EURx® MOLECULAR BIOLOGY PRODUCTS

Anaplasma/Ehrlichia qPCR Detection Kit

Kit Components

Component	Cat. No. E0456 -01 50 reactions of 20 µl	Cat. No. E0456-02 100 reactions of 20 µl
Master Mix	2 x 380 µl	4 x 380 µl
IC-internal control	1 x 500 µl	2 x 500 µl
PC—positive control	1 x 100 µl	1 x 200 µl
Water, nuclease free	1 x 500 µl	1 x 500 µl

Storage

Store the components at -20°C in the dark.
Repeated thawing and freezing (> 2x) should be avoided, as this may reduce the sensitivity.

Description

- Anaplasma/Ehrlichia qPCR Detection Kit is a ready-to-use system for the detection of *Anaplasma/Ehrlichia spp.* DNA in animal tissue samples (tick, mammal).
- The kit contains reagents and primers for the amplification of a region of the Anaplasma/Ehrlichia genome. The detection of the Anaplasma/Ehrlichia amplicon is based on using the specific FAM-labelled probe.
- In addition, the kit contains a second heterologous amplification system to monitor the DNA isolation procedure and identify possible PCR inhibition simultaneously. The internal control system consists of: primers, the HEX-labelled probe present in Master Mix and exogenous DNA fragment (IC-internal control). The amplification of the inner control does not interfere with the amplification of the specific Anaplasma/Ehrlichia DNA and confirms DNA isolation and/or PCR reaction correctness.
- IC-internal control can be added at the DNA isolation stage (point a) - then it is both a control of the DNA isolation and PCR inhibition, or only at the PCR stage (point b) - to assess the quality of the purified DNA and possible PCR inhibition.

a. Add IC to the lysis buffer or to the mixture of lysis buffer and sample material (do not add to the sample material directly) at a ratio 0,1 µl per 1 µl elution volume. For example, 5 µl of IC should be added initially using 50 µl elution volume. It is recommended to use carrier RNA when isolating DNA from cell-free body fluids and material low in DNA/RNA content. Carrier RNA increases the efficiency of DNA binding to silica membranes in the presence of small amounts of genetic material in the sample.

b. Add 0.5 µL of IC for every 20 µL of PCR reaction.

- Master Mix contains onTaq DNA Polymerase, optimized reaction buffer and dNTPs.
- onTaq DNA Polymerase is a modified “hot start” enzyme that is blocked at moderate temperatures and allows room temperature reaction setup.
- The polymerase activity is restored during the initial denaturation step when amplification reactions are heated at 95°C for 15 minutes.
- ROX passive reference dye included in the Master Mix allows fluorescence normalization on certain cyclers . The use of ROX passive reference dye is necessary for all real-time PCR cyclers from Applied Biosystems and optional for cyclers from Stratagene. ROX compensates for variations of fluorescent signal between wells due to slight differences in reaction volume and fluorescence fluctuations. ROX is not involved in PCR reaction and does not interfere with real-time PCR on any instrument.
- DNA isolation from animal tissues should be performed with dedicated, commercially available kits using silica/magnetic beads technology.

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Protocol

Preparation of PCR Reaction:

Component	Negative Amplification Control -NAC	Negative Extraction Control-NEC	Positive Extraction Control-PEC	Sample DNA	Positive Control Anaplasma/Ehrlichia -TPC
Master Mix	15 µl	15 µl	15 µl	15 µl	15 µl
IC-internal control (optional)	-	-	-	0.5 µl	0.5 µl
PC-positive control	-	-	-	-	5 µl
Sample of: extracted H_2O/ extracted IC extracted DNA	- - -	5 µl - -	- 5 µl -	- - 5 µl	- - -
Water, nuclease free	5 µl	-	-	-	-
Total volume	20 µl	20 µl	20 µl	20-20.5 µl	20-20.5 µl

Notes:

1. A reaction volume of 20 µl should be used with most real-time cyclers. Other reaction volumes may be used if recommended for a specific instrument.
2. Thaw, gently vortex and briefly centrifuge all solutions. Keep the Master Mix on ice.
3. Set up PCR reactions at room temperature according to the table above. Use of the Master Mix allows room temperature reaction setup.
4. Add:

- 5 µl of water (negative amplification control-NAC),
- 5 µl of water purified according to the DNA isolation procedure (negative extraction control-NEC),
- 5 µl of IC purified according to the DNA isolation procedure (positive extraction control-PEC),
- 5 µl of DNA (≤500 ng/reaction) purified according to the DNA isolation procedure (sample DNA),
- 5 µl of PC (positive control, Anaplasma/Ehrlichia-TPC).

5. Anaplasma/Ehrlichia-TPC gives a signal in the FAM channel only, when no IC-internal control was added to the reaction. To obtain a signal in both the FAM and HEX channels, add 0.5 µl of IC to the reaction. Increasing the PCR reaction volume for the Anaplasma/ Ehrlichia-TPC by 0.5 µL to 20.5 µL (when preparing one reaction mix for DNA samples and controls) does not adversely affect the efficiency of the PCR reaction.
6. Centrifuge briefly to settle down the reaction components and remove bubbles. Bubbles interfere with fluorescent detection.
7. Place the samples in the cycler and start the program.
8. Perform data analysis.

Thermal Cycling Conditions:

Step	Temperature	Time	Number of Cycles
Initial Denaturation	95°C	15 min	1
Denaturation	95°C	10 s	45
Annealing/Extension	60°C	60 s	
Cooling	4°C	Indefinite	1

Notes:

1. Run PCR reactions using standard ramp rate (standard mode).
2. During the initial denaturation step onTaq DNA Polymerase is activated. onTaq DNA polymerase requires 15 min incubation at 95°C to restore activity.
3. Fluorescence detection should be set at the end of the extension step and executed using FAM and HEX channels. FAM channel detects Anaplasma/Ehrlichia DNA and HEX channel detects the inner control.

Results Interpretation:

Sample type	FAM channel	HEX channel	Result
Negative Amplification Control -NAC	-	-	correct
Negative Extraction Control-NEC	-	-	correct
Positive Extraction Control-PEC	-	+	correct
Positive Control, Anaplasma/Ehrlichia-TPC (- IC) (+ IC)	+ +	- +	correct
1	-	+	negative Anaplasma/ Ehrlichia^{-}
2	+	+	positive Anaplasma/ Ehrlichia^{+}
3	+	-	positive Anaplasma/ Ehrlichia^{+}
4	-	-	incorrect

Notes:

1. A test can be considered valid/correct if:
- negative amplification control-NAC does not give a signal in both the FAM and HEX channels,
- negative extraction control-NEC does not give a signal in both the FAM and HEX channels,
- positive extraction control (when IC was purified according to the DNA isolation procedure) gives a signal only in the HEX channel,
- positive control, Anaplasma/Ehrlichia-TPC gives a signal only in the FAM channel when no IC was added to the reaction or in both the FAM and HEX channels when IC was added to the reaction.

2. The sample is positive (Anaplasma/Ehrlichia^{+}) and the test is valid if:
- the conditions of point 1 are met,
- the sample gives a signal in the FAM channel regardless of any signal n the HEX channel.

   In some circumstances, when the reaction is strongly positive (Anaplasma/Ehrlichia^{+}), the sample may give a weak or no signal in the HEX channel. The reason for this is competition between Anaplasma/Ehrlichia and the internal control DNA amplification reactions.

3. The sample is negative (Anaplasma/Ehrlichia^{-}) and the test is valid if:
- the conditions of point 1 are met,
- the sample gives a signal only in the HEX channel.

4. The presence of a fluorescence signal in the HEX channel for a given sample indicates that the DNA isolation process and PCR reaction were correct (in the case of adding IC to the DNA isolation process), or only that the PCR reaction was not inhibited (in the case of adding IC to the PCR reaction mixture). Very high C_T values (> 34) in the HEX channel may indicate a partial inhibition of the PCR reaction. In turn, the complete absence of a signal in the HEX channel with the simultaneous lack of a signal in the FAM channel may indicate complete inhibition of the PCR reaction or failure to add DNA to the PCR reaction with the simultaneous addition of IC in the DNA isolation process. In this case, it is recommended to dilute the sample 10 times or repeat the DNA isolation and rerun the PCR reaction.
5. Lack of signal for the Anaplasma/Ehrlichia-TPC indicates an error in PCR preparation.

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