Instructions for use

RealLine Neisseria gonorrhoeae
Fla-Format

ASSAY KIT FOR THE QUALITATIVE DETECTION OF NEISSERIA GONORRHOEAE DNA BY REAL-TIME PCR METHOD

In vitro diagnosticum

<table>
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<th>RealLine Neisseria gonorrhoeae (Fla-Format)</th>
<th>VBD4496</th>
<th>100 Tests</th>
</tr>
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<td>valid from</td>
<td>January 2016</td>
<td></td>
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# Explanation of symbols used in labeling

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<td>Consult instructions for use</td>
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<tr>
<td></td>
<td>Manufacturer</td>
</tr>
<tr>
<td></td>
<td>Keep out of sunlight</td>
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ASSAY KIT FOR THE QUALITATIVE DETECTION OF *NEISSERIA GONORRHOEAE* DNA BY REAL-TIME PCR METHOD

In vitro diagnosticum

1. **INTRODUCTION**

Clinical information:

*Neisseria gonorrhoeae* are gram-negative motile bacteria and the causative agent of gonorrhea (clap), one of the most common sexually transferred disease. In women gonorrhea can lead to severe symptoms such as infertility and abortion. In men gonorrhoea can lead to painful urethritis.

The RealLine Neisseria gonorrhoeae Fla-format kit has a high specificity, but a positive result is further recommended to confirm with the test using the RealLine Neisseria gonorrhoeae T2 (Fla format) kit (REF VBD4495). These kits use different specific regions of the Neisseria gonorrhoeae genome as targets; therefore, a comprehensive study allows to confirm the presence of this pathogen.

The RealLine Neisseria gonorrhoeae (Fla-format) assay kit is designed to detect *Neisseria gonorrhoeae* DNA isolated from clinical specimens using one of the following extraction kits:

- RealLine DNA-Express (REF VBC8899)
- RealLine DNA-Extraction 2 (REF VBC8897)
- RealLine DNA-Extraction 3 (REF VBC8889)
- RealLine Extraction 100 (REF VBC8896)

The RealLine Neisseria gonorrhoeae (Fla-format) kit is designed for the analysis of clinical materials: scrapings of the epithelial cells, semen, prostatic fluid, urine.

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

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

The kit is designed for use with block cyclers iQ™ iCycler, iQ™5 iCycler, CFX™96 (*Bio-Rad, USA*), DT96 (*DNA-Technology Research and Production Company ZAO, Russia*); and rotor type cyclers Rotor-Gene® 3000 and Rotor-Gene® 6000 (*Qiagen, Germany*).

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 Mastermix and 10 µl of extracted DNA, was validated. The protocol for using and cycling can be provided.
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

have 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, 2ml each;

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

I. Sensitivity:
Sensitivity control was performed on 5 samples containing 100 Neisseria gonorrhoeae DNA copies per sample, prepared from SRS (Standard Reference Sample containing Neisseria gonorrhoeae DNA), reg. No. 05-2-310.
The sensitivity equals 100%.

II. Specificity:
Specificity of Neisseria gonorrhoeae DNA detection was determined using negative DNA-extracts of the Standard Reference Panel (Reg. No.05-2-291), consisting of samples containing IC DNA and not containing DNA of STD agents. Specificity of Neisseria gonorrhoeae DNA detection equals 100%.

III. Diagnostic evaluation:
Diagnostic evaluation was performed on 50 clinical samples:
20 samples (No. 1-20), negative samples;
20 samples (No.21-40), positive samples containing Neisseria gonorrhoeae DNA;
10 samples (No. 41-50) obtained from individuals infected with Chlamydia trachomatis.

Determination of sensitivity was performed on 20 clinical samples obtained from the clinical material containing Neisseria gonorrhoeae with a CE-marked reference kit. The RealLine DNA Neisseria gonorrhoeae (Fla-format) kit determined all 20 samples as positive. Analysis by the reference kit proved all 20 samples containing Neisseria gonorrhoeae to be positive.
Diagnostics sensitivity equals 100%.

Determination of specificity was performed on 20 samples obtained from healthy individuals and 10 samples obtained from individuals infected with Chlamydia trachomatis, with the reference kit.
When studying clinical samples obtained from healthy individuals by RealLine DNA Neisseria gonorrhoeae (Fla-format), negative results were recorded for all 20 samples.
Analysis of similar samples by the reference kit confirms the results obtained in all cases.
When studying clinical samples obtained from individuals infected with Chlamydia trachomatis using RealLine DNA Neisseria gonorrhoeae (Fla-format), negative results were recorded for all 10 samples.
Analysis of the similar samples by the reference kit confirms the results obtained in all cases.

Thus, RealLine DNA Neisseria gonorrhoeae (Fla-format) and the CE-marked reference kit show a 100% agreement in results.
Specificity equals 100%
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 pathogens.
- To prevent contamination, the stages of DNA isolation and PCR test run must be spatially separated.
- Avoid microbial and nuclease 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.

6. ADDITIONAL MATERIALS AND DEVICES REQUIRED BUT NOT SUPPLIED

- real time PCR system, see paragraph 1
- DNA-Extraction Kit, RealLine DNA-Extraction 3 or see p.1 Extractions Kits with Internal control reagent
- Internal Control reagent (VBC8881) and Negative Control Sample or H₂O (molecular biology grade) 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
- safety laminar 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 ANALYSED SAMPLES AND REAGENTS

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 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 or H₂O (molecular biology grade).
- For the PC use **70 μl** of Negative Control Sample or H₂O (molecular biology grade) and **30 μl** of Positive Control to the tube marked PC.

7.1. Sample preparation

Prepare the samples for the assay using **RealLine DNA - Express, RealLine DNA - Extraction 2, RealLine DNA - Extraction 3** or **RealLine Extraction 100** extraction kits according their instruction manuals.

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 to 8°C for 2 days.*

*After initial opening shelf life of Positive Control sample at 2 to 8 ° C is 1 month.*

7.2. 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 samples and controls**). 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 the entire shelf life of the kit.*

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 2 weeks.*

*After initial opening shelf life of Recovery Solution at (2 – 8) °C is 3 months.*
8. PROCEDURE

8.1. Prepare an appropriate number of 0.2 ml tubes or a PCR plate. Label each tube for each specimen and control.

Attention! Labels should be placed on the caps of tubes for Rotor-Gene® 3000/6000 devices. For iQ™ iCycler, iQ™5 iCycler, CFX™96, DT-96 PCR devices labels should be placed on the lateral side of the tubes.

8.2. Add 25 µl of prepared Master Mix to each 0.2 ml tube.

8.3. Add 25 µl of corresponding isolated DNA solution to each tube using a separate pipette tip with filter. Do not touch the pellet! Tightly close the tubes.

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

8.5. Program real time PCR system.

For Rotor-Gene® 3000 (6000):

- Click New button.
- Select a Dual labeled Probe template from the Advanced tab of the New Run wizard. Click New button.
- Select 36-Well Rotor type, check that No Domed 0.2 ml Tubes are used. Click Next button.
- In the new window determine Reaction volume as 50 µl. Click Next button.
- The Temperature profile of real time PCR should be set. Click Edit Profile button.
- Then temperature profile is set, click OK button.
- In the New Run Wizard window click Calibrate (Gain optimisation) 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 FAM (Green), click Add. Set Tube Position 1, Min Reading 5, Max Reading 10, click OK.
- Tick off Perform Calibration Before 1st Acquisition. Click Close button.
- Click Next button, start the amplification process by clicking Start Run button.
- Save a file in the Rotor-Gene/templates named RealLine with *.ret extension. In subsequent work RealLine template would be presented in New run wizard.
- Save reaction result file with Rotor-Gene Run File *.rex extension.
- Record the positions of the controls and specimens according to the instruction manual of the operating device. Click Start run button.
8.6. Program real time PCR device according the instruction manual as follows:

<table>
<thead>
<tr>
<th>Stage 1:</th>
<th>50°C 2min</th>
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<tbody>
<tr>
<td>Stage 2:</td>
<td>95°C 2min</td>
</tr>
<tr>
<td>Stage 3:</td>
<td>94°C 10 sec 60°C* 20 sec</td>
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</tbody>
</table>

* Measure the fluorescence at 60°C

50 cycles
9. **DATA ANALYSIS AND INTERPRETATION**

For Rotor-Gene® 3000 (6000)

9.1. 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.

9.2. Results for *Neisseria gonorrhoeae* 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.

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

- increase of the IC DNA amplification signal along channel **FAM (Green)** and determine the threshold cycle, IC **Ct**;
- increase of the *Neisseria gonorrhoeae* DNA amplification signal along channel **ROX (Orange)** and determine the **Ct** value;

9.4. 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)** fluorescent increase should appear (*no Neisseria gonorrhoeae DNA amplification*).

When **Ct** value for NC through **ROX (Orange)** channel is **less than or equal to 40**, this indicates the presence of contamination (see paragraph 9.9.).
9.5. For each sample the program should detect the increase of the amplification signal of IC DNA along channel **FAM** (*Green*) and determine IC **Ct**.

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

9.7. The sample is considered **negative** (not containing *Neisseria gonorrhoeae* DNA), if **Ct** value via **ROX** (*Orange*) channels for this sample is **above 40** or is not determined. When IC **Ct** value for such sample differs from the \((\text{IC } \text{Ct})_{av}\) 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.

9.8. The sample is considered **positive**, i.e. contains *Neisseria gonorrhoeae* DNA, when **Ct** value via **ROX** (*Orange*) channel for this sample is **less than or equals to 40**.

9.9. 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.
For iQ™ iCycler, iQ™5 iCycler, CFX™96, DT-96

9.10. For PC the program should detect:
   • increase of the IC DNA amplification signal (channel FAM) and determine the threshold cycle, IC Ct;
   • increase of the Neisseria gonorrhoeae DNA amplification signal (channel ROX) and determine the Ct value;

9.11. For NC the program should detect the increase of the amplification signal of IC DNA (channel FAM) and determine the threshold cycle, IC Ct. No ROX fluorescent increase should appear (no Neisseria gonorrhoeae DNA amplification). When Ct value for NC through ROX channel is less than or equal to 40, this indicates the presence of contamination (see paragraph 9.16.).

9.12. For each sample the program should detect the increase of the amplification signal of IC DNA (channel FAM) and determine IC Ct.

9.13. 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.

9.14. The sample is considered negative (not containing Neisseria gonorrhoeae DNA), if Ct value via ROX channel for this sample 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.

9.15. The sample is considered positive (containing Neisseria gonorrhoeae DNA) when Ct value via ROX channel for this sample is less than or equals to 40.

9.16. 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.
10. STORAGE AND TRANSPORTATION

- Store the assay kit at (2 - 8) °C in the manufacturer’s packing. Transportation at 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.
  - Ready Master Mix (MM): unused MM at (2 - 8) °C for the entire shelf life of the kit.
  - Diluted MM: at (2 - 8) °C for 2 weeks.
  - Recovery Solution: at (2 - 8) °C for 3 months.

**Technical support:** techsupport@bioron.de