. The set-up generally followed the manufacturer's instructions (Organon Teknika Ltd). Primers (Entero1 and EnteroBK2, Table 1) were pre-mixed to give a concentration of 5 µM of each and stored at –20 °C until required. A final concentration of 70 mM KCl was used for the amplification. After transfer of the tubes to a water bath and amplification at 41 °C for 90–150 min (150 min in the

final protocol) the reaction was stopped by cooling on ice. The amplified products were either analysed immediately or stored at  $-80^{\circ}\text{C}$  for later study. In one set of experiments primer Entero2 (Table 2) replaced the EnteroBK2 primer to compare the amplification efficiency of a primer with and without the tail used for Basic Kit ECL detection.

### 2.11. Detection of 'in house' NASBA products by enzyme linked gel assay

Enzyme Linked Gel Assay (ELGA), a rapid non-radioactive 'in-solution' hybridisation assay, was performed according to the method of van der Vliet et al. (1993) with the modifications described previously (Darke et al., 1998; Samuelson et al., 1998). The dilution of each batch of peroxidase-labelled probe for ELGA (EnteroHRP, Table 1) was pre-optimised and analysed on a 7% acrylamide gel containing 0.04% w/v dextran sulphate.

### 2.12. Plate detection of NASBA products

This was undertaken as described previously (Darke et al., 1998; Samuelson et al., 1998). The optimum dilution of the biotinylated capture

probe and the digoxigenin-labelled detection probes (EnteroBIO2, EnteroDIG2, Table 1) was pre-determined. The cut-off in this assay was approximately 0.2 OD 450 nm (three times the mean value of OD for negative controls plus 3 S.D.).

### 2.13. ECL detection of NASBA products

Detection of Basic Kit NASBA products by ECL was undertaken in a similar way to that for the commercially-available assays [NucliSens<sup>®</sup> HIV-1 and cytomegalovirus (CMV) *pp67* assays] (van Gemen et al., 1994; Gerna et al., 1999) and utilised an ECL detection machine (NucliSens<sup>®</sup> Reader, Organon Teknika). The Basic Kit modified detection system utilises a common sequence in the amplified product so that a single ruthenium labelled probe can be utilised for detection of all amplified products. Specificity of the detection is dictated by use of a target-specific capture probe and the specificity of this approach was tested during the course of the study.

Coupling of the biotinylated capture probe (EnteroBIO2, Table 1) to streptavidin coated beads was undertaken according to well-established methods using molecular grade reagents supplied by Sigma Chemical Co, magnetic beads supplied in the Basic Kit and a magnetic particle concen-

Table 2  
Comparison of Basic Kit NASBA with RT-PCR on quality control samples

Expected result	'In house' RT-PCR		Basic Kit NASBA (general detection)	
	Gel detection	Plate hybridisation detection (OD 450 nm)	ECL Counts	Log <sub>10</sub> ECL counts
Strong positive	+	>2.00	340169	5.5
Strong positive	+	>2.00	188959	5.3
Strong positive	+	>2.00	343624	5.5
Strong positive	+	>2.00	146064	5.2
Positive	+	1.84	67853	4.8
Positive	+	1.51	139987	5.1
Positive	+	1.27	35996	4.6
Weak positive/equivocal	-	0.45*	1	0.0

Only samples with an expected or possible positive result are shown. The 14 samples which were expected to be negative did not give a signal in any of the assays. NASBA incubation was for 90 min for this experiment. Cut-off for plate hybridisation = 0.200. Cut-off ECL signal = 300 counts (log<sub>10</sub> = 2.5). \*Positive result, just above cut-off for the assay; ECL, electrochemiluminescence; OD, optical density.

trator (Dynal A.S., Oslo, Norway). A solution of 0.1% (w/v) BSA and 0.1% (w/v) 2-chloroacetamide in phosphate buffered saline (PBS) was made freshly (BSA buffer) and filtered (0.2 µm filter, Sigma Chemical Co). Streptavidin-coated magnetic beads (0.2 ml) were washed once and then reconstituted into 0.2 ml of the BSA buffer. A stock solution of biotin-labelled capture probe was then added to the bead-BSA buffer (equivalent to  $2 \times 10^{14}$  molecules of probe) and the mix incubated on a bench-top shaker at room temperature for 1 h. The bead-capture probe mix was then washed three times with BSA-buffer and finally reconstituted in 1 ml of the BSA buffer. The bead-capture probe mixture was kept at 4 °C until required.

Reagents required for ECL detection of NASBA products were supplied in the Basic Kit. A pre-optimised dilution of enterovirus NASBA product (1:10) was utilised in the detection using either the generic ECL probe supplied or an enterovirus-specific probe (EnteroECL, Table 1). The emission at 620 nm was read and the results analysed using the software supplied with the detection machine.

A weak positive enterovirus control (equivalent to 10 TCID<sub>50</sub> virus spiked into negative stool, CSF or respiratory material) and a negative control (negative stool, CSF or respiratory material) was included in each run and subjected to extraction, amplification and detection along with the test samples. The cut-off for the assay was set at  $0.01 \times$  reference solution (RS) value for the original 90 min assay and  $0.02 \times$  RS for the modified 150 min assay.

### 3. Results

#### 3.1. Extraction and sample preparation

Manual and automated extraction gave equivalent results for cultures and clinical samples evaluated during the course of the study (data not shown). Preparation of RNA using the NucliSens<sup>®</sup> extractor was less time consuming than the manual method (timings given in Fig. 1).

#### 3.2. Optimisation of NASBA using the Basic Kit

Initial experiments utilised enterovirus positive and negative culture material ( $n = 10$ ) to assess the effect of KCl concentration on amplification efficiency. The standard protocol with 70 mM KCl (final concentration) gave good results in this experiment and no benefit was seen in adjusting this. Amplification was initially undertaken for 90 min but was later changed to 150 min because of the slightly slow reaction kinetics for the primer/template combinations used (see below).

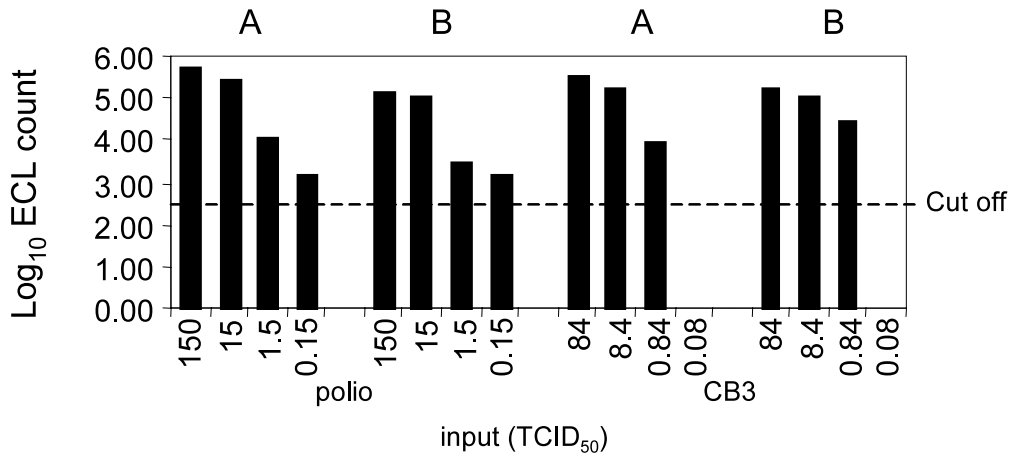
#### 3.3. Sensitivity of different enterovirus assay formats

The sensitivity of the semi-nested RT-PCR, tested on enterovirus samples of known titre, was  $\leq 1\text{TCID}_{50}$  input for all detection formats. Detection sensitivity was also  $\leq 1\text{TCID}_{50}$  input for each enterovirus in the Basic Kit and 'in house' NASBA assays.

In a series of experiments where dilutions of enteroviruses were subjected to extraction, amplification by RT-PCR or NASBA and detection by plate hybridisation or ECL, there was no more than a 10-fold difference in end point detection sensitivity (and no method that performed consistently better). The detection sensitivity for each assay was equivalent to approximately 10–100 TCID<sub>50</sub>/ml sample (depending on whether the concentration step was incorporated into the extraction). The preferred detection method was plate hybridisation ('in house' or kit based) for RT-PCR and ECL for NASBA because of the subjective reading of weak positive bands and laborious nature of plate hybridisation compared with ECL. It would be possible to modify the ECL detection for use with RT-PCR products and this may be a useful alternative detection procedure for some laboratories.

In no case did the Basic Kit NASBA with general ECL probe detection give different results to the enterovirus-specific ruthenium labelled probe. There was also no difference in amplification efficiency when using the Entero2 and EnteroBK2 primers with detection of amplified products using the enterovirus-specific ruthenium-

## Basic Kit NASBA



## 'In house' NASBA

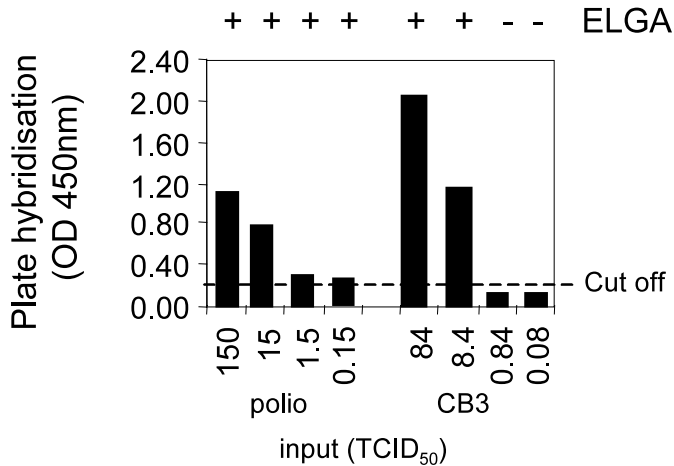


Fig. 2. ECL, electrochemiluminescence; CB3, coxsackievirus B3; polio, vaccine poliovirus; A, results for detection with enterovirus-specific ECL probe; B, results for general ECL probe (Basic Kit format); ELGA, enzyme-linked gel assay; OD, optical density. NASBA incubation was for 90 min for this experiment.

labelled probe. A comparison of results for diluted cultures using ruthenium-labelled general and enterovirus-specific probes is described below

and given in Fig. 2. All of the sensitivity experiments were conducted with a NASBA incubation time of 90 min.

### 3.4. Analysis of stool specimens

A series of 100 stool samples for which culture results were available had been stored as suspensions and were analysed by Basic Kit NASBA. Twenty-three samples had been reported as enterovirus culture positive (4 polioviruses, 10 Coxsackie B viruses, 2 echoviruses and 7 untyped enteroviruses), 25 contained detectable adenoviruses ( $n = 6$ ) or rotaviruses ( $n = 19$ ). The other 52 samples did not contain detectable viruses by 'conventional' culture, antigen detection or electron microscopy.

The molecular assays gave equivalent results on stored stool specimens. Of the 23 enterovirus culture positive stool samples, 21 were positive by all NASBA and RT-PCR protocols. One previously-reported culture positive sample had no detectable enterovirus RNA by any molecular method and one sample gave a positive result above cut-off in only one format RT-PCR. When these two samples were re-analysed by culture there was evidence that the samples had degraded on storage as virus was not culturable from the stored material. None of the stool sample suspensions in which either adenoviruses or rotaviruses had been identified had detectable enterovirus RNA by any molecular method. Of the 52 stool samples reported as containing no detectable virus by conventional methods, 48 were negative by all molecular methods. Two samples had detectable enterovirus RNA by all molecular methods; 1 had detectable enterovirus RNA by all molecular methods except 1 format RT-PCR and 1 sample was below but close to the cut-off in Basic Kit NASBA, 'in house' NASBA and both format RT-PCRs. This sample was investigated more closely with respect to Basic Kit NASBA results (see below). In this series of experiments the sensitivity and specificity of the different enterovirus molecular assays, as applied to stool specimens, was equivalent.

A 90 min incubation time was used for all NASBA formats and the ECL counts for positive and negative stool specimens are given in Fig. 3. The mean  $\log_{10}$  ECL count for the 24 positive samples was 5.1 (range = 3.8–5.7) whereas that for the 76 samples giving a reading below cut-off

was 0.9 (range = 0.0–2.3). The two samples falling closest to the cut-off in this assay were investigated further ( $\log_{10}$  ECL count 2.2 and 2.3 with cut-off at 2.5).

### 3.5. Evaluation of different NASBA formats

Typical results for diluted virus stocks analysed by the different format assays are given in Fig. 2. As detailed above, the two detection systems which utilised ECL detection (standard Basic Kit and analysis using an enterovirus-specific ruthenium labelled probe) were the methods of choice since the plate hybridisation was laborious to perform and the ELGA was difficult to interpret for weak positives. The software for detection and analysis of ECL signals allows the user to define values for appropriate controls (positive, negative, internal and external) which will determine whether the assay is valid, cut-off for wild-type and control signals. Thus, this is particularly suitable for use in a diagnostic setting. Once the assay has been developed the software provides a simple read-out of 'valid positive', 'valid negative' etc.

The sensitivity of the NASBA reactions was good for both formats of ECL detection. The Basic Kit format with general ruthenium-labelled

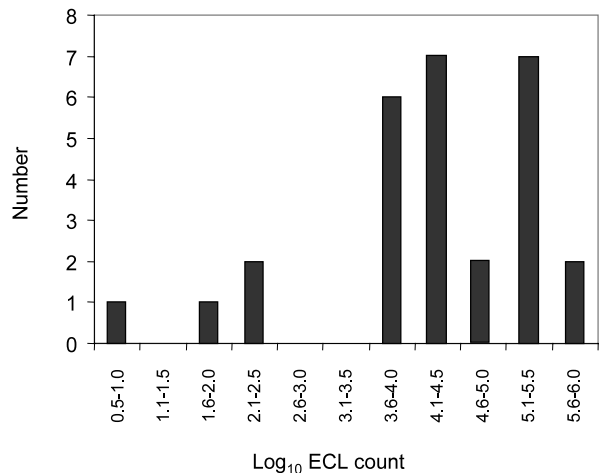


Fig. 3. Only those samples giving an ECL signal > 1 are included in the graph. 72 samples with a  $\log_{10}$  ECL count of 0 have not been plotted. NASBA incubation was for 90 min in this experiment and the cut-off ECL signal = 350 counts ( $\log_{10} = 2.6$ ,  $0.01 \times$  reference solution).

probe was considered to be the best approach for diagnostics since the system could be easily applied to other RNA targets with the design of a pair of target-specific primers and a biotinylated capture probe.

When serial dilutions of enterovirus targets were analysed it was noted that the amount of amplicons was not always independent of target input. This is illustrated in the results given in Fig. 2. The trail off in signal with dilution of target did not seem to be related to sequence variation in the primers and suggested that the amplification kinetics were not optimal. This was investigated further and led to a simple modification of the procedure (see below).

Primers and probes for Basic Kit NASBA were designed to amplify as wide a range of enteroviruses as possible without cross-reaction with rhinoviruses. Highly divergent parechoviruses would not be detected with these primers, as has been reported for other molecular assays using a single-set of target-specific oligonucleotides. Using the Basic Kit NASBA enterovirus positive cultures and samples gave signals at least 100–1000 fold higher than those for rhinovirus positive cultures and samples. In no case did a rhinovirus control (1000 TCID<sub>50</sub> input of the types listed in methods spiked into a negative respiratory specimen) give a signal above the cut-off for the assay. The range of log<sub>10</sub> ECL signals for nine rhinovirus cultures tested in a single experiment was 1.9–2.2 (mean = 2.1) compared with a range of 4.6–7.0 (mean = 5.8) for a series of 25 enterovirus positive culture preparations and stool specimens. The cut-off in this assay was log<sub>10</sub> ECL signal of 2.8.

### 3.6. Analysis of quality control samples by Basic Kit NASBA and RT-PCR

Twenty-two clinical samples and diluted controls were distributed between a number of different laboratories in order to assess the performance of different assays and methods. These samples were tested in Cardiff by Basic Kit NASBA and RT-PCR (gel and plate hybridisation detection). Results for the eight samples for which a positive result was expected or likely are given in Table 2; all other samples gave no de-

tectable signal in any of the enterovirus molecular assays. One sample gave a result just above cut-off in the RT-PCR with plate hybridisation detection but this was not positive by any other assay on any site. Thus results from this 'in house' comparison and distribution of the quality control panel confirmed that the Basic Kit NASBA gave very good results which were comparable with a range of different format RT-PCR methods. For other supplied quality control material ( $n = 108$ ) a comparison was made between the different 'in house' format RT-PCR results and NASBA (Basic Kit format). The results obtained were not significantly different for these different approaches.

### 3.7. Modification of the NASBA assay conditions

The occasional sample giving ECL readings close to cut-off and the trail off in signal when a dilution of target template was used (Fig. 2) led us to investigate whether the NASBA reaction kinetics were slower than expected. In vitro prepared poliovirus-specific RNA was utilised directly in an amplification reaction to determine the absolute sensitivity of the assay with ECL detection (Basic Kit format). When the original incubation time of 90 min was utilised the end-point detection sensitivity was approximately 100–1000 copies of target sequence with a similar trail off in signal as shown for cultured virus preparations (Fig. 2). When the incubation time was increased to 150 min the end-point detection gave a much clearer cut-off. Typical results are given in Fig. 4 and confirm that 50 copies of target RNA were detectable using the Basic Kit NASBA with this modified amplification time. On repeating these experiments the end-point was found to be stable between 10 and 50 copies of target sequence with only minimal trail off in signal as the target was diluted. Thus the modified assay uses an incubation time of 150 min; the reason for this slightly slower amplification than would normally be expected is currently being investigated. Where available, weak positive samples tested previously were re-tested using the longer incubation, all had an enhanced ECL signal relative to background using this modified approach.

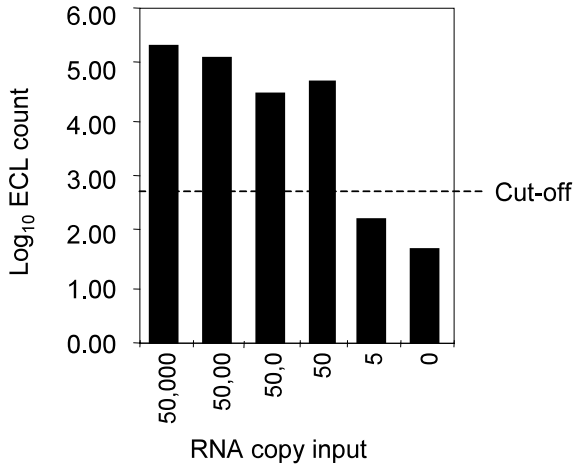


Fig. 4. ECL, electrochemiluminescence; NASBA incubation was increased to 150 min for this experiment.

### 3.8. Analysis of CSF and respiratory specimens by Basic Kit NASBA

The modified Basic Kit NASBA was used to screen 200 (unselected) CSF samples submitted for investigation of possible central nervous system disease and 80 pernasal and throat swab samples (taken because of current/recent respiratory symptoms). All CSF samples were subjected to virus culture and four enteroviruses were identified. These four culture positive samples and an additional two samples were found to contain detectable levels of enterovirus RNA by Basic Kit NASBA. All six of these samples also gave positive results by RT-PCR with gel and plate hybridisation detection. Of the 80 swab samples analysed four were positive by the enterovirus Basic Kit NASBA and RT-PCR with gel and plate hybridisation detection. Another six were confirmed as containing detectable rhinovirus RNA by the NASBA method of Samuelson et al. (1988). Only one of these respiratory samples was rhinovirus culture positive reflecting the low sensitivity of culture on swab material. The mean signal for positive CSF samples was  $\log_{10}$  ECL 4.1 (range 3.7–4.7) and for respiratory samples was 4.3 (range 3.7–5.0). Further prospective evaluation of the enterovirus assay on a range of clinical samples is being undertaken in Cardiff and collaborating laboratories.

## 4. Discussion

The enterovirus Basic Kit NASBA assay, in its optimised, final format, will be useful in a diagnostic setting. Many enterovirus types grow poorly in tissue culture and Basic Kit NASBA provides a time saving compared with most frequently used nucleic acid amplification procedures. Results can be made available in a time frame which is relevant to patient management. Rapid and sensitive diagnosis of enterovirus meningitis will avoid unnecessary and costly inappropriate antibiotic use. The enterovirus Basic Kit NASBA gave equivalent results to 'in house' RT-PCR on cultured virus preparations, clinical samples and quality control material but was easier to perform because of the use of pre-prepared, quality controlled master mixes. This is of particular importance in a diagnostic setting where there is a premium on assay robustness. The enterovirus Basic Kit NASBA assay is much less time consuming than 'in-house' RT-PCR (particularly where a plate or other hybridisation-detection system is incorporated) and even with the modified incubation times described, results are easily available within a working day.

Recently, a study to evaluate an enterovirus-specific kit-based RT-PCR was published (Carroll et al., 2000). This study confirmed the diagnostic utility of a kit-based assay and had a reduced turn-around time and enhanced sensitivity over culture for analysis of CSF samples. Unfortunately, this assay is no longer available commercially. The detection of enterovirus RNA using the Basic Kit has two main advantages above this methodology; the assay has been validated for a number of specimen types (i.e. CSF, respiratory and stool specimens) and the format is such that the reagents can be used in a flexible way (i.e. one kit can be used for a number of different RNA targets of interest). Thus, laboratories where small numbers of a range of samples need to be tested for enterovirus RNA on a regular basis can make use of the technology.

The next development in the enterovirus NASBA will be use of controls to help interpretation of samples which do not contain detectable levels of target RNA. Currently, the assay is run

only with weak positive and negative external controls. Such controls ensure primers, probes and other reagents are working but do not validate the extraction and amplification efficiency for each sample. Multiplex NASBA reactions have been developed and evaluated (van Deursen et al., 1999). A multiplex assay affords the opportunity to add a control with a separate set of target-specific primers. The amplified material could then be split at the detection stage and an assessment of the relative extraction and amplification efficiency made. This is likely to be sufficient for the majority of 'in house' assays but, where large numbers of samples are likely to be tested, (as in the routine diagnostic laboratory) an internal control (amplified with the same primers as wild type target but with a different internal sequence) is a major advantage to quality assurance. Such a control is being developed for the enterovirus assay. Thus in the final format it will be possible to spike an internal control into the sample prior to storage and then compare wild-type and control amplification at the ECL detection stage. For enterovirus positive samples a further refinement to the method could be an additional detection step to identify poliovirus sequences specifically. This will be essential as laboratories are required to identify poliovirus in order to inform WHO surveillance for the global poliomyelitis eradication programme. A poliovirus-specific NASBA has been developed and is currently being evaluated for use along-side the pan-enterovirus Basic Kit NASBA.

The ECL system is a great improvement over many hybridisation systems but as the demand for molecular assays increases it would be most convenient to avoid handling amplified products and to include the probe detection in the amplification stage. 'In tube, real-time' detection of NASBA products has already been reported for one RNA target (Leone et al., 1998) and will be the next major technology advance in the use of kit-based NASBA. Enterovirus specific molecular beacons have been designed and the likely success of this approach has been confirmed. Detection of NASBA products using a molecular beacon (or other fluorescent labelled probe) would have the advantage of giving information about the kinet-

ics of the reaction allowing us to investigate further the need for a longer amplification time than is usual for the enterovirus assay. This development will also offer the possibility for development of quantitative assays for enterovirus RNA in clinical samples.

In conclusion, the pan-enterovirus Basic Kit NASBA is a suitable diagnostic molecular amplification method for analysis of enterovirus sequences in stool, CSF and respiratory samples. The use of kit-based reagents and the fast turn around of results compared with 'in house' RT-PCR will enhance the cost-effectiveness and quality control of molecular-based diagnostic procedures for investigation of enterovirus meningitis and other infections. Further advances and modifications of the assay will include development of an internal control and the flexibility to use 'real-time' detection of amplified products.

The advantage of the Basic Kit format is that laboratories with only a small number of specific requests for RNA amplification methods could make full use of expensive amplification reagents by using a single kit for multiple targets which could include viral genomic RNA, rRNA or mRNA.

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