ENTEROVIRUS RNA

Qualitative Real-Time PCR for detection of Enterovirus spp

4BShoplab

Rev. 6

Enterovirus RNA

A. INTENDED USE

The **Enterovirus RNA** Real-Time PCR kit coded **ENTERORNA.CE** is intended for the qualitative detection of Enterovirus RNA in human plasma and CSF samples (Cerebral Spinal Fluid) with a simultaneous control of the amplification reaction through an **Internal Control (IC).**

The kit has been adapted for the use on the Real-Time Thermacyclers ABI 7500 Sequence Detection System® (Software SDS version 1.3.1, Applied Biosystems™*), or MX3000P® (Software MxPro version 4.01, Stratagene™**).

* Applied Biosystems is a registered trademark and ABI PRISM® is a trademark of Applera Corporation or its subsisdiaries in the US and/or certain other countries.
**Stratagene is a registered trademark.

IMPORTANT NOTE: The Enterovirus RNA Kit has been designed to detect the RNA genome of all enterovirus serotypes: Poliovirus 1-3, Coxsackievirus A1-A22, A-24, Coxsackievirus B1-B6, Echovirus 1-9, 11-21, 24-27, 29-33, Enterovirus 68-71. The Human Parechovirus (Echovirus 22 and 23) are not detected with this kit.

B. INTRODUCTION

Human Enteroviruses (until now there are 70 serotype known, belonging to the family of Picornaviridae) are ubiquitous pathogens with a high incidence worldwide. Although the majority of human enterovirus infections are asymptomatic, these viruses can cause a wide spectrum of clinical syndromes ranging from upper respiratory illness, febrile rash, aseptic meningitis, pleurodynia, encephalitis, acute flaccid paralysis, and neonatal sepsis-like disease. Human enterovirus may also be implicated in the pathogeneses of severe chronic diseases, including type 1 diabetes mellitus, myocarditis and congestive cardiomyopathy, and neuromuscular diseases. Furthermore, Enterovirus infections account for a substantial number of aseptic meningitis and encephalitis patients requiring hospitalization in summer and fall.

Human enteric viruses are excreted in the feces of infected patients in high concentrations and transmitted mainly by the fecal-oral route via contaminated food and water. They are estimated to cause about 30% to 90% of gastroenteritis cases worldwide.

Human enterovirus are small, non-enveloped viruses contain a linear single-stranded RNA genome (7.4 kb) comprising a 5' and a 3' noncoding region and a single, long open reading frame coding for a polyprotein of about 2200 amino acids. Cell culture is a common method to isolate viruses in water until the early 1990s. More recently, PCR has come to be a major tool for detection of these viruses.

C. PRINCIPLE OF THE TEST

The ENTERORNA.CE Kit is based on a Real Time chemistry which uses specific Primers and Probes.

Enterovirus RNA, recovered from the biological sample under investigation through an extraction step, is retrotrascribed to cDNA and amplified using Real Time amplification system. The amplified product is detected using a fluorescent reporter dye probe specific for an Enteroviruses unique genomic sequence.

An Heterologous Internal Control (IC) serves as an amplification control for each individually processed specimen aiming to the identification of reaction inhibitors.

An High Positive control (CTRL-H) and Low Positive control (CTRL-L) are supplied as controls for the PCR reaction.

D. COMPONENTS

The standard format of the product code ENTERORNA.CE contains reagents for 50 tests.

Component	Contents	ENTERORNA.CE 50 Reactions
A **CODED: ALL/MM-1** **COLOR CODE: LIGHT BLUE**	Master mix	N° 2 Vials / 0.4 ml
B **CODED: ENT/CB** **COLOR CODE: YELLOW**	Lyophilised Primers/Probes	N° 2 Vials (Dissolve with the volume of ALL/C indicated on the vial label)
C **CODED: ALL/C** **COLOR CODE: RED**	MG Water	N° 2 vial /1.5 ml
NTC **CODED: ALL/NTC** **COLOR CODE: WHITE**	Negative Control	N° 1 vial /1.5 ml
CTRL-H High Positive Control (10^4 Copies/ul) **CODED: ENT/CTRL-H** **COLOR CODE: VIOLET**	Lyophilised Qualitative High positive	N° 8 Vials (Dissolve with the volume of ALL/C indicated on the vial label)
CTRL-L Low Positive Control (50 Copies/ul) **CODED: ENT/CTRL-L** **COLOR CODE: PINK**	Lyophilised Qualitative Low positive	N° 8 Vials (Dissolve with the volume of ALL/C indicated on the vial label)
I.C. Internal Control **CODED: ENT/IC** **COLOR CODE: GREEN**	Lyophilised Internal Control	N° 2 Vials (Dissolve with the volume of ALL/C indicated on the vial label)
Package Insert	Instruction for Use	N° 1

Important note: Upon request, Dia.Pro can supply reagents for 25, 100, 150 tests, as reported below **:**

1. Component A 2. Component B 3. Component C 4. NTC 5. IC 6. CTRL-H 7. CTRL-L 8. Pack. insert	n°1 vial/0.4 ml n°1 vial n°1 vial/1.5 ml n°1 vial/1.5 ml n°1 vial n° 4 vial n° 4 vial n° 1	n°4 vials/0.4 ml n°4 vials n°2 vials/1.5ml n°1 vial/1.5 ml n°4 vials n° 4 vials n° 4 vials n° 1	n°6 vials/0.4 ml n°6 vials n°3 vials/1.5ml n°1 vial/1.5 ml n°6 vials n° 6 vials n° 6 vials n° 1
Number of tests	**25**	**100**	**150**
Code	**ENTERORNA.CE.25**	**ENTERORNA.CE.100**	**ENTERORNA.CE.150**

E.STORAGE AND STABILITY

The kit ENTERORNA.CE must be stored at +2…8 °C . Once dissolved Component B (coded ENT/CB) and Component IC (coded ENT/IC) are stable for 4 months at -20°C. Once dissolved components positive controls HIGH and LOW (coded ENT/CTRL-HIGH, ENT/CTRL-LOW) are stable for 2 weeks at -20°C. If the components are to be used only intermittently, they should be frozen in aliquots, repeated thawing and freezing should be avoided, Only one defreezing is allowed.

F. MATERIALS REQUIRED BUT NOT PROVIDED

1. Calibrated Micropipettes (0.5 µl < volume <1000 µl)
2. RNA extraction kit
3. MG EtOH
4. Thermal Block
5. Microcentrifuge
6. Tube racks
7. Sterile filtered tip with aerosol barrier
8. 0,2 ml Microtubes or Pcr Microplates recommended from the Real-Time PCR instruments manufacturers
9. Disposable gloves, powder-free
10. Thermalcycler
11. Real-Time PCR Thermalcycler (*)
12. Absorbent paper tissues.
13. Vortex or similar mixing tools.

(*) Attention: A valid calibration of the pure dyes (Pure Spectra Component File) and of the background (Background Component File) must be done routinely.

G. WARNINGS AND PRECAUTIONS

1. The kit has to be used by skilled and properly trained technical personnel only, under the supervision of a medical doctor responsible for the laboratory.
2. The technical personnel must be deeply trained in the use of Real-Time thermalcyclers, in the manipulation of Molecular Biology reagents and in the Real-Time PCR amplification protocols.
3. The kit has to be used in a laboratory certified and qualified by the national authority in that field (Ministry of Health or similar entity) to carry out this type of analysis.
4. All the personnel involved in performing the assay have to wear protective laboratory clothes, powder-free gloves and glasses. The use of any sharp (needles) or cutting (blades) devices should be avoided. All the personnel involved should be trained in biosafety procedures, as recommended by the Center for Disease Control, Atlanta, U.S. and reported in the National Institute of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
5. All the personnel involved in sample handling should be vaccinated for HBV and HAV, for which vaccines are available, safe and effective.
6. The laboratory environment should be controlled so as to avoid contaminants such as dust or air-born microbial agents.
7. Components A and B are light sensitive. Protect them from strong light exposition.
8. Avoid vibration of the bench surface where the test is undertaken.
9. Upon receipt, store the kit at 2..8°C into a temperature controlled refrigerator or cold room.
10. Do not interchange components between different lots of the kits. Moreover components of different kits coming from the same lot should not be interchanged.
11. Check that the reagents are clear and do not contain visible heavy particles or aggregates. If not, advise the laboratory supervisor to initiate the necessary procedures for kit replacement.
12. Avoid cross-contamination between samples by using disposable tips and changing them after each sample.
13. Avoid cross-contamination between kit reagents by using disposable tips and changing them between the use of each one.
14. Do not use the kit after the expiration date stated on the external container label.
15. Treat all specimens as potentially infective. All human urethral, cervical swabs and urine specimens should be handled at Biosafety Level 2, as recommended by the Center for Disease Control, Atlanta, U.S. in compliance with what reported in the Institutes of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
16. Store and extract specimens separately from the other reagents and use a separate room for their handling
17. Dissolve the lyophilised reagents with the correct amount stated in the label with Component C (Code: ALL/C) supplied in the kit.
18. Carry on all the working operations as quickly as possible maintaining the components on ice or in a cooling block.
19. The laboratory workflow must proceed in an unidirectional way, beginning in the Extraction Area and moving to the Amplification and Data Analysis Area. Do not return samples, equipment and reagents to the area where previous steps have been performed.
20. The use of disposable plastic-ware is recommended in the preparation of the liquid components or in transferring components into automated workstations, in order to avoid cross contamination.
21. Waste produced during the use of the kit has to be discarded in compliance with national directives and laws concerning laboratory waste of chemical and biological substances. In particular, liquid waste generated from sample extraction procedures, has to be treated as potentially infective material and inactivated before waste. Do not put in contact the extraction waste with bleach.
22. Accidental spills from samples and operations have to be adsorbed with paper tissues soaked with household bleach and then with water. Tissues should then be discarded in proper containers designated for laboratory/hospital waste.
23. Other waste materials generated (example: tips used for samples) should be handled as potentially infective and disposed according to national directives and laws concerning laboratory wastes.

H. SPECIMEN: PREPARATION AND RECOMMENDATIONS

1.CSF is drawn aseptically by lumbar puncture, it must be limpid and not haemolysed.
2.Blood is drawn aseptically by venepuncture and plasma is prepared using standard techniques of preparation of samples for clinical laboratory analysis.
3. No influence has been observed in the preparation of the sample with citrate, EDTA.
Attention: Heparin (≥10 IU/ml) affects the PCR reactions.
Samples, which has been collected in tubes containing heparin as an anticoagulant should not be used. Also, samples of heparinised patients must not be used.

4. Avoid any addition of preservatives to samples.
5. Samples have to be clearly identified with codes or names in order to avoid misinterpretation of results.
6.Haemolysed (red) and visibly hyperlipemic ("milky") samples have to be discarded as they could generate false results. Samples containing residues of fibrin or heavy particles or microbial filaments and bodies should be discarded as they could give rise to false results.
7.The plasma samples for RNA extraction must be collected according to the common laboratory procedures, transported and stored at +2 / +8 °C for a maximum period of 4 hours. The plasma samples can be stored frozen at – 20°C for a maximum period of 30 days or at -70 °C for longer periods.
8. CSF and plasma samples, if not used immediately, must be aliquoted and stored at -20°..-80°C after collection. Samples can be stored frozen at 20°..-80°C for several months. Any frozen samples should not be frozen/thawed more than once as this may affect the test result.
9.When using frozen samples, thaw the samples just immediately before the extraction in order to avoid possible cases of nucleic acid degradation.

I. PREPARATION OF COMPONENTS AND WARNINGS

Master Mix:

Component A. Ready to use. Mix well on vortex before use and centrifuge briefly to collect the whole volume.

WARNING: Component A is light sensitive. Protect it from strong light exposition.

Primers/Probes:

Component B.

- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized Component B with the volume of Component C (Code: ALL/C) indicated on the vial label.
- Keep it dissolve on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex

WARNING: Component B is light sensitive. Protect it from strong light exposition.

MG Water :

Component C. Ready to use.

Negative Control:

NTC. Ready to use.

Positive Controls:

Component CTRL-H.

- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized CTRL-H with the volume of Component C (Code: ALL/C) indicated on the vial label.
- Keep it dissolve on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex

Component CTRL-L.

- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized CTRL-L with the volume of Component C (Code: ALL/C) indicated on the vial label.
- Keep it dissolve on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex

Internal Control:

I.C.

- Centrifuge the vial at 11000 rpm for 1 min.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized I.C. with the volume of Component C (Code: ALL/C) indicated on the vial label.
- Keep it dissolve on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)
- Briefly vortex

L. INSTRUMENTS AND TOOLS USED IN COMBINATION WITH THE KIT

1. **Micropipettes** have to be calibrated to deliver the correct volume required by the assay and must be submitted to regular decontamination (household alcohol, 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample. They should also be regularly maintained in order to show a precision of 1% and a trueness of +/-5%.
2. **Extraction Device:** The ENTERORNA.CE Kit is intended to be used in combination only with QIAamp Viral RNA Code.52906 (QIAGEN), NucleoSpin RNA Virus Code 740956 (Macherey-Nagel) and NA Body Fluid Kit Code: D-2021 (Chemagen distributed by Dia.Pro).The end users must strictly follow the Instruction for use supplied by the manufacturer.
3. **Retro-transcription Step:** The ENTERORNA.CE Kit is intended to be used in combination only with RNA Retro-transcription Kit (Dia.Pro srl Code: RNART.CE).The end users must strictly follow the Instruction for use supplied by the manufacturer.
4. **Real-Time Thermocyclers.** The ENTERORNA.CE Kit is intended for the use in combination only with the Real Time Thermal cyclers ABI PRISM 7500, software SDS version 1.3.1 (Applied Biosystems), and MX3000P, software MxPro version 4.01 (Stratagene).
   The end users must strictly follow the Instruments Instruction for use supplied by the manufacturers.

M. PRE ASSAY CONTROLS AND OPERATIONS

1. Check the expiration date of the kit printed on the external label of the kit box. Do not use if expired.
2. Check that the liquid components are not contaminated by naked-eye visible particles or aggregates. Check that on the bottom of the Lyophilized components vials is present a well formed aggregate. Check that no breakage occurred in transportation and no spillage of liquid is present inside the box.
3. Dissolve the Lyophilized Components with the appropriate amount of Component C (Code: ALL/C) as described in the proper section (I).
4. Turn the Thermocyclers on, check settings and be sure to use the right assay protocol.
5. Follow strickly the Instruments Manual supplied by the manufacturers for the correct setting of the Real-Time Thermalcyclers.
6. Check that the micropipettes are set to the required volume.
7. Check that all the other equipment is available and ready to use.
8. In case of problems, do not proceed further with the test and advise the supervisor.

N. ASSAY PROCEDURE
The assay has to be carried out according to what reported below.

N.1 RNA extraction

The extraction step of the Enterovirus genomic RNA has to be carried out exclusively in combination with the following kits:

Manual Extraction tools

Material	Description	Kit code	manufacturer
CSF/Plasma	QIAamp Viral RNA mini kit®	52906	Qiagen™
CSF/Plasma	NucleoSpin RNA Virus	740956	MN™

Automatic Extraction tool in combination with DIA.FASTEX Instrument

Material	Description	Kit code	Manufacturer
CSF/Plasma	NA Body Fluid Kit	D-2021	Chemagen distributed by Dia.Pro

The RNA isolation must be carried out only according to the Instruction Manual (QIAGEN™, MN™, Dia.Pro).

Important Note : The following volumes have to be strictly used in the extraction procedures :

Description	Sample volume µl	Elution volume µl
QIAamp Viral RNA mini kit®	140	30
NucleoSpin RNA Virus	150	30
NA Body Fluid Kit	230	50

The RNA collected from the samples, not used in the run , has to be stored frozen (-80°C).

IMPORTANTE NOTE:
10 µl of RNA extracted for each sample must be used for retrotrascription with RNART.CE (Diapro).

N.2 Viral RNA Retro-transcription to cDNA

The Retro-transcription step of the Enterovirus genomic RNA has to be carried out exclusively in combination with the following kit:

Material	Description	Kit code	manufacturer
RNA	RNA Retro-transcription kit	RNART.CE	Dia.Pro srl

The RNA Retro-transcription must be carried out only according to the Manufacturer's instructions (Dia.Pro srl).

N.3 Setting up of the reaction

ENTERORNA.CE kit is intended to be used exclusively in combination with ABI PRISM 7500, software SDS version 1.3.1 (Applied Biosystem), and MX3000P, software MxPro version 4.01 (Stratagene).

N.3.1 Preparing the PCR

Important: *An example of dispensation scheme is reported in Section O. Please, refer to it before starting to read the instructions here below.*

- Prepare the components as described in Section I;
- Prepare the required number of reaction tubes or a 96-well reaction plate for the samples under evaluation and for positive controls (prepared as described in section I).

  Important note: *Use only optical tubes or microplates suggested by the Real-Time thermocyclers manufacturers.*
- Consider that the samples, if possible, should be tested in duplicate;
- Include at least 1 tube/well for the NTC (negative control)
- Prepare the **Amplification Mix** for **Samples, NTC and positive controls (CTRL-H, CTRL-L)** as table below:

Preparation of the Amplification Mix

Number of Reactions		x1	x12
A	Master mix	12,5 µl	150 µl
B	Primers/probes	2 µl	24 µl
C	MG Water	4.5 µl	54 µl
I.C.	Internal Control	1 µl	12 µl
Tot vol.		**20 µl**	**240 µl**

N.3.2 Amplification procedure

- Dispense 20 ul of the amplification mix in each reaction tube or microplate well
- Add 5 ul the **Samples, NTC and CTRL-H and CTRL-L** to the reaction tubes.
- Close firmly the reaction tubes
- Centrifuge briefly the reaction tubes at 2000rpm
- Don't leave the reaction tubes at room temperature (RT) for more than 30 minute and at light exposure (cover the tubes).
- Load the tubes in the Real-Time Thermacycler Thermalblock Holder.
- After the setting operations described in the Sections N4 (Instrument Programming) start the Thermacycler run.

Important note: The Components Lyophilized after dissolution in Component C (MG water) are stable no more than 3 hours kept in ice or at 2°…8° °C.

The not used volume of Component B, CTRL-H, CTRL-L and I.C. can be freeze at -20°C and used as described in Section E

N.4 Instrument programming

For programming the instrument refer to the Instruction Manual provided by the manufacturers.

Important Note: ***For Mx3000P set "Filter set gain settings" : ROX = x1, FAM = x4, JOE = x4. (see MxPro™ QPCR Software Instruction Manual, p.41)***

N.4.1 Thermal Profile

The thermal profile is reported in the table below:

Step	Cycle	Temp.	Time
1	1	50°C	2 min
1	1	95°C	10 min
2	50	95°C	15 sec
		60°C **(*)**	1 min

IMPORTANT NOTE: (*) step for the real time data collection

WARNING: Keep attention to set up the Real-Time Thermacycler with the correct Thermal Profile following the Instruments Manual supplied by the manufacturer.

N.4.2 Selection of the Detectors

Following the Instruction manuals of the Real-Time thermocyclers suggested (ABI 7500, MX3000P Stratagene) select the Detectors reported in the table here below:

Detection	Reporter	Quencher
Enterovirus	FAM	Non Fluorescent
Internal Control (I.C.)	JOE	Non Fluorescent
Passive Reference	ROX	Not Present

WARNING: Keep attention to set up the Real-Time Thermacycler with the correct settings following the Instruments Manual supplied by the manufacturer.

O. ASSAY SCHEME

An example of dispensation scheme for Qualitative Analysis is reported below:

Microplate or tubes

	1	2	3	.	.
A	**CTRL-H** 10^4copies/ µl	Sample 6			
B	**CTRL-L** 50copies/ µl	Sample 7			
C	NTC	Sample 8			
D	Sample 1	Sample 9			
E	Sample 2	Sample 10			
F	Sample 3	Sample 11			
G	Sample 4	Sample 12			
H	Sample 5	Sample 13			

Legenda: NTC = Negative Control CTRL-H, CTRL-L = Enterovirus RNA Positive Control, IC = Internal Control, Sample 1,2,3…13 = Samples under evaluation.

P. INTERNAL QUALITY CONTROL

P.1 Pre- Analysis setting

Before starting the analysis:

- Set the "Baseline" (the background fluorescence level) as reported below:

"Baseline"	
ABI™PRISM® 7500 SDS	**Manual Baseline: Start=3, End=15**
STRATAGENE™ MX3000P®	**Adaptive Baseline Do not use Mx4000 v1.00 to v3.00 algorithm**

- Set manually the FAM/JOE fluorescence "Threshold"

FAM fluorescence "Threshold"	
ABI™PRISM® 7500 SDS	**0.15**
STRATAGENE™ MX3000P®	**0.10**

JOE fluorescence "Threshold"	
ABI™PRISM® 7500 SDS	**0.02**
STRATAGENE™ MX3000P®	**0.02**

P.2 Data Analysis

A check is carried out on the High/Low Positive Controls any time the kit is used in order to verify whether their Ct values are as expected and reported in the table below.

Check	Requirements
CTRL-H	23 ≤ Ct (Threshold Cycle) <27
CTRL-L	31 ≤ Ct (Threshold Cycle) <35

Q. INTERPRETATION OF THE RESULTS AND TROUBLESHOOTING

For each samples FAM fluorescence (positive/negative Ct value) and Internal Control JOE fluorescence are assumed to validate Enterovirus RNA detection as described in the table below:

Enterovirus FAM	Internal Control JOE	Assay Result
SAMPLE POSITIVE	+	CORRECT
	-	CORRECT*
SAMPLE NEGATIVE	Ct < 42	CORRECT
	Ct ≥ 42 or undetrmined	INVALID**

* High Initial concentration of Enterovirus RNA in the sample (Positive FAM Signal) can lead to REDUCED or ABSENT Fluorescent Signal of Internal Control I.C. due to the reagents Competition.

** Problems may be occurred during the amplification step (inefficient or absent amplification) or during the extraction step (presence of inhibitors or initial sample containing an insufficient number of cells) leading to an incorrect result. The test procedure must be repeated starting from the Extraction step using a fresh sample coming from the patient.

The results obtained with this product must be interpreted taking consideration the clinical symptoms and the other laboratory parameters related to the patient conditions.

The following results are possible:

Troubleshooting table

	FAM	JOE	Result	CHECK
SAMPLE unknown	+	+/-	CORRECT RESULT *Positive*	***IMPORTANT***: High Initial concentration of Enterovirus RNA (Positive FAM Signal) can lead to REDUCED or ABSENT Fluorescent Signal of Internal Control I.C. due to the reagents Competition.
SAMPLE unknown	-	-	ATTENTION! POSSIBILITY OF: Inhibition, error in the procedure or misfunctioning of the Instruments	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. That the selected detection dyes are corrected FAM for the Enterovirus detection and JOE for the I.C. detection; 4. that the Analysis has been run with the correct Instrument settings; 5. that the kit has been stored correctly; 6. that no potential PCR inhibitors have been contaminated the tube 7. That the Extraction procedures have been executed correctly;
SAMPLE unknown	-	+	CORRECT RESULT *Negative*	
CTRL-H/CTRL-L	+	+/-	CORRECT RESULT	***IMPORTANT***: High Initial concentration of Enterovirus RNA (Positive FAM Signal) can lead to REDUCED or ABSENT Fluorescent Signal of Internal Control I.C. due to the reagents Competition.
CTRL-H/CTRL-L	-	-	ATTENTION ! POSSIBILITY OF: Error in the pipetting or in the procedure	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. That the selected detection dyes are corrected FAM for the Enterovirus detection and JOE for the I.C. detection; 4. that the Analysis has been run with the correct Instrument settings; 5. that the kit has been stored correctly; 6. that no potential PCR inhibitors have been contaminated the tube
CTRL-H/CTRL-L	-	+	ATTENTION ! POSSIBILITY OF: Error in the pipetting or in the procedure	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. That the selected detection dyes are corrected FAM for the Enterovirus detection and JOE for the I.C. detection; 4. that the Analysis has been run with the correct Instrument settings; 5. that the kit has been stored correctly;
NTC	-	+	CORRECT RESULT	
NTC	+	+/-	ATTENTION ! POSSIBILITY OF: Contamination	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. That the work space and Instruments are decontaminated at regular intervals; 4.;that the kit has been stored correctly;

Important notes:

1. *Interpretation of results should be done under the supervision of the responsible of the laboratory to reduce the risk of judgment errors and misinterpretations.*
2. *When test results are transmitted from the laboratory to an informatics centre, attention has to be done to avoid erroneous data transfer.*

If one of more of the problems described in the table above happen, after checking, report any residual problem to the supervisor for further actions.

R. PERFORMANCES

Evaluation of Performances has been conducted in accordance to what reported in the Internal Technical Specifications or ITS.
The performance evaluation was carried out in DiaPro's laboratories on materials supplied by the reference clinical lab.

R.1 ANALYTICAL SENSITIVITY

Analytical sensitivity may be expressed for qualitative methods as **limit of detection**,
Limit of detection (LOD): it is the lowest amount of target that can be detected by a test system with a stated probability.
For the NAT tests it is expressed as the smallest **analyte** concentration that tested in multiple repetitions gives positive results.
The **limit of detection (LOD)** is determined by testing serial dilutions containing known concentrations of the analyte.
The **LOD** is the lowest concentration of analyte that can be consistently detected (e.g. in $\geq$ 95% of samples under routine laboratory conditions).
In the kit code ENTERORNA.CE the **LOD** has been determined by testing 1:2 serial dilutions (8 replicates for three different runs) , of the highest dilution of the analyte that can be detected in 100% of them.

The results are the following:

Detection Limit	
ABI™PRISM® 7500 SDS	**2.5 copies/ µl**
MX3000P Stratagene	**2.5 copies/ µl**

This means that there is the 100% probability to detect a concentration of 2.50E+00 copies/ µl with the Instrument listed above.

R.2 ANALITYCAL SPECIFICITY

Analytical specificity is the ability of a method to detect only the target marker.
The analytical specificity of ENTEROVIRUS RNA assay has been studied as follow:

1. The primer/probe Set has been choose analysing the genome target sequence with an appropriate software (LionSoft v.1.0 supplied by Biotools and Primer Express v.3.0" supplied by Applied Biosystem Inc.).
2. The primer/probe Set and the target genome sequence has been controlled by the "BLAST" software, in order to check if any of the nucleotide sequences deposited in the worldwide genomic banks has any homology with Enterovirus, and by the "ClustalX" software, in order to compare the genome target sequences of the different genotypes of Enterovirus.
3. The specificity was improved through the selection of stringent reaction conditions.
4. Samples coming from patients suffering infections due to potential interfering organisms were obtained from Fondazione Ospedale Maggiore Policlinico Milano –Italy and tested.

The results are reported in the following table:

Organism	Result
Rhinovirus	negative

R.3 DIAGNOSTIC SPECIFICITY AND SENSITIVITY

R.3.1 Diagnostic Specificity:

Diagnostic specificity is the probability that the device gives a negative result in the absence of the target marker. So that **true negative** sample is a specimen known to be negative for the target marker and correctly classified by the device.
This parameter was studied by examining 33 Enterovirus RNA negative plasma samples extracts:

TRUE NEGATIVES	33
FALSE POSITIVES	0
TOTAL SAMPLES	33
SPECIFICITY %	**100**

On the basis of the results obtained Diagnostic Specificity of the system has been calculated ≥99%.

R.3.2 Diagnostic Sensitivity

Diagnostic sensitivity is the probability that the device gives a positive result in the presence of the target marker. So that **true positive** sample is a specimen known to be positive for the target marker and correctly classified by the device.
In the kit code ENTERORNA.CE this parameter was studied by examining Enterovirus RNA positive plasma samples in duplicates in the same run. Also QCMD 2008 and QCMD 2010 Enterovirus and Parechovirus (EVRNA08 – EVRNA10) panel samples were tested. Then it was been calculated the percentage (%) of positive samples.

TRUE POSITIVES	19
FALSE NEGATIVES	0
TOTAL SAMPLES	19
SENSITIVITY %	**100**

On the basis of the results obtained Diagnostic Sensitivity of the system has been calculated in the 100%.

Diagnostic Sensitivity	100 %
Diagnostic Specificity	> 99.5 %

R.4 PRECISION

Precision shows the degree of the system's reliability. Every measurement procedure has an inherent random variation called "random error". Random error does not have a number value but it is determined by dispersion of measurement as standard deviation (DevST) and coefficient variation (CV%). Usually precision of an assay refers to the agreement between replicate measurements of the same material.
In the kit code ENTERORNA.CE, **precision** was expressed as intra-assay variability and inter-assay variability. It were tested in the same run (intra-assay) and in two different runs (inter-assay), the CTRL-H and CTRL-L in 8 replicates.
Intra and inter-assay variability were then calculated.

In absence of established International parameters we have identified the following value of acceptability for the ENTERORNA.CE Kit:
Intra-Assay Coefficient Variation (CV%) ≤ 10%.
Inter-Assay Coefficient Variation (CV%) ≤ 10%.

S. LIMITATIONS

The user of this kit is advised to carefully read and understand this package insert. Strict adherence to the protocol is necessary in order to obtain reliable test results. In particular, accurate sample and reagent pipetting,application of a correct workflow along with careful programming of thermocycling step is essential for accurate and reproducible Enterovirus RNA detection.
It is recommended that confidentiality, appropriate counselling and medical evaluation be considered an essential aspect of the testing sequence.

T. BIBLIOGRAPHY

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3. Rapid and Sensitive routine detection of all members of the genus enterovirus in different clinical specimens by real-time PCR. M Nijhuis, N van Maarseveen, R Schuurman, S Verkuijlen, M de vos, K Hendriksen, and A M van Loon. J Clin Microbiol, Oct 2002, p3666-3670.
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5. Symbols

LEGENDA			
REF	Product code		Storage temperature
IVD	In Vitro Diagnostic Device		See use instructions
LOT	Lot number		Manufacturer
	Expiry date		Number of tests
CE	CE conformity mark		Date of manufacturing

All the IVD Products manufactured by the company are under the control of a certified Quality Management System in compliance with ISO 13485 rule. Each lot is submitted to a quality control and released into the market only if conforming with the EC technical specifications and acceptance criteria.

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