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Dirofilaria immitis PCR Detection Kit — Product Insert

Product # 44500

Pathogen Information

Heartworm (*Dirofilaria immitis*) is a parasitic roundworm that causes a serious and potentially fatal parasitic disease which primarily infects dogs, cats and a number of wild animals including wolves, coyotes, foxes, lions, raccoons. The parasite is transmitted from host to host by mosquito bites, whereby tiny heartworm larvae are injected into the animals bloodstream. Heartworms damage the blood vessels and reduce the heart's pumping ability, resulting in severe lung and heart disease. The signs of heartworm disease are usually detectable only after the disease has progressed and much damage has already been done to the internal organs. The animal may also tire easily during exercise and collapse due to heart failure.

Principle of the Test

Norgen's *Dirofilaria immitis* PCR Detection Kit constituents a ready-to-use system for the isolation and detection of *Dirofilaria immitis* using end-point PCR. The kit first allows for the isolation of *Dirofilaria immitis* DNA from the blood samples using spin-column chromatography. The *Dirofilaria immitis* DNA is isolated free from inhibitors, and can then be used as the template in a PCR reaction for *Dirofilaria immitis* detection using the provided *Dirofilaria immitis* Detection Master Mix. The *Dirofilaria immitis* Detection Mastermix contains reagents and enzymes for the specific amplification of a 276 bp region of the genome. In addition, Norgen's *Dirofilaria immitis* PCR Detection Kit contains a second Mastermix, the PCR Control Master Mix, which can be used to identify possible PCR inhibition and/or inadequate isolation via a separate 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	18 mL
Wash Solution	12 mL
Elution Buffer	6 mL
Proteinase K	0.6 mL
Spin Columns inserted Into Collection Tubes	25
Collection Tubes	25
Elution tubes (1.7 mL)	25
2x DIR Detection PCR Master Mix	0.35 mL
2x PCR Control Master Mix	0.35 mL
Isolation Control (IsoC)*[a]	0.3 mL
DIR Positive Control (PosC)*[b]	0.1 mL
Nuclease Free-Water	1.25 mL
Norgen's DNA Marker	0.1 mL
Product Insert	1

** IsoC = Isolation Control ; PosC= Positive Control*
[a] The isolation control is a cloned PCR product.
[b] The positive control is a fragment of Dirofilaria cloned in a plasmid

Customer-Supplied Reagents and Equipment

- Benchtop microcentrifuge
- 1.5 mL microcentrifuge tubes
- 96 – 100% ethanol
- Isopropanol
- 55°C water bath or incubator

Storage Conditions and Product Stability

All buffers should be kept tightly sealed and stored at room temperature (15-25°C). Buffers can be stored for up to 1 year without showing any reduction in performance. Norgen's *Dirofilaria immitis* PCR Detection Kit contains a ready-to-use Proteinase K solution, which is dissolved in a specially prepared storage buffer. The Proteinase K is stable for up to 1 year after delivery when stored at room temperature. To prolong the lifetime of the Proteinase K storage at 2–8°C is recommended.

The 2x DIR Detection PCR Master Mix, 2x PCR Control Master Mix, DIR Positive Control (*PosC*) and the Isolation Control (*IsoC*) should be kept tightly sealed and stored at -20°C for up to 1 year without showing any reduction in performance. Repeated thawing and freezing (> 2 x) should be avoided, as this may reduce the sensitivity. If the reagents are to be used only intermittently, they should be frozen in aliquots.

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 *Dirofilaria immitis* PCR Detection Kit, including the 2x DIR Detection PCR Master Mix, 2x PCR Control Master Mix, Isolation Control and DIR Positive Control are tested against predetermined specifications to ensure consistent product quality.

Product Use Limitations

Norgen's *Dirofilaria immitis* 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.

Disclaimers

The **Lysis Solution** contains guanidinium salts, and should be handled with care. Guanidinium salts form highly reactive compounds when combined with bleach, thus care must be taken to properly dispose of any of these solutions Blood of all human and animal subjects is considered potentially infectious. All necessary precautions recommended by the appropriate authorities in the country of use should be taken when working with whole blood.

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.

Protocol

Important Notes Prior to Beginning Protocol:

- Ensure that all isolation solutions are at room temperature prior to use, and that no precipitates have formed. If necessary, warm the solutions and mix well until the solutions become clear again.
- A variable speed centrifuge 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.
- For best results, the use of whole blood collected into tubes containing an anticoagulant is highly recommended.
- Both fresh and frozen anticoagulated blood may be used with this procedure. Ensure that frozen blood is thawed at room temperature prior to starting the protocol.
- Prepare a working concentration of Wash Solution by adding 28 mL of 96 – 100 % ethanol (provided by the user) to the supplied bottle containing concentrated Wash Solution. This will give a final volume of 40 mL. The label on the bottle has a box that can be checked to indicate that ethanol has been added.
- **Always** vortex the Proteinase K before use.
- **Isolation Control (*IsoC*)**
    - An Isolation Control (*IsoC*) is supplied. This allows the user to control the DNA isolation procedure. For this assay, add the Isolation Control *(IsoC)* to the lysate during the isolation procedure
    - The Isolation Control *(IsoC)* must not be added to the sample material directly.
    - Do not freeze and thaw the Isolation Control *(IsoC)* more than 2 times.
    - The Isolation Control *(IsoC)* must be kept on ice at all times during the isolation procedure.
- The PCR components of the *Dirofilaria immitis* PCR Detection Kit should remain at -20°C until DNA is extracted and ready for PCR amplification.
- It is important to work quickly during this procedure.

A. Lysate Preparation

1. Add 12 µL of **Proteinase K** to a microcentrifuge tube.
2. Transfer 500 µL of blood sample to the tube containing **Proteinase K**.
3. Add 600 µL of **Lysis Solution** to the blood and mix well by gentle vortexing for 10 seconds.
4. Briefly spin the tube to collect any drops of liquid from the inside of the lid.
5. Incubate at 55°C for 10 minutes.
6. If any debris is present in the sample, centrifuge for 2 minutes at 14,000 x g (~14,000 RPM) to precipitate. Transfer the clean supernatant to a microcentrifuge tube prior to **Step 7**.
7. Briefly spin the tube to collect any drops of liquid from the inside of the lid.
8. Add 240 µL of Isopropanol to the sample and mix well by gentle vortexing for 10 seconds.
9. Briefly spin the tube to collect any drops of liquid from the inside of the lid.

B. Specimen DNA Purification

Following the lysate preparation, DNA can be extracted from the patient specimens using the supplied buffers and solutions according to the following protocol:

1. Add 10 µL of **Isolation Control (IsoC)** to the lysate mixture.
2. Obtain a spin column assembled with its collection tube. Apply up to 650 µL of the lysate to the column and centrifuge for 1 minute at 6,000 x g (~8,000 RPM).
3. Discard the flowthrough. Reassemble the column and the collection tube.

    Note: Ensure that all of the lysate has passed through into the collection tube. If the entire lysate volume has not passed, centrifuge for an additional 2 minutes.

4. Repeat step **B2 and B3** with remaining lysate.
5. Discard the collection tube containing flow-through.
6. Assemble a spin column with a new collection tube.

7. Apply 500 µL of **Wash Solution** (ensure ethanol was added) to the column and centrifuge for 1 minute at 6,000 x g (~8,000 RPM). Discard the flowthrough and reassemble the spin column with its collection tube.
8. Wash column a second time by adding 500 µL of **Wash Solution** and centrifuging for 1 minute at 6,000 x g (~8,000 RPM). Discard the flowthrough and reassemble the spin column with its collection tube.
9. Spin the column for 2 minutes in order to thoroughly dry the resin at 14,000 x g (~14,000 RPM). Discard the collection tube.
10. Place the column into a provided 1.7 mL elution tube.
11. Add 200 µL of **Elution Buffer** to the column.
12. Centrifuge for 1 minute at 6,000 x g (~8,000 RPM)
13. The purified DNA sample may be stored at 4°C for a few days. It is recommended that samples be placed at –20°C for long term storage.

C. *Dirofilaria immitis* PCR Assay Preparation

Notes:

- Before use, suitable amounts of all PCR components should be completely thawed at room temperature, vortexed and centrifuged briefly.
- The amount of 2X DIR Detection PCR Master Mix and 2X PCR Control Master Mix provided is enough for up to 32 PCR reactions (24 sample PCR, 4 positive control PCR and 4 no template control PCR).
- For each sample, one PCR reaction using the 2X DIR Detection PCR Mastermix and one PCR reaction using 2X PCR Control Mastermix should be set up in order to have a proper interpretation of the results.
- For every PCR run, one reaction containing DIR Positive Control and one reaction as no template control must be included for proper interpretation of results.
- The recommended minimum number of DNA samples tested per PCR run is 6.
- Using a lower volume from the sample than recommended may affect the sensitivity of DIR Limit of Detection.

1. Prepare the PCR reaction for sample detection (Set #1, using **2X DIR Detection PCR Mastermix**) and the PCR reaction for control detection (Set #2, using **2X PCR Control Mastermix**) as shown in Table 1 below. The recommended amount of sample DNA to be used is 2.5 µL. However, a volume between 1 and 5 µL of sample DNA may be used as template. Ensure that one DIR detection reaction and one control reaction is prepared for each DNA sample. Adjust the final volume of the PCR reaction to 20 µL using the Nuclease-Free Water provided.

Table 1. PCR Assay Preparation

PCR Components	Volume Per PCR Reaction
2X DIR PCR Master Mix Or 2X PCR Control Master Mix	**10 µL**
Sample DNA	**2.5 µL**
Nuclease-Free Water	**7.5 µL**
Total Volume	**20 µL**

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

Table 2. PCR Positive Control Preparation

PCR Components	Volume Per PCR Reaction
2X DIR PCR Master Mix Or 2X PCR Control Master Mix	**10 µL**
DIR *Positive Control (PosC)*	**10 µL**
Total Volume	**20 µL**

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

Table 3. PCR Negative Control Preparation

PCR Components	Volume Per PCR Reaction
2X DIR PCR Master Mix Or 2X PCR Control Master Mix	**10 µL**
Nuclease-Free Water	**10 µL**
Total Volume	**20 µL**

D. *Dirofilaria immitis* PCR Assay Programming

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

Table 4. *Dirofilaria immitis* Assay Program

PCR Cycle	Step	Temperature	Duration
Cycle 1	**Step 1**	**95°C**	**3 min**
Cycle 2 (35x)	**Step 1**	**94°C**	**15 sec**
	Step 2	**60°C**	**15 sec**
	Step 3	**72°C**	**30 sec**
Cycle 3	**Step 1**	**72°C**	**5 min**
Cycle 4	**Step 1**	**4°C**	**∞**

E. *Dirofilaria immitis* PCR Assay Results Interpretation

1. For the analysis of the PCR data, the entire 15-20 µL PCR Reaction should be loaded on a 1X TAE 1.5% Agarose DNA gel along with 10 µL of Norgen's DNA Marker (provided).
2. The 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:

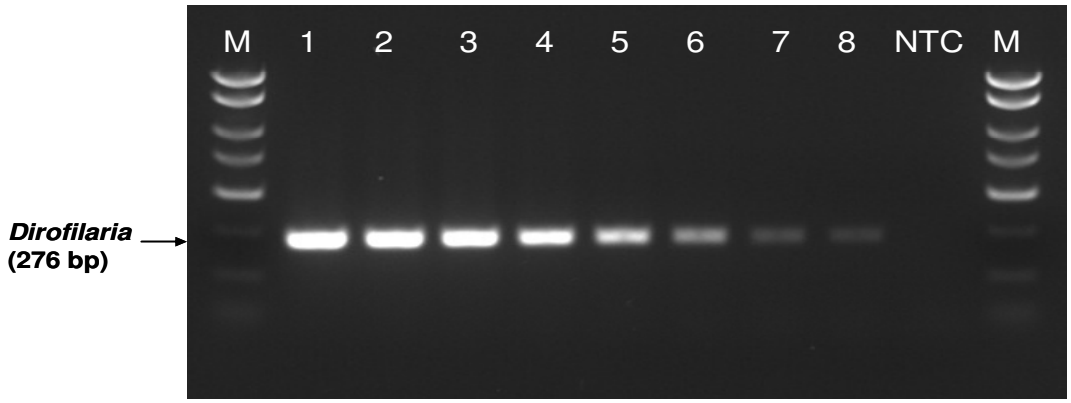

Figure 1: A representative 1X TAE 1.5% agarose gel showing the amplification of *Dirofilaria* at different concentrations (*Dirofilaria* Target). The size of the *Dirofilaria* target amplicon corresponds to 276 bp as represented by the provided DNA Marker (M). **NTC** = Negative Control.

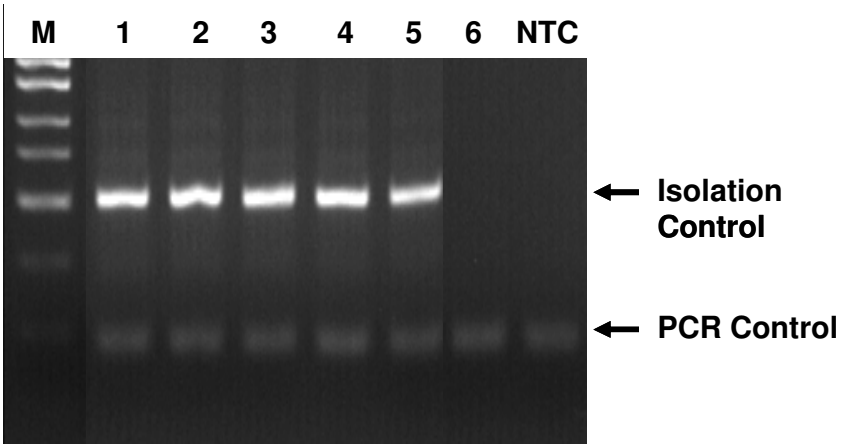

Figure 2: A representative 1X TAE 1.5% agarose gel showing the amplification of **Isolation Control** and **PCR Control** under different conditions using the **2X PCR Control Mastermix**. The size of the Isolation Control amplicon and PCR Control amplicon correspond to 499 bp and 150 bp, respectively, as represented by the provided DNA Marker (M). Lanes 1 to 5 showed detection of both Isolation Control and PCR Control, suggesting that the DNA isolation as well as the PCR reaction was successful. Lane 6 showed only the detection of PCR Control suggesting that while the PCR was successful, the isolation failed to recover even the spiked-in Isolation control. **NTC** = Negative Control.

Table 5. Interpretation of PCR Assay Results

Input Type	Target Reaction	Control Reaction		Interpretation
	***Dirofilaria* Target Band (276 bp)**	***IsoC* Band (499 bp)**	***PCRC* Band (150 bp)**	
Positive Control	X	X	X	Valid
Negative Control			X	Valid
Sample	X	X	X	Positive
Sample		X	X	Negative
Sample			X	Re-test
Sample				Re-test
Sample		X		Negative
Sample	X		X	Positive
Sample	X	X		Positive
Sample	X			Re-test

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

E. *Dirofilaria immitis* PCR Assay Specificity and Sensitivity

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

F. Linear Range

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

Frequently Asked Questions

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

- Norgen's *Dirofilaria immitis* 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 *DIR* PCR control nor the *DIR* Isolation Control (*IsoC*) amplifies?

- If neither the *DIR* PCR control nor the *DIR* 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 *DIR* PCR control showed amplification but neither the *DIR* target nor the *DIR* 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 *DIR* Isolation Control (*IsoC*) was amplified in a sample?

- The sample tested can be considered as *Dirofilaria immitis* negative.

5. How should it be interpreted if the *DIR* PCR control and the *DIR* 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 *DIR* target and the *DIR* PCR control were amplified in a sample?

- The sample tested can be considered as *Dirofilaria immitis* positive.

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

- It is recommended that the isolation is repeated.

8. How should it be interpreted if only the *DIR* PCR control and the *DIR* 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 DNA elution will be contaminated with the Wash Solution. This may dilute the DNA 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 *DIR* Isolation Control (*IsoC*) during the isolation?

- It is recommended that the isolation is repeated.

11. What if I forgot to run the Control PCR for the sample and I only ran the Detection PCR and I obtained a positive result?

- The result can be considered positive. However, any negative result must be verified by running the associated control PCR to ensure that it is a true negative and not a false negative due to problems with the RNA isolation or the PCR reactions.

Related Products	Product #
Blood Genomic DNA Isolation Kit	46300
Toxoplasma gondii PCR Detection Kit	44700
Leptospira interrogans PCR Detection Kit	44600

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 *Dirofilaria immitis* PCR Detection Kit 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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