

# Development of a sensitive and specific real-time RT-PCR System for the qualitative detection of Enterovirus and Rhinovirus RNA

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## Introduction

The genus *Enterovirus* (family *Picornaviridae*) contains 12 different species (9 enterovirus and 3 rhinovirus species). They carry positive, single-stranded RNA genomes of around 7500 nucleotides. Non-polio enterovirus infections often occur seasonally and are commonly associated with cardiac and respiratory symptoms, cutaneous and mucosal infections, neonatal sepsis or viral meningitis and encephalitis. Rhinovirus infections are usually restricted to the respiratory tract with symptoms of a common cold but more severe illness like pneumonia can occur. The Poliovirus group is most closely associated with poliomyelitis.

The clinical symptoms are mostly unspecific, which makes infections caused by enteroviruses difficult to distinguish from those caused by other agents. Therefore, there is need for reliable enterovirus *in vitro* diagnostics.

The RealStar<sup>®</sup> Enterovirus RT-PCR Kit facilitates detection of all human entero- and rhinoviruses in different types of patient specimens. It detects all currently known strains, including emerging C-group enteroviruses. The assay contains an internal control for reliable interpretation of results.

Here we present data on the analytical specificity and sensitivity of RealStar<sup>®</sup> Enterovirus RT-PCR Kit. Furthermore, 20 pretested samples were analyzed with the RealStar<sup>®</sup> Enterovirus RT-PCR Kit in comparison to different real-time RT-PCR assays.

## Methods

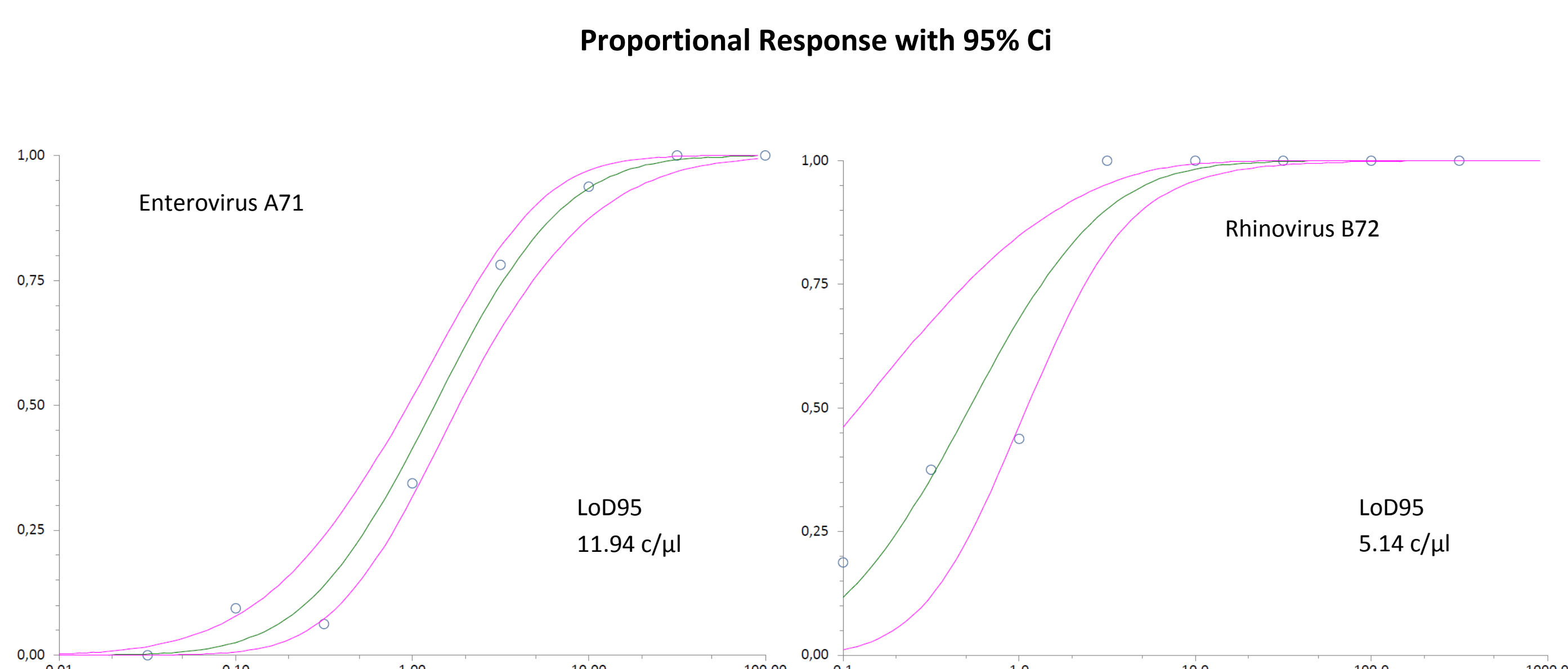
The **analytical sensitivity** was determined by probit analysis. *In vitro* transcripts containing the respective target region of Enterovirus A71 and Rhinovirus B72 were diluted in half-logarithmic steps and tested in replicates with the RealStar<sup>®</sup> assay. Hit rates were determined and the 95% limit of detection (LoD95) was calculated.

**Reactivity** was confirmed by testing QCMD proficiency panels and viral RNA of different entero- and rhinoviruses.

Possible **cross-reactivity** of the RealStar<sup>®</sup> Enterovirus RT-PCR Kit was assessed by testing nucleic acids from various viruses and bacteria that may cause similar symptoms or are present in the same sample type as entero- and rhinoviruses. Nucleic acid extraction was performed using the QIAamp<sup>®</sup> Viral RNA Mini Kit (Qiagen, Hilden, Germany) accordance manufacturer's instructions.

## Results

### Analytical Sensitivity



**Figure 1**  
Probit analysis for the RealStar<sup>®</sup> Enterovirus RT-PCR Kit.

The limit of detection (LoD95) was calculated using probit analysis and was determined by testing replicates of half-logarithmic dilutions specific *in vitro* transcribed RNA of Enterovirus A71 and Rhinovirus B72. The X-axis shows the concentration of RNA and the Y-axis the proportion of positive results. The LoD95 is 11.94 c/µl (95% confidence interval 7.32 c/µl to 24.24 c/µl) for Enterovirus A71 and 5.14 c/µl (95% confidence interval 2.59 c/µl to 18.59 c/µl) for Rhinovirus B72.

### Analytical Specificity

The specificity of primers and probes selected for the specific detection of all enterovirus and rhinovirus serotypes was experimentally determined by testing strains of genus *Enterovirus* (Table 1). All strains were successfully detected with the RealStar<sup>®</sup> Enterovirus RT-PCR Kit.

**Table 1:** Enterovirus and rhinovirus types tested for reactivity.

Species	Serotype
Enterovirus A	Coxsackievirus A3
	Coxsackievirus A9
	Coxsackievirus A16
	Enterovirus A71
Enterovirus B	Echovirus 11
	Echovirus 30
	Coxsackievirus B3
	Coxsackievirus B4
Enterovirus C	Coxsackievirus A24
	Enterovirus C99
	Enterovirus C104
Enterovirus D	Enterovirus D68
Rhinovirus A	Rhinovirus A8
	Rhinovirus A16
	Rhinovirus A39
	Rhinovirus A43
	Rhinovirus A51
	Rhinovirus A58
	Rhinovirus A61
	Rhinovirus A71
	Rhinovirus A90
Rhinovirus B	Rhinovirus B3
	Rhinovirus B5
	Rhinovirus B42
	Rhinovirus B89
	Rhinovirus B72
Rhinovirus C	unknown serotype

**Table 2:** European Enterovirus & Rhinovirus Proficiency Panel (QCMD 2014):

21 of 22 (95.4%) tested samples were in agreement with expected results. Only one educational enterovirus sample was not detected. The results show the sensitivity and specificity of RealStar<sup>®</sup> Enterovirus RT-PCR Kit.

Sample Code	Sample Content	Sample Status	Detection Frequency	Expected Result	Result RealStar <sup>®</sup> Kit
EVRNA14-01	Enterovirus A71	Core	Frequently Detected	+	+
EVRNA14-02	EV Negative	Core	Negative	-	-
EVRNA14-03	Coxsackievirus A24	Core	Detected	+	+
EVRNA14-04	Enterovirus A71	Educational	Detected	+	+
EVRNA14-05	Coxsackievirus B3	Educational	Detected	+	+
EVRNA14-06	Echovirus 11	Educational	Detected	+	-
EVRNA14-07	Coxsackievirus A9	Core	Detected	+	+
EVRNA14-08	EV Negative	Core	Negative	-	-
EVRNA14-09	Coxsackievirus A16/B5	Core	Frequently Detected	+	+
EVRNA14-10	Enterovirus D68	Core	Detected	+	+
EVRNA14-11	Coxsackievirus B3	Core	Frequently Detected	+	+
EVRNA14-12	Echovirus 11	Core	Frequently Detected	+	+
RVRNA14-01	Rhinovirus - 90	Educational	Detected	+	+
RVRNA14-02	Rhinovirus - 16	Core	Detected	+	+
RVRNA14-03	Rhinovirus - Type C	Core	Frequently Detected	+	+
RVRNA14-04	Rhinovirus - 8	Core	Detected	+	+
RVRNA14-05	Rhinovirus - 16	Educational	Infrequently Detected	+	+
RVRNA14-06	Rhinovirus - 90	Core	Detected	+	+
RVRNA14-07	Rhinovirus - 42 B	Core	Detected	+	+
RVRNA14-08	Rhinovirus - 5 B	Educational	Detected	+	+
RVRNA14-09	RV Negative	Core	Negative	-	-
RVRNA14-10	Rhinovirus - 5 B	Core	Detected	+	+

The cross reactivity was tested with DNA/RNA of CMV, EBV, HHV6 (A/B), HSV (1/2), VZV, HIV, HCV, HAV, BKV, JCV, Adenovirus, Parvovirus B19, Influenza (A H1N1, H3N2; B), PIV (1-4), RSV (A/B), hMPV, mumps virus, measles virus, parechovirus, Coronavirus, Norovirus (GI/GII), Astrovirus, Rotavirus, *Neisseria meningitidis*, *Bordetella pertussis*, *Bordetella parapertussis*, *Streptococcus pneumoniae*, *Mycoplasma pneumoniae* and *Escherichia coli*. No unspecific cross-reactivity was observed.

## Diagnostic validation

**Table 3: Ct values of tested clinical Samples:** Preanalyzed samples of different sample materials were analyzed with RealStar<sup>®</sup> Enterovirus RT-PCR Kit 1.0, FastTrack Diagnostics EPA, FastTrack Diagnostics Respiratory pathogens 21, Cepheid Xpert<sup>®</sup> and with a published In-House RT-PCR method of Salveraju et al.[1] as reference tests. 17 of 20 specimens were detected enterovirus and rhinovirus positive with the RealStar<sup>®</sup> Enterovirus RT-PCR Kit 1.0. 20 of 20 (100%) samples tested were in agreement with expected results. The reference tests show equivalent results.

ID	Material	RealStar <sup>®</sup> Enterovirus RT-PCR Kit 1.0	Fast Track Diagnostics EPA	Fast Track Diagnostics Respiratory pathogens 21	Cepheid Xpert <sup>®</sup> EV	In-house RT-PCR [1]	Sequencing
#1	Serum	negative	negative	nd*	negative	negative	nd*
#2	Serum	ct 23	ct 25	nd*	ct 25	ct 25	EV 68
#3	Serum	ct 32	ct 30	nd*	ct 27	ct 28	Cox B3
#4	Serum	ct 26	ct 27	nd*	ct 24	ct 24	Cox A21
#5	Swab	ct 31	ct 31	ct 35	nd*	nd*	EV D68
#6	Swab	ct 31	ct 29	ct 37	nd*	nd*	EV E11
#7	Swab	ct 29	ct 28	ct 34	nd*	nd*	Cox B1
#8	Swab	ct 23	ct 23	ct 31	nd*	nd*	Cox A5
#9	Swab	ct 29	ct 31	ct 37	nd*	nd*	Rhino
#10	Plasma	ct 31	ct 27	nd*	nd*	ct 27	Echo E9
#11	Stool	ct 23	ct 21	nd*	nd*	ct 20	Echo E18
#12	Stool	ct 27	ct 27	nd*	nd*	ct 26	Cox A2
#13	Swab	ct 27	ct 27	ct 33	nd*	nd*	Cox B5
#14	Stool	ct 33	ct 30	nd*	nd*	ct 28	EV
#15	Swab	ct 27	nd*	ct 29	nd*	nd*	Rhino
#16	NPS*	ct 27	nd*	ct 30	nd*	nd*	Rhino
#17	Swab	ct 31	nd*	ct 33	nd*	nd*	Rhino
#18	BAL	ct 31	nd*	ct 33	nd*	nd*	Rhino
#19	CSF	negative	negative	nd*	nd*	nd*	nd*
#20	CSF	negative	negative	nd*	nd*	nd*	nd*

\* nd = not done; NPS = nasopharyngeal swab

## Conclusion

The results presented here show that the RealStar<sup>®</sup> Enterovirus RT-PCR Kit is highly sensitive and allows the detection of low-level of virus RNA in different sample types. The Internal Control included in the assay enables reliable diagnostics. Therefore the RealStar<sup>®</sup> Enterovirus RT-PCR Kit will be a valuable tool for the detection of enterovirus and rhinovirus RNA in patients samples.

## References

[1] Selvarajua, S.B., Nix, W. A., Obersteb, M.S. and Rangaraj Selvarangana, R. (2013). Optimization of a Combined Human Parechovirus-Enterovirus Real-Time Reverse Transcription-PCR Assay and Evaluation of a New Parechovirus 3-Specific Assay for Cerebrospinal Fluid Specimen Testing. J. Clin. Microbiol. vol. 51 no. 2 452-458.

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