

# Rapid and highly sensitive qualitative real-time assay for detection of respiratory syncytial virus A and B using NASBA and molecular beacon technology

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## Abstract

The performance of a sensitive and specific qualitative respiratory syncytial virus (RSV) assay based on NASBA technology and real-time molecular beacon detection is presented. Very low detection limits for both RSV A and RSV B were determined: 95% detection hit-rate of 95 and 47 copies/input in isolation for RSV A and RSV B, respectively. RSV was detected in a wide variety of clinical samples including respiratory swabs, nasopharyngeal aspirates (NPA), bronchoalveolar lavages (BAL), endotracheal secretions, and sputum samples. In total 779 clinical samples were tested and a valid result was obtained for 765 (RSV NASBA assay), 765 (cell culture), and 529 (rapid direct immunofluorescence testing (IF)) samples. Of these samples, 229 (RSV NASBA assay), 61 (cell culture), and 122 (IF) samples were positive for RSV. In addition, 106 samples were reported as RSV negative using the NOW<sup>®</sup> RSV assay (Binax). Subsequent testing using the RSV NASBA assay demonstrated that 32 (30%) of these samples were RSV positive. The RSV NASBA assay includes a homologous internal control, which offers a high degree of standardization and quality control. When the RSV NASBA assay was performed on the NucliSens EasyQ platform (bioMérieux), test results of 48 sample extracts were obtained in less than 2 h.

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## 1. Introduction

Respiratory syncytial virus (RSV) is a major cause of severe lower respiratory tract infections in infants, vulnerable older children, and adults with underlying conditions. It is associated with annual epidemics in the winter months in temperate climates leading to high rates of morbidity and hospitalization in these patient groups. Fast and accurate diagnosis is crucial for appropriate patient management and infection control measures to prevent nosocomial transmission. Antigen detection assays, such as direct immunofluorescence assays (DFA), enzyme immunoassays (EIA), and more recently immunochromatography point of care tests (POCTS) on nasopharyngeal aspirates (NPA) have become common rapid methods for RSV detection particularly in samples from infants (Lipson, 2002;

Ohm-Smith et al., 2004; Aldous et al., 2004). With these assays, results are obtained within an hour of sample receipt and the associated costs are moderate (Barenfanger et al., 2000; Lipson, 2002). However, these assays often have limitations in sensitivity and specificity as compared to molecular tests (Abels et al., 2001; Liolios et al., 2001; Falsey et al., 2002; Boivin et al., 2004; Kuroiwa et al., 2004), particularly on samples of poor quality or those from adult patients, and DFA, the rapid method of choice in many laboratories, requires good technical skills, high quality reagents, and equipment for optimal sensitivity. Because of the higher costs and longer turnaround time of the molecular tests, as compared to the antigen-detection tests, some laboratories use antigen detection to screen incoming clinical samples and only perform a more sensitive molecular test on the negative samples (Gruteke et al., 2004). Some laboratories use an 'in-house' developed assay (Abels et al., 2001; Falsey et al., 2002; Whiley et al., 2002; Hu et al., 2003; Boivin et al., 2004; Dewhurst-Maridor et al., 2004; Templeton et al., 2004). However, many laboratories do not have the appropriate facilities to

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develop their own molecular test for RSV or prefer to work with a quality controlled standardized molecular test, emphasizing that there is a need for commercial molecular RSV assays.

This paper describes the performance of a qualitative real-time assay for detection of RSV A and B using NASBA (for review see Deiman et al., 2002) and real-time molecular beacon detection technology. The RSV assay includes a homologous internal control (IC) to control both the isolation and amplification step. The assay is highly sensitive and specific, and delivers a same-day result, which leads directly to rapid appropriate patient management and the implementation of infection control measures to prevent further transmission events.

## 2. Materials and methods

### 2.1. Alignments

Alignments were made, using DNAMAN software Version 3.2 (Lynnon BioSoft), on sequences taken from EMBL/GenBank database. The accession numbers that were used for the selection of the primers and molecular beacon are as follows: RSV A: AF512538, AY198177, Z26524, L25351, U39661, U31562, U50362, M22643, X02221, AY198176, AY198175, AY114151, AY114150, AY114149, U39662, U63644, AF035006, U31561, U31560, U31559, U31558, D00151, M74568, M11486, AF067125 and for RSV B: NC\_001781, AF013254, AF013255, and D00334.

### 2.2. Primer and molecular beacon design

Primers and molecular beacons were designed in a conserved part of the F-gene region of RSV A and RSV B. One set of primers was used to amplify both RSV A and RSV B and the IC. One generic molecular beacon (MB) probe was designed to detect both RSV A and RSV B. An additional beacon, RSV MB IC was designed for detection of the IC. The generic RSV beacon was labeled with FAM and the IC beacon was labeled with ROX at the 5' ends and Dabsyl was used as the quencher at the 3' ends. The molecular beacons were designed making use of the DNA folding program Mfold to predict the structures of the resulting molecules (<http://www.bioinfo.rpi.edu/applications/mfold>). The purity of the primers and beacons was determined to be at least 90% by capillary gel electrophoresis. For theoretical determination of specificity, primers and beacons were aligned in NCBI BLAST (<http://www.ncbi.nlm.nih.gov/blast/>).

### 2.3. Wild type and internal control RNA

RSV A wild type (WT) and RSV B wild type plasmids were constructed for the *in vitro* production of the corresponding wild type transcripts. Supernatant of tissue culture material of RSV A was obtained from the Wales specialist virology center of the University Hospital of Wales (Cardiff, United Kingdom) and of RSV B (no. V3976) was obtained from the *Centre d'Immunologie Pierre-Fabre (Saint-Julien en Genevois, France)*. An RNA fragment of 249 bp of the F-gene was multiplied by RT-PCR and cloned in-between the *EcoRI* and *Csp45I*

sites of a pGEM-3X-based vector. Sequencing of the plasmid showed that the sequence of the RSV A fragment corresponded to EMBL accession number Hru39662, position 6640–6889, and the RSV B fragment corresponded to EMBL accession number D00334, position 1014–1271.

The RSV A wild type plasmid was used for the construction of the IC plasmid from which the IC RNA could be transcribed. The IC plasmid is identical to the WT plasmid except for a fragment of 20 bp including the molecular beacon-binding site for the generic RSV detection which was replaced by a random sequence, not related to RSV, of 20 bp including the IC molecular beacon-binding site, using fusion PCR. The correct sequence was confirmed by sequencing (Baseclear).

The WT and IC plasmids were digested with Bam HI prior to run-off T7 transcription (Ribomax™ large scale RNA production system T7, Promega). The transcripts were purified (RNeasy mini kit, Qiagen), and the concentration was determined by OD ( $A_{260}$ ) measurement. The length of the RNA (1178 nt for both the WT and IC) was checked by gel electrophoresis and bioanalyzer (Agilent Technologies). All transcripts were stored at  $-70^{\circ}\text{C}$ .

### 2.4. Isolation

Nucleic acid was isolated from clinical samples or transcript samples using the NucliSens miniMAG (bioMérieux), in combination with the NucliSens magnetic extraction reagents (bioMérieux) and NucliSens Lysis buffer (bioMérieux) according to the manufacturers instructions. For transcripts in water, 1 ml defibrinated human plasma (Seracon I, Serologicals corporation) was added to mimic sample background. Fifty to two hundred microlitres of sample, was added to 2 ml of Lysis buffer. The mixture was incubated at RT for 10 min, and 3000 cp IC was added. To this, 50  $\mu\text{l}$  of magnetic silica was added and the mixture incubated for another 10 min at RT. The silica was then washed several times and the nucleic acid eluted in 25  $\mu\text{l}$  elution buffer following incubation for 5 min at  $60^{\circ}\text{C}$ . The extracts were used directly in amplification or stored at  $-80^{\circ}\text{C}$ .

### 2.5. Amplification, detection, and data analysis

A primer mixture was prepared using the reagents from the NucliSens EasyQ basic kit Version 2 (bioMérieux) and the RSV primers and probes. The WT and IC targets are amplified with the same RSV related primer pair. Amplification reactions were performed at 100 mM KCl. The final concentration in the reaction mixture of each primer was 0.2  $\mu\text{M}$ , and the final concentrations of the FAM and ROX molecular beacons were at 0.01  $\mu\text{M}$  and 0.1  $\mu\text{M}$ , respectively. For each amplification reaction, 5  $\mu\text{l}$  of extract was added directly to the primer mixture. Removal of secondary RNA structure and primer hybridization was performed by a two-step incubation of 2 min at  $65^{\circ}\text{C}$  and 2 min at  $41^{\circ}\text{C}$ . To each reaction, 5  $\mu\text{l}$  of enzyme mixture was added, amplification and simultaneous detection was then performed using the NucliSens EasyQ analyzer (bioMérieux) at  $41^{\circ}\text{C}$  for 90 min. The fluorescence signal was measured with an interval time of 30 s for each independent reaction at two wavelengths using

the accompanying NucliSens EasyQ Director software (Version 2.0). For data analysis the QL1 calculation engine was used and the following parameters were defined: ‘target detection threshold’: 1.1, ‘IC lambda threshold’: 3.0, ‘Growth threshold’: 50, ‘Maximum time to primer depletion’: 60.

## 2.6. Assay sensitivity

For determination of assay sensitivity, a dilution series of RSV A and RSV B transcript of 125, 100, 75, 50, 35, 25, and 12.5 copies/input in isolation (independent dilutions), were tested. The highest input concentrations (125 and 100 copies/input) were replicated 12 times. The other inputs were tested in 24 replicates. To each dilution of WT RNA, 3000 cp IC RNA, 2 ml Lysis buffer and 1 ml defibrinated human plasma were added. To control for contamination and inhibition from extraction through to amplification and detection, each isolation run ( $n = 24$ ) included two no template controls (NT) (=no WT, no IC input) and three no sample controls (NS) (=no WT, normal IC input) in 2 ml Lysis buffer and 1 ml defibrinated human plasma. In all amplification runs extra NT (amplification NT\*) were tested. For these NT\* 5  $\mu$ l NASBA water were used instead of eluted clinical or control material.

## 2.7. Sample type comparison study

Twenty-four RSV negative nasopharyngeal swab samples obtained from the National flu center (Geneva) were pooled together. From this 200  $\mu$ l aliquots were spiked with 45 copies of RSV A transcript, and tested in 20 replicates using the RSV NASBA assay, from this the 80% hit-rate as determined by probit analysis.

In addition, six RSV negative bronchoalveolar lavages (BAL) samples were obtained from the *Centre national de référence des légionelles* (hôpital Edouard-Herriot, Lyon, France) and 200  $\mu$ l aliquots of these were spiked with 3000 copies IC and tested for RSV.

To determine the assay sensitivity in BAL samples, 11 RSV negative BAL samples obtained from the ‘*Centre national de référence des légionelles*’ (hôpital Edouard-Herriot, Lyon, France) were pooled. From this pool, 100  $\mu$ l was treated with 10 units DNase I (Promega, Southampton, UK) in DNase I buffer (provided with the enzyme) for 1 h at 37 °C or with 20 units of DNase I in DNase buffer for 30 min at 37 °C to degrade cellular DNA before isolation. The BAL samples were then spiked with 45 copies (80% hit-rate) of RSV A transcript and tested in 20-fold. For comparison, 1 ml defibrinated human plasma samples were spiked with the same input and tested in 20 replicates. To all samples IC (3000 copies) and 2 ml Lysis buffer was added before isolation. Three types of NT have been included: 2 ml Lysis buffer with 1 ml defibrinated human plasma, 200  $\mu$ l of RSV negative nasopharyngeal swab pool, or 100  $\mu$ l DNase-treated BAL.

## 2.8. Cross-reactivity

Assay specificity was tested using the following targets: parainfluenza virus (PIV 1: V4036: from ATCC ref

VR-907 strain sendai/cantell, PIV 2: V4035: from ATCC ref VR-92 strain greer, PIV 3: V4029: strain unknown), human metapneumovirus (hMPV A1: NL/1/00, hMPV A2: NL/17/00, hMPV B1: NL/1/99, hMPV B2: NL/1/94) (acquired from Vironovative (Rotterdam, the Netherlands)), Measles (V4158 from ATCC ref VR-24 strain Edmonster 97-08). In addition a ‘respiratory viruses’ panel (diagnostic hybrids, Athens, OH, USA) was tested including: RSV (RSV long, RSV9320, and RSV/B/wash/18537/62), influenza virus (Influenza A: A/WS/33, A/Mal/302/54, A/Victoria/3/75 and A/PortChalmers/1/73, Influenza B: B/Taiwan/2/62, B/GL/1734/54), PIV (PIV 1: C-35, PIV 2: Greer, PIV 3: C234), Adenovirus (Type 1: Adenoid 71, Type 5: Adenoid 75). For each sample, 200  $\mu$ l was used for the extraction.

## 2.9. Clinical samples

Seventeen respiratory clinical samples (nasopharyngeal swabs) were obtained from the Rockefeller virology laboratory (Lyon, France). These samples were found to be RSV positive, and some were further characterized as RSV A or RSV B. The samples were received frozen and were stored at  $-70$  °C until testing. For RNA extraction, 200  $\mu$ l of each sample was added to 2 ml of Lysis buffer. Before extraction, 3000 cp IC RNA was added to the sample once added to the Lysis buffer.

In addition, 116 respiratory swabs, 586 nasopharyngeal aspirates, 12 endo-tracheal secretions, 59 bronchoalveolar lavages, and six sputum samples, collected during the winter 2004–2005 season, were tested in the Wales specialist virology center (UHW, Cardiff, United Kingdom). Most samples were received in virus transport medium comprising of Hanks BBS  $\times$  1 (BioWhittaker<sup>TM</sup>, Workingham, Berkshire, USA), 7.5% bovine albumen (Sigma-Aldridge, Poole, Dorset, Surrey, UK), penicillin (Britannia pharmaceuticals, Redhill, Surrey, UK) and streptomycin (Sigma-Aldridge). All samples were tested as soon as they arrived in the laboratory and were not stored for any length of time. The samples were incubated with 20 units of DNase I (Promega, Southampton, UK) at 37 °C for 30 min in DNase buffer (provided with the enzyme) prior to isolation. Some of the samples were initially screened in external laboratories using the NOW<sup>®</sup> RSV Assay (Binax). Samples from which cells could be obtained were screened with IFA using Simulfluor<sup>®</sup> immunofluorescence screening assay (Light diagnostics, Chemicon Europe Ltd, Hampshire, UK). In addition, most samples were tested by cell culture using primary monkey kidney cells, human hepatoma cells (PLC), MRC-5 cells or Hep-2 cells (for details see Materials and methods of the retrospective study, Moore et al., 2006).

## 3. Results

### 3.1. Analytical sensitivity of the real-time RSV NASBA assay

Based on the alignment of sequences of RSV A and RSV B, a real-time RSV NASBA assay was designed on the F-gene of

the viral genome. The assay includes an IC to control the entire process from nucleic acid isolation through to amplification and detection. To determine assay sensitivity a dilution series of *in vitro* transcribed WT RNA of RSV A and RSV B of concentrations of 12.5, 25, 35, 50, 75, 100, and 125 copies/input in isolation were tested. To mimic sample type background, commercially available defibrinated human plasma was added to the transcript dilution series. To each dilution 3000 copies/input in isolation of IC RNA was added. By probit analysis, a 50% hit-rate of 21 copies/input in isolation and a 95% detection hit-rate of 95 copies/input in isolation for RSV A (Fig. 1A), and a 50% hit-rate of 15 copies/input in isolation and 95% detection hit-rate of 47 copies/input in isolation for RSV B (Fig. 1B) was determined. By testing a dilution series of *in vitro* transcribed WT RNA of RSV A and RSV B directly in amplification, it was shown that this corresponds to a 95% hit-rate of approximately 10 and 5 copies/amplification reaction of RSV A and RSV B, respectively. This Factor 10 is explainable as, when using the NucliSens miniMAG for isolation, only 1/5th of the extract is used in amplification, and some of the nucleic acid can be lost in extraction (van Deursen et al., 2004).

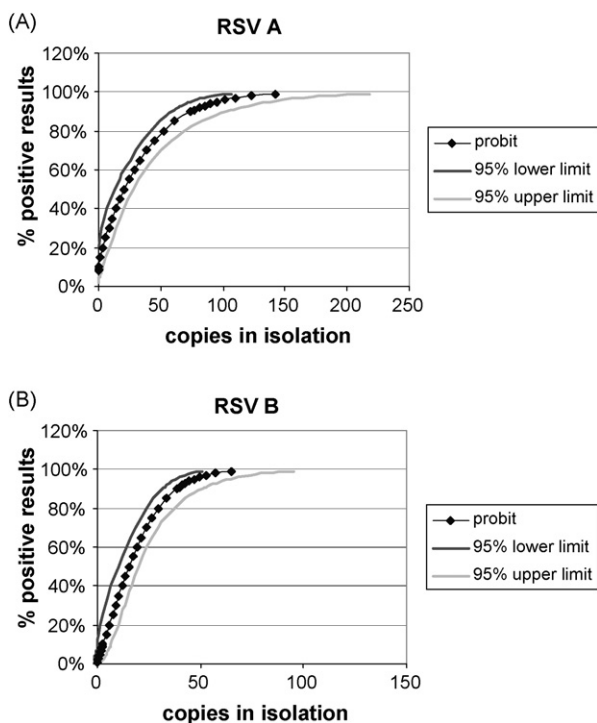


Fig. 1. An assay sensitivity for RSV. (A) *In vitro* transcribed WT RNA of RSV A of input concentrations of 12.5, 25, 35, 50, 75, 100, and 125 copies/input in isolation was tested. The highest inputs (125 and 100 copies/input in isolation) were tested in 12-fold. The other inputs were tested in 24-fold. The percentage of positive results per input is presented. The probit and the 95% lower limit and 95% upper limit are presented. Fig. 1. (B) Assay sensitivity for RSV B. *In vitro* transcribed WT RNA of RSV B of input concentrations of 12.5, 25, 35, 50, 75, 100, and 125 copies/input in isolation was tested. The highest inputs (125 and 100 copies/input in isolation) were tested in 12-fold. The other inputs were tested in 24-fold. The percentage of positive results per input is presented. The probit and the 95% lower limit and 95% upper limit are presented.

Table 1  
Sample type comparison

Matrix	Hit-rate (% positive reactions) <sup>a</sup>
Defibrinated human plasma/nasopharyngeal swab	
Defibrinated human plasma I	72
Nasopharyngeal swab	70
Defibrinated human plasma/BAL	
Defibrinated human plasma I	70
BAL	90

<sup>a</sup> Input used is 45 copies/input in isolation of RSV A, which is the 80% hit-rate in a defibrinated human plasma background as determined by probit analysis.

### 3.2. Sample type comparison study

To determine assay sensitivity, defibrinated human plasma was added to the transcript dilution series to mimic sample type background. To investigate assay sensitivity in RSV samples types, nasopharyngeal swab was compared to defibrinated human plasma. A pool of RSV negative nasopharyngeal swabs samples was used. To both matrices 45 copies/input in isolation of RSV A transcript, that is, 80% hit-rate in defibrinated human plasma as determined by probit analysis, was added. This input was tested in 20-fold. Results show that the number of positive reactions in nasopharyngeal swab background is 70%, which is comparable to that in defibrinated human plasma background, determined to be 72% in this experiment (Table 1). This indicates that assay sensitivity in nasopharyngeal swab samples is comparable to that in defibrinated human plasma background.

In addition, assay sensitivity was determined in BAL samples. Initially, six RSV negative BAL samples were spiked with 3000 copies/input in isolation of RSV IC. However, in only two out of six sample, IC was detectable (result not shown). BAL samples may contain a large amount of cellular DNA that could inhibit NASBA. Therefore, a pool of BAL samples was treated with DNase I. To investigate assay sensitivity, this pool was compared to defibrinated human plasma. To both matrices 45 copies/input in isolation of RSV A transcript (80% hit-rate), was added and tested in 20-fold. Results showed that the percentage of positive reactions in BAL samples treated with DNase I is 90%, which was not significantly different from that in defibrinated human plasma background, determined to be 70% in this experiment (Table 1).

Additional experiments showed that the DNase I sample pretreatment also improved RSV detection in nasopharyngeal aspirate samples and sputum samples containing high levels of mucus.

### 3.3. Assay specificity

To investigate assay specificity of the RSV NASBA assay, pathogens isolated from the same sample types were tested. No cross-reactivity could be observed for PIV1-3, Influenza A and B, measles, Adenovirus Types 1 and 5, hMPV A1, A2, B1, and B2, indicating that the assay was specific for RSV.

Table 2  
Clinical samples

Sample type	Number	Number of RSV positives by method			
		Culture <sup>a</sup>	Binax NOW	IFA	NASBA
Respiratory swabs	116	2	Not tested	Not tested	12
NPA	586	59	0	109 <sup>b</sup>	208
Endo-tracheal secretions	12	0	Not tested	1	1
BAL-directed	32	0	Not tested	2	4
BAL-non directed	27	0	Not tested	4	4
Sputum	6	0	Not tested	0	0
Samples tested	779	765 <sup>c</sup>	106 <sup>d</sup>	529 <sup>e</sup>	765
Total positive		61	0	122	229
Percentage positives		8	0	23.1	29.9

<sup>a</sup> All samples positive by culture were also positive in the RSV NASBA assay.

<sup>b</sup> One sample was tested negative for RSV by the RSV NASBA assay. This sample was tested three times by IFA and only once the result was positive for RSV.

<sup>c</sup> 29.5% of these samples were tested RSV positive by the RSV NASBA assay.

<sup>d</sup> 30.2% of these samples were tested RSV positive by the RSV NASBA assay.

<sup>e</sup> 32.7% of these samples were tested RSV positive by the RSV NASBA assay.

### 3.4. Evaluation of clinical samples

Initially seventeen clinical nasopharyngeal swab samples, indicated to be positive for RSV of unknown concentration, were tested. As shown in Fig. 2, RSV (RSV A, RSV B or nontyped RSV) was detectable in all samples.

In addition, clinical respiratory swabs, nasopharyngeal aspirates, endo-tracheal secretions, bronchoalveolar lavages and sputum samples were tested as part of a prospective study at the Wales specialist virology center (UHW, Cardiff, United Kingdom). In this study traditional laboratory techniques were directly compared with the RSV NASBA assay. The samples were obtained from various external laboratories referring samples to Cardiff for testing. Some samples were initially tested by, the NOW<sup>®</sup> RSV assay (Binax, Inc., Portland, Maine) and all samples from which cells could be obtained such as nasopharyngeal aspirates and bronchoalveolar lavages were screened by the simulfluor immunofluorescence-screening assay (Light diagnostics, Chemicon Europe Ltd, Eastleigh, UK). In addition

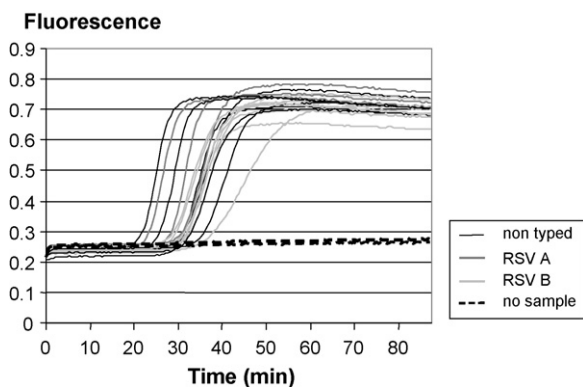


Fig. 2. Clinical samples. Seventeen nasopharyngeal swab samples indicated to be positive for RSV (A, B or non-typed) were tested. The fluorescence curves of real-time NASBA are presented.

most samples were tested in cell culture. As BAL and sputum samples were included, all samples were pretreated with DNase I before isolation. The number of RSV positive samples, as determined by the different methods, is presented by sample type (Table 2). A total of 779 samples were tested during this study, of which 106 were initially screened with the NOW<sup>®</sup> RSV assay (Binax), and 529 were screened with immunofluorescence (IF). Of these, cell culture results were available for 765 samples. All samples were tested with the RSV NASBA assay. A result was obtained for 765 samples giving an inhibitory rate of 1.8%. From a group of 106 NPA samples initially tested to be negative for RSV with NOW<sup>®</sup> RSV Assay (Binax), RSV was detectable with the RSV NASBA assay in 32 samples. Two of these samples were also found positive by cell culture. Samples initially determined to be positive for RSV by IF, were also positive for RSV using the RSV NASBA assay, except for one sample. However, this sample was tested three times by DFA and was only found RSV positive once. The greatest discrepancy of results came from NPAs tested by DFA, in total 354 were found to be negative by DFA in the initial screen, however on repeat testing using the RSV NASBA assay 62 (17%) were found to be RSV positive. In addition, 30 BAL samples that were initially reported as being negative for RSV by DFA and cell culture, an RSV positive result was obtained for another two samples using the RSV NASBA assay. Only 61 out of 765 samples were found RSV positive using cell culture whereas 229 out of 765 samples were found RSV positive using the RSV NASBA assay.

### 3.5. Clinical significance of clinical samples found to be RSV positive by NASBA alone

Of the clinical samples tested by all methods, 102 were found to be RSV positive by NASBA but not by any other method. In most cases a second sample from the patient was tested to confirm the initial positive result. These samples came from

89 patients. Nine of these patients had repeat samples tested during the study that were only RSV positive by NASBA. All nine patients presented with haematological malignancies and respiratory tract infections. In all cases RSV was the most significant pathogen repeatedly detected. A further 13 patients had a previous sample tested positive for RSV by both NASBA and traditional assays. The detection of RSV by NASBA alone in this group of patients probably represents the end of the infection and a low viral load detected only by molecular method. Conversely eight patients had repeat samples taken following this first test positive which were subsequently positive by other methods as well as NASBA. Together this data suggests that NASBA is effective in detecting early and late infection when compared to the less sensitive traditional methods. Again these 21 patients had underlying haematological malignancies highlighting the importance of RSV infection in this patient group. The remaining 59 patients had a single test RSV positive. By looking at the clinical picture of the patients and the results of other tests it is possible to determine the clinical significance of the RSV result. As described, RSV has its greatest burden in infants below the age of one year. Of the 59 patients, 49 were in this age group, all were hospitalized and of these, the primary clinical diagnosis in 36 was bronchiolitis. As all of the cases fell within the RSV season of 2004–2005 and without the detection of any other pathogen, it would be difficult to rule out RSV as the causative pathogen. The remaining infants presented with a range of respiratory symptoms including wheeze, shortness of breath cough, coryzal symptoms, and poor feeding. One infant was preterm and two required mechanical ventilation. Again as all presented within the defined RSV season and with the absence of an alternative diagnosis, the RSV result was considered clinically significant in all cases. The remaining 10 patients were older children and adults presenting with a range of respiratory symptoms, apart from one patient, all had underlying chronic conditions or trauma. Four were children below the age of 16 with haematological malignancy, one child presented with a confirmed parainfluenza Type 3 dual infection who required ITU treatment. The remaining six patients included one community patient presenting with influenza like illness and five hospitalized patients. Two patients had chronic obstructive pulmonary disease, one patient subsequently died. The remaining patients were haematology patients with respiratory tract infections. In all cases the RSV result was considered significant and the patients were managed accordingly.

#### 4. Discussion

Routine screening for RSV by molecular methods is becoming increasingly popular due to the low sensitivity and specificity of traditional assays, particularly in samples taken from adult patients, where the viral load was shown to be reduced when compared to infant samples. This paper describes the performance of a highly sensitive and specific RSV A + B real-time NASBA assay utilizing molecular beacon technology. By testing dilution series of transcripts of both RSV A and RSV B it is demonstrated that assay sensitivity for RSV A and RSV B transcripts is comparable, a 95% detection hit-rate of 95

and 47 copies per input in isolation for RSV A and RSV B, respectively. This assay sensitivity was confirmed in two RSV sample types: nasopharyngeal swab samples and BAL samples.

For most BAL material sample pretreatment with DNase I was required to remove extraneous DNA that inhibited the NASBA reaction. DNase I pretreatment also improved the detection of RSV in some nasopharyngeal aspirate and sputum samples.

The assay was shown to be highly specific for RSV, when tested against a panel of common respiratory pathogens including; PIV1-3, Influenza A and B, measles, Adenovirus Types 1 and 5, hMPV A1, A2, B1, and B2. No cross-reactivity was observed with the RSV assay. This was in agreement with results published previously (Moore et al., 2006) showing that a number of clinical sample tested negative with the RSV NASBA assay were tested positive for Influenza A, parainfluenza Type 1, parainfluenza Type 3, adenovirus, human metapneumovirus or herpes simplex Type 1.

RSV could be detected in a wide range of clinical sample types including nasopharyngeal swabs, and as part of a prospective study, respiratory swabs, NPAs, BALs, endotracheal secretions, and sputum samples. Of the 779 samples tested in the prospective study, a valid result was obtained with the RSV NASBA assay for 765 samples, giving an overall inhibition rate of 1.8%. Of these 765 samples, a positive result was obtained for 229 samples (29.9%) by the RSV NASBA assay. In comparison, the RSV isolation rate was only 8% in cell culture (61/765). Direct immunofluorescence on appropriate samples gave a detection rate of 23.1% (122/529) although some samples gave insufficient cells for an immunofluorescence test to be performed. The detection rate in these samples was increased using the NASBA assay giving an overall detection rate of 32.7% compared to 23.1% when using DFA alone. POCT are becoming routine in many centers. Of the 106 samples that were tested by the NOW<sup>®</sup> RSV assay (Binax) for which a negative result was obtained, 32 tested positive by the RSV NASBA assay (30.2%), and RSV was isolated in cell culture from two of these samples.

In total, 102 samples were tested RSV positive by the RSV NASBA alone. For most of these samples a second patient sample was tested to confirm the initial result. For all of these samples the validity of this positive result was supported by the information of the condition of the patient and the fact that samples were taken during the RSV season period and no other pathogen was detectable. In addition, some of these samples are part of a sample collection obtained from one and the same patient used for repeat testing during infection of which the previous samples or the subsequent samples were tested RSV positive also by the traditional methods, proving that the patient was indeed infected with RSV. In addition, the results of a retrospective study using the same RSV NASBA (Moore et al., 2006) showed that the presence of RSV RNA in samples tested positive by the RSV NASBA assay but negative by the traditional methods, could be confirmed by RT-PCR. However, as for all test systems, it should be mentioned that there is always a potential for any of these results to be false positives by NASBA.

In conclusion, the data presented in this paper demonstrated the RSV NASBA assay to be highly sensitive compared with cell culture, DFA, and the NOW<sup>®</sup> RSV assay (Binax). In addition, most respiratory sample types could be processed, using a combination of the NucliSens magnetic extraction reagents (bioMérieux) and the NucliSens EasyQ miniMAG (bioMérieux). It is recommended that a DNase I sample pretreatment step is used for samples containing high levels of mucus such as BAL, NPAs, and sputum samples.

The results of a retrospective study that was recently published (Moore et al., 2006), using the RSV A + B NASBA assay also demonstrated that this assay is indeed highly specific for RSV and highly sensitive compared to traditional assays. This retrospective study was a pilot study to show the performance of the RSV A + B NASBA assay on a large amount of clinical samples that had been frozen and tested some months after collection. The prospective study as presented in this paper shows how well the assay works in practice in a routine laboratory on freshly received samples. Together, it could be concluded that assay sensitivity was not affected by sample storage.

The RSV NASBA assay includes a homologous IC, which offers a high degree of standardization and quality control. In addition, using the NucliSens EasyQ Director software used for data analysis requires the specification of various assay specific software parameters what leads to a high degree of validity control and prevention of false negative results.

Using the RSV NASBA assay on the NucliSens EasyQ system, test results from 48 sample extracts were obtained in less than 2 h. Using the NucliSens EasyQ miniMAG as front end, 48 samples were processed and extracted within 2 hours and test results from 48 samples were obtained within 4 h. In case a DNase I pretreatment needs to be performed, the total sample to result time is approximately 4 h and 30 min. This rapid turnaround time and the ease of use of the assay, makes it an attractive alternative for routine RSV testing and a valuable addition to traditional testing.

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Except for USA, the RSV NASBA assay is worldwide commercially available as NucliSens EasyQ RSV A + B assay (bioMérieux). For USA, the NucliSens EasyQ RSV A + B ASR (bioMérieux) is commercially available.

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