RealLine Anaplasma / Ehrlichia Fla-Format

Instructions for Use

RealLine Anaplasma / Ehrlichia Fla-Format

KIT FOR THE QUALITATIVE DETECTION OF DNA OF *ANAPLASMA PHAGOCYTOPHILUM*, *EHRLICHIA MURIS* AND *EHRLICHIA CHAFFEENSIS* BY REAL TIME PCR

In vitro Diagnostics

CE

RealLine Anaplasma / Ehrlichia (Fla-Format)	**VBD5399**	**50 Tests**
valid from	**September 2019**	

BIORON Diagnostics GmbH
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RealLine Anaplasma / Ehrlichia Fla-Format

Explanation of symbols used in labeling

IVD	*In vitro* diagnostic medical device
LOT	Batch code
REF	Catalogue number
	Contains sufficient for <n> tests
	Use-by-date
	Temperature limit
	Consult instructions for use
	Keep away from sunlight
	Manufacturer

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KIT FOR THE QUALITATIVE DETECTION OF DNA OF *ANAPLASMA PHAGOCYTOPHILUM*, *EHRLICHIA MURIS* AND *EHRLICHIA CHAFFEENSIS* BY REAL TIME PCR

In vitro diagnostic

1. INTENDED USE

Clinical information:

HGA - human granulocytic anaplasmosis - is a disease which is caused by *Anaplasma phagocytophilum* and **Ehrlichiosis (HME)** is caused by *Ehrlichia chaffeensis* or *Ehrlichia muris*. Humans and other mammals are infected by bites of ticks. If the disease is symptomatic it can come to flu-like symptoms up to serious symptoms like meningoencephalitis especially in immunosupressed patients.

RealLine Anaplasma / Ehrlichia (Fla-format) assay kit is designed to detect DNA of *Anaplasma phagocytophilum* and DNA of *Ehrlichia muris* and *Ehrlichia chaffeensis* isolated from clinical specimens using DNA extraction kit **RealLine Extraction 100 (REF VBC8896)**.

The assay is based on the real-time polymerase chain reaction (PCR) method with fluorescent detection of the amplified product.

RealLine Anaplasma / Ehrlichia kit is designed for the analysis of clinical materials such as, leukocyte fractions, blood serum (plasma), biopsy material, and tick suspension samples.
The results of PCR analysis are taken into account in complex diagnostics of disease.

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The **Fla-format** Kit contains 5 vials with the lyophilized Mastermix, each vial with 10 reactions, for volume of 50 µl per reaction. The kit contains reagents required for 50 tests, including the positive control samples.

The kit is designed for use with block cyclers iQ iCycler, iQ5 iCycler, CFX96 (Bio-Rad, USA), RealLine Cycler (BIORON Diagnostics GmbH), DT96 (DNA-Technology, Russia); and rotor type cyclers Rotor-Gene® 3000, Rotor-Gene® 6000 and Rotor-Gene® Q (Qiagen, Germany).

The use of:

- **Extraction Kits for nucleic acids from clinical specimen from other supplier**
- **other real-time PCR devices**
- **appropriate reaction volumes, other than 50 µl**

has to be validated in the lab by the user. The special notes regarding the internal control IC have to be strongly followed.

2. KIT CONTENTS

Positive Control Sample (PC)	1 tube, 1 ml
Master Mix (MM) for PCR, lyophilized	5 tubes (10 tests each)
Recovery Solution (RS)	1 vial, 2 ml
Solution for Sample Preparation (SSP)	4 vials, 4 ml each

3. PRINCIPLE OF THE METHOD

Real time PCR is based on the detection of the fluorescence, produced by a reporter molecule, which increases as the reaction proceeds. Reporter molecule is dual-labeled DNA-probe, which specifically binds to the target region of pathogen DNA. Fluorescent signal increases due to the fluorescent dye and quencher separating by Taq DNA-polymerase exonuclease activity during amplification. PCR process consists of repeated cycles: temperature denaturation of DNA, primer annealing and complementary chain synthesis.

Threshold cycle value - Ct – is the cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold, a fluorescent signal rises significantly above the background fluorescence. Ct depends on initial quantity of pathogen DNA template.

The use of **Internal Control (IC)** prevents generation of false negative results associated with possible loss of DNA template during specimen preparation. IC indicates if PCR inhibitors occur in the reaction mixture. IC template should be added in each single sample (including control samples) prior to DNA extraction procedure. The amplification and detection of IC does not influence the sensitivity or specificity of the target DNA PCR.

Note: IC is a component of the NA extraction kits of RealLine series. Internal Control is added to the sample during NA isolation step and is used throughout the whole process of NA extraction, amplification, detection.

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4. SPECIFICATIONS

4.1. Sensitivity:

Sensitivity control was performed on 5 samples containing 100 copies of *Anaplasma phagocytophilum* DNA and *Ehrlichia muris, Ehrlichia chaffeensis* DNA per sample, prepared from SRS (Standard Reference Sample containing *A.phagocytophilum* DNA and *E.muris, E.chaffeensis* DNA). The sensitivity equals 100 %.

4.2. Specificity:

Specificity of *Anaplasma phagocytophilum* DNA and *Ehrlichia muris, Ehrlichia chaffeensis* DNA detection was determined using Standard Reference Panel of negative samples. Specificity equals 100 %.

All samples were analyzed in **RealLine Anaplasma** / **Ehrlichia (Fla-format)** assay kit and a CE-marked Reference Kit.

5. PRODUCT USE LIMITATIONS

- This assay must not be used on the clinical specimen directly. Appropriate nucleic acids extraction methods have to be conducted prior to using this assay.
- The presence of PCR inhibitors (e.g. heparin) may cause false negative or invalid results.
- When monitoring a patient the same extraction method must be used in all determinations. Otherwise, results may not be comparable.
- The kit is designed for use in patients with a clinical history and/or symptoms consistent with *Anaplasma* and/or *Ehrlichia.* infections. The kit may be used for screening purposes.
- Diagnostic sensitivity of the kit may vary depending on the pathogen prevalence and characteristics of the enrolled cohort.
- Reliable results depend on adequate specimen sampling.
- Positive results indicate active or asymptomatic infection; clinical history and symptoms should be taken into account.
- Negative results indicate lack of detectable DNA but do not exclude the infection or disease.
- Potential mutations within the target regions of the *Anaplasma phagocytophilum* and/or *Ehrlichia chaffeensis* and *E. muris* genome covered by the primers and/or probes used in the kit may result in failure to detect the presence of the pathogens.
- The kit is not intended to replace culture and other methods (e.g., cervical exam) for diagnosis of infections.

6. WARNING AND PRECAUTIONS

- For in vitro use only.
- The kits must be used by skilled personnel only.
- When handling the kit, follow the national safety requirements for working with pathogens.
- To prevent contamination, the stages of DNA isolation and PCR test run must be spatially separated.
- Wear protective disposable gloves, laboratory coats and eye protection when handling specimens and kit reagents.
- Every workplace must be provided with its own set of variable-volume pipettes, necessary auxiliary materials and equipment. It is prohibited to relocate them to other workplaces.
- To conduct real-time amplification reaction with PCR products detection, use only disposable tips with filters.
- Never use the same tips for different samples.
- Do not pool reagents from different lots or from different vials of the same lot.
- Dispose unused reagents and waste in accordance with country, federal, state and local regulations.
- Do not use the kit after the total expiration date at the side label of the box.

7. ADDITIONAL MATERIALS AND DEVICES REQUIRED BUT NOT SUPPLIED

- Real time PCR system, like described in paragraph 1
- DNA-Extraction Kit: RealLine Extraction 100
- Internal Control reagent (VBC8881), if the kit is used with the extraction kits of other supplier;
- Negative Control Sample, if the kit is used with the extraction kits of other supplier;
- Plates or Tubes suitable for the used device with caps or a sealing foil for PCR
- laminar safety box;
- refrigerator;
- microcentrifuge for 1.5 - 2 ml tubes;
- vortex mixer with adjustable rotation speed;
- half-automatic variable-volume single-channel pipettes with disposable tips;
- disposable medical non-sterile powder-free gloves;
- disposable pipette tips with filters;
- biohazard waste container.

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8. PREPARATION OF THE ANALYSED SAMPLES

The assay is performed on extracted DNA specimens obtained from the clinical material using the RealLine Extraction 100 kit, according the Instruction Manuals.

*Each group of samples undergoing the procedure of DNA isolation must include a **Positive Control sample (PC)** from this kit and a **Negative Control sample (NC)** that is a component of the DNA extraction kit.*
We strongly recommend the implementation of the Internal Control IC, the Negative Control NC and Positive Control PC samples to the extraction procedure.

When using a kit of another supplier for the extraction of nucleic acids as recommended in chapter 1, add **20 µl** of **IC (VBC8881)** to each tube.

- For the Negative Control NC use **100 µl** of the Negative Control Sample
- For the PC use **70 µl** of Negative Control Sample and **30 µl** of Positive Control to the tube marked PC.

If samples of isolated DNA were stored frozen prior the assay, thaw them and keep at least 30 minutes at a temperature of (18 – 25) °C.

The isolated DNA can be stored at (2 – 8) °C for no more than 24 hours.
After initial opening shelf life of Positive Control sample (CP) at (2 - 8) °C is 1 month or in 50 µl aliquots at (-18 …-60) 3 months.

8.1 Preparation of tick suspensions is described in Annex I.

8.2 Preparation of leukocyte fraction

Add 1.5 - 2.0 ml of the EDTA-stabilized whole blood to Eppendorf-type tube and centrifuge for 10 min at 800 rpm. Collect upper layer of plasma with leukocytes (approximately 500 µl); transfer it to a new clean tube and centrifuge at 13000 rpm for 10 min. Remove most of the supernatant, and use the cell pellet and 200 µl of the remaining supernatant to extract the NA using the **RealLine Extraction 100** kit according to the instruction manual.

8.3 Preparation of cerebrospinal fluid

Centrifuge the cerebrospinal fluid (1 - 1.5 ml) at 13000 rpm for 10 min. Transfer most of the supernatant to a container with disinfecting solution, and use the cell pellet and 200 µl of the remaining supernatant to extract the NA using the **RealLine Extraction 100** kit according to the instruction manual.

9. PROCEDURE

9.1. Preparation of the reagents

Prior the test take the kit out of the refrigerator and keep the Master Mix (MM) closed in the package at (18 – 25) °C for at least 30 minutes. Then open the package and take the necessary number of tubes with MM (including prepared specimen and controls). Put the remaining tubes immediately back into the foil pouch, squeeze the air out and tightly close with the clip.

Attention! 1 test tube with MM is intended for 10 assays.

Store the unused MM at (2 – 8) °C for 3 month

To prepare the diluted MM add **300 µl of recovery solution (RS)** to the each vial with MM, mix gently and keep at room temperature (18 – 25) °C for 15 min. Mix and collect the tube content by brief centrifugation..
*Store the recovered MM at (2 – 8) °C for no more **than 7 days**.*
After initial opening shelf life of Recovery Solution at (2 – 8) °C is 3 months.

9.2. Label the necessary number of 0.2 ml tubes (including necessary controls). Or prepare a plate for PCR.
***Attention!** Labels should be placed on the caps of tubes for rotor-type cyclers, for Blockcyclers labels should be placed on the lateral side of the tubes.*

9.3. Add **25 µl** of recovered MM to each tube using pipette tip with filter.

9.4. Add **25 µl** of corresponding isolated DNA solution to each tube using a separate pipette tip with filter. Do not capture the sediment! Add 25 µl of NC and PC to the corresponding tubes. Tightly close the tubes with caps, or the plates with PCR foils.

9.5. Place the tubes into the real-time PCR system

9.6. Program the real-time PCR sytem:
For Rotor-Gene 3000 (6000, Q):

Step 1:	50°C	2 min	
Step 2:	95°C	2 min	
Step 3:	94°C	10 sec	50 Cycles
	60°C*	40 sec	

* measurement of fluorescent at 60 °C

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For iQ iCycler, iQ5 iCycler, CFX96, DT-96 and RealLine Cyclers:

Step 1:	50°C	2min	
Step 2:	95°C	2min	
Step 3:	94°C	10 sec	50 cycles
	60°C*	20 sec	

* Measure the fluorescence at 60°C

9.7. Select the amplification detection channels:

- Collect data through the **FAM** channel (iQ iCycler, iQ5 iCycler, CFX96, RealLine Cycler, DT-96, Rotor-Gene 3000) and **Green** channel (Rotor-Gene 6000, Rotor-Gene Q) for detection of amplification signal of IC DNA;

- Collect data through the **HEX** channel (iQ iCycler, iQ5 iCycler, RealLine Cycler, CFX96, DT-96) and **JOE/Yellow** (Rotor-Gene 3000, Rotor-Gene 6000, Rotor-Gene Q) for detection of amplification signal of *Anaplasma phagocytophilum* DNA;

- Collect data through the **ROX** channel (iQ iCycler, iQ5 iCycler, CFX96, DT-96, Rotor-Gene 3000) and **Orange** (Rotor-Gene 3000, Rotor-Gene 6000, Rotor-Gene Q) for detection of amplification signal of *Ehrlichia muris* and *Ehrlihia chaffeensis* DNA.

9.8. Program the positions of test tubes with samples, positive and negative controls according to the instruction manual for the real time PCR system in use.

9.9. Run the program.

10. DATA ANALYSIS AND INTERPRETATION

10.1 The program for the Positive Control sample PC should detect:

- increase of the IC DNA amplification signal along channel **FAM/Green** and determine the threshold cycle, IC **Ct**;
- increase of the *Anaplasma phagocytophilum* DNA amplification signal along channel **JOE/HEX/Yellow** and determine the **Ct** value;
- increase of the *Ehrlichia muris* and *Ehrlichia chaffeensis* DNA amplification signal along channel **ROX/Orange** and determine the **Ct** value;

10.2 For **NC** the program should detect the increase of the amplification signal of **IC DNA** along channel **FAM/Green** and determine the threshold cycle, IC **Ct**. No significant **ROX/Orange** and **JOE/HEX/Yellow** fluorescent increase should appear (*no Anaplasma phagocytophilum, Ehrlichia muris or Ehrlichia chaffeensis DNA amplification*).

When **Ct** value for NC through **ROX/Orange** or **JOE/HEX/Yellow** channel **is less than or equal to 40,** this indicates the presence of contamination (see paragraph 9.10).

10.3 For each sample the program should detect an increase of the amplification signal of IC DNA along channel **FAM/Green** and determine IC **Ct**.

10.4 Calculate (IC **Ct**)$_{av}$ as an average IC **Ct** of all analyzed samples (including PC and NC). IC **Ct** values that differ by more than 2 from the (IC **Ct**)$_{av}$ should be ignored. Recalculate the (IC **Ct**)$_{av}$ for the remaining values after the screening.

10.5 The sample is considered **negative** (not containing *Anaplasma phagocytophilum, Ehrlichia muris* or *Ehrlichia chaffeensis* DNA), if **Ct** value via **ROX/Orange** and **JOE/HEX/Yellow** channels is **above 40** or is not determined.

When IC **Ct** value for such sample differs from the (IC **Ct**)$_{av}$ value by more than 2, the result is regarded as equivocal. A repeated analysis of the sample, starting with the DNA isolation step is necessary.

10.6 The sample is considered **positive**, i.e. contains *Anaplasma phagocytophilum* DNA, when **Ct** value via **JOE/HEX/Yellow** channel for this sample is **less than or equals to 40.**

The sample is considered **positive**, i.e. contains *Ehrlichia muris* and/or *Ehrlichia chaffeensis* DNA, when **Ct** value via **ROX/Orange** channel for this sample is **less than or equals to 40.**

10.7 In case of contamination all positive results of this individual PCR run are considered equivocal. Actions are required to identify and eliminate the source of contamination, and repeat the analysis of all samples of this run that were identified as positive. Samples that showed negative results in this run should be considered as negative.

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11. STORAGE AND TRANSPORTATION

- Store and transport the assay kit at (2 - 8) °C in the manufacturer's packing.
- Transport at (2 - 8) °C. Transportation at up to 25 °C for up to 10 days is allowed.
- Do not freeze the kit!
- Do not pool reagents from different lots or from different vials of the same lot.
- Strictly follow the Instruction manual for reliable results.
- Do not use kits with damaged inner packages and get in contact with BIORON Diagnostics GmbH.

Storage and shelf life of solutions and components of the kit after initial opening:

Positive Control sample: 1 month at (2 – 8) °C or aliquoted at (-18…-60) °C for 3 month.
Ready Master Mix (MM): unused MM at (2 – 8) °C for no more than 3 month
Diluted MM: at (2 – 8) °C for 7 days
Recovery Solution: at (2 – 8) °C for 3 months.

Technical support: techsupport@bioron.de

ANNEX I: PREPARATION OF TICK SUSPENSIONS

Equipment required:

- Laminar box;
- 1.5 ml tube rack;
- IsoFreeze double-sided refrigerator SSI-5610-43 or floating stand SSI-5100-43 (“SSI”, USA);
- Cooling element or a container with ice;
- Metal pestles for grinding ticks.

Place ticks or pools of ticks (*up to 10 unfed ticks; up to 3 soaked ticks*) of *Ixodes* genus into the numbered 1.5 ml tubes. *Dermacentor* genus ticks are studied separately.
To clean the ticks from contamination by substances used for the removal of attached insects, wash them before preparation of suspension (see p. a). In case of analysis of free ticks, the suspension can be prepared immediately (see p. b).

a) Preliminary washing of ticks:

Add 300 µl of 96% ethanol to each tube with a tick (or 500 µl when analyzing pools), vortex the tubes, and then centrifuge briefly. Remove ethanol using a pipette or an aspirator with a new tip for each specimen without touching the tick. Add 500 µl of 0.15 M sodium chloride to tubes, vortex the tubes and spin for 15 sec at 7000 rpm to collect any drops; discard the supernatant using a pipette or an aspirator with a new tip for each specimen.

b) Preparation of tick suspensions:

Attention! *To prevent degradation of NA extracted from the ticks, avoid heating of the specimen to a temperature above 8 °C at all stages of preparation of tick suspensions.*
To prepare tick suspension, use SSP pre-cooled to a temperature of (2 - 8) °C.

Method 1. Freeze the tubes with ticks in liquid nitrogen (for at least 5 min) or freezing chamber at minus (50-80) °C, for 20 min). Take one frozen tube and immediately carefully grind the tick with a separate sterile pestle combining rotational movements and pressing. To prepare suspension, add the appropriate amount of SSP to the tube with crushed tick, do not remove the pestle. For single *Ixodes* genus tick, add 250 µl of SSP. For pool of *Ixodes* genus ticks, add 500 µl of SSP. For single *Dermacentor* genus tick, add 500 µl of SSP.
Gently rinse the pestle in the tube and keep it in the disinfectant solution for at least 2 hours. Rinse the pestle with water, wipe it with a tissue moistened with 70% ethanol and let dry. Before work, the metal part of the pestle should be heated over the flame of the spirit lamp.
Vortex the tube for 5-10 sec. Centrifuge briefly to collect the drops from the inside tube walls. Perform the grinding procedure with other specimens. Without touching the pellet, take 100 µl of the *Ixodes* genus ticks suspension specimen or 50 µl of *Dermacentor* genus ticks suspension for nucleic acids extraction and further assay.
Carry out NA extraction from ticks using "RealLine extraction 100" according to the instruction manual. At the final step, dissolve the extracted NA specimen in 200 µl of the Specimen Diluent.

Method 2.
Add 20 µl of SSP to the tubes with *Ixodes* genus ticks (in case of analysis of full, large ticks, *Dermacentor* genus ticks or pools of ticks add 30-50 µl of SSP so that the solution would fully cover them). Spin the tubes briefly to collect any drops. Place the tubes into a pre-chilled to minus 20 °C thermal rack and keep for at least 20 min in the freezer at minus 30 °C. Place the rack with analyzed specimens into a container with ice or refrigerant. Take a tube with the tick (pool of ticks) frozen in the SSP, and crush the tick thoroughly with a sterile pestle before the solution thaws. Then follow the same protocol as in method 1 except that when the volume of the suspension is adjusted by the SSP solution, it is necessary to make a correction for the volume of SSP (20-50 µl) originally added into the tube for tick grinding.

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Carry out NA extraction from ticks using **"RealLine Extraction 100"** according to the instruction manual.
Transportation and storage of ticks and tick suspension specimens:

- At (2 - 8) °C – alive ticks – for no more than 1 week, tick suspensions - for no more than 2 hours ;
- At minus (18 - 60) °C – for no more than 2 weeks.
- At minus 70 °C and below – for no more than 1 year.

Attention! Do not freeze-thaw specimens repeatedly!

ANNEX II: Settings for RealLine Cycler and DT96:
for these cyclers the measurement exposure must be adjusted. Choose the **Operation with the device** mode in the **Settings** menu, select the item **Measurement exposition:**

- **FAM** to **250**
- **HEX** and **ROX** to **1000**

Confirm that the current exposure value is saved by pressing **YES**

Attention! The specified exposure values are applicable only for RealLine kits and, if necessary, must be changed for other purposes.

ANNEX III: Setting for RotorGene Cyclers: Rotor-Gene® 3000 (6000, Q):
Hereinafter, detection channels and terms corresponding to different versions of devices and software are listed in the following order: Rotor-Gene 3000 (Rotor-Gene 6000, Rotor-Gene Q).

1) Click **New** button.
2) Select an **Advanced** template from the tab of the New Run wizard. Click **New** button.
3) Select **36-Well Rotor** type, check that No Domed 0.2 ml Tubes are used. Click **Next** button.
4) In the new window, determine Reaction volume as **50 µl**. Click **Next** button.
5) The temperature profile of real time PCR should be set. Click **Edit Profile** button.

Step 1:	50°C	2min	
Step 2:	95°C	2min	
Step 3:	94°C	10 sec	50 cycles
	60°C*	40 sec	

* Measure the fluorescence at 60°C

6) Then temperature profile is set, click **OK** button.
7) In the **New Run Wizard** window click **Calibrate** *(**Gain optimization**)* button. The window **Auto Gain Calibration Setup** opens. In the line **Channel Settings** choose **ROX** *(Orange)*, click **Add**. Set **Tube Position** 1, **Min Reading** 5, **Max Reading** 10, click **OK**. In the line **Channel Settings** choose **JOE** *(Yellow)*, click **Add**. Set **Tube Position** 1, **Min Reading** 5, **Max Reading** 10, click **OK**. In the line **Channel Settings** choose **FAM** *(Green)*, click **Add**. Set **Tube Position** 1, **Min Reading** 5, **Max Reading** 10, click **OK**.
8) Tick off **Perform Calibration Before 1st Acquisition**. Click **Close** button.
9) Click **Next** button, start the amplification process by clicking **Start Run** button.
10) Save a file in the Rotor-Gene/templates folder, named RealLine with **.ret* extension. In subsequent work RealLine template would be presented in New run wizard.
11) Save reaction result file with Rotor-Gene Run File **.rex* extension.
12) Record the positions of the controls and specimens according to the instruction manual of the operating device. Click **Start run** button.

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Results for Internal Control DNA amplification

- Click **Analysis** button, choose **Quantitation** from the list, choose **Cycling A. FAM** *(Cycling A. Green)*, click **Show** button.
- Click **OK** button, and cancel automatic **Threshold** determination.
- Click **Linear scale** button. Settings should change to **Log. scale**.
- In the ***Quantitation analysis*** menu buttons **Dynamic tube** and **Slope Correct** should be pressed.
- Click **More Settings** *(Outlier Removal)* **button,** determine **NTC threshold** value as **5%**.
- In the column **CT Calculation** *(right part of the window)* determine **Threshold** value as **0,04**.
- In the result table *(**Quant. Results** window)* **Ct** will be displayed.

Results for Anaplasma phagocytophilum DNA amplification

- Click **Analysis** button, choose **Quantitation** from the list, choose **Cycling A. JOE** *(Cycling A. Yellow)* click, **Show** button.
- Click **OK** button, and cancel automatic **Threshold** determination.
- Click **Linear scale** button. Settings should change to **Log. scale**.
- In the ***Quantitation analysis*** menu buttons **Dynamic tube** and **Slope Correct** should be pressed.
- Click **More Settings** *(Outlier Removal)* **button,** determine **NTC threshold** value as **10%**.
- In the column **CT Calculation** *(right part of the window)* determine **Threshold** value as **0,04**.
- In the result table *(**Quant. Results** window)* **Ct** will be displayed.

Results for Ehrlichia muris and Ehrlichia chaffeensis DNA amplification

- Click **Analysis** button, choose **Quantitation** from the list, choose **Cycling A. ROX** *(Cycling A. Orange)* click, **Show** button.
- Click **OK** button, and cancel automatic **Threshold** determination.
- Click **Linear scale** button. Settings should change to **Log. scale**.
- In the ***Quantitation analysis*** menu buttons **Dynamic tube** and **Slope Correct** should be pressed.
- Click **More Settings** *(Outlier Removal)* **button,** determine **NTC threshold** value as **5%**.
- In the column **CT Calculation** *(right part of the window)* determine **Threshold** value as **0,04**.
- In the result table *(**Quant. Results** window)* **Ct** will be displayed.

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