

***Cryptosporidium* RT-PCR Detection Kit**

Product # 39100

Product Insert

Pathogen Information

Cryptosporidium is a parasite found in water that causes an infection in mammals termed cryptosporidiosis. It is one of the most common water-borne diseases and is found world-wide. It affects the intestines of mammals and typically causes an acute short-term infection. The most common symptom is self-limiting diarrhea in healthy individuals, however in immunocompromised individuals the symptoms are particularly severe and often fatal. There is no specific treatment for cryptosporidiosis other than fluid rehydration and management of any pain. Therefore early detection of *Cryptosporidium* in water is the foremost action to prevent the infection.

Principle of the Test

Norgen's *Cryptosporidium* RT-PCR Detection Kit constitutes a ready-to-use system for the isolation and detection of *Cryptosporidium* using end-point one step RT-PCR. The kit first allows for the isolation of *Cryptosporidium* RNA from the water samples using spin-column chromatography based on Norgen's proprietary resin. The *Cryptosporidium* RNA is isolated free from inhibitors, and can then be used as the template in a one step RT-PCR reaction for *Cryptosporidium* detection using the provided *Cryptosporidium* Master Mix. The *Cryptosporidium* Mastermix contains reagents and enzymes for the specific amplification of a 218 bp region of the viral genome. In addition, Norgen's *Cryptosporidium* RT-PCR Detection Kit contains a second Mastermix, the Control 2X RT-PCR Master Mix, which can be used to identify possible PCR inhibition and/or inadequate isolation via a separate RT-PCR reaction with the use of the provided *PCR control (PCRC)* or *Isolation Control (IsoC)*, respectively. This kit is designed to allow for the testing of 24 samples.

Kit Components:

Component	Contents
Lysis Solution	25 mL
Wash Solution	11 mL
Elution Buffer	2 mL
Bead Tubes	24
Mini Spin Columns	24
Collection Tubes	24
Elution tubes (1.7 mL)	24
CRYP 2x RT-PCR Master Mix	0.35 mL
Control 2x RT-PCR Mastermix	0.35 mL
Isolation Control (IsoC)^{*a}	0.3 mL
CRYP Positive Control (PosC)^{*b}	0.1 mL
<i>Nuclease Free-Water</i>	1.25 mL
Norgen's DNA Marker	0.1 mL
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* IsoC = Isolation Control ; PosC= Positive Control

^a The isolation control is a cloned PCR product.

^b The positive control is *Cryptosporidium* RNA transcript

Customer-Supplied Reagents and Equipment

- Benchtop microcentrifuge
- 1.5 mL microcentrifuge tubes
- 96 – 100% ethanol
- 70% ethanol

Storage Conditions and Product Stability

- The **CRYP 2X Detection RT-PCR Mastermix** (green cap) and the **Control 2X RT-PCR Mastermix** (yellow cap) should be stored at -20°C upon receipt (-70°C for long-term). Make appropriate aliquots and store at -20°C if needed.
- All other kit components may be stored at room temperature
- The **CRYP 2X Detection RT-PCR Mastermix** and the **Control 2X RT-PCR Mastermix**, Positive Control and Isolation Control should not undergo repeated freeze-thaw (a maximum freeze-thaw of three times).
- For RT-PCR
 - Allow reagents to thaw at room temperature prior to use
 - When thawed, mix the components and centrifuge briefly
 - Work quickly on ice
 - After addition of RT-PCR Mastermix use within one hour

General Precautions

The user should exercise the following precautions when using the kit:

- Use sterile pipette tips with filters.
- Store and extract positive material (specimens, controls and amplicons) separately from all other reagents and add it to the reaction mix in a spatially separated facility.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Work quickly on ice.

Quality Control

In accordance with Norgen's ISO 9001 and ISO 13485-certified Quality Management System, each lot of Norgen's CRYP RT-PCR Detection Kit, including the CRYP 2x RT-PCR Master Mix, Control 2X RT-PCR Mastermix, Isolation Control and CRYP Positive Control are tested against predetermined specifications to ensure consistent product quality.

Product Use Limitations

Norgen's *Cryptosporidium* RT-PCR Detection Kit is designed for research purposes only.

Product Warranty and Satisfaction Guarantee

NORGEN BIOTEK CORPORATION guarantees the performance of all products in the manner described in our product manual. The customer must determine the suitability of the product for its particular use.

Safety Information

Ensure that a suitable lab coat, disposable gloves and protective goggles are worn when working with chemicals. For more information, please consult the appropriate Material Safety Data Sheets (MSDSs). These are available as convenient PDF files online at www.norgenbiotek.com.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Working with RNA

RNases are very stable and robust enzymes that degrade RNA. Autoclaving solutions and glassware is not always sufficient to actively remove these enzymes. The first step when preparing to work with RNA is to create an RNase-free environment. The following precautions are recommended as your best defense against these enzymes.

- The RNA area should be located away from microbiological work stations.
- Clean, disposable gloves should be worn at all times when handling reagents, samples, pipettes, disposable tubes, etc. It is recommended that gloves are changed frequently to avoid contamination.
- There should be designated solutions, tips, tubes, lab coats, pipettes, etc. for RNA only.
- All RNA solutions should be prepared using at least 0.05% DEPC-treated autoclaved water or molecular biology grade nuclease-free water.
- Clean all surfaces with commercially available RNase decontamination solutions.
- When working with purified RNA samples, ensure that they remain on ice during downstream applications.

Protocol

A. *Cryptosporidium* Total RNA Isolation

Important Notes Prior to Beginning Protocol:

- 0.22 or 0.45 μM Filter Columns (please see Related Products table) must be purchased separately and used with this procedure. Users may also provide their own Filter Columns.
- All centrifugation steps are carried out in a benchtop microcentrifuge at 14,000 x g (~14,000 RPM) except where noted. All centrifugation steps are performed at room temperature.
- A variable speed microcentrifuge should be used for maximum kit performance. If a variable speed centrifuge is not available a fixed speed centrifuge can be used, however reduced yields may be observed.
- Ensure that all solutions are at room temperature prior to use.
- Prepare a working concentration of the **Wash Solution** by adding 25 mL of 95 - 100 % ethanol (provided by the user) to the supplied bottle containing the concentrated **Wash Solution**. This will give a final volume of 36 mL. The label on the bottle has a box that may be checked to indicate that the ethanol has been added.
- Pre-heat a water bath or an incubator to 65 °C
- For optimal kit performance we recommend processing 10 mL to 100 mL of water through Norgen's Filter Columns (sold separately, see related product table), depending on the sample. For clear water samples, the maximum input of 100 mL can be processed. For turbid water samples we recommend processing smaller volumes, as the suspended solids or sediment may clog the filter.
- It is important to work quickly when purifying RNA.
- ***Cryptosporidium* Isolation Control (IsoC)**
 - A CRYP Isolation Control (*IsoC*) is supplied. This allows the user to control the RNA isolation procedure. For this assay, add the CRYP Isolation Control (*IsoC*) to the lysate during the isolation procedure
 - The CRYP Isolation Control (*IsoC*) must not be added to the sample material directly.
 - Do not freeze and thaw the CRYP Isolation Control (*IsoC*) more than 2 times.
 - The CRYP Isolation Control (*IsoC*) must be kept on ice at all times during the isolation procedure.
- The RT-PCR components of the *Cryptosporidium* RT-PCR Detection Kit should remain at -20°C until RNA is extracted and ready for RT-PCR amplification.
- It is important to work quickly during this procedure.

1. Lysate Preparation using a Vacuum Apparatus

- a. Obtain a water sample that is 10 mL – 100 mL, depending on the water source (see Notes Prior to Use above).
- b. Place the Filter Column (Sold separately, see related product table) onto a vacuum apparatus.
- c. Transfer up to 20 mL of water to the filter and allow the total volume to pass. This may be repeated as required in order to allow the entire water sample (up to 100 mL) to pass through the filter.
- d. Cut the filter out by running a sterile scalpel blade along with edge of O-ring, located on top of the filter. Alternatively, remove the O-ring from the filter column by using tissue forceps which have 1x2 teeth (e.g. VWR Tissue forceps Cat. No. 25607-608).
- e. Carefully remove the filter from the filter column using sterile forceps, and transfer to a provided Bead Tube.

Note: Remove the filter by picking it up by the edges/corner. Avoid touching the center of the filter. Ensure when placing the filter into the Bead Tube that the upper surface of the filter is facing the center of the tube.

- f. Add 500 µL of Lysis Solution to the Bead Tube and secure the tube horizontally on a flat-bed vortex pad with tape, or in any commercially available bead beater equipment (e.g. Scientific Industries' Disruptor GenieTM). Vortex for 5 minutes at maximum speed.

Note: The appearance of some white foam during the homogenization is common. This is due to detergents present in the Lysis Buffer and will not affect the protocol.

- g. Incubate at 65 °C for 10 minutes. Occasionally mix the lysate 2 or 3 times during incubation by inverting the tube. Ensure that the filter does not become dry.
- h. Centrifuge the tube for 1 minute at 14000 × g (~14,000 RPM).
- i. Transfer the lysate to another RNase-free microcentrifuge tube (provided by the user). Note the volume.
- j. Add an equal volume of 70% ethanol (provided by the user) to the lysate collected above (100 µL of ethanol is added to every 100 µL of lysate). Vortex to mix. Proceed to Step 2.

2. Binding Nucleic Acids to Column

- a. Assemble a spin column with one of the provided collection tubes.
- b. Add 10 µL of **Isolation Control (IsoC)** to the lysate mixture.
- c. Apply up to 600 µL of the lysate with ethanol onto the column and centrifuge for 1 minute at 14,000 × g (~14,000 RPM). Discard the flowthrough and reassemble the spin column with the collection tube

Note: Ensure the entire lysate volume has passed through into the collection tube by inspecting the column. If the entire lysate volume has not passed, spin for an additional minute.

- d. Depending on your lysate volume, repeat step 2c if necessary.

3. Column Wash

- a. Apply 500 µL of Nucleic Acid Wash Solution to the column and centrifuge for 1 minute.

Note: Ensure the entire wash solution has passed through into the collection tube by inspecting the column. If the entire wash volume has not passed, spin for an additional minute.

- b. Discard the flowthrough and reassemble the column with its collection tube.
- c. Repeat step 3a to wash column a second time.
- d. Discard the flowthrough and reassemble the spin column with its collection tube.

- e. Spin the column for 2 minutes in order to thoroughly dry the resin. Discard the collection tube.

4. RNA Elution

- a. Place the column into a fresh 1.7 mL Elution tube provided with the kit.
- b. Add 50 μL of **Elution Buffer** to the column.
- c. Centrifuge for 2 minutes at **200 x g (~2,000 RPM)**, followed by a 1 minute spin at **14,000 x g (~14,000 RPM)**. Note the volume eluted from the column. If the entire volume has not been eluted, spin the column at **14,000 x g (~14,000 RPM)** for 1 additional minute.
- d. The purified RNA sample could be used immediately for RT-PCR as described below. It is recommended that samples be placed at -70°C for long term storage.

5. Storage of DNA and RNA

The purified nucleic acids may be stored at -20°C for a few days. It is recommended that samples be placed at -70°C for long term storage.

B. *Cryptosporidium* RT-PCR Assay Preparation

Notes:

- Before use, suitable amounts of all RT-PCR components should be completely thawed at room temperature, vortexed and centrifuged briefly.
 - The amount of *Cryptosporidium* 2X RT-PCR Master Mix provided is enough for up to 32 RT-PCR reactions (24 sample RT-PCR, 4 positive control RT-PCR and 4 no template control RT-PCR).
 - For each sample, one RT-PCR reaction using the ***Cryptosporidium* 2X Detection RT-PCR Mastermix** and one RT-PCR reaction using **Control 2X RT-PCR Mastermix** should be set up in order to have a proper interpretation of the results.
 - For every RT-PCR run, one reaction containing *Cryptosporidium* Positive Control (**CRYP PosC**) and one reaction as no template control (*Nuclease Free-Water*) must be included for proper interpretation of results.
 - The recommended minimum number of RNA samples tested per RT-PCR run is 6.
 - Using a lower volume from the sample than recommended may affect the sensitivity of *Cryptosporidium* Limit of Detection.
1. Prepare the RT-PCR reaction for sample detection (Set #1, using **CRYP 2X Detection RT-PCR Mastermix**) and the RT-PCR reaction for control detection (Set #2, using **Control 2X RT-PCR Mastermix**) as shown in Table 1 below. The recommended amount of sample RNA to be used is 2 μL . Ensure that one *Cryptosporidium* detection reaction and one control reaction is prepared for each RNA sample. Adjust the final volume of the RT-PCR reaction to 20 μL using the Nuclease-Free Water provided.

Table 1. RT-PCR Assay Preparation

RT-PCR Components	Volume Per RT-PCR Reaction
CRYP 2X RT-PCR Master Mix Or Control 2X RT-PCR Master Mix	10 μL
Sample RNA	2 μL
Nuclease-Free Water	8 μL
Total Volume	20 μL

2. For every RT-PCR run, prepare **one** positive control RT-PCR as shown in Table 2 below:

Table 2. RT-PCR Positive Control Preparation

RT-PCR Components	Volume Per RT- PCR Reaction
CRYP 2X RT-PCR Master Mix Or Control 2X RT-PCR Master Mix	10 µL
CRYP <i>Positive Control (PosC)</i>	10 µL
<i>Total Volume</i>	20 µL

3. For every RT- PCR run, prepare **one** no template control RT-PCR as shown in Table 3 below:

Table 3. RT-PCR Negative Control Preparation

RT-PCR Components	Volume Per RT-PCR Reaction
CRYP 2X RT- PCR Master Mix Or Control 2X RT-PCR Master Mix	10 µL
<i>Nuclease-Free Water</i>	10 µL
<i>Total Volume</i>	20 µL

Therefore, at a minimum, each PCR run will contain 6 separate RT-PCR reactions.

C. One Step RT- *Cryptosporidium* PCR Assay Programming

1. Program the thermocycler according to the program shown in Table 4 below.
2. Run one step RT-PCR.

Table 4. *Cryptosporidium* Assay Program

One Step RT-PCR Cycle	Step	Temperature	Duration
<i>Cycle 1</i>	Step 1	50°C	30 min
<i>Cycle 2</i>	Step 1	95°C	3 min
<i>Cycle 3 (40x)</i>	Step 1	94°C	15 sec
	Step 2	60°C	30 sec
	Step 3	72°C	45 sec
<i>Cycle 4</i>	Step 1	72°C	5 min
<i>Cycle 5</i>	Step 1	4°C	∞

D. *Cryptosporidium* One Step RT- PCR Assay Results Interpretation

1. For the analysis of the RT-PCR data, the entire 15-20 μL RT-PCR Reaction should be loaded on a 1X TAE 1.5% Agarose RNA gel along with 10 μL of Norgen's DNA Marker (provided).
2. The RT-PCR products should be resolved on the 1X TAE 1.5% Agarose gel at 150V for 20 minutes (Gel running time will be vary depending on an electrophoresis apparatus).
3. Sample results are provided below:

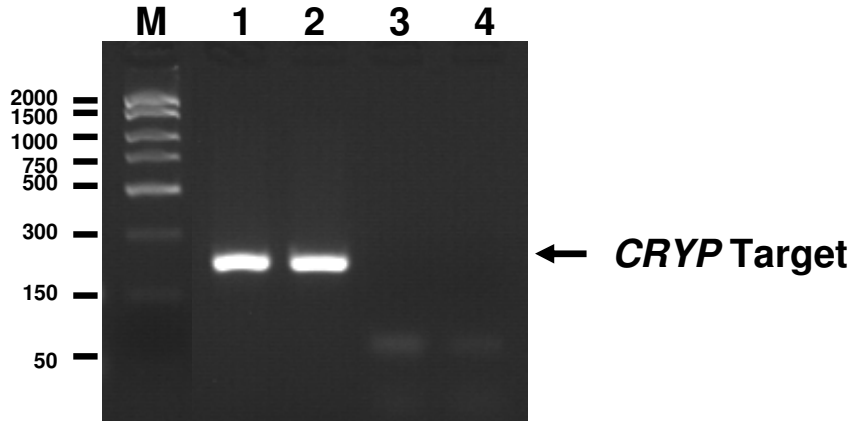


Figure 1: A representative 1X TAE 1.5% agarose gel showing the amplification of CRYPT positive (lane 1 and 2) negative (lane 3 and 4) controls. The size of the CRYPT target amplicon corresponds to 218 bp as represented by the provided DNA Marker (M).

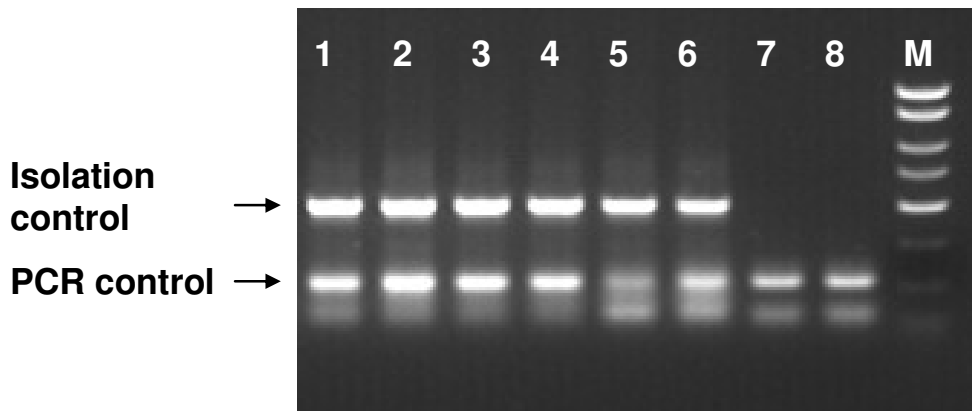


Figure 2: A representative 1X TAE 1.5% agarose gel showing the amplification of **Isolation Control** and **PCR Control** under different conditions using the **Control 2X RT-PCR Mastermix**. The size of the Isolation Control amplicon and PCR Control amplicon correspond to 499 bp and 150 bp, respectively, as represented by the provided DNA Marker (M). Lanes 1 to 6 showed detection of both Isolation Control and PCR Control, suggesting that the RNA isolation as well as the RT-PCR reaction was successful. Lane 7 and 8 showed only the detection of PCR Control suggesting that while the RT-PCR was successful, the isolation failed to recover even the spiked-in Isolation control.

Table 5. Interpretation of One Step RT-PCR Assay Results

Input Type	Target Reaction	Control Reaction		Interpretation
	CRYP Target Band (218 bp)	CRYP <i>IsoC</i> Band (499 bp)	CRYP <i>PCRC</i> Band (150 bp)	
Positive Control	X	X	X	Valid
Negative Control		X	X	Valid
Sample	X	X	X	Positive
Sample		X	X	Negative
Sample		X		Negative
Sample	X		X	Positive
Sample	X	X		Positive
Sample	X			Positive

** For results obtained that are not covered in Table 5 above, please refer to the Troubleshooting Section.

E. *Cryptosporidium* RT-PCR Assay Specificity and Sensitivity

- The specificity of Norgen's *Cryptosporidium* RT-PCR Detection Kit is first and foremost ensured by the selection of the *Cryptosporidium*-specific primers, as well as the selection of stringent reaction conditions. The *Cryptosporidium* universal primers were checked for possible homologies to all in GenBank published sequences by sequence comparison analysis and published *Cryptosporidium* strains.

F. Linear Range

- The linear range of Norgen's *Cryptosporidium* RT-PCR Detection Kit was determined by analysing a dilution series of a *Cryptosporidium* quantification standards ranging from 100 ag to 1 pg.
- Each dilution has been tested in replicates (n = 4) using Norgen's *Cryptosporidium* RT-PCR Detection Kit on a 1X TAE 1.7% agarose gel.
- The linear range of Norgen's *Cryptosporidium* RT-PCR Detection Kit has been determined to cover concentrations from 100 ag to 1 ng
- Under the conditions of the Norgen's *Cryptosporidium* RNA Isolation procedure, Norgen's *Cryptosporidium* RT-PCR Detection Kit covers a linear range from 100 copies to 1×10^6 copies.

Frequently Asked Questions

1. How many samples should be included per RT-PCR run?

- Norgen's *Cryptosporidium* RT-PCR Detection Kit is designed to test 24 samples. For every 6 samples, a non-template control (Nuclease Free Water) and a Positive Control must be included. It is preferable to pool and test 6 samples at a time. If not, the provided Positive Control is enough to run 3 samples at a time.

2. How can I interpret my results if neither the *Cryptosporidium* RT-PCR control nor the Isolation Control (*IsoC*) amplifies?

- If neither the *Cryptosporidium* PCR control nor the Isolation Control (*IsoC*) amplifies, the sample must be re-tested. If the positive control showed amplification, then the problem occurred during the isolation, where as if the Positive control did not amplify, therefore the problem has occurred during the setup of the PCR assay reaction.

3. How should it be interpreted if only the *Cryptosporidium* PCR control showed amplification but neither the *Cryptosporidium* target nor the Isolation control amplified for a sample?

- This indicates a poor isolation. The isolation procedure must be repeated.

4. How should it be interpreted if only the Isolation Control (*IsoC*) was amplified in a sample?

- The sample tested can be considered as *Cryptosporidium* negative.

5. How should it be interpreted if the *Cryptosporidium* PCR control and the *Cryptosporidium* target showed amplification in a sample?

- The sample tested can be considered positive. It could happen when too much template was added to the reaction.

6. How should it be interpreted if only the *Cryptosporidium* target and the *Cryptosporidium* PCR control were amplified in a sample?

- The sample tested can be considered as *Cryptosporidium* positive.

7. How should it be interpreted if only the *Cryptosporidium* target was amplified in a sample?

- The sample tested should be considered as *Cryptosporidium* positive. At high *Cryptosporidium* input, the *Cryptosporidium* amplicon will be predominant and thus the *Cryptosporidium* PCR control as well as the Isolation control may not amplify as they compete for PCR resources.

8. How should it be interpreted if only the *Cryptosporidium* PCR control and the Isolation control showed amplification in a sample?

- The sample tested can be considered negative

9. What if I forgot to do a dry spin after my third wash?

- Your first RNA elution will be contaminated with the Wash Solution. This may dilute the RNA yield in your first elution and it may interfere with the PCR detection, as ethanol is known to be a PCR inhibitor.

10. What if I forgot to add the Isolation Control (*IsoC*) during the isolation?

- It is recommended that the isolation is repeated.

Related Products	Product #
Water RNA/DNA Purification Kit (0.45 µm)	26450
0.45 µm Filter Maxi Columns (20/bag)	40006
Water RNA/DNA Purification Kit (0.22 µm)	26400
0.22 µm Filter Maxi Columns (20/bag)	40004

Technical Assistance

NORGEN's Technical Service Department is staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of NORGEN products. If you have any questions or experience any difficulties regarding Norgen's Urine RNA Isolation Mini Kit (Slurry Format) or NORGEN products in general, please do not hesitate to contact us.

NORGEN customers are a valuable source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at NORGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please contact our Technical Support Team between the hours of 8:30 and 5:30 (Eastern Standard Time) at (905) 227-8848 or Toll Free at 1-866-667-4362 or call one of the NORGEN local distributors (www.norgenbiotek.com) or through email at techsupport@norgenbiotek.com.

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