Instructions for use

RealLine Ureaplasma urealyticum / U. parvum Fla-Format

A QUALITATIVE ASSAY KIT FOR THE DIFFERENTIAL DETECTION OF *UREAPLASMA UREALYTICUM* AND *UREAPLASMA PARVUM* DNA BY REAL TIME PCR

In vitro Diagnostics

CE

RealLine Ureaplasma urealyticum / - parvum (Fla-Format)	**VBD2295**	**100 Tests**
valid from	**September 2019**	

RealLine Ureaplasma urealyticum / U. parvum Fla-Format

Explanation of symbols used in labeling

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

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RealLine Ureaplasma urealyticum / U. parvum Fla-Format

Table of content

RealLine Ureaplasma urealyticum / U. parvum Fla-Format

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1. INTRODUCTION

Ureaplasma urealyticum is part of the normal genital flora of men and women and is found in about 70% of sexually active humans. *U. urealyticum* can be associated with a number of diseases in humans, including non-specific urethritis (NSU), infertility, chorioamnionitis, stillbirth, premature birth, and, in the perinatal period, pneumonia, bronchopulmonary dysplasia and meningitis.

Ureaplasma parvum colonized in women up to 57 % in the vagina and can result during a pregnancy - caused by inflammation - to premature rupture of membranes, premature labor, chorioamnionitis, and the child to sepsis and bronchopulmonary dysplasia and to meningitis.

RealLine Ureaplasma urealyticum / **parvum (Fla-format)** assay kit is designed for differential detection of *Ureaplasma urealyticum* DNA and *Ureaplasma parvum* DNA isolated from specimens using extraction kits:
RealLine DNA-Express (REF VBC8899)
RealLine DNA-Extraction 2 (REF VBC8897)
RealLine DNA-Extraction 3 (REF VBC8889)
RealLine Extraction 100 (REF VBC8896)

RealLine Ureaplasma urealyticum / **parvum (Fla-format)** kit is designed for the analysis of clinical materials: urogenital and cervical swabs, semen, prostate fluid, urine.
The assay is based on real-time polymerase chain reaction (PCR) method with fluorescent detection of amplified products.

The **Fla-format** Kit contains 10 vials with the lyophilized Mastermix, each vial with 10 reactions, for volume of 50 µl per reaction. The kit contains reagents required for 100 tests, including the positive control samples.

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

For the Eco48™ Realtime PCR System (PCRmax, UK) RealLine Fla-format kits can be recommended. The practice with this cycler to use 10 µl of the diluted Mastemix and 10 µl of extracted DNA, was validated. The protocol for using and cycling can be provided.

RealLine Ureaplasma urealyticum / U. parvum Fla-Format

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

Universal Positive Control Sample (PC)	1 vial, 1 ml
Master Mix (MM), lyophilized	10 tubes, 10 tests each
Recovery Solution (RS)	2 vials, 2 ml each

RealLine Ureaplasma urealyticum / U. parvum Fla-Format

3. PRINCIPLE OF THE METHOD

The 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.

4. SPECIFICATIONS

4.1. Sensitivity:

Sensitivity of the detection of *Ureaplasma urealyticum* DNA is determined on five samples prepared from *Ureaplasma urealyticum* DNA SRS (Standard Reference Sample), containing 100 copies of *Ureaplasma urealyticum* DNA per sample. Sensitivity equals 100%.

Sensitivity of *Ureaplasma parvum* DNA detection is determined on five samples prepared from *Ureaplasma parvum* DNA SRS, containing 100 copies of *Ureaplasma parvum* DNA per sample. Sensitivity equals 100%.

4.2. Specificity:

Specificity of *Ureaplasma urealyticum* DNA detection equals 100 %. It is determined on five samples prepared from *Ureaplasma parvum* DNA SRS, containing 100 copies of *Ureaplasma parvum* DNA per sample.

Specificity of *Ureaplasma parvum* DNA detection equals 100 %. It is determined on five samples prepared from *Ureaplasma urealyticum* DNA SRS, containing 100 copies of *Ureaplasma urealyticum* DNA per sample.

4.3. Diagnostic sensitivity:

Diagnostic sensitivity of *Ureaplasma urealyticum* DNA detection: clinical trials conducted on 47 positive samples showed 100 % *sensitivity (interval 93.8 % -100 %, with a confidence level of 90 %); Diagnostic sensitivity of Ureaplasma* parvum DNA detection: clinical trials conducted on 75 positive samples showed 100% sensitivity (interval 96.1 % -100 %, with a confidence level of 90 %);

4.4. Diagnostic specificity:

Diagnostic specificity of *Ureaplasma urealyticum* DNA detection: clinical trials conducted on 130 negative samples showed 100 % specificity (97.7 % -100 % interval, with a 90 % confidence level). Diagnostic specificity of *Ureaplasma parvum* DNA detection: clinical trials conducted on 113 negative samples showed 100% specificity (interval 97.4 % -100 %, with a confidence level of 90 %).

Analysis by the CE-marked reference kit showed full match of results.

RealLine Ureaplasma urealyticum / U. parvum Fla-Format

5. 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 pathogensTo prevent contamination, the stages of DNA isolation and PCR test run must be spatially separated.
- Avoid microbial and ribonuclease contamination of reagents when removing aliquots from reagent vials.
- 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.
- The use of sterile disposable pipette tips is recommended.
- 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 expiration date at the side label of the kit.

6. ADDITIONAL MATERIALS AND DEVICES REQUIRED BUT NOT SUPPLIED

- real time PCR system, like described in p.1
- DNA-Extraction Kit: **RealLine DNA-Express, RealLine DNA-Extraction 2, RealLine DNA-Extraction 3** or **RealLine Extraction 100**
- Internal Control reagent (VBC8881) and 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;
- half-automatic variable-volume single-channel pipettes;
- disposable medical non-sterile powder-free gloves;
- disposable pipette tips with aerosol barrier;
- biohazard waste container.

7. PREPARATION OF THE SPECIMENS

*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)** which 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 p1., add **20 µl** of **IC (VBC8881)** to each tube.

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

Prepare the samples from clinical specimens for the assay using an Extraction kit named in p.1 according to the instruction manual.
Store the extracted DNA at (2 – 8) °C for no more than 24 hours.
After initial opening of the tube, store PC at (2 – 8) °C for no more than 1 month or in 50 µl aliquots at minus (18 – 60) °C during 3 months.

RealLine Ureaplasma urealyticum / U. parvum Fla-Format

8. PROCEDURE

8.1. Preparation of the kit components:

Prior to 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 samples and controls: 1 NC and 1 PC*). Each tube is intended for 10 tests.
Put the remaining tubes immediately back into the foil pouch, squeeze the air out and tightly close with the clip.
After initial opening, store MM at (2 – 8) °C for no more than 3 months.

To prepare diluted Master Mix, add **300 µl of Recovery Solution (RS)** to each tube with MM. Mix gently, hold at room temperature for 15 minutes, and then carefully re-mix.
Store diluted MM at (2 – 8) °C for no more than 7 days.
After initial opening, store RS at (2 – 8) °C for no more than 3 months.

8.2. Prepare an appropriate number of 0.2 ml tubes. Label each tube for each specimen and control.

Attention! *Labels should be placed on the caps of tubes for rotor-type cyclers; for block-type cyclers labels should be placed on the lateral side of the tubes.*

8.3. Add **25 µl** of diluted Master Mix to each 0.2 ml tube.

8.4. Add **25 µl** of corresponding isolated DNA solution to each tube using a separate pipette tip with filter. Tightly close the tubes.

8.5. Place the tubes into the Real Time PCR system.

8.6. Program Real Time PCR system.

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	

* Measure the fluorescence at 60 °C.

For RealLine Cycler, iQ™ iCycler, iQ™5 iCycler, CFX96™, DT-96:

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

RealLine Ureaplasma urealyticum / U. parvum Fla-Format

8.7. Select the amplification detection channels:

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

- Collect data through **ROX** channel (iQ5 iCycler, CFX96, RealLine Cyclers, DT-96, Rotor-Gene 3000), and **Orange** channel (Rotor-Gene 6000, Rotor-Gene Q) for the detection of amplification signal of ***Ureaplasma parvum* DNA**

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

8.8. Program the positions of test tubes with samples, PC and NC according to the instruction manual for the Real Time PCR system in use.

8.9. Run the program.

RealLine Ureaplasma urealyticum / U. parvum Fla-Format

9. DATA ANALYSIS AND INTERPRETATION

9.1. The program should detect in **Positive Control** sample:

- an increase in IC DNA amplification signal (**FAM** / *Green* channeland determine IC **Ct**;
- an increase of the *Ureaplasma parvum* DNA amplification signal (**ROX** / *Orange* channel) and determine the PC **Ct** value;
- an increase of the *Ureaplasma urealyticum* DNA amplification signal (**JOE** / **HEX** / *Yellow* channel) and determine the PC **Ct** value.

9.2. For **NC** the program should detect the increase inthe amplification signal of IC DNA (**FAM** / *Green channel)*) and determine the IC **Ct**. No significant HEX/**JOE**/*Yellow* and **ROX**/*Orange* fluorescent increase should appear.

9.3. For each speciment the program should detect the increase in the amplification signal of IC DNA in channel **FAM** (*Green*) and determine IC **Ct**.

9.4. 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.

9.5. The specimen is considered **negative**, if **Ct** value via JOE/HEX/*yellow* or ROX/*orange* channel for this sample is **> 40** or is not determined.

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

9.6. The specimen is considered **positive** and contains ***Ureaplasma urealyticum*** **DNA** if **Ct** value in **JOE**/**HEX**/***yellow*** channel for this sample is **less than or equals to Ct 40.** ,

The specimen is considered **positive** and contains ***Ureaplasma parvum*** DNA, if the **Ct** value in **ROX**/***Orange*** channel for this sample is **less than or equals to Ct 32.** The specimen is considered **positive** and contains ***Ureaplasma parvum*** DNA in clinically insignificant concentrations, if the **Ct** value in **ROX**/***Orange*** channel for this sample is **above 32 but less than or equals to 40.**

Note: *Ureaplasma parvum* is a commensal bacterium in the uterus as part of the microbiome in healthy women. In low concentrations these bacteria do not necessarily need a treatment.

The **RealLine Ureaplasma urealyticum** / **Ureaplasma parvum** kit detects even lowest amounts of *Ureaplasma parvum*. For results with **Ct 32 to 40** it is advisable to re-check the samples regarding the subsequent treatment decisions, by taking the clinical data, course and appearance of the patient, into account

9.7. If Ct value for NC through HEX/ JOE/ Yellow channel **is less than or equal to 40,** and through ROX/ Orange channel **is less than or equal to 32,** this indicates the presence of contamination. 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 specimens of this run that were identified as positive. Specimens that showed negative results in this run should be considered negative.

10. STORAGE AND TRANSPORTATION

- Transport and store the assay kit at (2 – 8) ° C in the manufacturer's packing.
- 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 in 50 µl aliquots 3 months at (-18 …-24) °C..
  Ready Master Mix (MM): unused MM at (2 – 8) ° C for not more than 3 month
  Diluted MM: at (2 – 8) ° C for up to 7 days.
  Recovery Solution: at (2 – 8) ° C for 3 months.

ANNEX I: 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.

RealLine Ureaplasma urealyticum / U. parvum Fla-Format

ANNEX II: Programming the cycler and analysis of results using rotor-type cyclers: Rotor-Gene 3000, Rotor-Gene 6000 (Corbett Research, Australia), Rotor-Gene Q (Qiagen, Germany)

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).

Program real-time PCR cycler.

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

* Measure the fluorescence at 60°C.

*measure fluorescence at 60 °C through channels FAM, JOE, ROX (Green, Yellow, Orange).

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 RealBest with **.ret* extension. In subsequent work RealBest 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 control samples and specimens according to the instruction manual of the PCR cycler. Click **Start run** button.

Results of IC DNA amplification

1) Click **Analysis** button, choose **Quantitation** from the list, choose **Cycling A. FAM** *(«Cycling A. Green»)*, click **Show** button.
2) Click **OK** button, and cancel automatic **Threshold** determination.
3) Click **Linear scale** button. Settings should change to **Log. scale**.
4) In the ***Quantitation analysis*** menu buttons **Dynamic tube** and **Slope Correct** should be pressed.
5) Click **More Settings** *(Outlier Removal)* **button,** determine **NTC threshold** value as **5 %**.
6) In the column **CT Calculation** *(right part of the window)* determine **Threshold** value as **0.04**.
7) In the result table *(**Quant. Results** window)* **Ct** will be displayed.

Results of *Ureaplasma parvum* DNA amplification

1) Click **Analysis** button, choose **Quantitation** from the list, choose **Cycling A. ROX** *(Cycling A.*

Orange) click, **Show** button.

2) Click **OK** button, and cancel automatic **Threshold** determination.
3) Click **Linear scale** button. Settings should change to **Log. scale**.
4) In the ***Quantitation analysis*** menu buttons **Dynamic tube** and **Slope Correct** should be pressed.
5) Click **More Settings** *(Outlier Removal)* **button,** determine **NTC threshold** value as **5 %**.
6) In the column **CT Calculation** *(right part of the window)* determine **Threshold** value as **0.04**.
7) In the result table *(**Quant. Results** window)* Ct will be displayed.

Results of *Ureaplasma urealyticum* DNA amplification

8) Click **Analysis** button, choose **Quantitation** from the list, choose **Cycling A. JOE** *(Cycling A. Yellow)* click, **Show** button.
9) Click **OK** button, and cancel automatic **Threshold** determination.
10) Click **Linear scale** button. Settings should change to **Log. scale**.
11) In the ***Quantitation analysis*** menu buttons **Dynamic tube** and **Slope Correct** should be pressed.
12) Click **More Settings** *(Outlier Removal)* **button,** determine **NTC threshold** value as **10 %**.
13) In the column **CT Calculation** *(right part of the window)* determine **Threshold** value as **0.04**.
14) In the result table *(**Quant. Results** window)* Ct will be displayed.

RealLine Ureaplasma urealyticum / U. parvum Fla-Format

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