

MBPCR252

Hi-PCR[®] Protozoan Parasite Multiplex Probe PCR Kit

Description:

Haemoparasitic infections pose serious problems for the health and management of domestic cattle in tropical and subtropical regions of the world. Among these diseases, babesiosis, tropical theileriosis, anaplasmosis and trypanosomosis caused by *Babesia bigemina*, *Theileria annulata*, *Anaplasma marginale* and *Trypanosoma evansi* respectively, are the most economically important vector borne diseases encountered by cattle. These diseases are responsible for high level of morbidity and mortality in susceptible population and thereby causing huge economic losses to the dairy industry. Clinical signs associated with above diseases are overlapping and may include pyrexia, anorexia, anemia, icterus, hemoglobinuria, weight loss, reduced milk production. Co-infection of these parasites in animals is a common feature in most parts of the world. Hence, timely and specific diagnosis of the causative agent before implementation of appropriate treatment and related control measures is very important. Polymerase Chain Reaction (PCR) technology will help to provide a quick, accurate and highly specific diagnosis of economically important diseases of livestock. Among the different PCR protocols for pathogen detection, multiplex PCR assay offers an advantage of detecting multiple pathogens in a single reaction and has been employed to detect mixed infections of haemoparasites.

NOTE: Hi-PCR Protozoan Parasite Multiplex Probe PCR Kit is for *in vitro* use only.

Intended Use:

Hi-PCR[®] Protozoan Parasite Multiplex Probe PCR Kit is intended for use by qualified laboratory personnel trained in Real-Time PCR. The kit is recommended for sensitive and specific detection of *Anaplasma*, *Babesia*, *Theileria* and *Trypanosoma* in a single tube assay.

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. Hi-PCR[®] Protozoan Parasite Multiplex Probe PCR Kit is designed to detect *Theileria*, *Anaplasma*, *Trypanosoma* and *Babesia* in **FAM, Texas Red, Cy5 and Cy5.5 channel, respectively, and the Internal Control in JOE channel** in a single tube reaction. The kit allows sensitive and specific detection of all targeted Protozoan Parasites.

Negative Control

A negative control is needed to ensure that the reagents, equipment, and environment used in the assay is not contaminated. In this reaction, nuclease free water is used as the template.

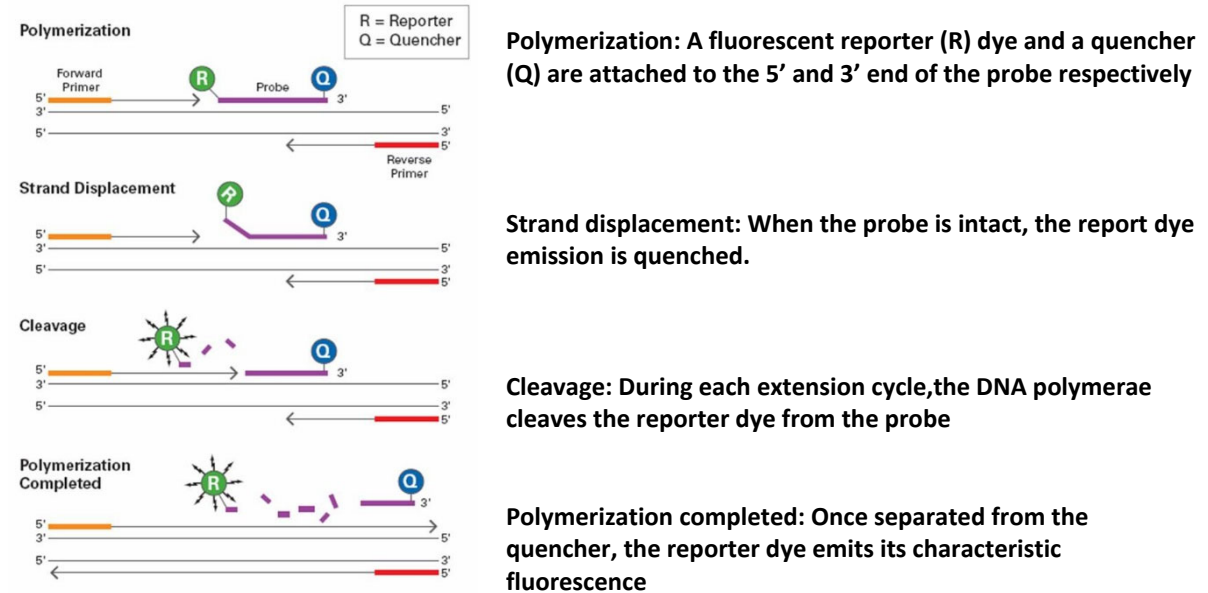
Positive control

This is a control reaction using a known template (target pathogen). A positive control is usually used to ensure proper and intended functioning of all the reagents and is recommended to be used in every run to assess optimal performance.

Internal Control

This is a control sequence which is amplified in the same reaction tube along with the target sequence 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.

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



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 cycle 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

- Simultaneous detection of *Anaplasma*, *Babesia*, *Theileria* and *Trypanosoma* in a single assay
- Fast and reliable results
- Includes all reagents & controls for validity of the test
- Good sensitivity and specific results

Sample Source: EDTA Blood samples, Blood sample on InstaDNA™ Card

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.

Storage and Shelf life

The provided kit has a shelf-life of 12 months when stored between -10°C 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 DNA 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 (reactions)* (µL)	
		25R	50R
Protozoan Parasite Master mix	DS1976	162	324
Protozoan Parasite Primer-Probe Mix	DS2002	81	162
Protozoan Parasite Positive Control	DS2003	27	54
Molecular Biology Grade Water for PCR	ML065	297	594

*For a 24 µL PCR reaction

Materials needed but not provided

All materials are available through www.himedialabs.com

Product name	Product Code
Real-Time PCR Instrument and equipment	
Insta Q96® Real time PCR System, 96 well block, 5 channels	LA1012
Insta Q96® Plus Real time PCR System, 96 well block, 5 channels	LA1073
Insta Q96® - 6.0 Real time PCR System, 96 well block, 6 channels	LA1074
TabSpin™ Microcentrifuge	LA1089/LA1090
Automated nucleic acid extraction system and materials	
Insta NX® Instrument - fully automated nucleic acid purification system utilizing the Innovative Super -S membrane column method	LA1056
Kits and Reagents	
HiPurA® Pre-filled Clinical Multi-purpose Magnetic Nucleic Acid Purification kit (Cartridges)	MB583PC16200
HiPurA® Pre-filled Clinical Multi-purpose Magnetic Nucleic Acid Purification kit (Plates)	MB583MPF16200
Tubes, plates and other consumables	
Varivol II Micropipette-10 (Capacity: 0.5 to 10 µl)	LA611
Varivol II Micropipette-100 (Capacity: 10 to 100 µl)	LA615
Varivol II Micropipette-1000 (Capacity: 200 to 1000 µl)	LA614
Barrier Tips, Maximum capacity 10 µl	LA749/LA749A
Barrier Tips, Maximum capacity 200 µl	LA751/LA751A
Barrier Tips, Maximum capacity 1000 µl	LA859/LA859A
8-strip tubes & optically clear flat caps for PCR	PR17, PR22, PR23
PCR Tubes, 0.2 mL; PCR Plates	PW1255/ PR2/PR3/PR19
Optical Sealing film	PR18

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.

Protocol for PCR Master Mix Preparation (For one reaction)

1. In the “Master mix Preparation” area, thaw all components from the kit on ice, mix by inverting the tubes and centrifuge the reagents for several seconds. Keep on ice for later use.
2. Based on the number of specimens to be tested (N), including the PC and NC, calculate the volume of the components to be added as N* volume of 1X

Components	Volume (µL) to be added for 1R (for 24 µL reaction)
Protozoan Parasite Master mix	6.0
Protozoan Parasite Primer-Probe Mix	3.0
Molecular Biology Grade Water for PCR	10.0
Protozoan Parasite Positive Control/Template/Water	5.0
Total volume	24.0

3. Use 0.5 mL Nuclease free centrifuge tube(s) for the preparation of the reaction system. After all the reagents are added, mix them thoroughly and centrifuge for several seconds.
4. Load 19 µL of master mix into the 0.1/0.2 mL PCR reaction tube/plate/strips, compatible to the instrument to be used. In Negative control tube, add 19 µL of master mixture and 5 µL Nuclease free water.
5. In the “Nucleic acid handling” area, add 5 µL Positive Control and extracted test DNA into respective tubes in the plate/strip.
6. Tightly cap the tubes/strips or seal the plate using an optically clear adhesive film.
7. Briefly, spin the strips/tubes to settle the reagent to the bottom of the tube.
8. Place the plate/strips/tubes in Real-time PCR machine and set the PCR program.

Recommended PCR program

- | | | |
|-------------------------|---|---------------------|
| 1. Initial denaturation | : 95°C for 10 minutes | } No. of cycles: 45 |
| 2. Denaturation | : 95°C for 15 seconds | |
| 3. Annealing | : 55°C for 20 seconds (Plate Read) | |
| Channel | : FAM/Texas red/Cy5/Cy5.5/JOE | |

Selection of channels:

Target	Channels
Theileria	FAM
Anaplasma	Texas Red/ROX
Trypanosoma	Cy5
Babesia	Cy5.5
IC	JOE/HEX/VIC

Please select ‘Passive reference dye’ and Quencher as ‘None’ wherever applicable

Threshold value set up

For the Hi-PCR® Protozoan Parasite Multiplex Probe PCR Kit, the threshold value for some popular thermal cyclers are as follows:

Sr. No.	Real-Time PCR instrument	Threshold set value
1.	HiMedia Insta Q96	FAM, Cy5.5: 200 TexRed, Cy5, Joe: 400
2.	Bio-Rad CFX-96	For all fluorophores: 100

The threshold range value varies between different instruments depending upon the age, model and the calibration. Please contact our technical team for any queries.

Data Analysis

The following conditions should be met for a valid test:

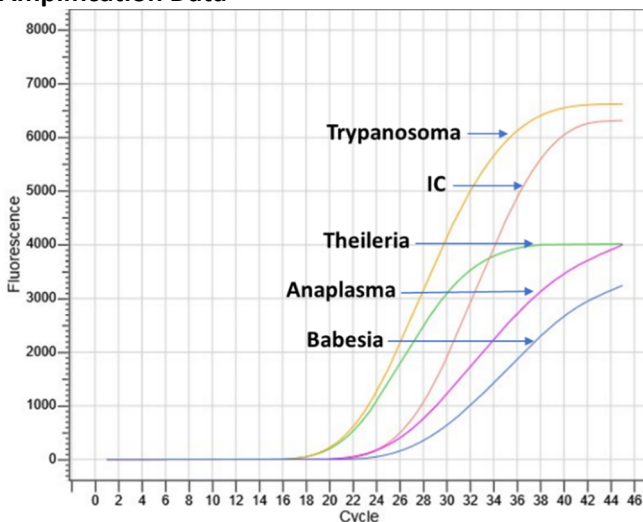
Control	Target				
	Theileria (FAM)	Anaplasma (Texas Red)	Trypanosoma (Cy5)	Babesia (Cy5.5)	Internal Control (JOE)
Positive Control (PC)	+	+	+	+	+
Negative Control (NC)	-	-	-	-	+

Data Interpretation

Detection Channel					Result Interpretation
Theileria (FAM)	Anaplasma (Texas Red)	Trypanosoma (Cy5)	Babesia (Cy5.5)	Internal Control (JOE)	
+	+	+	+	+	Positive for all Protozoan Parasites
+	-	-	-	+	Positive for Theileria
-	+	-	-	+	Positive for Anaplasma
-	-	+	-	+	Positive for Trypanosoma
-	-	-	+	+	Positive for Babesia
-	-	-	-	+	Negative for all Protozoan Parasites
-	-	-	-	-	PCR inhibition or reagent failure. Repeat PCR or repeat extraction from original sample

	Ct value	Result	Ct value	Result
Theileria	≤ 39	(+) Detected	> 39 or N/A	(-) Not Detected
Anaplasma	≤ 39	(+) Detected	> 39 or N/A	(-) Not Detected
Trypanosoma	≤ 38	(+) Detected	> 38 or N/A	(-) Not Detected
Babesia	≤ 39	(+) Detected	> 39 or N/A	(-) Not Detected

Amplification Data



Sr. No	Sample	Ct value
1	Positive Control (Theileria)	20.1
2	Positive Control (Anaplasma)	26.04
3	Positive Control (Trypanosoma)	21.16
4	Positive Control (Babesia)	26.61
5	Internal Control	25.51

Representative image showing amplification plot of the specific gene targets and the Internal Control with Ct values in the positive control sample, using Hi-PCR® Protozoan Parasite Multiplex Probe PCR Kit.

Performance evaluation

Analytical Sensitivity - Limit of Detection (LoD)

Sensitivity assay for the Hi-PCR® Protozoan Parasite Multiplex Probe PCR Kit was conducted using synthetic DNA of target genes of *Theileria*, *Anaplasma*, *Trypanosoma* and *Babesia* on InstaQ96® Real Time PCR systems. The detectable limit of the Hi-PCR® Protozoan Parasite Multiplex Probe PCR Kit on Real Time instrument was determined to be **5 copies/μL** for *Theileria*, **100 copies/μL** for *Anaplasma*, **10 copies/ μL** for *Trypanosoma* and **25 copies/ μL** for *Babesia* gene targets.

Analytical Specificity

Inclusivity

In silico analysis for the assessment of inclusivity for the Hi-PCR® Protozoan Parasite Multiplex Probe PCR Kit was conducted by mapping the primers and probes against all the available Protozoan Parasite sequences in GenBank. The Hi-PCR® Protozoan Parasite Multiplex Probe PCR Kit targets 100% of the known *Anaplasma*, *Babesia*, *Theileria* and *Trypanosoma* strains.

Exclusivity / Cross-reactivity analysis

In silico analysis was performed using NCBI nucleotide and Primer BLAST. The primers used were analyzed against other bacteria, yeast and viruses and determined to be 100% specific for the detection of the protozoan parasite targets only.

Quality Control

Each lot of HiMedia's Hi-PCR® Protozoan Parasite Multiplex Probe PCR Kit is functionally tested in DNA amplification assays.

Warning and 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.

Limitations

Although rare, mutations within the highly conserved regions of the target genes covered by the kit's primers and/or probe may result in under quantitation or failure to detect the presence of the target regions in these cases. Validity and performance of the assay design are revised at regular intervals.

Performance and Evaluation

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

Troubleshooting Guide

Sr. No.	Problem	Cause	Solution
1.	No amplification in samples	Degraded samples	1. Check the integrity of DNA using agarose gel electrophoresis. 2. Use freshly prepared DNA to ensure the availability of intact template sequence for efficient amplification.

		Error in protocol setup	Verify that the correct reagent volumes, dilutions and storage conditions have been used.
2.	Variability between replicates	Error in reaction set-up	Prepare a large volume master mix, vortex thoroughly and aliquot into reaction tubes.
		Air bubbles in reaction mix	Briefly centrifuge reaction samples/plate prior to running on a real-time PCR instrument.
		Pipetting error	C _t values of replicates can show increased variation due to poor laboratory technique or imprecise pipettes.
3.	Amplification of targets other than IC in negative control	Reagents contaminated	1. Replace all critical solutions. 2. Repeat the analysis of all tests with fresh aliquots of critical reagents.
4.	No signal with positive control	Incorrect programming of the temperature profile of the thermocycler	Compare the temperature profile to the manual.
		Degradation of Positive control due to improper storage condition	Check the storage condition and number of freeze thaw cycles for the tube

Safety Information

HiMedia's Hi-PCR® Protozoan Parasite Multiplex 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
	Batch code		Temperature limit
	Date of manufacture (YYYY-MM)		Consult instructions for use
	Use-by date (YYYY-MM)		Catalogue number

Identification No.: PIMBPCR252

Rev.No.:03

Date of Issue: 2025-02

Disclaimer :

User must ensure suitability of the product(s) in their application prior to use. Products conform solely to the information contained in this and other related HiMedia™ publications. The information contained in this publication is based on our research and development work and is to the best of our knowledge true and accurate. HiMedia™ Laboratories Pvt Ltd reserves the right to make changes to specifications and information related to the products at any time. Products are not intended for human or animal or therapeutic use but for laboratory, diagnostic, research or further manufacturing use only, unless otherwise specified. Statements contained herein should not be considered as a warranty of any kind, expressed or implied, and no liability is accepted for infringement of any patents.

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