Instructions for Use

RealLine HPV 6 / 11
Fla-Format

QUALITATIVE ASSAY KIT FOR THE DIFFERENTIAL DETERMINATION OF DNA FROM HUMAN PAPILLOMAVIRUS TYPES 6 AND 11 BY REAL TIME PCR METHOD

In-vitro Diagnostics

CE

RealLine HPV 6 / 11 (Fla-Format)	**VBD8481**	**100 Tests**
valid from	**October 2019**	

BIORON Diagnostics GmbH
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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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Trademarks:
Rotor-Gene® is a registered trademark of Qiagen Group, Germany.

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1. INTENDED USE

Clinical information:
Human Papilloma Viruses HPV are DNA-Viruses and more than 100 different types are known at the moment. While the majority of HPVs cause no symptoms, some can cause low severe symptoms like warts and a few are known to cause cancer. HPV types that are more likely to lead to the development of cancer are referred as High-Carcinogenic-Risk types HPV. The HPV Types 6 and 11 are known as Low-Carcinogenic-Risk-Types and cause genital warts.

RealLine HPV 6 / 11 (Fla-format) assay kit is designed to detect DNA of human papillomavirus type 6 and 11 isolated from clinical specimens using the extraction kits:
RealLine DNA-Express (REF VBC8899)
RealLine DNA-Extraction 3 (REF VBC8889)
RealLine Extraction 100 (REF VBC8896)

RealLine HPV 6 / 11 (Fla-format) kit is designed for the analysis of clinical materials: scrapings of epithelial cells, semen, prostatic fluid and 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 master mix, each vial with 10 reactions, for a volume of 50 µl per reaction. The kit contains reagents required for 100 tests, including the positive control samples.

The kit is designed for use with block cyclers iQ™ iCycler, iQ5™ iCycler, CFX96™ (Bio-Rad, USA), DT96 (DNA-Technology, Russia) and RealLine Cyclers (BIORON Diagnostics GmbH); and rotor type cyclers Rotor-Gene® 3000, 6000, 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 vial, 1 ml
Master Mix (MM), lyophilized	10 tubes, 10 tests each
Recovery Solution (RS)	2 vials, 2 ml 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.

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4. SPECIFICATIONS

4.1. Sensitivity:

Sensitivity control was performed on 5 samples containing 100 HPV 6 and 11 types DNA copies per sample, prepared from Standard Reference Samples containing DNA of HPV 6 and 11 types, (HPV 6 DNA SRS; HPV 11 DNA SRS).
The sensitivity equals 100 %.

4.2. Specificity:

Specificity of HPV 6 and 11 types DNA detection was determined using 5 negative DNA-extracts samples not containing DNA of STD agents or herpes virus infections, including HPV types 6 and 11 DNA, and containing IC DNA. Specificity of HPV 6/11 DNA detection equals 100 %.

4.3. Diagnostic evaluation:

Diagnostic evaluation was performed on 50 clinical samples:
10 clinical samples obtained from healthy donors;
8 clinical samples obtained from patients with STD symptoms;
3 clinical samples obtained from patients with diagnosed type of HPV 16, 18, 35;
18 clinical samples obtained from patients with cytological signs of HPV infection.

All samples were analyzed with the **RealLine HPV 6** / **11** assay kit (BIORON Diagnostics) and the CE-marked reference kit.

Results obtained show total coincidence between the RealLine DNA HPV 6/11 assay kit and the CE-marked reference kit – 100% sensitivity and specificity according the 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 HPV Low carcinogenic Risk Types 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 HPV Low carcinogenic Risk Types 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.

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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.
- 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 at the side label of the box.

7. ADDITIONAL MATERIALS AND DEVICES REQUIRED BUT NOT SUPPLIED

- real time PCR system, like described in p.1
- **DNA-Extraction Kit**, RealLine DNA-Extraction 3, RealLine DNA-Express or RealLine Extraction 100
- **Internal Control reagent** (VBC8881) and Negative Control Sample, if the kit is used with the extraction kits of other supplier.
- **Tubes or plates** for the PCR according to the used cycler
- 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.

8. PREPARATION OF THE SPECIMEN

The assay is performed on extracted DNA samples obtained from the clinical material using one of the DNA extraction kits listed in p.1, according to the Instruction Manual to the kit.

*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 kits 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 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 24 hours.
After initial opening shelf life of Positive Control sample is 1 month at (2 – 8) °C or for 50 µl aliquots 3 month at (-18 … -60) °C

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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 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.
Attention! Each tube is intended for 10 tests.
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 Soltion RS** to each tube with MM. Mix gently, keep at (18 - 25) °C during 15 min, and mix thoroughly one more time. Collect the tube contents by brief centrifugation.
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.
9.2. 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.*

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

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

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

9.6. 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	2 min	
Step 2:	95°C	2 min	
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 **FAM** channel (iQ5 iCycler, CFX96, RealLine Cyclers, DT-96, Rotor-Gene 3000) or **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), or **Orange** channel (Rotor-Gene 6000, Rotor-Gene Q) for the detection of amplification signal of **HPV 11;**

- Collect data through **HEX** channel (iQ5 iCycler, CFX96, RealLine Cyclers, DT-96, Rotor-Gene 3000), or **JOE/Yellow** channel (Rotor-Gene 6000, Rotor-Gene Q) for the detection of amplification signal of **HPV 6;**

9.8. Program the position of the tubes with the specimens, PC and NC according to the Instruction Manual for the cycler in use.

9.9. Run the program.

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10. DATA ANALYSIS AND INTERPRETATION

10.1 Data analysis:

For PC the program should detect:

- an increasing **HPV 6 DNA** amplification signal (**HEX/JOE/Yellow** channel) and determine the **PC Ct** threshold cycle value;
- an increasing **HPV 11 DNA** amplification signal (**ROX/Orange** channel) and determine the **PC Ct** threshold cycle value;
- an increasing **IC DNA** amplification signal (**FAM/Green** channel) and determine the **IC Ct** threshold cycle

10.2 In **NC**, the programme should record:

- an increasing **IC DNA** amplification signal (**FAM/Green** channel) and determine **IC Ct**;
- at the same time, the programme should not record an increasing signal of the specific product of DNA amplification in the **HEX/JOE/Yellow/ and ROX/Orange** channels.

10.3 **For each test sample**, the programme should record an increasing **IC DNA** amplification signal (**FAM/Green** channel) and determine IC **Ct** .

10.4 Calculate (IC **Ct**)$_{av}$ as an average value of IC **Ct** for all the test samples (*including PC and NC*). Discard the IC **Ct** values differing by more than 2 from the (IC **Ct**)$_{av}$ value. After discarding them, recalculate (IC **Ct**)$_{avg}$ for the remaining values.

10.5 The test sample is considered **positive**, i.e. containing **HPV 6 DNA**, if, for this sample, the **Ct** value in the **HEX/JOE/Yellow channel is less than or equal to 35**.

10.6 The test sample is considered **positive**, i.e. containing **HPV 11 DNA**, if, for this sample, the **Ct value in the ROX/Orange channels is less than or equal to 35**.

10.7 The result of sample assay is considered **negative**, if the **Ct** value for this sample in the **HEX/JOE/Yellow and ROX/Orange channels exceeds 35 or cannot be determined**.

If, for such sample, the IC **Ct** value exceeds the (IC **Ct**)$_{avg}$ value by more than 2, the result for this sample shall not to be analysed and evaluated as negative. A repeated assay for this sample starting with isolation is required.

10.8 If the Ct value for **NC** in the **HEX/JOE/Yellow** and/or **ROX/Orange** channels is less **than or equal to 35**, this is indicative of the presence of **contamination** in the system. In this case, the positive results of this individual PCR test run are considered invalid. Take measures to detect and eliminate the source of contamination and repeat the assay for all the samples of this test run which had a positive result. The samples for which the test has yielded negative results should be considered negative.

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 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 at minus (18 - 60) °C for up to 3 months.
  Ready Master Mix (MM): unused MM at (2 - 8) °C for no more than 3 months
  Diluted MM: at (2 - 8) °C for 7 days in a dark surrounding
  Recovery Solution: at (2 - 8) °C for 3 months.

Technical support: techsupport@bioron.de

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.

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ANNEX II: Programming the device and analysis of results using Rotor-Gene 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).

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.

Analysing the 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.

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Analysing the results of HPV 6 DNA amplification:

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

Analysing the results of HPV 11 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.

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