MBPCR201 Hi-PCR® Enterovirus Probe PCR Kit

Instructions For Use

Enteroviruses are a genus of the family Picornaviridae and are a large and diverse group of small RNA viruses characterized by a ssRNA molecule of positive sense. To date, over 70 serotypes of Enterovirus have been isolated from man. The human enteroviruses include poliovirus, Coxsackie A and Coxsackie B viruses, enterocytopathic human orphan (ECHO) viruses and other enteroviruses. Enteroviruses infect millions of people worldwide each year, and the infection can result in a wide variety of symptoms depending on the serotype of the enterovirus. Symptoms of infection may include, but are not limited to, mild respiratory illness (common cold), hand, foot and mouth disease, Rubelliform rashes, acute hemorrhagic conjunctivitis, aseptic meningitis, myocarditis, and neonatal infections. Nucleic acid amplification-based assays or Polymerase Chain Reaction (PCR) is an alternative method of Enterovirus diagnosis that allows for sensitive and specific detection of Enterovirus RNA from stool samples. Real-Time PCR technique is considerably simple and fast with respect to the standard PCR technique. This technique has been successfully used for the rapid detection and identification of a variety of infectious pathogens.

NOTE: HiMedia's Hi-PCR® Enterovirus Probe PCR Kit is for *in-vitro* use only.

Intended Use

Recommended for sensitive and specific detection of Enterovirus in clinical samples.

Principle

Real-time polymerase chain reaction, also called quantitative Polymerase Chain Reaction (qPCR) or kinetic Polymerase Chain Reaction, is a laboratory technique based on the principle of PCR. This technique is used to amplify a targeted DNA sequence by use of hydrolysis probes that are short oligonucleotides that have a fluorescent reporter dye attached to the 5' end and a quencher dye to the 3' end. HiMedia's Hi-PCR® Enterovirus Probe PCR Kit is designed to detect the **5'untranslated region (5'UTR) of Enterovirus in FAM channel** with **Internal Control in HEX channel** in a single tube reaction. The kit allows sensitive and specific detection of Enterovirus in a single tube reaction.

Positive control

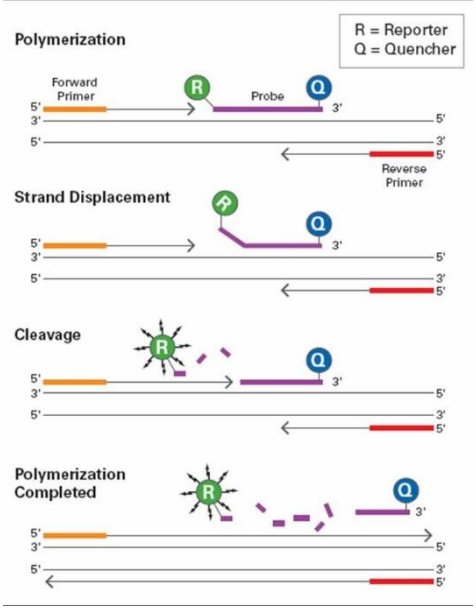
This is a control reaction using a known template (target pathogen). A positive control is usually used to check that the primers have been designed properly and the PCR conditions have been set up correctly.

Internal Control

This is a control sequence which is amplified in the same reaction tube along with the target sequence (target species) but detected with a different primer (i.e. Multiplex PCR). An internal control is often used to detect the failure of amplification in cases where the target sequence is not amplified.

Registered Office
HiMedia Laboratories Pvt Ltd.
Plot No. C-40, Road No. 21Y, MIDC, Wagle Industrial Area,
Thane, (West) 400604, Maharashtra, INDIA.
Customer Care No.: 00-91-22-6116 9797
Tel : 00-91-22-6147 1919, 6903 4800
Fax : 6147 1920
Web : www.himedialabs.com
Email : info@himedialabs.com
mb@himedialabs.com

Diagrammatic representation of preferential binding of probe specific to DNA fragments in Real-time PCR



Polymerization: A fluorescent reporter (R) dye and a quencher (Q) are attached to the 5' and 3' end of the probe respectively

Strand displacement: When the probe is intact, the report dye emission is quenched.

Cleavage: During each extension cycle,the DNA polymerae cleaves the reporter dye from the probe

Polymerization completed: Once separated from the quencher, the reporter dye emits its characteristic fluorescence

While the probe is intact, the proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET). The probes are designed such that they anneal within a DNA region amplified by a specific set of primers. During PCR amplification, these probes will hybridize to the target sequences located in the amplicon i.e. the DNA. As the *Taq* DNA polymerase replicates the template with the bound probe, the 5'- nuclease activity of the polymerase enzyme cleaves the fluorescent probe. The end result in cleavage of the probe is separation of the reporter dye from the quencher dye and increasing the reporter dye signal. As the probe is removed from the target strand, primer extension continues to the end of the template strand. Hence, fluorescence detected in the quantitative PCR thermal cycler is directly proportional to the fluorophore released and the amount of DNA template present in the PCR. Thus, inclusion of the probe does not inhibit the overall PCR process.

Features

- Fast and simple
- Good sensitivity and specific results
- Guaranteed reproducible results
- Rapid detection of all relevant clinical pathogens

Sample Source: Blood / Serum / Water / Virus cultures

Storage and Shelf life

The provided kit has a shelf-life of 12 months when stored between -10° to -20°C. Repeated thawing and freezing of PCR reagents should be avoided, as this may reduce the sensitivity. If the reagents are to be used multiple times, we recommend storing reagents as aliquots to avoid repeated freeze and thaw. Degradation of sample RNA specimens can also reduce the sensitivity of the assay. HiMedia Laboratories does not recommend using the kit after the expiry date stated on pack.

Kit Contents: The provided PCR kit contains:

Components	Product code	Reagents provided for * (µL)	
		25R	**50R**
RT Buffer	DS0221	135	270
10X solution H	DS0222	68	135
M-MuLV Reverse Transcriptase	DS0220	27	54
Enterovirus Primer-Probe Mix	DS0784	27	54
Enterovirus-Internal control Primer Probe Mix	DS3133	27	54
Enterovirus-Internal control DNA	DS3134	27	54
Molecular Biology Grade Water for PCR	ML065	300	600
Enterovirus Positive Control	DS0785	25	50

***** For a 25 µL PCR reaction

Specimen collection and Handling

Follow appropriate techniques for handling specimens; after use, contaminated materials must be sterilized by autoclaving before discarding. Standard precautions as per established guidelines should be followed while handling clinical specimens and items contaminated with blood and other body fluids. Safety guidelines may be referred in individual safety data sheets.

Sample Preparation

Various samples are routinely examined. For extraction and purification of pure RNA for high yield, perform the nucleic acid purification using HiMedia's extraction kits as instructed in the protocol.

Materials needed but not provided

- PCR tubes (Product code: PW1255) or PCR Strips (Product code: PR17) or PCR Plates (Product code: PR2 / PR3 / PR19) & Sealing film (PR18)
- Insta Q Real Time PCR System (Product Code: LA1012 / LA1023 / LA1024 / LA1073 / LA1074)
- Barrier Micropipette Tips (Product Code: LA749 / LA749A / LA751 / LA751A / LA750 / LA750A / LA859 / LA859A)
- Micropipettes
- For blood / serum / viral cultures: HiPurA® Viral RNA Purification Kit (MB615)

General Preparation Instructions

- Before use all PCR components should be completely thawed on ice (4°C).
- Perform the amplification reactions in a clean area, preferably in a biosafety cabinet.
- Use of aerosol barrier pipette tips is recommended to reduce contamination risks from extraneous DNA templates.
- Extract and store positive control sample (if used) separately from all other reagents to avoid contamination and add it to the reaction mix in a separate area.

A. Protocol for PCR Master Mix Preparation

Components	Product code	Volume to be added for 1R (for a 25 µL reaction)
RT Buffer	DS0221	5 µL
10X solution H	DS0222	2.5 µL
M-MuLV Reverse Transcriptase	DS0220	1 µL
Enterovirus Primer-Probe Mix	DS0784	1 µL
Enterovirus-Internal control Primer Probe Mix	DS3133	1 µL
Enterovirus-Internal control DNA	DS3134	1 µL
Molecular Biology Grade Water for PCR	ML065	8.5 µL
Template RNA / Positive Control / Negative Control	-	5 µL
Total volume	-	Upto 25 µL

Centrifuge the tube briefly at 6000 rpm for about 10 seconds. Place the tubes in Real-time PCR machine and set the recommended PCR program (mentioned below). Interpret the data from the amplification plot (observe the Ct values).

B. Recommended PCR program

1. Reverse Transcription : 50°C for 15 minutes
2. Initial denaturation : 95°C for 2 minutes 30 seconds
3. Denaturation : 95°C for 15 seconds
4. Annealing & Extension : 60°C for 30 seconds (Sampling) — No. of cycles: 40
   Channels : FAM/HEX
5. Hold : 4°C for ∞

Quality Control

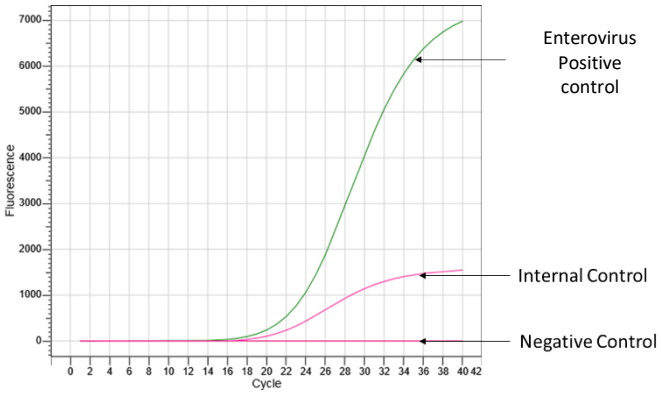
Each lot of HiMedia's Hi-PCR® Enterovirus Probe PCR Kit is assayed for contaminating endonuclease, exonuclease and non-specific DNase activities. Functionally tested in DNA amplification.

Data Analysis

The following conditions should be met for a valid diagnostic test:

Control	Detection channel	
	FAM (Enterovirus)	HEX (Internal Control)
Positive Control	+	+
Negative Control	-	+

Amplification Data



Sr. No.	Sample	C_t value
1.	Enterovirus positive control	22.53
2.	Internal Control	21.90

Image representing amplification plot of Enterovirus RNA with Ct values using HiMedia's Hi-PCR® Enterovirus Probe PCR Kit. The results completely depend upon sample types.

Data Interpretation

Detection Channel		Result Interpretation
FAM (Enterovirus)	HEX (Internal Control)	
≤ 35 Ct	15 – 35 Ct*	Positive for Enterovirus
-	15 – 35 Ct	Negative for Enterovirus
-	-	PCR inhibition or reagent failure. Repeat PCR or repeat extraction from original sample

*The presence or absence of a signal in the HEX channel is not relevant for the validity of the test run due to competition between the test template and Internal Control template.

Warning

Certified for *in vitro* Diagnostic Use (IVD). Not for Medicinal Use.

Precautions

Read the procedure carefully before beginning the protocol. Wear protective gloves/protective clothing/eye protection/face protection. Follow good clinical laboratory practices while handling clinical samples. Standard precautions should be followed as per established guidelines. Safety guidelines may be referred in safety data sheets of the product.

Performance and Evaluation

Each lot of HiMedia's Hi-PCR® Enterovirus Probe PCR Kit is tested against predetermined specifications to ensure consistent product quality.

Analytical sensitivity

Limit of detection (LOD)

The analytical sensitivity or the Limit of Detection (LOD) of Hi-PCR® Enterovirus Probe PCR Kit is defined as the concentration of Enterovirus RNA molecules that can be detected with a positivity rate of ≥ 95%. The analytical sensitivity for Hi-PCR® Enterovirus Probe PCR Kit was conducted using Quantitative Genomic RNA from Enterovirus 68 strain Fermon (VR-1826DQ). The preliminary LoD was determined by testing a 10-fold dilution series in triplicates per concentration, and then confirmed with 20 replicates of the concentration determined to be the detectable LoD. The data revealed that the Hi-PCR® Enterovirus Probe PCR Kit detects 20 copies /µL. Thus, the detectable Limit of Detection (LoD) was determined to be 20 copies/µL. The sensitivity analysis of the Hi-PCR® Enterovirus Probe PCR Kit was carried out on HiMedia's InstaQ 96 series, InstaQ 96 series AG, Biorad's CFX Series and Thermo Fisher's QuantStudio™ 5 Real-Time PCR System.

Analytical Specificity

Inclusivity – In silico

The analytical specificity of the Hi-PCR® Enterovirus Probe PCR Kit was validated through in silico analysis of the Enterovirus primers and probes using NCBI BLAST, along with optimization of stringent PCR conditions. The primers and probes were examined for potential homologies with all sequences available in the NCBI database using multiple sequence alignment tools, ensuring that the kit detects all relevant EV strains.

Analytical Reactivity

The analytical reactivity of the Hi-PCR® Enterovirus Probe PCR Kit was verified through wet lab testing of the oligonucleotides (primers and probes) against commercially available Enterovirus control- ATCC Genomic RNA from Enterovirus 68 strain fermon (VR-1826DQ).

Cross-Reactivity

Wet testing was conducted on genomic or synthetic DNA/RNA of various pathogens (from ATCC) using the InstaQ 96® Plus Real-Time PCR System to assess potential cross-reactivity. None of the pathogens listed in the table exhibited any reactivity with the primers and probes of the Hi-PCR® Enterovirus Probe PCR Kit.

Hepatitis E virus (3258SD)	*Escherichia coli* (10798DQ)
Hepatitis A virus (VR-3257SD)	*Leptospira* (BAA-1198D-5)
Hepatitis B virus (VR-3232SD)	Human adenovirus 1 strain Adenoid 71 (VR-1DQ)
Hepatitis C virus (NIBSC code: 18/184)	*Pseudomonas aeruginosa* (47085DQ)
Rotavirus (VR-2018DQ)	Genomic DNA extracted from *Enterococcus faecalis* (ATCC: 51299)
Cryptosporidium parvum (PRA-67DQ)	Human parainfluenza virus 1 (94DQ)
Giardia intestinalis (30888D)	Human parainfluenza virus 2 (92DQ)
Norovirus (VR-3234SD)	Human parainfluenza virus 3 (93DQ)
Genomic DNA extracted from *Campylobacter jejuni* (BAA-1153)	Rhinovirus (VR-283DQ)
Genomic DNA extracted from *Shigella flexineri* (ATCC: 12022)	Human coronavirus 229E (740DQ)
Legionella pneumophila (33152DQ)	Influenza B Virus (1804DQ)
Chlamydophila pneumoniae (1360DQ)	Respiratory Syncytial Virus (1580DQ)
Mycoplasma pneumoniae (29342DQ)	Rotavirus (VR-2018DQ)

Troubleshooting Guide

Sr. No.	Problem	Possible Cause	Solution
1.	No amplification in test and/or control wells	Degraded samples or poor-quality template	Use freshly extracted, high-quality nucleic acids. Avoid repeated freeze-thaw cycles for stored RNA.
		Missing or incorrect addition of reagents	Verify all reagents were added in the correct volumes and order. Recheck reaction setup steps. Use a master mix to reduce pipetting errors.
		Incorrect thermal cycling conditions	Cross-check the PCR cycling profile with the IFU.
		Incorrect fluorophore/dye setting	Refer the selection of channels table and set the correct fluorophore in the real-time PCR instrument
		Expired or improperly stored reagents	Confirm the expiry date and proper storage. Avoid using reagents that have undergone multiple freeze-thaw cycles
		Instrument malfunction	Verify that the real-time PCR instrument is functioning properly and calibrated.
2.	Variability between replicates	Inconsistent pipetting or error in reaction setup	Prepare a single master mix for all replicates to minimize variation. Vortex thoroughly and aliquot carefully. Use calibrated pipettes and consistent technique.
		Air bubbles in reaction mix	Briefly centrifuge PCR tubes or plate before placing in the instrument to eliminate air bubbles.
		Uneven mixing of reagents	Ensure all reagents are fully thawed and mixed by vortexing before use. Spin down before pipetting.
		Edge effect (thermal variation across plate)	Avoid using outer wells in PCR plates if not temperature-uniform; use a plate seal and consistent plate layout.
3.	Amplification in negative control	Reagent contamination	Replace all critical reagents. Clean workspace, pipettes, and repeat analysis with fresh aliquots. Use filter tips and maintain a unidirectional workflow.
		Cross-contamination or aerosol contamination during reaction setup	Maintain strict unidirectional workflow. Set up reactions in a PCR hood or clean bench. Use aerosol-resistant filter tips for all pipetting steps. Avoid fast, forceful pipetting. Regularly clean work surfaces and equipment with DNA/RNA-decontaminating agents. Minimize opening of positive control tubes and avoid splashing.
		Template contamination in workspace or pipettes	Decontaminate work surfaces, pipettes, and equipment using DNA/RNA decontamination solutions. Perform regular cleaning.
		Improper sealing of PCR plate/tubes	Ensure plates/tubes are properly sealed to prevent cross-well contamination during

			thermal cycling. Use optical-grade seals if required.
4.	No signal with positive control or partial target amplification	Degradation of the positive control material due to improper storage or repeated freeze-thaw cycles	Use a fresh aliquot of positive control. Ensure storage conditions follow IFU and avoid repeated freeze-thaw. Discard expired or compromised controls.
		Incorrect thermal cycling conditions	Cross-verify cycler settings with IFU. Ensure annealing/extension temperature and time are as specified.
		Improper mixing of PC before use	Vortex and briefly spin down the positive control before adding it to the PCR mix. Mix well to ensure homogeneity.
5.	Early Ct value with non-sigmoidal amplification	Instrumental noise, background fluorescence, or non-specific amplification	Visually inspect amplification plots. Disregard flat or atypical (non-sigmoidal) curves that show a sudden rise in fluorescence with very early Ct values (e.g., <6). These may not indicate true amplification. Repeat the assay if necessary. Adjust threshold setting in software within exponential phase. Refer to instrument-specific guidance.
6.	Non-specific amplification of targets in samples and/or negative control	Improper threshold setting in the real-time PCR software	Visually inspect all amplification plots. Do not rely on Ct values alone. Manually adjust the threshold as recommended in the threshold setting table. Refer to the instrument-specific guidelines or user manual for proper threshold adjustment.
		Contamination of reagents or workspace	Use fresh aliquots of reagents. Prepare reactions in a contamination-free environment using separate areas for pre- and post-PCR steps. Use aerosol-resistant filter tips and routinely decontaminate surfaces and pipettes.
7.	No Internal Control (IC) amplification	High target load may suppress IC amplification	If valid Ct for target is observed, it is still considered a valid result. Test repetition with freshly extracted sample is recommended.
		Presence of PCR inhibitor	Dilute the extracted nucleic acid in molecular biology grade water and repeat the PCR assay.
		Sample inhibition, extraction failure, or expired reagents	Repeat extraction or test with fresh sample. Check IC amplification to rule out PCR inhibition. Use validated extraction method and fresh reagents.
		Instrument malfunction	Verify that the real-time PCR instrument is functioning properly and calibrated.
8.	High Ct values in positive samples (late amplification)	Low pathogen load or suboptimal sample	Results near the cut-off should be interpreted with caution. Repeat test using freshly extracted nucleic acid. Confirm with orthogonal test if clinically significant.
9.	Signal in only one replicate (of	Pipetting error or borderline positivity	Repeat the test. If consistent upon retesting, interpret cautiously in context of clinical findings. Borderline cases (close to Ct cutoff)

	duplicate or triplicate reactions)		may require repeat sampling or orthogonal testing (e.g., NGS). Use calibrated pipettes and proper technique.

Safety Information

HiMedia's Hi-PCR® Enterovirus Probe PCR Kit is for laboratory use only, not for drug, household or other uses. Take appropriate laboratory safety measures and wear gloves when handling.

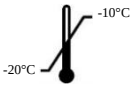
Disposal

User must ensure safe disposal by autoclaving and/or incineration of used or unusable preparations of this product. Follow established laboratory procedures in disposing of infectious materials and material that comes into contact with clinical sample must be decontaminated and disposed of in accordance with current laboratory techniques.

Technical Assistance

At HiMedia, we pride ourselves on the quality and availability of our technical support. For any kind of technical assistance, mail at mb@himedialabs.com.

Symbols:

	Manufacturer		Do not use if package is damaged
	Authorized representative in the European Community		Temperature limit
	Date of manufacture (YYYY-MM)		Consult instructions for use
	Use-by date (YYYY-MM)		In vitro diagnostic medical device
	Batch code		CE marking of conformity
	Catalogue number		

Authorized representative (AR) Address:

	**AR Experts B.V.** Boeingavenue 209, 1119 PD, Schiphol-Rijk, The Netherlands

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HiMedia Laboratories Pvt. Ltd. Reg.office : Plot No. C-40, Road No. 21Y, MIDC, Wagle Industrial Estate, Thane, (West) 400604, Maharashtra, INDIA. Customer Care No.: 00-91-22-6116 9797 Tel: 00-91-22-6147 1919, 6903 4800 Email:mb@himedialabs.com Website: www.himedialabs.com