

Cryptosporidium Real Time PCR Kit User Manual

REF MBS598073 - Instrument I, II



For use with LightCycler1.0/2.0 Instrument



1. Intended Use

Cryptosporidium real time PCR kit is used for the detection of Cryptosporidium in stool, vomit or water samples by using real time PCR systems.

2. Principle of Real-Time PCR

The principle of the real-time detection is based on the fluorogenic 5' nuclease assay. During the PCR reaction, the DNA polymerase cleaves the probe at the 5' end and separates the reporter dye from the quencher dye only when the probe hybridizes to the target DNA. This cleavage results in the fluorescent signal generated by the cleaved reporter dye, which is monitored real-time by the PCR detection system. The PCR cycle at which an increase in the fluorescence signal is detected initially (Ct) is proportional to the amount of the specific PCR product. Monitoring the fluorescence intensities during Real Time allows the detection of the accumulating product without having to re-open the reaction tube after the amplification.

3. Product Description

Cryptosporidium is a coccidian protozoan parasite that has gained much attention in the last 20 years as a clinically important human pathogen. Electron microscopic examination of the intestinal mucosa led to the discovery that Cryptosporidium parvum was the infectious species in humans. In the early 1980s, the strong association between cases of cryptosporidiosis and immunodeficient individuals (such as those with AIDS-acquired immunodeficiency syndrome) brought Cryptosporidium to the forefront as a ubiquitous human pathogen. Little is known about the pathogenesis of the parasite, and no safe and effective treatment has been successfully developed to combat cryptosporidiosis. Unlike other intestinal pathogens, Cryptosporidium can infect several different hosts, can survive most environments for long periods of time due to its "hardy cyst", and inhabits all climates and locales.

Cryptosporidium real time PCR kit contains a specific ready-to-use system for the detection of the Cryptosporidium through polymerase chain reaction in the real-time PCR system. The master contains reagents and enzymes for the specific amplification of the Cryptosporidium DNA. Fluorescence is emitted and measured by the real time systems' optical unit during the PCR. The detection of amplified Cryptosporidium DNA fragment is performed in fluorimeter channel 530nm with the fluorescent quencher BHQ1. In addition, the kit contains a system to identify possible PCR inhibition by measuring the 560nm fluorescence of the internal control (IC). An external positive control defined as 1x10⁷ copies/ml is supplied which allow the determination of the gene load. For further information, please refer to section 9.3 Quantitation.

4. Kit Contents

Ref.	Type of Reagent	Presentation 25rxns
1	DNA Extraction Buffer	2 vials, 1.5ml
2	CPS Reaction Mix	1 vial, 450µl
3	PCR Enzyme Mix	1 vial, 12µl
4	Molecular Grade Water	1 vial, 400µl
5	Internal Control	1 vial, 30µl
6	CPS Positive Control(1x10 ⁷ copies/ml)	1 vial, 30µl

Analysis sensitivity: 1x10³ copies/ml; LOQ: 2x10³~1x10⁸ copies/ml

Note: Analysis sensitivity depends on the sample volume, elution volume, nucleic acid extraction methods and other factors. If you use the DNA extraction buffer in the kit, the analysis sensitivity is the same as it declares. However, when the sample volume is dozens or even hundreds of times greater than elution volume by some concentrating method, it can be much higher.

5. Storage

- All reagents should be stored at -20°C. Storage at +4°C is not recommended.
- All reagents can be used until the expiration date indicated on the kit label.
- Repeated thawing and freezing (>3x) should be avoided, as this may reduce the sensitivity of the assay.
- Cool all reagents during the working steps.
- Reaction mix should be stored in the dark.

6. Additionally Required Materials and Devices

- Biological cabinet
- Vortex mixer
- Cryo-container
- Sterile filter tips for micro pipets
- Disposable gloves, powderless
- Refrigerator and Freezer
- Desktop microcentrifuge for "eppendorf" type tubes (RCF max. 16,000 x g)
- Real time PCR system
- Real time PCR reaction tubes/plates
- Pipets (0.5µl - 1000µl)
- Sterile microtubes
- Biohazard waste container
- Tube racks

7. Warnings and Precaution

- Carefully read this instruction before starting the procedure.
- For in vitro diagnostic use only.
- This assay needs to be carried out by skilled personnel.
- Clinical samples should be regarded as potentially infectious materials and should be prepared in a laminar flow hood.
- This assay needs to be run according to Good Laboratory Practice.
- Do not use the kit after its expiration date.
- Avoid repeated thawing and freezing of the reagents, this may reduce the sensitivity of the test.
- Once the reagents have been thawed, vortex and centrifuge briefly the tubes before use.
- Quickly prepare the reaction mix on ice or in the cooling block.
- Set up two separate working areas: 1) Isolation of the RNA/ DNA and 2) Amplification/ detection of amplification products.
- Pipets, vials and other working materials should not circulate among working units.
- Use always sterile pipette tips with filters.
- Wear separate coats and gloves in each area.

8. Sample Collection, Storage and transportation

- Collect samples in sterile tubes;
- Specimens can be extracted immediately or frozen at -20°C to -80°C.
- Transportation of clinical specimens must comply with local regulations for the transport of etiologic agents

9. Procedure

9.1. DNA-Extraction

DNA extraction buffer is contained in the kit. Please thaw the buffer thoroughly and spin down briefly in the centrifuge before use. It's better to use commercial kits for nucleic acid extraction.

9.1.1 Stool or vomit samples

- Take about 50mg samples to a 1.5ml tube; add 1.0ml normal saline then vortex vigorously. Centrifuge the tube at 13000rpm for 2 minutes, carefully remove and discard supernatant from the tube without disturbing the pellet.
- Add 100µl DNA extraction buffer, close the tube then resuspend the pellet with vortex vigorously. Spin down briefly in a table centrifuge.
- Incubate the tube for 10 minutes at 100°C.
- Centrifuge the tube at 13000rpm for 5 minutes. The supernatant contains the DNA extracted and can be used for PCR template.

9.1.2 Water samples

- Take 3 ml water to a tube, Centrifuge the tube at 13000rpm for 2 minutes, carefully remove and discard supernatant from the tube without disturbing the pellet.
- Add 100µl DNA extraction buffer, close the tube then vortex for 10 seconds. Spin down briefly in a table centrifuge.
- Incubate the tube for 10 minutes at 100°C.
- Centrifuge the tube at 13000rpm for 5 minutes. The supernatant contains the DNA extracted and can be used for PCR template.

Attention:

- During the incubation, make sure the tube is not open, as the vapor will volatilize into the air and may cause contamination if the sample is positive.
- The extraction sample should be used in 3 hours or store at -20°C for one month.
- Different DNA extraction kits are available. You may use your own extraction systems or the commercial kit based on the yield. For the DNA extraction, please comply with the manufacturer's instructions.

9.2 Internal Control

It is necessary to add internal control (IC) in the reaction mix. Internal Control (IC) allows the user to determine and control the possibility of PCR inhibition. Add the internal control (IC) 1µl/rxn and the result will be got in the 560nm channel.

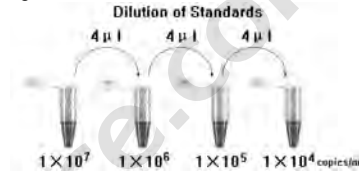
9.3 Quantitation

The kit can be used for quantitative or qualitative real-time PCR. A positive control defined as 1x10⁷ copies/ml is supplied in the kit.

For performance of quantitative real-time PCR, Standard dilutions must prepare first as follows. Molecular Grade Water is used for dilution.

The step of dilution is not needed for performance of qualitative real-time PCR.

Take positive control (1x10⁷ copies/ml) as the starting high standard in the first tube. Respectively pipette 36µl of Molecular Grade Water into next three tubes. Do three dilutions as the following figures:



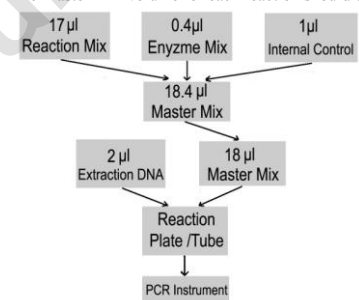
To generate a standard curve on the real-time system, all four dilution standards should be used and defined as standard with specification of the corresponding concentrations.

Attention:

- Mix thoroughly before next transfer.
- The positive control (1x10⁷ copies/ml) contains high concentration of the target DNA.

9.4 PCR Protocol

The Master Mix volume for each reaction should be pipetted as follows:



※PCR system without 560nm channel may be treated with 1µl Molecular Grade Water instead of 1µl IC.

- The volumes of Reaction Mix and Enzyme Mix per reaction multiply with the number of samples, which includes the number of the controls, standards and sample prepared. Molecular Grade Water is used as the negative control. For reasons of unprecise pipetting, always add an extra virtual sample. Mix the master mix completely then spin down briefly in a centrifuge.
- Pipet 18µl Master Mix with micropipets of sterile filter tips to each Real time PCR reaction plate/tube. Then separately add

- 2µl DNA sample, positive and negative controls to different reaction plate/tubes. Immediately close the plate/tubes to avoid contamination.
- Spin down briefly in order to collect the Master Mix in the bottom of the reaction tubes.
- Perform the following protocol in the instrument:

37°C for 2min	1cycle
94°C for 2min	1cycle
93°C for 5sec, 60°C for 30sec (Fluorescence measured at 60°C)	40cycles

Selection of fluorescence channels	
530nm	Target Nucleic Acid
560nm	IC

10. Threshold setting: Choose Arithmetic as back ground and none as Noise Band method, then adjust the Noise band just above the maximum level of molecular grade water, and adjust the threshold just under the minimum of the positive control.

11. Calibration for quantitative detection: Input each concentration of standard controls at the end of run, and a standard curve will be automatically formed.

12. Quality control: Negative control, positive control, internal control and QS curve must be performed correctly, otherwise the sample results is invalid.

Control	Channel	Crossing point value	
		530nm	560nm
Molecular Grade Water		Blank	25~35
Positive Control(qualitative assay)		≤35	---
QS (quantitative detection)		Correlation coefficient of QS curves ≤ -0.98	

13. Data Analysis and Interpretation

The following results are possible:

	Crossing point value		Result Analysis
	530nm	560nm	
1#	Blank	25~35	Below the detection limit or negative
2#	≤35	---	Positive; and the software displays the quantitative value
3#	35~40	25~35	Re-test; If it is still 35~40, report as 1#
4#	Blank	Blank	PCR Inhibition; No diagnosis can be concluded.

For further questions or problems, please contact our technical support

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(For Research Use Only In USA & China)

Cryptosporidium Real Time PCR Kit User Manual

REF MBS598073 - Instrument III, IV



For use with ABI Prism®7000/7300/7500/7900/Step One Plus; iCycler iQ™4/iQ™5; Smart Cycler II; Bio-Rad CFX 96; Rotor Gene™ 6000; Mx3000P/3005P; MJ-Option2/Chromo4; LightCycler®480 Instrument

EC REF

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3. Product Description

Cryptosporidium is a coccidian protozoan parasite that has gained much attention in the last 20 years as a clinically important human pathogen. Electron microscopic examination of the intestinal mucosa led to the discovery that Cryptosporidium parvum was the infectious species in humans. In the early 1980s, the strong association between cases of cryptosporidiosis and immunodeficient individuals (such as those with AIDS-acquired immunodeficiency syndrome) brought Cryptosporidium to the forefront as a ubiquitous human pathogen. Little is known about the pathogenesis of the parasite, and no safe and effective treatment has been successfully developed to combat cryptosporidiosis. Unlike other intestinal pathogens, Cryptosporidium can infect several different hosts, can survive most environments for long periods of time due to its "hardy cyst", and inhabits all climates and locales. Cryptosporidium real time PCR kit contains a specific ready-to-use system for the detection of the Cryptosporidium through polymerase chain reaction in the real-time PCR system. The master contains reagents and enzymes for the specific amplification of the Cryptosporidium DNA. Fluorescence is emitted and measured by the real time systems' optical unit during the PCR. The detection of amplified Cryptosporidium DNA fragment is performed in fluorimeter channel FAM with the fluorescent quencher BHQ1. An external positive control defined as 1×10⁷ copies/ml is supplied which allow the determination of the gene load. For further information, please refer to section 9.3 Quantitation.

9.3 Quantitation.

4. Kit Contents

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2	CPS Reaction Mix	1 vial, 950µl
3	PCR Enzyme Mix	1 vial, 12µl
4	Molecular Grade Water	1 vial, 400µl
5	Internal Control	1 vial, 30µl
6	CPS Positive Control(1×10 ⁷ copies/ml)	1 vial, 30µl

Analysis sensitivity: 1×10⁶copies/ml;

LOQ: 2×10⁷~1×10⁸ copies/ml

Note: Analysis sensitivity depends on the sample volume, elution volume, nucleic acid extraction methods and other factors. If you use the DNA extraction buffer in the kit, the analysis sensitivity is the same as it declares. However, when the sample volume is dozens or even hundreds of times greater than elution volume by some concentrating method, it can be much higher.

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- Cool all reagents during the working steps.
- Reaction mix should be stored in the dark.

6. Additionally Required Materials and Devices

- Biological cabinet
- Vortex mixer
- Cryo-container
- Sterile filter tips for micro pipets
- Disposable gloves, powderless
- Refrigerator and Freezer
- Desktop microcentrifuge for "ependorf" type tubes (RCF max. 16,000 x g)
- Real time PCR system
- Real time PCR reaction tubes/plates
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- Sterile microtubes
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- Add 100µl DNA extraction buffer, close the tube then resuspend the pellet with vortex vigorously. Spin down briefly in a table centrifuge.
- Incubate the tube for 10 minutes at 100°C.
- Centrifuge the tube at 13000rpm for 5 minutes. The supernatant contains the DNA extracted and can be used for PCR template.

9.1.2 Water samples

- Take 3 ml water to a tube, Centrifuge the tube at 13000rpm for 2 minutes, carefully remove and discard supernatant from the tube without disturbing the pellet.
- Add 100µl DNA extraction buffer, close the tube then vortex for 10 seconds. Spin down briefly in a table centrifuge.
- Incubate the tube for 10 minutes at 100°C.
- Centrifuge the tube at 13000rpm for 5 minutes. The supernatant contains the DNA extracted and can be used for PCR template.

Attention:

- During the incubation, make sure the tube is not open, as the vapor will volatilize into the air and may cause contamination if the sample is positive.
- The extraction sample should be used in 3 hours or store at -20°C for one month.
- Different DNA extraction kits are available. You may use your own extraction systems or the commercial kit based on the yield. For the DNA extraction, please comply with the manufacturer's instructions.

9.2 Internal Control

It is necessary to add internal control (IC) in the reaction mix. Internal Control (IC) allows the user to determine and control the possibility of PCR inhibition.

Add the internal control (IC) 1µl/rxn and the result will be got in the HEX/VIC/JOE channel.

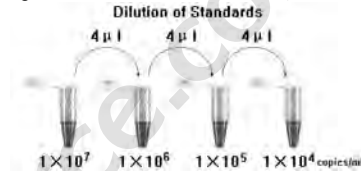
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The step of dilution is not needed for performance of qualitative real-time PCR.

Take positive control (1×10⁷copies/ml) as the starting high standard in the first tube. Respectively pipette 36µl of Molecular Grade Water into next three tubes. Do three dilutions as the following figures:



To generate a standard curve on the real-time system, all four dilution standards should be used and defined as standard with specification of the corresponding concentrations.

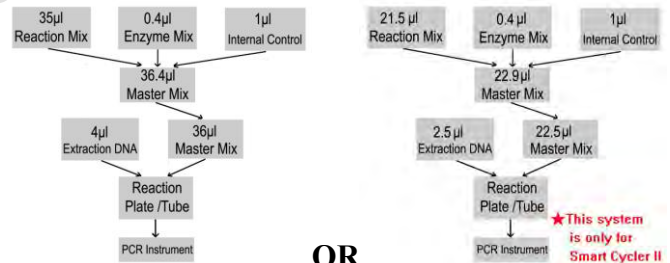
Attention:

- Mix thoroughly before next transfer.
- The positive control (1×10⁷copies/ml) contains high concentration of the target DNA.

Therefore, be careful during the dilution in order to avoid contamination.

9.4 PCR Protocol

The Master Mix volume for each reaction should be pipetted as follows:



OR

※PCR system without HEX/VIC/JOE channel may be treated with 1µl Molecular Grade Water instead of 1µl IC.

- The volumes of Reaction Mix and Enzyme Mix per reaction multiply with the number of samples, which includes the number of the controls, standards and sample prepared. Molecular Grade Water is used as the negative control. For reasons of unprecise pipetting, always add an extra virtual sample. Mix the master mix completely then spin down briefly in a centrifuge.
- Pipet 36µl (22.5µl for SmartCycler II) Master Mix with micropipets of sterile filter tips to each Real time PCR reaction plate/tube. Then separately add 4µl (2.5µl for SmartCycler II) DNA sample, positive and negative controls to different reaction plate/tubes. Immediately close the plate/tubes to avoid contamination.
- Spin down briefly in order to collect the Master Mix in the bottom of the reaction tubes.
- Perform the following protocol in the instrument:

37°C for 2min	1cycle
94°C for 2min	1cycle
93°C for 15sec, 60°C for 1min	40cycles
(Fluorescence measured at 60°C)	

Selection of fluorescence channels	
FAM	Target Nucleic Acid
HEX/VIC/JOE	IC

5) ⚠ If you use ABI Prism® system, please choose "none" as passive reference and quencher.

10. Threshold setting: just above the maximum level of molecular grade water.

11. Calibration for quantitative detection: Input each concentration of standard controls at the end of run, and a standard curve will be automatically formed.

12. Quality control: Negative control, positive control, internal control and QS curve must be performed correctly, otherwise the sample results is invalid.

Channel	Ct value	
	Control	FAM
Molecular Grade Water	UNDET	25-35
Positive Control(qualitative assay)	≤35	—
QS (quantitative detection)	Correlation coefficient of QS curve≤-0.98	

13. Data Analysis and Interpretation : The following results are possible:

	Ct value		Result Analysis
	FAM	HEX/VIC/JOE	
1#	UNDET	25-35	Below the detection limit or negative
2#	≤35	—	Positive; and the software displays the quantitative value
3#	35~40	25-35	Re-test; If it is still 35-40, report as 1#
4#	UNDET	UNDET	PCR Inhibition; No diagnosis can be concluded.

For further questions or problems, please contact our technical support