Development of a new diagnostic tool for the detection of Rhinovirus/Enterovirus and Cellular control in a duplex RT PCR

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INTRODUCTION

A large number of respiratory agents involved in respiratory tracts infections, including viruses and bacteria share similar clinical features and symptoms. Enteroviruses and Rhinoviruses are the viruses most commonly implicated in respiratory infections in young children, the elderly, and in patients with depressed immune systems. Symptoms may be mild but the most common Upper and Lower Respiratory Infections (URTI and LRTI) in children and adults include tracheobronchitis, pharyngitis, laryngitis, sinusitis and also more severe illness like pneumonia.
Their rapid detection is difficult to obtain with immunological tests or cultures as these tests lack sensitivity and certain serotypes are not cultivable.
Now, the Nucleic Acid Amplification Techniques (NAATs) with Real-Time PCR techniques have many benefits for the detection of respiratory pathogens like high sensitivity and specificity and are much faster. We propose a new real-time PCR based diagnostic tool for Rhinovirus and Enterovirus diagnosis.
Rhino&EV/Cc r-gene™ helps to simplify the search for agents of respiratory infections. This kit allows for the detection of numerous serotypes not detectable by immunofluorescence and/or with greater sensitivity.
The Cellular control (Cc) included in duplex in this assay, assesses the quality of sample collection by validating the presence of cells, thus preventing a false negative result.

MATERIALS & METHODS

Extraction :
Nucleic acids were extracted from nasopharyngeal specimens (200µL or 400µL) by using NucliSENS® easyMAG™ (bioMérieux) and eluted in 50µL or 100µL respectively. For both 50µL of magnetic silica are used. A Proteinase K (Novagen) pre-treatment was performed with 10µL (for 200µL of sample) of PK at 20mg/mL and incubated for 15 min at 56°C.
Amplification :
0.15µL of reverse transcriptase was added to 15µL of Rhino&Ev/Cc amplification premix. Then 10µL of purified nucleic acids were added. Rhinovirus or Enterovirus were detected at 530nm and the Cell control was detected at 560nm. Amplification was performed on ABI 7500 Fast (Applied Biosystems), Dx Real Time System (Bio-Rad), LightCycler 480 (Roche) or Versant kPCR AD (Siemens).

QCMD European Proficiency Panel
Rhinovirus RNA 2011 :
This Panel was extracted on NucliSENS® easyMAG™ extraction Specific B of 200µL of sample eluted in 50µL. Subsequently, the samples were analysed by real-time PCR using Rhino&EV/Cc r-gene™ - ref.: 71-042 on ABI 7500 Fast.
Enterovirus RNA 2012 :
This Panel was extracted on NucliSENS® easyMAG™ extraction Specific B of 200µL of sample eluted in 50µL. Subsequently, the samples were analysed by real-time PCR using Rhino&EV/Cc r-gene™ - ref.: 71-042 on LC480.

Specificity
The specificity of the kit Rhino&EV/Cc r-gene™ assay was determined experimentally on samples containing various viruses/bacteria that may be involved in respiratory diseases or present in respiratory samples at high viral/bacterial load.
NucliSENS® easyMAG™ extraction from 400µL of sample eluted in 100µL was performed then amplification was done on Versant kPCR AD.

Analytical sensitivity
The analytical sensitivity of the kit Rhino&EV/Cc r-gene™ assay was determined from stock solution of Rhinovirus 14 cell cultures (at 3.71x 10^{+5} TCID50/mL).
Six serial dilutions were performed in a nasopharyngeal (NP) negative sample for Rhinoviruses. Each dilution was extracted 15 times using NucliSENS® easyMAG™ extraction with 200µL of sample eluted in 50µL. PK pre-treatment was performed. Each extract was amplified with Rhino&EV/Cc r-gene™ kit on ABI 7500 Fast and Dx Real Time System.

RESULTS

Rhinovirus RNA EQA QCMD Panel 2011

Rhinovirus RNA EQA QCMD European Proficiency Panel 2011 Results					Rhino&EV/Cc r-gene™ Results on ABI 7500 Fast	
Panel Code	**Sample Content**	**Sample Type**	**Dilution Factor**	**Expected Results QCMD real time in-house PCR ABI7500 (CT)**	**Rhino&EV (CT)**	**Cells (Presence or Absence)**
RV 2011-01	Rhinovirus - 90		1.0×10^{-3}	**Positive RV (42.23)**	**34.67**	**Presence**
RV 2011-02	Rhinovirus – 5 B	Core	1.0×10^{-3}	**Positive RV (31.08)**	**26.63**	**Presence**
RV 2011-03	Rhinovirus - 16		1.0×10^{-5}	**Positive RV (39.09)**	**41.76**	**Absence**
RV 2011-04	Rhinovirus - 90	Core	1.0×10^{-4}	**Positive RV (38.02)**	**30.92**	**Presence**
RV 2011-05	Rhinovirus - 16		1.0×10^{-6}	**Positive RV (41.04)**	**Negative**	**Absence**
RV 2011-06	Rhinovirus – 42 B		1.0×10^{-2}	**Positive RV (33.98)**	**29.42**	**Presence**
RV 2011-07	Rhinovirus - 8	Core	$1.0 \times 10^{-3.5}$	**Positive RV (35.37)**	**32.22**	**Presence**
RV 2011-08	Enterovirus 68		1.0×10^{-3}	**Positive EV (35.40)**	**28.95**	**Presence**
RV 2011-09	Rhinovirus - 16	Core	1.0×10^{-4}	**Positive RV (33.69)**	**33.54**	**Presence**
RV 2011-10	Negative	Core	N.A	**Negative (neg)**	**Negative**	**Presence**
RV 2011-11	Rhinovirus - Type C	Core	1.0×10^{-2}	**Positive RV (32.49)**	**28.83**	**Presence**
RV 2011-12	Rhinovirus - Type C		1.0×10^{-3}	**Positive RV (36.13)**	**32.96**	**Presence**

The 5 “Core” positive Rhinovirus samples of QCMD panel RV 2011 are detected with Rhino&EV/Cc r-gene™ assay including the Rhinovirus type C.
The “Core” negative sample is undetected as expected with Rhino&EV/Cc r-gene™
4 on 5 “Challenging samples” are detected with Rhino&EV/Cc r-gene™ including Rhinovirus type C.
The lack of cells of sample RV 2011-05 does not allow to validate the status of this sample. A new extraction and amplification are necessary. As claimed, sample Enterovirus 68 (RV 2011-08) is detected.

Enterovirus RNA EQA QCMD Panel 2012

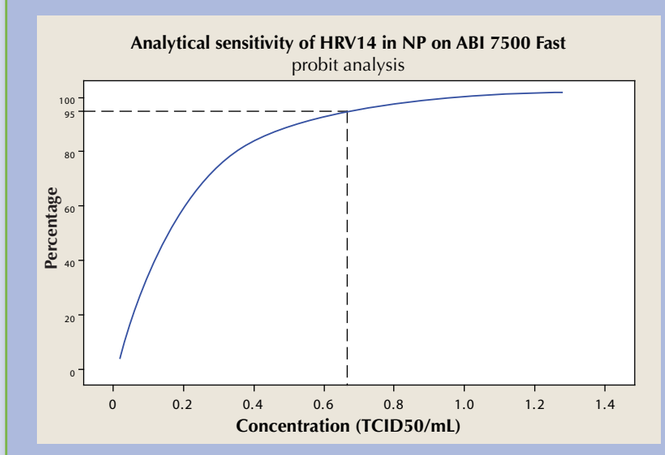
Enterovirus RNA EQA QCMD European Proficiency Panel 2012 Results					Rhino&EV/Cc r-gene™ Results on LC480	
Panel Code	**Sample Content**	**Sample Type**	**TCID50/0.05mL stock solution (Dilution Factor sample test)**	**Expected Result**	**Rhino&EV (CT)**	**Cells (Presence or Absence)**
EV 2012-01	Coxsackievirus B3		$5.0 \times 10^{+4}$ (1.0×10^{-2})	**Positive** (Infrequently detected)	**42.54**	**Presence**
EV 2012-02	Coxsackievirus A24	Core	$1.5 \times 10^{+4}$ (1.0×10^{-3})	**Positive** (Detected)	**35.32**	**Presence**
EV 2012-03	Echovirus 30	Core	$2.7 \times 10^{+5}$ (4.0×10^{-4})	**Positive** (Frequently detected)	**31.66**	**Presence**
EV 2012-04	Coxsackievirus A9	Core	$3.0 \times 10^{+6}$ (1.0×10^{-1})	**Positive** (Detected)	**30.93**	**Presence**
EV 2012-05	Echovirus 11	Core	$2.5 \times 10^{+7}$ (1.0×10^{-1})	**Positive** (Detected)	**32.54**	**Presence**
EV 2012-06	Enterovirus 68	Core	$1.6 \times 10^{+4}$ (1.0×10^{-1})	**Positive** (Detected)	**28.20**	**Presence**
EV 2012-07	Enterovirus 71	Core	$1.0 \times 10^{+5}$ (1.0×10^{-1})	**Positive** (Detected)	**30.71**	**Presence**
EV 2012-08	Coxsackievirus A16	Core	$4.0 \times 10^{+5}$ (1.0×10^{-1})	**Positive** (Frequently detected)	**28.67**	**Presence**
EV 2012-09	Enterovirus 68		$1.6 \times 10^{+4}$ (1.0×10^{-1})	**Positive** (Infrequently detected)	**42.30**	**Presence**
EV 2012-10	Echovirus 11		$2.5 \times 10^{+7}$ (1.0×10^{-2})	**Positive** (Infrequently detected)	**36.96**	**Presence**
EV 2012-11	Coxsackievirus B3	Core	$5.0 \times 10^{+6}$ (1.0×10^{-1})	**Positive** (Detected)	**33.38**	**Presence**
EV 2012-12	Negative (VTM)	Core	N.A	**Negative**	**Negative**	**Presence**

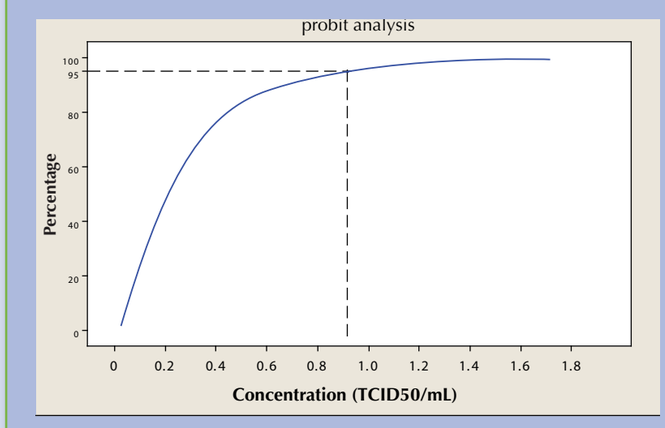
The 8 “Core” positive Enterovirus samples of QCMD panel EV 2012 are detected with Rhino&EV/Cc r-gene™ assay.
The “Core” negative sample is undetected as expected with Rhino&EV/Cc r-gene™
3 on 3 “Challenging samples”, EV 2012-01, EV 2012-09 & EV 2012-10 are detected with Rhino&EV/Cc r-gene™.

The results show the sensitivity and specificity of the Rhino&EV/Cc r-gene™

Analytical Sensitivity

The limit of detection of **Rhinovirus 14** with Rhino&EV/Cc r-gene™ assay is :
+ 0.699 TCID50/mL at 95% and 0.038 TCID50/mL at 5% using NucliSENS® easyMAG™ and Dx Real Time System.
+ 0.936 TCID50/mL at 95% and 0.051 TCID50/mL at 5% using NucliSENS® easyMAG™ and ABI7500 Fast.





Specificity

Nine specific viruses (in green) were detected as expected.
None of following viruses or bacteria were amplified with Rhino&EV/Cc r-gene™, which proves the good specificity of the assay.

Viruses	Quantification (copies/mL) or Crossing Threshold (cycles)	Rhino&EV (FAM - 530nm)
Rhinovirus 14	N.A	23.13 cycles
Rhinovirus 87	N.A	26.07 cycles
Rhinovirus 1B	N.A	32.96 cycles
Echovirus 25	N.A	25.18 cycles
Coxsackievirus B2	N.A	26.74 cycles
Coxsackievirus A9	N.A	27.06 cycles
Echovirus 9	N.A	29.42 cycles
Poliovirus S3	N.A	32.61 cycles
Echovirus 30	N.A	28.57 cycles
Adenovirus 12	2E+05	-
Adenovirus 3	7E+04	-
Adenovirus 11	6E+04	-
Adenovirus 5	4E+04	-
Adenovirus 8	3E+04	-
Adenovirus 4	6E+05	-
Adenovirus 40	6E+05	-
Cytomegalovirus	4E+04	-
Epstein Barr Virus	1E+06	-
BK Virus	3E+06	-
Herpes Simplex Virus 1	2E+05	-
Herpes Simplex Virus 2	3E+05	-
Varicella Zoster Virus	2E+05	-
Human Herpes Virus 6	5E+03	-
Human Herpes Virus 7	30.50 cycles	-
Human Herpes Virus 8	5E+04	-
Influenza A/PR/8/34	23.70 cycles	-
Influenza B/Ann Arbor	22.31 cycles	-
Respiratory Syncytial Virus A	24.14 cycles	-
Respiratory Syncytial Virus B	23.75 cycles	-
Human Metapneumovirus type A	26.24 cycles	-
Human Metapneumovirus type B	24.23 cycles	-
Human Bocavirus 1	26.42 cycles	-
Parainfluenza Type 1	21.09 cycles	-
Parainfluenza Type 2	25.34 cycles	-
Parainfluenza Type 3	22.86 cycles	-
Parainfluenza Type 4	24,54 cycles	-
NL63	30.67 cycles	-
Parechovirus 1	29.05 cycles	-
Parechovirus 2	27.49 cycles	-
Parvovirus B19	27.02 cycles	-

Bacteria	Quantification (copies/mL) or Crossing Threshold (cycles)	Rhino&EV (FAM - 530nm)
Bordetella pertussis	28.88 cycles	-
Bordetella parapertussis	27.63 cycles	-
Legionella pneumophila	1E+06	-
Bordetella bronchiseptica	2E+06	-
Escherichia coli	4E+06	-
Staphylococcus epidermidis	1E+06	-
Klebsiella pneumoniae	1E+06	-
Haemophilus influenzae	3E+06	-
Serratia marcescens	6E+06	-
Staphylococcus aureus	3E+06	-
Proteus mirabilis	3E+07	-
Klebsiella oxytoca	3E+06	-
Pseudomonas aeruginosa	1E+05	-
Stenotrophomonas maltophilia	7E+06	-
Bordetella pertussis	3E+05	-
Legionella pneumophila	4E+05	-
Pseudomonas aeruginosa	8E+05	-
Klebsiella pneumoniae	6E+05	-
Staphylococcus aureus	5E+05	-
Klebsiella oxytoca	2E+06	-
Enterobacter kobei	3E+06	-
Morganella morganii	9E+06	-
Branhamella catarrhalis	2E+05	-
Citrobacter freundi	2E+06	-
Citrobacter koseri	7E+06	-
Streptococcus constellatus	2E+06	-
Citrobacter freundi	7E+05	-
Raoultella ornithinolytica	3E+05	-
Serratia marcescens	3E+06	-
Haemophilus parainfluenzae	3E+06	-
Haemophilus influenzae	3E+06	-
Enterobacter cloacae	6E+05	-
Stenotrophomonas maltophilia	7E+05	-
Morganella morganii	1E+06	-
Acinetobacter baumanii	9E+06	-
Pseudomonas aeruginosa	2E+05	-
Branhamella catarrhalis	1E+05	-
Streptococcus agalactiae	7E+05	-
Mycoplasma pneumoniae	25.92 cycles	-
Chlamydophila pneumoniae	27.01 cycles	-

CONCLUSION

The high quality associated with its compatibility with the major extraction and real time PCR platforms allows an immediate integration of Rhino&EV/Cc r-gene™ 71-042 assay in most routine diagnostic laboratories. The cellular control checks for the presence of cells in the samples, thus preventing a false negative result due to a lack of cells.
This tool belongs to Respiratory MWS r-gene™ brand range which represents an innovative solution in response to the challenges in respiratory infections.