Manual

MutaPLEX® Ehrlichia

real time PCR kit

For the in vitro detection of Ehrlichia and Anaplasma DNA, extracted from clinical specimens and ticks.

Valid from 2018-11-19

REF **KG192532**
KG192596

Σ
32/96

-20 °C

IVD

CE

Immundiagnostik AG, Stubenwald-Allee 8a, 64625 Bensheim, Germany
Tel.: +49 6251 70190-0 Fax: + 49 6251 70190-363
e.mail: info@immundiagnostik.com www.immundiagnostik.com

Table of Contents

1 INTENDED USE

The MutaPLEX® Ehrlichia real time PCR kit is a screening assay for the detection of Ehrlichia and Anaplasma DNA (*E. chaffeensis, E. ewingii, E. canis, A. phagocytophilum, A. platys*), extracted from clinical specimens and ticks

2 BACKGROUND INFORMATION

Ehrlichia chaffeensis, an obligate intracellular bacterium, is the causative agent of human monocytotropic ehrlichiosis (HME). The pathogen is transmitted by hard ticks such as *Amblyomma americanum*.

The most common symptoms of HME are fever, headache, malaise, and muscle aches (myalgia). Compared to human granulocytic ehrlichiosis (HGE), rash is more common. Laboratory abnormalities include thrombocytopenia, leukopenia, and elevated liver tests. The severity of the illness can range from minor or asymptomatic to life-threatening. CNS involvement may occur. A serious septic or toxic shock-like picture can also develop, especially in patients with impaired immunity.

Anaplasma phagocytophilum (formerly *Ehrlichia phagocytophila, E. equi*) and *Ehrlichia ewingii* causes human granulocytic ehrlichiosis. This disease was first identified in 1990, although this pathogen was known to cause veterinary disease since 1932. Since 1990, incidence of this disease has increased, and it is now recognised in Europe. This disease was first identified due to a Wisconsin patient who died with a severe febrile illness two weeks after a tick bite. During the last stage of the infection, a group of small bacteria were seen within the neutrophils in the blood. Other symptoms include fever, headache, absence of skin rash, leucopenia, thromboctytopenia and mild injury to the liver.

Analysis of ticks offers the possibility to identify the risk of infection very quickly, and therefore minimising the delay of an antibiotics treatment.

3 PRINCIPLE OF THE TEST

The MutaPLEX® Ehrlichia real time PCR contains specific primers and dual-labelled probes for the amplification and detection of Ehrlichia/Anaplasma DNA, extracted from clinical specimens and ticks.

The presence of nucleic acids is detected by an increase in fluorescence due to hydrolysis of the probes during amplification.

The fluorescence of the pathogen-specific probes is measured in the FAM channel.

Furthermore, MutaPLEX® Ehrlichia real time PCR kit contains a control DNA, which is added during DNA extraction and detected in the same reaction by a differently labelled probe.

The control DNA allows the detection of PCR inhibition and acts as control for the isolation of the nucleic acid from the clinical specimen.

The fluorescence of the control DNA is measured in the VIC/HEX/JOE/TET channel.

4 PACKAGE CONTENTS

The reagents supplied are sufficient for 32 or 96 reactions respectively.

Table 1: Components of the MutaPLEX® Ehrlichia real time PCR kit

Label	Lid colour	Content	
		KG192532	KG192596
Reaction mix	yellow	1 x 512 µl	2 x 768 µl
Positive control	red	1 x 50 µl	1 x 100 µl
Negative control	green	1 x 50 µl	1 x 100 µl
Control DNA	colourless	1 x 160 µl	2 x 240 µl

5 EQUIPMENT AND REAGENTS TO BE SUPPLIED BY USER

- DNA isolation kit (e.g. MutaCLEAN® Universal RNA/DNA, KG1038, or NukEx® Complete Mag RNA/DNA, KG1020)
- PCR grade water
- Sterile microtubes
- Pipets (adjustable volume)
- Sterile, DNase/RNase-free disposable pipet tips with aerosol barriers
- Table centrifuge
- Vortex
- Real time PCR instrument
- Optical PCR reaction tubes or optical PCR reaction plates
- Optional: Liquid handling system for automation

6 TRANSPORT, STORAGE AND STABILITY

The MutaPLEX® Ehrlichia real time PCR kit is shipped on dry ice or cool packs. All components must be stored at maximum < -20 °C in the dark immediately after receipt. Do not use reagents after the date of expiry printed on the package. Up to 20 freeze and thaw cycles are possible.

For convenience, opened reagents can be stored at 2–8 °C for up to 6 months.

Protect kit components from direct sunlight during the complete test run.

7 WARNINGS AND PRECAUTIONS

- The MutaPLEX® Ehrlichia real time PCR must be performed by qualified personnel only.
- Good Laboratory Practice (GLP) has to be applied.
- Clinical samples must always be regarded as potentially infectious material and all equipment used has to be treated as potentially contaminated.
- Stick to the protocol described in the instruction for use.
- Set up different laboratory areas for the preparation of samples and for the set up of the PCR in order to avoid contaminations.
- Pipettes, tubes and other materials must not circulate between those different laboratory areas.
- Always use filter tips.
- Regulary decontaminate equipment and benches with ethanol-free decontaminant.
- Do not combine MutaPLEX® Ehrlichia real time PCR kit components of different lot numbers.

8 SAMPLE PREPARATION

Starting material for the MutaPLEX® Ehrlichia real time PCR is DNA isolated from clinical specimens (e.g. EDTA-blood, plasma, serum, cerebrospinal fluid and tissue samples) or from ticks.

The MutaPLEX® Ehrlichia real time PCR is suitable for the detection of Ehrlichia/ Anaplasma DNA isolated from clinical specimens or ticks with appropriate isolation methods.

Commercial kits for DNA isolation such as the following are recommended:

- MutaCLEAN® Universal RNA/DNA, KG1038
- NukEx® Complete Mag RNA/DNA, KG1020

It is recommended to use mechanical disruption of ticks before DNA extraction. Please follow the instructions for use of the respective extraction kit.

Important:

In addition to the samples always run a water control in your extraction. Treat this water control analogously to a sample.

Comparing the amplification of the control DNA in the samples to the amplification of the internal control in the water control will give insights on possible inhibitions

of the real time PCR. Furthermore, possible contaminations during DNA extraction will be detectable.

Please note the chapter "Control DNA".

If the real time PCR is not performed immediately, store extracted DNA according to the instructions given by the manufacturer.

9 CONTROL DNA

A control DNA is supplied and can be used as extraction control or only as inhibition control. This allows the user to control the DNA isolation procedure and to check for possible real time PCR inhibition.

DNA isolation from EDTA-blood, plasma, serum, cerebrospinal fluid and tissue samples

9.1 Control DNA used as extraction control

MutaPLEX® Ehrlichia real time PCR control DNA is added to the DNA extraction.

Add **5 µl** control DNA per extraction (5 µl x (N+1)). Mix well. Perform the DNA isolation according to the manufacturer's instructions. Please follow protocol A.

The Control DNA must be added to the lysis buffer of the extraction kit.

9.2 Control DNA used as internal control of the real time PCR

If only inhibition will be checked please follow protocol B.

10 REAL TIME PCR

10.1 Important points before starting:

- Please pay attention to the chapter **„**Warnings and Precautions**"**.
- Before setting up the real time PCR, familiarise yourself with the real time PCR instrument and read the user manual supplied with the instrument.
- The programming of the thermal profile should take place before the PCR set up.
- In every PCR run, a positive control and a negative control should be included.
- Before each use, all reagents should be thawed completely at room temperature, thouroughly mixed, and centrifuged very briefly.

- We recommend to keep reagents and samples at 2–8 °C (e. g. on ice or a cooling block) at all times.

10.2 Procedure

If the control DNA is used to control both, the real time PCR and the DNA isolation procedure, please follow protocol A. If the control DNA is solely used to detect possible inhibition of the real time PCR, please follow protocol B.

Protocol A

The control DNA was added during DNA extraction ("Control DNA"). In this case, prepare the master mix according to table 2.

The master mix contains all of the components needed for PCR except the sample. Prepare a volume of master mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 2: Preparation of the master mix (control DNA was added during DNA extraction)

Volume per reaction	Volume master mix
16 µl reaction mix	16 µl x (N+1)

Protocol B

The control DNA is used for the control of the real time PCR only ("Control DNA"). In this case, prepare the master mix according to table 3.

The master mix contains all of the components needed for real time PCR except the sample. Prepare a volume of master mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 3: Preparation of the master mix (control DNA is added directly to the master mix)

Volume per reaction	Volume master mix
16.0 µl reaction mix	16.0 µl x (N+1)
0.5 µl control DNA*	0.5 µl x (N+1)*

*The increase in volume caused by adding the control DNA is not taken into account when preparing the PCR assay.

Protocol A and B: real time PCR set up

- Place the number of optical PCR reaction tubes needed into the respective tray of the real time PCR instrument.
- Pipet **16 µl** of the master mix into each optical PCR reaction tube.
- Add **4 µl** of the eluates from the DNA isolation (including the eluate of the water control), the positive control and the negative control to the corresponding optical PCR reaction tube (table 4).

- Close the optical PCR reaction tubes immediately after filling in order to reduce the risk of contamination.

Table 4: Preparation of the real time PCR component volume

Component	Volume
Master mix	16.0 µl
Sample	4.0 µl
Total volume	20.0 µl

10.3 Instrument settings

For the real time PCR use the thermal profile shown in table 5.

Table 5: real time PCR thermal profile

Description	Time	Temperature	No of cycles
Initial Denaturation	10 min	95 °C	1
Amplification of DNA			45
Denaturation	10 s	95 °C	
Annealing	20 s	60 °C	
	Aquisition at the end of this step		
Extension	10 s	72 °C	

If in the same run samples should be tested for pathogens with RNA genome, e.g. with the MutaPLEX® TBE real time RT-PCR kit, use the thermal profile shown in table 6.

Table 6: real time RT-PCR thermal profile

Description	Time	Temperature	No of cycles
Reverse Transcription	20 min	45 °C	1
Initial Denaturation	5 min	95 °C	1
Amplification of DNA			45
Denaturation	10 s	95 °C	
Annealing	40 s	60 °C	
	Aquisition at the end of this step		
Extension	10 s	72 °C	

Dependent on the real time instrument used, further instrument settings have to be adjusted according to table 7.

Table 7: Overview of the instrument settings required for the MutaPLEX® Ehrlichia real time PCR.

Real time PCR instrument	Parameter	Detection channel	Notes	
LightCycler 480I	Ehrlichia	483-533	pre-installed universal CC FAM (510) – VIC (580)	
	Control DNA	523-568		
LightCycler 480II	Ehrlichia	FAM (465-510)		
	Control DNA	HEX (533-580)		
Stratagene Mx3000P / Mx3005P	Ehrlichia	FAM	Gain 8	Reference Dye: None
	Control DNA	HEX	Gain 1	
ABI 7500	Ehrlichia	FAM	Option Reference Dye ROX: NO	
	Control DNA	JOE		
Rotor-Gene Q, 3000 and 6000	Ehrlichia	Green	Gain 5	
	Control DNA	Yellow	Gain 5	
mic qPCR Cycler	Ehrlichia	Green	Gain 8	
	Control DNA	Yellow	Gain 10	

11 DATA ANALYSIS

The Ehrlichia/Anaplasma specific amplification is measured in the FAM channel. The amplification of the control DNA is measured in the VIC/HEX/JOE/TET channel.

Following results can occur:

A signal in the FAM channel is detected:

The result is positive, the sample contains Ehrlichia/Anaplasma DNA.

In this case, detection of a signal of the Control DNA in the VIC/HEX/JOE/TET channel is inessential, as high concentrations of bacterialDNA may reduce or completely inhibit amplification of the control DNA.

No signal in the FAM channel, but a signal in the VIC/HEX/JOE/TET channel is detected:

The result is negative, the sample does not contain *Ehrlichia/Anaplasma* DNA.

The signal of the control DNA excludes the possibilities of DNA isolation failure (in case the control DNA is being used as an extraction control) and/or real time PCR inhibition. If the C_T value of a sample differs significantly from the C_T value of the water control, a partial inhibition occured, which can lead to negative results in weak positive samples (see chapter „Troubleshooting“).

Neither in the FAM nor in the VIC/HEX/JOE/TET channel a signal is detected:

A diagnostic statement cannot be made.

The DNA isolation was not successful or an inhibition of the PCR has occurred. In case the control DNA was added during DNA isolation and not directly to the PCR master mix, the negative control is negative in both channels.

Figure 1 and Figure 2 show examples for positive and negative real time PCR results.

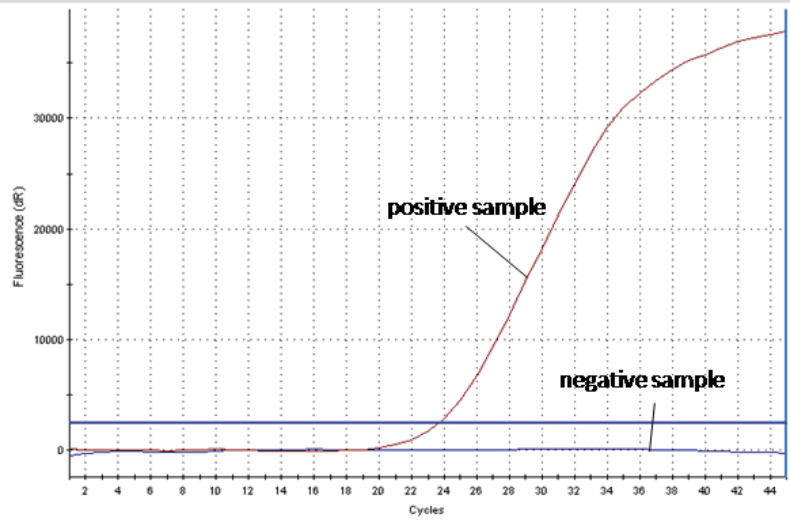

Figure 1: The positive sample shows specific amplification in the FAM channel, whereas no fluorescence signal is detected in the negative sample.

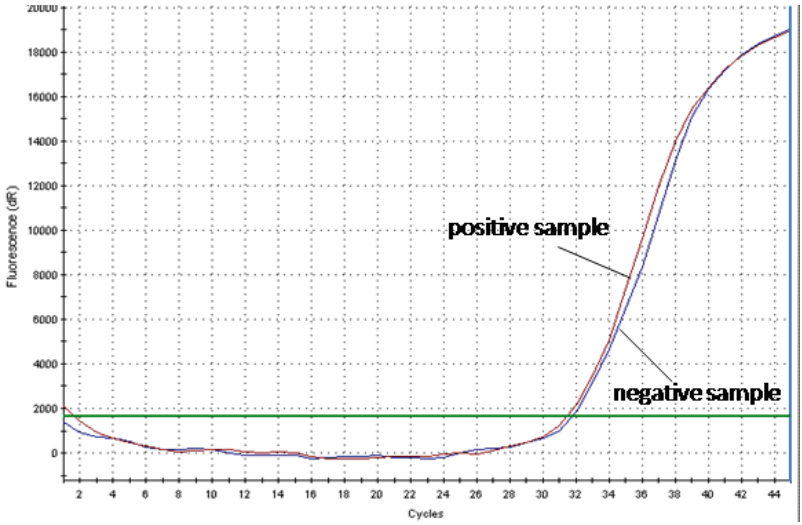

Figure 2: The positive sample as well as the negative sample show a signal in the control DNA-specific VIC/HEX/JOETM/TET channel. The amplification signal of the control DNA in the negative sample shows, that the missing signal in the bacteria-specific FAM channel is not due to PCR inhibition or failure of DNA isolation, but that the sample is a true negative.

12 ASSAY VALIDATION

Set a threshold as follows:

Negative controls

All negative controls should be below the threshold. If there is a potential contamination (appearance of a curve in the negative control or a cluster of curves in specimens at high C_T – for example above 36), results obtained are not interpretable and the whole run (including extraction) has to be repeated.

Positive controls

All the positive controls must show a positive (i.e. exponential) amplification curve. The positive controls must fall below a C_T of 30.

Internal controls

All internal controls must show a positive (i.e. exponential) amplification curve. The internal control must fall below a C_T of 33. If the internal control is above C_T 34, this points to a purification problem or a strong positive sample that can inhibit the internal control. In the latter case, the assay is valid. If a water control run is performed, the internal control must fall below a C_T of 33.

13 LIMITATIONS

The results must always be considered in relation to the clinical symptoms. Therapeutical consequences should be made in consideration of clinical data.

A negative test result does not exclude an Ehrlichia/Anaplasma infection.

14 TROUBLESHOOTING

The following troubleshooting guide is included to help you with possible problems that may arise when performing a real time PCR. If you have further questions, please do not hesitate to contact our scientists on info@immundiagnostik.com.

No fluorescence signal in the FAM channel of the positive control	
The selected channel for analysis does not comply with the protocol	Select the FAM channel for analysis of the bacteria specific amplification and the VIC/HEX/JOE/TET channel for the amplification of the control DNA.
Incorrect configuration of the real time PCR	Check your work steps and compare with "Procedure"
The programming of the thermal profile is incorrect	Compare the thermal profile with the protocol (table 5).
Incorrect storage conditions for one or more kit components or kit expired	Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in "Transport, Storage and Stability"

Weak or no signal of the Control DNA and simultaneous absence of a signal in the bacteria specific FAM channel.	
real time PCR conditions do not comply with the protocol	Check the real time PCR conditions.
real time PCR inhibited	Make sure that you use an appropriate isolation method (see chapter 'Sample Preparation') and follow the manufacturer's instructions. Make sure that the ethanol-containing washing buffers have been completely removed. An additional centrifugation step at high speed is recommended before elution of the DNA.
DNA loss during isolation process	In case the control DNA was added before extraction, the lack of an amplification signal can indicate that the DNA isolation was not successful. Make sure that you use an appropriate isolation method (commercial kits are recommended) and stick to the manufacturer's protocol.
Incorrect storage conditions for one or more components or kit expired	Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in "Transport, Storage and Stability".

Detection of a fluorescence signal in the FAM channel of the negative control	
Contamination during preparation of the PCR	Repeat the real time PCR in replicates. If the result is negative in the repetition, the contamination occured when the samples were pipetted into the optical PCR reaction tubes. Make sure to pipet the positive control last and close the optical PCR reaction tube immediately after adding the sample. If the same result occurs, one or more of the kit components might be contaminated. Make sure that work space and instruments are decontaminated regularly. Use a new kit and repeat the real timePCR.

15 KIT PERFORMANCE

15.1 Analytical sensitivity

The limit of detection (LoD) of MutaPLEX® Ehrlichia real time PCR was determined using serial dilutions of a synthetic DNA-fragment containing the Ehrlichia target sequence in a Stratagene Mx3000 real time PCR instrument. The results of the determinations in triplicates for the Ehrlichia system within the MutaPLEX® Ehrlichia real time PCR kit are shown in table 8.

The LoD of MutaPLEX® Ehrlichia real time PCR for Ehrlichia is > 10 genome copies per reaction each.

Table 8: Determination of the analytical sensitivity of the Ehrlichia system within the MutaPLEX® Ehrlichia real time PCR kit.

Copies per reaction	C_T-value FAM	Mean C_T FAM
1.000.000	18,55	18,08
	18,15	
	17,53	
100.000	20,97	20,64
	20,39	
	20,57	
10.000	25,07	24,55
	24,00	
	24,59	
1.000	27,74	27,67
	27,66	
	27,62	
100	31,21	31,16
	31,69	
	30,57	
10	34,07	33,95
	34,79	
	32,98	

15.2 Analytical specificity

The specificity of the MutaPLEX® Ehrlichia real time PCR was evaluated with different other relevant viruses and bacteria found in clinical samples.

Results:

The MutaPLEX® Ehrlichia real time PCR kit showed a positiv result for the sample containing Ehrlichia and Anaplasma, whereas samples containing other pathogens were reliably tested negative. The results are shown in table 9.

Table 9: Bacterial and viral pathogens tested for the determination of the analytical specificity of MutaPLEX® Ehrlichia real time PCR.

Strain	Expected result	Result
Enterovirus 68	negative	negative
Coxsackievirus B3	negative	negative
Influenza virus A A/ Brisbane H1N1 59/2007 E40/08	negative	negative
Influenza virus B B/ Brisbane 60/2008 E09/09	negative	negative
TBEV (Tick Borne Encephalitis virus)	negative	negative
Coxiella burnetii	negative	negative
West Nile virus	negative	negative
Borrelia burgdorferi strain 4681	negative	negative
Borrelia burgdorferi sensu stricto	negative	negative
Borrelia afzelii	negative	negative
Borrelia garinii	negative	negative
Borrelia spielmanii	negative	negative
Borrelia bavariensis	negative	negative
Borrelia bisettii	negative	negative
Borrelia lustianae	negative	negative
Borrelia valaisiana	negative	negative
Borrelia kurtenbachii	negative	negative

Strain	Expected result	Result
Borrelia japonica	negative	negative
Borrelia miyamotoi	negative	negative
Treponema phagedenis	negative	negative
Leptospira	negative	negative
Babesia caballi	negative	negative
Babesia canis canis	negative	negative
Babesia canis vogeli	negative	negative
Babesia duncani	negative	negative
Babesia equi	negative	negative
Babesia gibsoni	negative	negative
Babesia divergens	negative	negative
Babesia microti	negative	negative
Babesia sp. EU1	negative	negative
Ehrlichia chaffeensis	positive	positive
Ehrlichia ewingii	positive	positive
Ehrlichia canis	positive	positive
Ehrlichia phagocytophilum	positive	positive
Anaplasma platys	positive	positive

15.3 Diagnostic sensitivity

The diagnostic sensitivity of real time (RT-) PCR assays is mainly dependent on the DNA/RNA extraction method used to isolate DNA and RNA from various clinical specimens. DNA/RNA extraction reagents are not part of the MutaPLEX® real time (RT-) PCR kits. MutaPLEX® real time (RT-) PCR kits include an extraction control and guidelines for the validation criteria of the extraction control in each reaction. The extraction control indicates inhibition of the real time (RT-) PCR and/or inefficient nucleic acid extraction. It cannot be used as a calibrator.

Therefore, Immundiagnostik AG guarantees the analytical sensitivities and specificities of the real time (RT-) PCR kits, performed with eluted DNA and RNA from reference materials and ring trial samples and with synthetic nucleic acid fragments. Immundiagnostik AG does not guarantee diagnostic sensitivities. If diagnostic sensi-

tivities are mentioned in manuals of MutaPLEX® real time (RT-) PCR kits, the data are strictly correlated to a specific nucleic acid extraction method that has been used during the validation of the respective kits and cannot be transferred to other extraction methods.

It is the responsibility of the user, to qualify the extraction methods used for DNA/RNA isolation from clinical samples.

16 ABBREVIATIONS AND SYMBOLS

DNA	Deoxyribonucleid acid	REF	Catalog number
PCR	Polymerase chain reaction	→REF	To be used with
IVD	*In vitro* diagnostic medical device	Σ	Contains sufficient for <n> test
CONT	Content		Upper limit of temperature
LOT	Lot number		Manufacturer
CONTROL +	positive control		Use by YYYY-MM-DD
CONTROL –	negative control		Consult instructions for use
CONTROL DNA IC	control DNA	CE	European conformity
REACTION MIX	Reaction mix		

17 LITERATURE

1. Dumler (J.S.), Barbet (A.F.), Bekker (C.P.J.), Dasch (G.A.), Palmer (G.H.), Ray (S.C.), Rikihisa (Y.) and Rurangirwa (F.R.): Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of Ehrlichia with Anaplasma, Cowdria with Ehrlichia and Ehrlichia with Neorickettsia, description of six new species combinations and designation of Ehrlichiaequi and 'HGE agent' as subjective synonyms of Ehrlichiaphagocytophila. Int. J. Syst. Evol. Microbiol. 2001, 51, 2145-2165.

Immundiagnostik AG

Stubenwald-Allee 8a
64625 Bensheim, Germany

Tel.: +49 6251 70190-0
Fax: +49 6251 70190-363

info@immundiagnostik.com
www.immundiagnostik.com