MBPCR165 Hi-PCR® Theileria Probe PCR Kit

Description

Theileriosis is a disease caused by a species of *Theileria*, a blood-borne parasite, that mainly affects cattle and is primarily transmitted by ticks. It is a disease of mammals and birds caused by a protozoal pathogen which resides within the lymphocytes and macrophages. It causes anaemia in cattle and can sometimes be fatal. Cows during calving and young calves (2-3 months) are at most risk from this infection. Cattle are at risk of infection when moved to areas where infected ticks are present. Likewise, if an infected animal is transported, it can spread the infection to ticks in the new location, in turn spreading the disease to uninfected animals. The diagnosis of theileriosis is usually carried out by blood smear staining technique, which is not sufficiently sensitive to detect the piroplasms in the carrier animals. The advent of molecular diagnostic tests like polymerase chain reaction (PCR) has paved the way to efficient diagnosis than the conventional techniques. The Hi-PCR® Theileria Probe PCR Kit is designed for specific and sensitive detection of *Theileria* spp. responsible for theileriosis.

NOTE: Hi-PCR® Theileria Probe PCR Kit is for *in-vitro* use only.

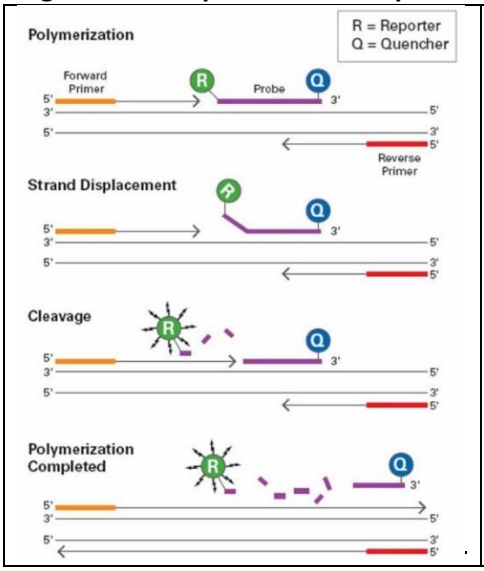
Intended Use

The Hi-PCR® Theileria Probe PCR Kit is intended for use by qualified clinical laboratory personnel trained in the techniques of PCR and *in vitro* diagnostic procedures. The kit is recommended for sensitive and specific detection of *Theileria* spp. in clinical samples.

Principle

Hi-PCR® Theileria Probe PCR Kit, a multiplex Probe PCR Kit, is based on the principle of real-time PCR. The technique is designed to amplify targeted nucleic acid sequences using hydrolysis probes that are short oligonucleotides with a fluorescent reporter dye attached to the 5' end and a quencher dye to the 3' end. The assay includes primers and probes specific for **18S ribosomal RNA gene** of Theileria spp., with probes labeled with FAM fluorophore. Additionally, the kit incorporates an exogenous internal control (IC) amplification system (probe labelled with JOE fluorophore) to ensure efficient PCR amplification.

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 polymerase cleaves the reporter dye from the probe

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

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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.

Controls

Positive Control

A Positive control (PC) is a control reaction which contain the target DNA sequence that the PCR is designed to amplify. It is usually used to ensure proper and intended functioning of all the PCR reagents and is recommended to be used in every run to assess optimal assay performance.

Negative Control

A Negative Control (NC) is essential to verify that the reagents, equipment, and environment used in the assay are free from contamination with target nucleic acid. In this control reaction, nuclease-free water is used as the template. It is recommended to include at least one negative control reaction per run to ensure the reliability of the results.

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-probe set. An internal control is often used to detect the failure of amplification in cases where the target sequence is not amplified, identify potential PCR inhibition, and ensuring the integrity of the reagents used in the assay.

Features

- Fast and reliable results within 90 minutes
- High sensitivity and specificity for accurate detection of *Theileria* spp.
- Includes all necessary reagents and controls to ensure test validity
- Compatible with any 2-channel, 4-channel, 5-channel and 6-channel qPCR cyclers
- Wet-lab assays validated on the Bio-Rad CFX Opus 96, Applied Biosystems QuantStudio 5, Insta Q96® AG and Insta Q96® Plus Real Time PCR Systems

Types of Specimen: Blood, Tissue samples, Parasitic culture

Specimen collection and Handling

When handling specimens for *Theileria* detection, it is essential to follow appropriate procedures to prevent contamination and ensure safe handling. After use, all contaminated materials must be sterilized by autoclaving before disposal. Standard precautions as per established guidelines should be followed while handling clinical specimens and items contaminated with 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 and -20°C. Repeated thawing and freezing of PCR reagents should be avoided, not more than 5 freeze-thaw cycles, 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. Exposure to light, heat or humidity may also affect the shelf life of certain kit components and should be avoided. Degradation of specimen/ extracted RNA can also hamper 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
Theileria Probe PCR Master mix	DS2443	108	216
Thelieria spps. Primer-Probe Mix	DS0551B	54	108
Thelieria Probe Positive Control	DS2509	40	80
Molecular Biology Grade Water for PCR	ML065	187	374

***** For a 20 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®AG Real time PCR System, 96 well block, 5 channels	MBLA027
Insta Q96®AG 6.0 Real time PCR System, 96 well block, 6 channels	MBLA028
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
Insta Q96® Real time PCR System, 96 well block, 5 channels	LA1012
Insta Q48® M4 Real time PCR System, 96 well block, 4 channels	LA1023
Insta Q48® M2 Real time PCR System, 96 well block, 2 channels	LA1024
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
Insta NX® Mag16, Insta NX® Mag16^{Plus}	LA1118, MBLA018
Insta NX® Mag32, Insta NX® Mag32^{Plus}	LA1096, MBLA019
Insta NX® Mag96	LA1097
Extraction Kits	
HiPurA® Pre-filled Cartridges for Viral Nucleic Acid Purification	MB582PC16
HiPurA® Pre-filled Plates for Viral Nucleic Acid Purification	MB582MPF16
HiPurA® Pre-filled Plates for Viral Nucleic Acid Purification [For Insta NX® Mag32]	MB582MPF-32
HiPurA® Prefilled Plates for Viral Nucleic Acid Purification [For Insta NX® Mag96]	MB582MPF-96
HiPurA® Viral DNA/RNA Purification Kit	MB582
HiPurA® Multi-Sample DNA Purification Kit	MB554
Tubes, plates and other consumables	
Varivol II Micropipettes (Capacity: 0.5 to 10 µL/10 to 100 µL/200 to 1000 µL)	LA611/LA614/LA615
µPet Autoclavable Micropipettes (Capacity: 0.5 - 10 µL/10 - 100 µL/20 - 200 µL/100 - 1000 µL)	LA955/LA958/LA959/LA960
Q4Pet Autoclavable Micropipette (Capacity: 0.5 to 10 µL/10 to 100 µL/100 - 1000 µL)	MBLA009/MBLA011/MBLA008
Barrier Tips, Maximum capacity 10 µL	LA749A
Barrier Tips, Maximum capacity 200 µL	LA751A
Barrier Tips, Maximum capacity 1000 µL	LA859A
8-strip tubes & optically clear flat caps for PCR	PR17, PR22, PR23
PCR Tubes, 0.1mL, 0.2 mL; PCR Plates	PW1255/PR2/PR3/PR19
Optical Sealing film	PR18

Kit compatibility with Real-Time PCR Systems

Hi-PCR® Theileria Probe PCR Kit contains fluorophores that are compatible to the following PCR systems:

Real-Time PCR system	Company	Dye 1	Dye 2
Insta Q96®AG/ Insta Q96®AG 6.0/Insta Q96® - 6.0/Insta Q96® Plus/ Insta Q48® M4	HiMedia Laboratories Pvt. Ltd.	FAM	JOE
BioRad CFX Opus 96/CFX96 Touch/ CFX384 Touch	Bio-Rad Laboratories, Inc.	FAM	JOE/VIC/HEX
QuantStudio™ 5 / Quant Studio™ 6 and 7 Flex Real-Time PCR Systems / QuantStudio™ Dx	Applied Biosystems	FAM	JOE/VIC/HEX
ABI® Prism SDS 7500	Applied Biosystems	FAM	JOE/VIC/HEX
QIAquant 96 & 384 5plex	QIAGEN	FAM	JOE/HEX
Rotor-Gene®6000 & Q	QIAGEN	Green	Yellow
LightCycler® 96	Roche	FAM	JOE/VIC/HEX
LightCycler® 480	Roche	FAM	JOE/VIC/HEX
qTOWER^3	Analytik Jena	FAM	JOE/VIC/HEX

Note: Ensure that the Real-Time PCR system is calibrated for dyes mentioned above and maintained according to the manufactureŕs instructions and recommendations

Warning and Precautions

Not for Medicinal Use. 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.

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 nucleic acid 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 Reaction Mix Preparation

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 5 seconds. Keep on ice for later use.
2. Based on the total number of specimens (including PC and NC) to be tested (N), calculate the volume of the components to be added as **N X volume of "1R"**
3. Use 1.5 mL Nuclease free centrifuge tube(s) for the preparation of the PCR reaction mix. Refer the following table. After all the reagents are added, mix them thoroughly and centrifuge for 5 seconds.

Components	Product code	Volume for "1R" (one reaction)
Preparation of PCR Reaction Mix		
Theileria Probe PCR Master mix	DS2443	4.0 µL
Thelieria spps. Primer-Probe Mix	DS0551B	2.0 µL
Molecular Biology Grade Water for PCR	ML065	6.0 µL
Total PCR Reaction Mix	-	**12.0 µL**
Template addition		
Template (Extracted DNA)		8.0 µL
Total reaction volume	**-**	**20.0 µL**

4. Aliquot **12 µL** of **Total PCR Reaction Mix** into 0.1/0.2mL PCR tube/plate/strips, compatible to the PCR instrument to be used.
5. In the "Nucleic acid handling" area, add **8 µL** of extracted nucleic acid of test specimen into the plate/strip to respective wells.
6. For positive and negative control, template nucleic acid is replaced by Positive control mix and nuclease free water respectively. Refer the following table.

Set up of **Positive controls (PC)** for the PCR run		
Components	**Product code**	**Volume for "1R" (one reaction)**
Total PCR Reaction Mix	-	12.0 µL
Thelieria Probe Positive Control	DS2509	8.0 µL
Total reaction volume	**-**	**20.0 µL**

Set up of **Negative controls (NC)** for the PCR run		
Components	**Product code**	**Volume for "1R" (one reaction)**
Total PCR Reaction Mix	-	12.0 µL
Molecular Biology Grade water for PCR	ML065	8.0 µL
Total reaction volume	**-**	**20.0 µL**

7. Tightly cap the tubes/strips or seal the plate using an optically clear adhesive film.
8. Centrifuge the tube briefly at 6000 rpm for about 10 seconds.
9. 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).

Recommended PCR program

Sr. No	Step	Temperature	Time	Sampling	No. of cycles
1.	Initial denaturation	94°C	5 minutes	---	1
2.	Denaturation	94°C	30 seconds	---	45
3.	Annealing & Extension	59°C	40 seconds	YES	

[Note: Instruments not calibrated for JOE, refer section 'Kit compatibility with Real-Time PCR Systems' for dye selection]

Selection of channels:

Target	**Channels**	**Quencher**
Theileria spp.	FAM	None
Internal Control	JOE/ VIC/ HEX	None

Please select 'Passive reference dye' as 'None' wherever applicable

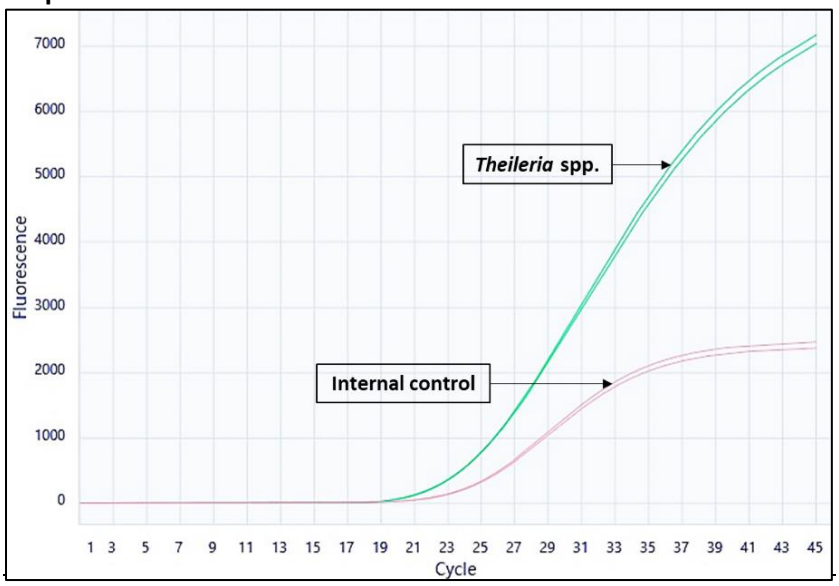
Threshold value set up

For the Hi-PCR® Theileria Probe PCR Kit, the threshold values to be set for some popular thermal cyclers are as follows:

Sr. No.	**Real-Time PCR instrument**	**Threshold range**
1.	HiMedia Insta Q96® plus	200
2.	Himedia Insta Q96®AG	300
3.	Applied Biosystems QuantStudio 5	20,000
4.	Bio-Rad CFX-96	100

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

Amplification Data



Sr. No.	Sample	C_t value
1.	Theileria spp.	22.64
2.	Internal Control	24.45

Image representing probe based Real-Time amplification of Theileria positive control (Ct values provided in table are for representation). run on Insta Q96® AG thermal cycler.

Data Analysis

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

Control	Detection channel	
	FAM (*Theileria* spp.)	JOE (Internal Control)
Positive Control	+	+
Negative Control	-	+

Target	Ct value	Result/Interpretation
***Theileria* spp. (FAM)**	≤ 40	Detected (+)

Data Interpretation

Detection Channel		Result Interpretation
FAM (*Theileria*)	JOE (Internal Control)	
+	+/-*	Positive for *Theileria* spp.
-	+	Negative for *Theileria* spp.
-	-	Inconclusive test**

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

** When an inconclusive result is obtained, repeat PCR or re-test the extracted template or re-extract the specimen and test the newly extracted template.

Note: All negative findings must be correlated with clinical observations. Negative results do not exclude infections because of other variants of these viruses.

Performance Evaluation

Analytical Sensitivity - Limit of Detection (LoD)

The Limit of Detection (LoD) is defined as the concentration (copies per µl of the eluate) of target molecule that can be detected at 95% or greater probability according to CLSI EP17-A2. The LoD assay of the Hi-PCR® Theileria Probe PCR Kit was performed using 20 replicates each on Biorad CFX Opus 96,

Applied Biosystems QuantStudio 5, Insta Q96® AG and Insta Q96® Plus Real Time PCR Systems using synthetic DNA of 18S gene. The detectable limit of the Hi-PCR® Theileria Probe PCR Kit was determined to be **10 copies/µL**.

Analytical Specificity

Inclusivity

The ability of the Hi-PCR® Theileria Probe PCR Kit to detect a wide range of related target organisms has been assessed in the inclusivity parameter by two ways (i) *in silico* analysis of the oligonucleotides (primers and probes) and (ii) wet lab testing using nucleic acids of related target organisms. The oligonucleotide sequences of all the targets were checked by sequence comparison against all the relevant sequences of Theileria in the GenBank database. The primers and probes showed significant hits for various isolates and clones of *Theileria annulata, Theileria parva, Theileria orientalis, Theileria ovis, Theileria buffeli, Theileria equi, Theileria cervi, Theileria capreoli, Theileria sinensis, Theileria velifera, Theileria lestoquardi* and *Theileria luwenshuni.*

Exclusivity / Cross-Reactivity Analysis

The ability of the Hi-PCR® Theileria Probe PCR Kit to distinguish the target organisms from similar but genetically distinct non-target organisms has been assessed by (i) *in silico* analysis of the oligonucleotides (primers and probes) and (ii) wet lab testing using nucleic acids of non-related target organisms.

Wet lab testing of the Hi-PCR® Theileria Probe PCR Kit for potential cross-reactivity was performed using DNA/RNA from various pathogens available in the laboratory, on InstaQ 96 system. None of the pathogens listed in the table below exhibited any reactivity with the primers and probes of the Hi-PCR® Theileria Probe PCR Kit.

Quantitative Genomic DNA from Staphylococcus aureus subsp. Aureus (ATCC: 43300DQ)	Quantitative Genomic RNA from Enterovirus 68 strain Fermon (ATCC: 1826DQ)
Quantitative Genomic DNA from Streptococcus pyogenes strain Bruno (ATCC: 19615DQ)	Quantitative Genomic DNA from Mycoplasma pneumoniae strain M129-B7 (ATCC: 29342DQ)
Quantitative Genomic DNA from Cryptosporidium parvum (ATCC: 67DQ)	Quantitative Genomic RNA from Influenza A virus (H3N2) strain A/Wisconsin/15/2009 (ATCC: 1882DQ)
Quantitative Genomic DNA from Pseudomonas aeruginosa strain PAO1-LAC (ATCC: 47085DQ)	Influenza B virus (ATCC: VR-1804DQ)
Quantitative Genomic DNA from Legionella pneumophila subsp. Pneumophila (ATCC: 33152DQ)	Quantitative Genomic DNA from Aspergillus flavus strain SN 3 (ATCC: 9643DQ)
Salmonella enterica subsp. enterica serovar Typhimurium strain (ATCC: 14028)	Aspergillus fumigatus (ATCC: 204305)
Genomic DNA from Giardia intestinalis strain Portland-1 (ATCC: 30888D)	Quantitative genomic DNA from Candida albicans strain SC5314 (ATCC: 2876DQ)
Quantitative Synthetic DNA from Plasmodium malariae (ATCC: 3001SD)	Quantitative Genomic DNA from Plasmodium falciparum strain 3D7 (ATCC: 405DQ)
Quantitative Synthetic Plasmodium vivax DNA (ATCC: 3004SD)	Quantitative Genomic DNA from Escherichia coli (ATCC: 10798DQ)
Quantitative Genomic DNA from Bordetella pertussis (ATCC: 9797DQ)	Genomic DNA from Leptospira interrogans serovar Copenhageni strain Fiocruz L1-130 (ATCC: 1198D-5)

Evaluation

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

Quality Control

Each lot of Hi-PCR® Theileria Probe PCR Kit has been functionally tested in amplification assay.

General Precautions

- Strict compliance with the Instructions for Use is required for optimal results and the use of the kit is limited to staff qualified clinical laboratory personnel trained in the techniques of real-time PCR and in vitro diagnostic procedures.
- Appropriate specimen collection, transport, storage and processing procedures are required for the optimal performance of this test.
- This assay must not be performed on the specimen directly. RNA extraction should be performed using appropriate nucleic acid extraction method.
- Presence of PCR inhibitors and other interferences may lead to false negative or invalid results.

Limitations

Although rare, mutations within the highly conserved regions of the targets 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.

Troubleshooting Guide

Sr. No.	Problem	Cause	Solution
1.	No amplification	Degraded samples	Check the integrity of nucleic acid using agarose gel electrophoresis.
			Use freshly prepared RNA to ensure the availability of intact template sequence for efficient amplification.
		Error in protocol setup	Check whether all components are added in correct volume as per the manual.
		Inappropriate storage conditions	Store the reagents at recommended temperature for its optimal performance. Check expiry of the reagents and use new lot of reagents if necessary.
		Incorrect PCR programming	Ensure selection of appropriate fluorescence channel as detailed in the manual. Compare the PCR program to the manual.
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. Use calibrated pipettes. Repeat the run.
3.	Amplification of pathogen targets in Negative control	Cross contamination during handling	Replace all critical solutions. Repeat the analysis of all tests with fresh aliquots of critical reagents. Follow good laboratory practices to avoid contamination issues.

Safety Information

Hi-PCR® Theileria 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.

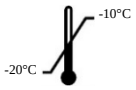
Disposal

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

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