Instructions for Use

LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0

LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0

For detection and subtyping of Human Enteroviruses, Enterovirus A71, Coxsackievirus A6 and Coxsackievirus A16 from human specimens

For use with

CFX96™ (BioRad)
CFX Opus 96 (BioRad)

IVD For *in vitro* diagnostic use

REF Product No.: 881303

96 reactions

Please refer to Storage and Shelf Life in this IFU

Ver. 01 / June 2025

AstronDX Technologies Sdn Bhd
Unit 307, Block B, Phileo Damansara 1, 9, Jalan 16/11,
46350 Petaling Jaya, Selangor, Malaysia

Content

1. Intended Use

The LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 is intended for the specific detection and subtyping of Enterovirus RNA in human throat, vesicles, lesions or rectal swabs in VTM, and blood and cerebrospinal fluid (CSF) specimens. The LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 is a four-target assay comprising a screening assay targeting pan-Enterovirus 5' UTR region, and a typing assay that further differentiates Enterovirus serotypes A71 (EV71), via targeting of the *VP3-VP1* genes, and Coxsackievirus A16 (CVA16) and Coxsackievirus A6 (CVA6), via targeting the *VP1* gene.

The LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 is for professional use only.

2. Kit Components

Catalog no.	881303
Master A	2 x 360 µl
Master B	2 x 360 µl
Internal Control (IC)	800 µl
Positive Control (PC)	200 µl
PCR grade water	500 µl

3. Storage and Shelf Life

- The LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 has a shelf life of 12 months from the manufacturing date.
- Store all reagents at -15°C to -25°C upon arrival.
- Repeated thawing and freezing should be avoided, as this might affect the performance of the assay. Master B should be frozen in aliquots, if they are to be used intermittently.
- Mix Master A thoroughly by vortex mixing, and centrifuge briefly before use.
- Protect Master B from light.
- All frozen reagents should be completely thawed to room temperature before use. Immediately return unused portions to the freezer for storage.

4. Quality Control

In compliance with AstronDX Technologies' EN ISO 13485 certified Quality Management System, each lot of the LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 is tested against pre-determined specifications to ensure consistent product quality.

5. Product Use Limitations and Precautions

- Use of this product is limited to personnel specially instructed and trained in the techniques of real-time PCR procedures.
- Specimens should always be treated as if infectious and/or biohazardous in accordance with safe laboratory procedures.
- Wear protective disposable powder-free gloves, a laboratory coat and eye protection when handling specimens.
- Avoid microbial and nuclease (DNAse/RNAse) contamination of the specimen and the components of the kit.
- Always use DNAse/RNAse-free disposable pipette tips with aerosol barriers.
- Always wear protective disposable powder-free gloves when handling kit components.
- Use separated and segregated working areas for (i) specimen preparation, (ii) reaction set-up and (iii) amplification/detection activities.
- Workflow in the laboratory should proceed in unidirectional manner.
- Always wear disposable gloves in each area and change them before entering different areas.
- Dedicate supplies and equipment to the separate working areas and do not move them from one area to another.
- Store positive and/or potentially positive material separated from all other components of the kit.
- Do not open the reaction tubes/plates post amplification, to avoid contamination with amplicons.
- Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organizations.
- Do not use components of the kit that have passed their expiration date.
- Discard sample and assay waste according to your local safety regulations.

- Wash hands thoroughly after handling specimens and test reagents.
- Do not use kits from different lots together.
- Do not use an expired kit.
- In case of damage to the packaging and leaking vials, do not use the kit (possible contamination or deterioration that can cause false interpretation)
- Use of this kit is not recommended if patient has recently used Betadine Nasal Spray (Kid). Carragelose present in this nasal spray may cause PCR interference.
- Due to highly conserved 5'UTR region between Enteroviruses and Rhinoviruses, cross-reactivity with some Rhinovirus serotypes may occur if viral load is high.

6. Product Warranty

AstronDX Technologies guarantees the performance of the LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 for applications as described in the manual. The user must determine the suitability of the product for the particular intended use. Should the product fail to perform satisfactorily in the described applications, please contact AstronDX Technologies Technical Support (**16. Technical Support**) for trouble-shooting.

AstronDX Technologies reserves the right to change, alter, or modify any product to enhance its performance and design.

7. Product Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles/face masks. For more information, please consult the appropriate material safety data sheets (MSDSs).

8. Introduction

Human Enteroviruses (HEVs) are a group of positive sense, single stranded RNA viruses belonging to the picornavirus family. The genome of approximately 7.4 kb consists of an open reading frame flanked by 5' and 3' untranslated regions. The long single open reading frame encodes a polyprotein which is then being cleaved into viral capsid proteins, VP1 to VP4; and also non-capsid proteins, P2 and P3. The nucleotide sequence of the VP1 coding region has been utilised for phylogenetic analysis due to its serotype specificity [1]. HEVs can be subdivided into Human

Coxsackievirus (CVA and CVB), Human Polio virus (PV type 1, 2 and 3), non-polio Enterovirus (EV) and Echovirus.

Another picornavirus, Human Rhinovirus (HRV), also exhibits high genomic similarity with the HEVs but it is a casual agent for common colds and other upper respiratory tracts syndromes [2]; whereas EVs and Coxsackieviruses are well-recognised causal agents for the hand, foot and mouth disease (HFMD) which is highly contagious, especially among children.

HFMD mode of transmission is from person to person through contact. Infected discharge of mucus from throat, nose, saliva and fluids from the ruptured blisters as well as infected faeces from the patient are contagious, especially during the first week of infection. The incubation period for HFMD ranges from 3 to 7 days. Children below 5 years old are most susceptible to HFMD, although adolescents and adults can also be infected. HFMD is generally a mild disease, but fatal neurologic and systemic complications can occur in severe HFMD cases. Common symptoms of HFMD include fever, sore throat, rashes on hands, feet, and mouth [3].

There are at least 15 HEV serotypes which have been associated with the development of HFMD. Among the diverse serotypes, EV71, CVA16, CVA6 and CVA10 are noticeably causing outbreaks of HFMD in countries such as Malaysia, Singapore, Thailand, China and Taiwan.

Malaysia has experienced multiple major HFMD outbreaks since the year 1997. The serotype associated with the major outbreaks of HFMD in Malaysia was identified as EV71 and has caused fatalities. Serotype CVA16 has not led to large outbreaks thus far and severe diseases were only observed sporadically [4]. In more recent years, increasing numbers of HFMD cases caused by CVA6 were reported in Kuala Lumpur and Selangor HFMD outbreaks and this serotype was found to infect predominantly infants below 12 months old [5]. According to the Ministry of Health, HFMD major outbreaks dating back to 2018 have caused more than 76,000 cases. More recently, in June 2022, there were altogether 95,924 cases of HFMD reported. This amounted to a 36-fold increase in cases compared to the same period of time in year 2021 (2654 reported cases).

Both EV71 and CVA16 are actively circulating in Malaysia and other Asia Pacific countries while causing acute outbreaks from time to time, with CVA6 rising to be another causal agent of HFMD [3]. Nevertheless, fatalities and severe neurological complications were still mostly observed in EV71 infections rather than CVA16 or CVA6. Hence, the identification and subtyping of the EV serotypes found in the patient samples at early stages are crucial in order to identify potentially severe HFMD cases. Various real-time RT-PCR assays have been published to detect and

subype HFMD-causing Enteroviruses. The LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 was developed based on assays previously described. The kit comprises of four assays targeting the 5'UTR region of pan-Enterovius, VP3-VP1 gene of Enterovirus A71 (EV71), and VP1 gene of Coxsackievirus A16 (CVA16) and Coxsackievirus A6 (CVA6), for specific detection and differentiation of respective Enterovirus serotypes.

[1] Hu, Y. F., Yang, F., Du, J., Dong, J., Zhang, T., Wu, Z. Q., Xue, Y., & Jin, Q. (2011). Complete genome analysis of coxsackievirus A2, A4, A5, and A10 strains isolated from hand, foot, and mouth disease patients in China revealing frequent recombination of human enterovirus A. *Journal of Clinical Microbiology*, 49(7), 2426–2434.

[2] Hayden F. G. (2004). Rhinovirus and the lower respiratory tract. *Reviews in Medical Virology*, 14(1), 17–31.

[3] Fong, S. Y., Mori, D., Rundi, C. Yap, J. F., Jikal, M., Abd Latip, A.L.L., Johnny, V. & Ahmed, K. (2021) A five-year retrospective study on the epidemiology of hand, foot and mouth disease in Sabah, Malaysia. *Scientific Reports*, 11: 17814.

[4] Chan, Y. F., Wee, K. L., Chiam, C. W., Khor, C. S., Chan, S. Y., Amalina W, M. Z., & Sam, I. C. (2012). Comparative genetic analysis of VP4, VP1 and 3D gene regions of enterovirus 71 and coxsackievirus A16 circulating in Malaysia between 1997-2008. *Tropical Biomedicine*, 29(3), 451–466.

[5] Lee, M., Chong, Y. M., Tay, C. G., Koh, M. T., Chem, Y. K., Noordin, N., Jahis, R., Sam, I. C., & Chan, Y. F. (2021). Detection of enteroviruses during a 2018 hand, foot and mouth disease outbreak in Malaysia. *Tropical Biomedicine*, 38(1), 150–153.

9. Product Description

The LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 is an *in vitro* diagnostic test system, based on real-time PCR technology, for the qualitative detection of Human Enteroviruses (including Enterovirus A to D, Coxsackievirus A and B, Poliovirus 1, 2 and 3, and Echovirus; excluding Human Rhinoviruses) and subtyping of Enterovirus A71 (EV71), Coxsackievirus A16 (CVA16) and Coxsackievirus A6 (CVA6). The LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 consists of a single tube assay with four targets, namely pan-Enterovirus (panEV), EV71, CVA16 and CVA6. The LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 includes a heterologous amplification system (Internal Control) to identify possible RT-PCR inhibition and to confirm the integrity of the reagents of the kit. The Internal Control

used in the LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 is an *in vitro* transcribed RNA of an artificial sequence with no homology to any known genomes. Real-time RT-PCR technology utilizes reverse transcriptase (RT) reaction to convert RNA into complementary DNA (cDNA), and polymerase chain reaction (PCR) for the amplification of specific target sequences, and target specific probes for the detection of the amplified DNA. The probes are labelled with fluorescent reporter and quencher dyes.

In the assay, the probe for the screening of panEV is labelled with the fluorophore Cy5™, and probes specific for the typing of EV71, CVA16 and CVA6 are labelled with the fluorophores FAM, Tex615 and Tye705, respectively. The pan-Enterovirus probe specifically detects Human Enteroviruses only (Enterovirus A to D, Coxsackievirus A and B, Poliovirus 1, 2, and 3, and Echovirus) and does not cross-react with Human Rhinoviruses. The probes for EV71, CVA16 and CVA6 are specific only to Enterovirus serotype A71 (EV71), Coxsackievirus A16 (CVA16), and Coxsackievirus A6 (CVA6), respectively. The probe specific for the Internal Control (IC) is labelled with the fluorophore HEX™. Using probes linked to distinguishable dyes enables the parallel detection of Human Enteroviruses, typing of serotype EV71, CVA16, CVA6 and also the Internal Control in the corresponding detector channels of the real-time PCR instrument.

The oligonucleotides included in the four assays were designed, modified and based on sequences/targets published in the articles listed below:

Target	**Publication**
Pan-Enterovirus	Chen *et al*. (2006), Verstrepen e*t al*. (2001), Zhang e*t al*. (2014),
Enterovirus A71 (EV71)	NCBI GenBank (2023)
Coxsackievirus A16 (CVA16)	Cui *et al*., (2013)
Coxsackievirus A6 (CVA6)	Puenpa *et al*., (2017)
Internal Control	Deer *et al*., (2010)

The test consists of three processes in a single tube assay:

- Reverse transcription of target and Internal Control RNA to cDNA
- PCR amplification of target and Internal Control cDNA
- Simultaneous detection of PCR amplicons by fluorescent dye labelled probes

The LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 consists of:

- Two Master reagents (Master A and Master B)
- The template of the Internal Control (IC)
- The template of the Positive Control (PC)
- PCR grade water (for setting up of “No Template Control”, NTC)

Master A and Master B reagents contain all components (buffer, enzymes, primers and probes) to allow PCR mediated reverse transcription, amplification and target detection of 5’UTR region of pan-Enterovirus specific RNA, *VP3-VP1* gene for specific detection and differentiation of Enterovirus A71 (EV71), and *VP1* gene for the specific detection and differentiation of Coxsackievirus A16 (CVA16) and Coxsackievirus A6 (CVA6), and Internal Control in one reaction setup.

The Positive Control (PC) contains *in vitro* transcripts of synthesized target genes of pan-Enterovirus, Enterovirus A71, Coxsackievirus CVA16 and Coxsackievirus A6.

The Internal Control used in the LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 is an *in vitro* transcribed RNA of an artificial sequence with no homology to any known genomes.

The LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 was developed and validated to be used with the following real-time PCR instruments:

- CFX96™ (BioRad)
- CFX Opus 96 (BioRad)

10. Material and Devices required but Not Provided

- Appropriate real-time PCR instrument
- Appropriate nucleic acid extraction system or kit
- 1.5 ml microcentrifuge tubes (with safe-lock or screw cap)
- Microcentrifuge (with speed ≥ 13,000 rpm)
- Pipettes, adjustable (range: 10 µl, 100 µl, 200 µl, 1000 µl)
- Pipette tips (with aerosol barriers)
- Disposable gloves (powder-free)
- Heating block for lysis of specimens during extraction
- Vortex mixer

- Appropriate 96-well reaction plates or reaction tubes with corresponding (optical) closing material. **(Clear tubes are recommended. Do not use white tubes)**

11. Specimen Storage

- Suitable specimens include human throat, vesicles, lesions or rectal swabs in VTM, and blood and cerebrospinal fluid (CSF) specimens.

- Follow specimen transport and storage conditions outlined in the following guidelines:

  - World Health Organization (2000). Guidelines for the collection of clinical specimens during field investigation of outbreaks. https://iris.who.int/handle/10665/66348
  - World Health Organization (2011). A Guide to clinical management and public health response for hand, foot and mouth disease (HFMD). https://iris.who.int/handle/10665/207490
  - Ministry of Health Malaysia (2007). Hand Foot and Mouth Disease (HFMD) Guidelines. https://www.moh.gov.my/moh/resources/auto%20download%20images/589d71f714d23.pdf

12. Instructions for Use

12.1. Sample Preparation

Extracted RNA is the starting material for the LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0. The quality of the extracted RNA has a profound impact on the performance of the whole test system. It has to be ensured that the nucleic acid extraction system used is compatible with real-time PCR technology.

The following nucleic acid extraction kits / systems are suitable for use with the LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0:

- SpinStar™ Viral Nucleic Acid Kit (AstronDX Technologies)
- MagCore® Plus II Automated Nucleic Acid Extractor (RBC Bioscience)
- QIAamp® MinElute Virus Spin Kit (Qiagen)
- QIAamp® Viral RNA Mini Kit (Qiagen)
- QIAsymphony® (Qiagen)

Alternative nucleic acid extraction systems and kits might also be appropriate. The suitability of the nucleic acid extraction procedure for use with LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 has to be validated by the user.

If using a spin column based sample preparation procedure including washing buffers containing ethanol, an additional centrifugation step for 10 min at approximately 17000 x g (~ 13000 rpm), using a new collection tube, prior to the elution of the nucleic acid is highly recommended.

NOTE

Ethanol is a strong inhibitor in real-time PCR. If your sample preparation system is using washing buffers containing ethanol, you need to make sure to eliminate any traces of ethanol prior to elution of the nucleic acid.

12.2. Master Mix Setup

1. All reagents and samples should be thawed completely, mixed (by gentle vortex mixing) and centrifuged briefly before use. Prepare a marginal excess (additional 0.5 reaction) of the required Master Mix volume.

2. The LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 contains a heterologous Internal Control (IC), which can either be used as (i) a PCR inhibition control or as (ii) a control of the sample preparation procedure (nucleic acid extraction) and PCR inhibition control.

   (i) If the IC is used as a PCR inhibition control, but not as a control for the sample preparation procedure, the Master Mix is set up according to the following pipetting scheme:

Number of Reactions	1	12
Master A	7.5 µl	90 µl
Master B	7.5 µl	90 µl
IC	0.5 µl	6 µl
Volume Master Mix	**15.5 µl**	**186 µl**

(ii) If the IC is used as a control for the sample preparation procedure and as a PCR inhibition control, the IC has to be added during the nucleic acid extraction procedure.

No matter which method/system is used for nucleic acid extraction, the IC **must not** be added directly to the specimen. The IC should always be added to the specimen/lysis buffer mixture.

The volume of the IC which has to be added depends always and only on the elution volume. It represents **10% of the elution volume**. For instance, if the nucleic acid is going to be eluted in 60 µl of elution buffer or water, 6 µl of IC per sample must be added into the specimen/lysis buffer mixture.

If the IC was added during the sample preparation procedure, the Master Mix is set up according to the following pipetting scheme:

Number of Reactions	1	12
Master A	7.5 µl	90 µl
Master B	7.5 µl	90 µl
Volume Master Mix	**15 µl**	**180 µl**

NOTE

⚠ ***Never add the Internal Control directly to the specimen.***

12.3. Reaction Setup

1. Pipette 15 µl Master Mix into each required well of an appropriate optical 96-well reaction plate or an appropriate optical reaction tube.

2. Add 5 µl of the sample (eluate from the nucleic acid extraction) or 5 µl of the controls (Positive Control; or water as No Template Control, NTC).

3. Make sure at least one Positive Control and one NTC are used per run.

4. Thoroughly mix the samples and controls with the Master Mix by pipetting up and down.

5. Close the 96-well reaction plate with an appropriate optical adhesive film and the reaction tubes with appropriate caps.

6. Centrifuge the 96-well reaction plate at 1,000 xg (~3,000 rpm) for 30s.

Reaction Setup	
Master Mix	15 µl
Sample or Control	5 µl
Total Volume	**20 µl**

13. Programming the Real-Time PCR Instrument

13.1 Settings

• Define the following settings:

Settings	
Reaction Volume	20 µl
Ramp Rate	Default

13.2 Fluorescent Detectors (Dyes)

• Define the following fluorescent detectors:

Detection	Detector Name	Reporter	Quencher
Pan-Enterovirus (5'UTR region) specific RNA	panEV	Cy5	IBFQ
EV71 (*VP3-VP1* genes) specific RNA	EV71	FAM	IBFQ
CVA16 (*VP1* gene) specific RNA	CVA16	Tex615	IBRQ
CVA6 (*VP1* gene) specific RNA	CVA6	Quasar705	BHQ1
Internal Control	IC	HEX	IBFQ

13.3 Temperature Profile and Dye Acquisition

• Define the temperature profile and dye acquisition:

	Stage	Cycle Repeats	Acquisition	Temperature	Time
Reverse-transcription	Hold	1	-	50 °C	10:00 min
Denaturation	Hold	1	-	95 °C	2:00 min
Amplification	Cycling	45	-	95 °C	5 sec
			√	60 °C	40 sec

√ Signal acquisition: activate Cy5, FAM, Tex615, Quasar705 and HEX channels in all runs

14. Data Analysis

For basic information regarding data analysis on specific real-time PCR instruments, please refer to the manual of the respective instrument. For detailed instructions regarding data analysis of the LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 on different real-time PCR instruments please contact our Technical Support (**16.Technical Support**).

14.1. Validity of Diagnostic Test Runs

14.1.1 Valid Diagnostic Test Runs (Qualitative)

For a **valid** diagnostic test run (qualitative), the following control conditions must be met:

Control ID	Cy5 / FAM / Tex615 / Quasar705 Detection Channel	HEX Detection Channel
Positive Control	POSITIVE	POSITIVE
Negative Control	NEGATIVE	POSITIVE

14.1.2 Target CT values of PC and IC

	Positive Control *(panEV)*	Positive Control *(EV71)*	Positive Control *(CVA16)*	Positive Control *(CVA6)*	Internal Control
Target CT value	< 35 cycles	< 35 cycles	< 35 cycles	< 35 cycles	≤ 40 cycles*

*Required for unknown samples that do not amplify in Cy5, FAM, Tex615 and Quasar705 channels

Note: The above CT target values are exclusively given for **monitoring the integrity of the product and validated assay conditions** and should be achieved **ONLY for the provided Positive Control (PC) and Internal Control (IC)** when used as per the instructions given under section 12.3. Reaction set up. **The target CT values for PC MUST NOT be misinterpreted as the diagnostic cut-off values for clinical samples.**

14.1.3 Invalid Diagnostic Test Runs (Qualitative)

A **qualitative** diagnostic test run is **invalid**, (i) if the run has not been completed or (ii) if any of the control conditions for a **valid** diagnostic test run are not met.

In case of an **invalid** diagnostic test run, **repeat testing by using the remaining purified nucleic acids** or start from the original samples again.

14.2 Interpretation of Results

Cy5 *PanEV*	FAM *EV71*	Tex615 *CVA16*	Quasar 705 *CVA6*	HEX *Internal Control*	Result Interpretation
+	+	+	+	+*	Pan-Enterovirus, Enterovirus A71, Coxsackievirus A16 and Coxsackievirus A6 specific RNA detected *Positive for human Enteroviruses, Enterovirus A71, Coxsackievirus A16, and Coxsackievirus A6*
-	+	+	+	+*	Enterovirus A71, Coxsackievirus A16 and Coxsackievirus A6 specific RNA detected *Positive for Enterovirus A71, Coxsackievirus A16, and Coxsackievirus A6* [A]
+	-	+	+	+*	Pan-Enterovirus, Coxsackievirus A16 and Coxsackievirus A6 specific RNA detected. *Positive for human Enteroviruses, Coxsackievirus A16, and Coxsackievirus A6*
+	+	-	+	+*	Pan-Enterovirus, Enterovirus A71 and Coxsackievirus A6 specific RNA detected. *Positive for human Enteroviruses, Enterovirus A71 and Coxsackievirus A6*
+	+	+	-	+*	Pan-Enterovirus, Enterovirus A71 and Coxsackievirus A16 specific RNA detected. *Positive for human Enteroviruses, Enterovirus A71 and Coxsackievirus A16*

+	+	-	-	+*	Pan-Enterovirus and Enterovirus A71 specific RNA detected. *Positive for human Enteroviruses and Enterovirus A71*
+	-	+	-	+*	Pan-Enterovirus and Coxsackievirus A16 specific RNA detected. *Positive for human Enteroviruses and Coxsackievirus A16*
+	-	-	+	+*	Pan-Enterovirus and Coxsackievirus A6 specific RNA detected. *Positive for human Enteroviruses and Coxsackievirus A6*
-	+	+	-	+*	Enterovirus A71 and Coxsackievirus A16 specific RNA detected. *Positive for Enterovirus A71 and Coxsackievirus A16* [A]
-	+	-	+	+*	Enterovirus A71 and Coxsackievirus A6 specific RNA detected *Positive for Enterovirus A71 and Coxsackievirus A6* [A]
-	-	+	+	+*	Coxsackievirus A16 and Coxsackievirus A6 specific RNA detected *Positive for Coxsackievirus A16 and Coxsackievirus A6* [A]
+	-	-	-	+*	Pan-Enterovirus specific RNA detected. *Positive for human Enteroviruses*

-	+	-	-	+*	Enterovirus A71 specific RNA detected *Positive for Enterovirus A71* [A]
-	-	+	-	+*	Coxsackievirus A16 specific RNA detected *Positive for Coxsackievirus A16* [A]
-	-	-	-	+*	Coxsackievirus A6 specific RNA detected *Positive for Coxsackievirus A6* [A]
-	-	-	-	+	Pan-Enterovirus, Enterovirus A71, Coxsackievirus A16 and Coxsackievirus A6 specific RNA not detected. The samples do not contain detectable amounts of human Enteroviruses, Enterovirus A71, Coxsackievirus A16 and Coxsackievirus A6 specific RNA
-	-	-	-	-	PCR inhibition or reagent failure. Repeat testing from original sample or collect and test a new sample.

Note: For pan-Enterovirus 5'UTR (Cy5 channel), Enterovirus A71 *VP3-VP1* genes (FAM channel), Coxsackievirus A16 *VP1* gene (Tex615 channel) and Coxsackievirus A6 *VP1* gene (Quasar705 channel) "+" refers to amplification curve detected at CT ≤ 45 cycles. "-" refers to no amplification / no CT obtained.

* Detection of the Internal Control in the HEX channel is not required for positive results in the Cy5/FAM/Tex615/Quasar705 detection channels. A high Enterovirus viral load in the sample can lead to reduced or absent Internal Control signals.

[A] Due to different detection sensitivity of pan-Enterovirus (Cy5) and the Enterovirus A71 (FAM), Coxsackievirus A16 (Tex615) and Coxsackievirus A6 (Quasar705) assays, in rare cases weak positive samples may show a signal in the FAM, Tex615 and/or Quasar705 channels but not in the Cy5 channel.

14.2.1 Baseline Settings for Cycler Software

After the run is completed, normalize fluorescence signals in all channels by selecting “Apply Fluorescence Drift Correction” from the Baseline Setting menu.

14.2.2 Threshold Settings for Cycler Software

Cycler	Threshold				
	Cy5 panEv **Channel**	**FAM** EV71 **Channel**	**Tex615** CVA16 **Channel**	**Quasar705** CVA6 **Channel**	**HEX** IC **Channel**
CFX96™	100 RFU	100 RFU	100 RFU	100 RFU	100 RFU
CFX Opus 96	100 RFU	100 RFU	100 RFU	100 RFU	100 RFU

14.2.3 CT Cut-Off Values for Clinical Samples

	Cy5 panEV **Channel**	**FAM** EV71 **Channel**	**Tex615** CVA16 **Channel**	**Quasar705** CVA6 **Channel**
CT Cut-Off Value	< 45 cycles	< 45 cycles	< 45 cycles	< 45 cycles

15. Performance Evaluation

The analytical performance evaluation of the LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 was accomplished using quantified panEV, EV71, CVA16 and CVA6 specific RNA.

15.1 Analytical Sensitivity

The analytical sensitivity (limit of detection: LoD) of the LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 is defined as the concentration of panEV, EV71, CVA16, and/or CVA6 RNA molecules that can be detected with a positivity rate of ≥ 95%.

The analytical sensitivity was determined by analyzing panEV, EV71, CVA16, and/or CVA6 genomic RNA of known concentration.

A dilution series of Quantitative Genomic RNA from Human Enterovirus 71, and Coxsackievirus A6 and Coxsackievirus A16 specific *in vitro* transcripts (IVT) of known concentration was prepared by using 1× TE buffer as diluent. Dilutions of panEV, EV71, CVA16, and CVA6 RNA were tested with LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0. Results were analyzed by Probit analysis (Table 1, Table 2, Table 3, and Table 4).

The analytical sensitivity of the LyteStar™ Enterovirus HFMD RT-PCR Kit 1.0 in combination with the CFX Opus 96 (BioRad) platform was determined at 2.44 copies/µl for pan-Enterovirus 5'UTR region target, 1.79 copies/µl for Enterovirus A71 *VP3-VP1* gene target, 4.79 copies/µl for Coxsackievirus A6 *VP1* gene target, and 3.44 copies/µl for Coxsackievirus A16 *VP1* gene target ($p \leq 0.05$).

Table 1. PCR results used for the calculation of the analytical sensitivity of pan-Enterovirus 5'UTR region target for the LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 in combination with the CFX Opus 96 (BioRad) platform.

Concentration (copies/µl)	No. of Samples Tested	No. of Samples Positive	Hit rate (%)
20.0	12	12	100
6.33	12	12	100
2.0	12	11	91.7
1.0	12	6	50.0
0.63	12	4	33.3
0.20	12	0	0
0.063	12	0	0
0.020	12	0	0
0.0063	12	0	0

Table 2. PCR results used for the calculation of the analytical sensitivity of Enterovirus A71 *VP3-VP1* gene target for the LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 in combination with the CFX Opus 96 (BioRad) platform.

Concentration (copies/µl)	No. of Samples Tested	No. of Samples Positive	Hit rate (%)
20.0	12	12	100
6.33	12	12	100
2.0	12	12	100
1.0	12	11	91.7
0.63	12	7	58.3
0.20	12	6	50.0
0.063	12	2	16.7
0.020	12	0	0
0.0063	12	0	0

Table 3. PCR results used for the calculation of the analytical sensitivity of Coxsackievirus A6 *VP1* gene target for the LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 in combination with the CFX Opus 96 (BioRad) platform.

Concentration (copies/µl)	No. of Samples Tested	No. of Samples Positive	Hit rate (%)
20.0	12	12	100
6.33	12	12	100
2.0	12	10	83.3
1.0	12	9	75.0
0.63	12	4	33.3
0.20	12	5	41.7
0.063	12	1	8.3
0.020	12	0	0
0.0063	12	0	0

Table 4. PCR results used for the calculation of the analytical sensitivity Coxsackievirus A16 *VP1* gene target for the LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 in combination with the CFX Opus 96 (BioRad) platform.

Concentration (copies/µl)	No. of Samples Tested	No. of Samples Positive	Hit rate (%)
20.0	12	12	100
6.33	12	12	100
2.0	12	12	100
1.0	12	5	41.7
0.63	12	3	25.0
0.20	12	2	16.7
0.063	12	0	0
0.020	12	0	0
0.0063	12	0	0

15.2 Analytical Specificity

The analytical specificity of the LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 is ensured by the thorough selection of the oligonucleotides (primers and probes). The oligonucleotides were checked by sequence comparison analysis against publicly available sequences to ensure that the applied primer/probes in LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 specifically detect human Enteroviruses (including Enterovirus A to D, Coxsackievirus A and B, Poliovirus 1, 2, and 3 and Echovirus; excluding Rhinoviruses), Enterovirus A71, Coxsackievirus A16 and Coxsackievirus A6.

The specificity of the LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 was evaluated by testing genomic RNA/DNA extracted from other pathogens likely to be present in the same sample material as human Enteroviruses, or that cause similar symptoms to these viruses (Table 5).

Table 5. Microorganisms tested to demonstrate the analytical specificity of the LyteStar™ Enterovirus HFMD Typing RT-PCR 1.0 Kit.

LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0					
Organisms	***panEV (Cy5 channel)***	***EV71 (FAM channel)***	***CVA16 (Tex615 channel)***	***CVA6 (Quasar 705 channel)***	***Internal Control (HEX channel)***
Human respiratory syncytial virus type A	negative	negative	negative	negative	valid
Human respiratory syncytial virus type B	negative	negative	negative	negative	valid
Human coronavirus OC43	negative	negative	negative	negative	valid
Human coronavirus strain NL63	negative	negative	negative	negative	valid
Human coronavirus SARS-CoV-2	negative	negative	negative	negative	valid
MERS coronavirus	negative	negative	negative	negative	valid
Influenza A virus (H1N1)	negative	negative	negative	negative	valid
Influenza A virus (H3N2)	negative	negative	negative	negative	valid
Influenza B virus	negative	negative	negative	negative	valid
Human parainfluenzavirus 2	negative	negative	negative	negative	valid
Human parainfluenzavirus 3	negative	negative	negative	negative	valid
Bordetella parapertussis	negative	negative	negative	negative	valid
Bordetella pertussis	negative	negative	negative	negative	valid
Moraxella catarrhalis	negative	negative	negative	negative	valid
Mycobacterium tuberculosis	negative	negative	negative	negative	valid
Mycoplasma pneumoniae	negative	negative	negative	negative	valid
Chlamydophila pneumoniae	negative	negative	negative	negative	valid
Human bocavirus	negative	negative	negative	negative	valid
Epstein Barr virus	negative	negative	negative	negative	valid
Dengue virus type 1	negative	negative	negative	negative	valid
Dengue virus type 2	negative	negative	negative	negative	valid
Dengue virus type 3	negative	negative	negative	negative	valid
Japanese Encephalitis Virus	negative	negative	negative	negative	valid
West Nile Virus	negative	negative	negative	negative	valid
Ross River Virus	negative	negative	negative	negative	valid

Zika virus	negative	negative	negative	negative	valid
Chikungunya virus	negative	negative	negative	negative	valid
Legionella pneumophilia	negative	negative	negative	negative	valid
Parvovirus B19	negative	negative	negative	negative	valid
Borrelia burgdorferi	negative	negative	negative	negative	valid
Orientia tsutsugamushi	negative	negative	negative	negative	valid
Rickettsia conorii	negative	negative	negative	negative	valid
Human herpesvirus 5	negative	negative	negative	negative	valid
Enterovirus A71	positive	positive	negative	negative	valid
Enterovirus D68	positive	negative	negative	negative	valid
Enterovirus D68 B3	positive	negative	negative	negative	valid
Coxsackievirus A9	positive	negative	negative	negative	valid
Coxsackievirus A16	positive	negative	positive	negative	valid
Coxsackievirus A24	positive	negative	negative	negative	valid
Coxsackievirus B3	positive	negative	negative	negative	valid
Echovirus 6	positive	negative	negative	negative	valid
Echovirus 18	positive	negative	negative	negative	valid
Echovirus 25	positive	negative	negative	negative	valid
Echovirus 30	positive	negative	negative	negative	valid
Rhinovirus A8	negative	negative	negative	negative	valid
Rhinovirus A16	negative	negative	negative	negative	valid
Rhinovirus A90	negative	negative	negative	negative	valid
Rhinovirus B5	negative	negative	negative	negative	valid
Rhinovirus A72	negative	negative	negative	negative	valid
RhinovirusType C	negative	negative	negative	negative	valid

The LyteStar™ Enterovirus HFMD Typing RT-PCR 1.0 did not cross-react with any pathogen or genotype/subtype other than its own target.

15.3 Diagnostic Sensitivity and Specificity

The clinical performance of the LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0, in regards to diagnostic sensitivity and specificity, was evaluated through retrospective studies at various public health laboratories. The diagnostic sensitivity and specificity of the LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 was compared to the clinical site's reference method.

In comparison to the reference test, the LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0 achieved a diagnostic sensitivity of 93.75%, 100%, 93.55% and 100% for the detection of pan-Enterovirus, Enterovirus A71, Coxsackievirus A16 and Coxsackievirus A6, respectively, and a diagnostic specificity of 100% for the detection of pan-Enterovirus, Enterovirus A71 and Coxsackievirus A6, and 93.10% for Coxsackievirus A16.

		Reference Method			
		panEV (n=16)	EV71 (n=10)	CVA16 (n=31)	CVA6 (n=13)
		Positive			
LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0	Detected	15	10	29	13
	Not Detected	1	0	2	0
	Total	**16**	**10**	**31**	**13**
Overall Concordance		93.75 % 15 / 16 Sensitivity	100 % 10 / 10 Sensitivity	93.55 % 29 / 31 Sensitivity	100 % 13 / 13 Sensitivity
		Reference Method			
		panEV (n=29)	EV71 (n=25)	CVA16 (n=29)	CVA6 (n=25)
		Negative			
LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0	Detected	0	0	2	0
	Not Detected	29	25	27	25
	Total	**29**	**25**	**29**	**25**
Overall Concordance		100 % 29 / 29 Specificity	100 % 25 / 25 Specificity	93.10 % 27 / 29 Specificity	100 % 25 / 25 Specificity

To calculate the diagnostic sensitivity of LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0:

$$\text{Diagnostic Sensitivity [\%]} = \frac{\text{“correct positive”}}{\text{(“correct positive” + “false positive”)}} \times 100$$

To calculate the diagnostic specificity of LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0:

$$\text{Diagnostic Specificity [\%]} = \frac{\text{“correct negative”}}{\text{(“correct negative” + “false negative”)}} \times 100$$

16. Technical Support

For customer support, please contact our Technical Support:

e-mail: **techsupport@astrondx.com**
phone: **+603 7931 6760**

17. Appendix

Explanation of Symbols

Symbol	Explanation
	*In vitro* diagnostic medical device
	Product Number
	Batch Code
	Manufacturer
	Date of Manufacture
	Contains sufficient for “n” tests/rxns
	Temperature limitation
	Version
	Use-By Date

18. Ordering Information

Products	Packing (reactions)	Order No.
LyteStar™ Enterovirus HFMD Typing RT-PCR Kit 1.0	96	881303
SpinStar™ Viral Nucleic Acid Extraction Kit 1.0	100	811803
MagCore® Viral Nucleic Acid Extraction Kit, High Sensitivity (200µl/400µl), CART CODE 203	96	MVN400-06

NOTES

NOTES

NOTES

NOTES

AstronDX Technologies Sdn Bhd [200901035495 (878612-H)]
Unit 307, Block B, Phileo Damansara 1,
9, Jalan 16/11, 46350 Petaling Jaya,
Selangor, Malaysia

phone +603 7931 6760
fax +603 7931 5352
email info@astrondx.com

www.astrondx.com