Instructions for Use

RealLine HPV HCR Genotype
Str-Format

REAL TIME PCR QUALITATIVE ASSAY KIT FOR THE DIFFERENTIAL DETERMINATION OF DNA OF THE HIGH CARCINOGENIC RISK TYPES OF THE HUMAN PAPILLOMA VIRUS 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 AND 59

In vitro Diagnostics

CE

RealLine HPV HCR Genotype (Str-Format)	**VBD8479**	**96 Tests**
valid from	**September 2023**	

BIORON Diagnostics GmbH
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Explanation of symbols used in labelling.

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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QUALITATIVE ASSAY KIT FOR THE DIFFERENTIAL DETECTION OF DNA OF THE HIGH CARCINOGENIC RISK TYPES 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 AND 59 OF THE HUMAN PAPILLOMA VIRUS BY REAL TIME PCR

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1. INTENDED USE

Clinical information:

Human Papilloma Viruses HPV are DNA-Viruses and in between more than 100 different types are known. 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”. High-carcinogenic-risk HPV-types are known to cause the vast majority of cervical cancers which cause death in women with an annual incidence of around half a million and a mortality of almost 50 %.

The **RealLine HPV HCR Genotype** assay kit is intended for differential determination of DNA of high carcinogenic cancer human papillomavirus (HPV) types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 isolated from clinical specimens using the validated extraction kits:

- **RealLine DNA-Express (REF VBC8899-R)**
- **RealLine Extraction 100 (REF VBC8896-R)**
- **INSTANT Virus RNA/DNA Kit – IPC16 (Roboscreen GmbH, Germany)**
- **INSTANT Virus RNA/DNA Kit – FX (Roboscreen GmbH, Germany)**
- **VeriLab UMag (Laboveritas SIA)**

When using DNA extraction kits of other manufacturers, it is necessary to use Internal Control sample (IC) (VBC8881, BIORON Diagnostics).

RealLine HPV HCR Genotype assay kit is intended for the differential detection of high-risk human papillomavirus (HR HPV) types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 DNA in clinical specimens (cervical swabs) using real-time polymerase chain reaction (PCR) with fluorescence detection.

The results of PCR analysis are taken into account in complex diagnostics of disease.

The **Str-Format Kit** contains 4 x 96 tubes (0.2 ml each) in strips with lyophilized Mastermix. For the test 50 µl of extracted DNA have to be pipetted into the tube and the ready Mastermix is diluted. The kit contains reagents required for 96 tests, including control samples and a Positive Control sample. For each clinical specimen there is a differential detection of the described high carcinogenic risk viruses (paragraph.3).

The kit is validated for use with: iQ™5 iCycler (Bio-Rad, USA). The kit is compatible with real-time PCR systems such as RealLine Cycler (BIORON Diagnostics GmbH), iQ™ iCycler, CFX™96 (Bio-Rad, USA) and DT-96 (DNA-Technology, Russia).

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),	**2 tubes, 1 ml each**
Ready Master Mix 1 - 4 (RMM HPV HCR genotype), lyophilized	**4 microplates, 96 test-tubes each**
The kit is additionally supplied with PCR optical-quality film	

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 to 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.

To analyse each sample for the content of high-carcinogenic-risk HPV DNA one half of the strip has to be used. Amplification is carried out in four tubes (**RMM1, RMM2, RMM3, RMM4**) for detection of DNA; three HPV types can be detected in each tube (see Table 1).

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Table 1

Test-tube	HPV types
RMM1	16, 18, 39
RMM2	33, 45, 56
RMM3	31, 35, 58
RMM4	52, 51, 59

In order to validate the quality of sampling from patient and improve the reliability of the results, the Mastermix **MM 4** include the additional detection for the presence of human DNA (β-actin) in the analysed samples.

4. SPECIFICATIONS

4.1. Sensitivity:

Sensitivity of detection 100 copies of DNA of HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 of high cancer risk the standard reference samples equals 100%.

4.2. Specificity:

Specificity of detection DNA of HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 of high cancer risk (on samples, not containing DNA of given types of HPV, but containing DNA of HPV of 8 other types) on equals 100 %.

4.3. Diagnostic evaluation:

Diagnostic evaluation was performed on the following clinical samples:
10 clinical samples obtained from healthy donors.
5 clinical samples obtained from patients with STD symptoms but without laboratory signs of the papillomavirus infection.
5 clinical samples containing human papillomavirus types 6 and 11.
44 clinical samples obtained from patients with diagnosed types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 of HPV HCR.

All samples were analyzed in “RealLine HPV HCR genotype” assay kit and the CE-marked reference kit. The obtained results have shown total coincidence between the “RealLine HPV HCR genotype” assay kit and the CE-marked reference kit – 100 % sensitivity and specificity according to 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 *Human Papilloma Virus* 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* 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.
- 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 total expiration date on the side label of the box.

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7. ADDITIONAL MATERIALS AND DEVICES REQUIRED BUT NOT SUPPLIED

- real time PCR system,-as described in p.1
- DNA-Extraction Kits listed in p.1
- Internal Control reagent (VBC8881) and Negative Control Sample, if the kit is used with the extraction kits from another supplier;
- 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;
- Razor or scalpel

8. PREPARATION OF THE ANALYSED SAMPLES

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 from another supplier for the extraction of nucleic acids as recommended in paragraph 1, 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 for the assay using the extraction kits according to their instruction manuals. If an extraction kit with magnetic particles is used, keep the tubes with extracted NA in a magnetic rack.

When using **Verilab UMag DNA extraction kit**, add 100 µl of Elution Buffer to each well after elution.
Store the extracted DNA at (2–8) °C for no more than 24 hours.
After initial opening shelf life of Positive Control sample at (2 – 8) °C is 1 month or in 50 µl aliquots at (-18 - -60) °C for up to 3 months.

9. PROCEDURE

9.1 Preparation of the reagents.

Prior to the test take the kit out of the refrigerator and keep the **RMM HPV HCR Genotype** closed in the package at (18 – 25) °C for at least 30 min. (One **RealLine HPV HCR Genotype** microplate is sufficient for analysis of **22** specimens). Open the package and cut the necessary number of tubes with RMM HPV HCR genotype (including the specimens and control samples: 1 NC and 1 PC) with the scissors. Cut the tubes together with the covering film.

Analysis of each specimen (including control samples) is performed using four tubes with RMM HPV HCR genotype:

- Half of the strip, containing RMM1, RMM2, RMM3, RMM4).
- The tube with RMM1 is marked with a line.

Put the remaining tubes immediately back into the foil pouch, squeeze the air out and tightly close with the clip.
After initial opening, store RMM at (2 - 8) °C for no more than 3 months.

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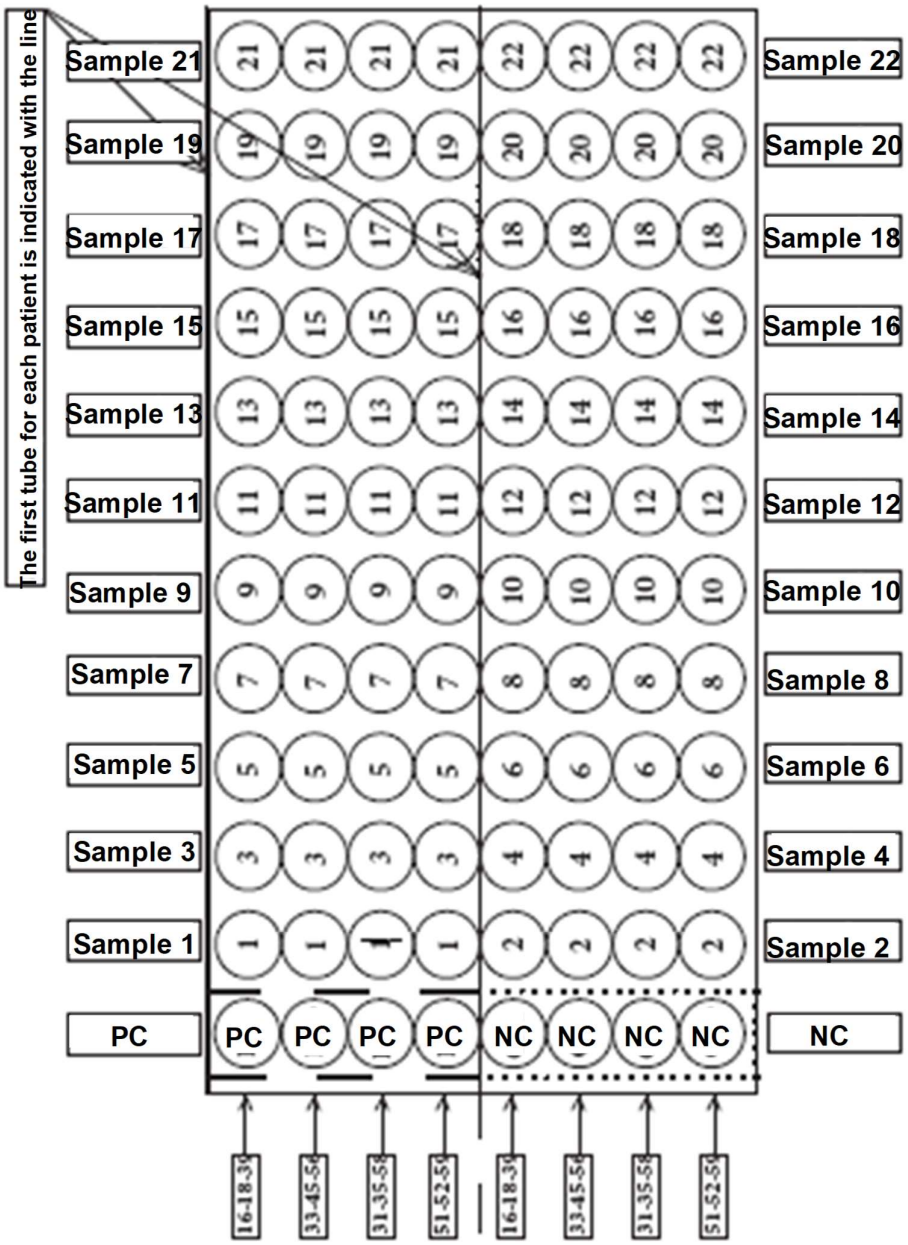
Recommended order of tubes in the cycler



9.2 Label the tubes with RMM for each specimen and control.
Attention! *Labels should be placed on the lateral side of the tubes.*

9.3 Carefully remove the covering film so that RMM remains in the tube.

9.4 Add **50 µl** of corresponding isolated DNA solution to each tube using a separate pipette tip with filter. Tightly close the tubes with caps or seal with the PCR transparent film. Start the PCR no later than 30 min after the DNA addition.
Attention! *The optical film should remain clean (free of any labels or marks).*

9.5 Place the tubes into the Real Time PCR system.

Program Real Time PCR system as follows:

Stage 1:	50°C	2min	
Stage 2:	95°C	2min	
Stage 3:	94°C	10 sec	50 cycles
	60°C*	20 sec	
* Measure the fluorescence at 60 °C in FAM, HEX, ROX and Cy5			

9.6 Select the amplification detection channels.

Mastermixes RMM 1 - 3:	
FAM **HEX** **ROX**	registration of DNA signal of HPV of high cancer risk.
Cy5	registration of IC DNA signal
Mastermix RMM 4:	
FAM **HEX** **ROX**	registration of DNA signal of HPV of high cancer risk.
Cy5	registration of **human β-actin gene** DNA signal

9.7 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.

9.8 Run the program.

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10. DATA ANALYSIS AND INTERPRETATION

10.1 For PC in each of RMM 1- 3 the program should detect:

- increase of the IC DNA amplification signal (channel **Cy5**) and determine the threshold cycle, IC **Ct**;
- increase of the HPV DNA amplification signal (channels **FAM, HEX, ROX**) and determine the PC **Ct** value for each channel.

For PC in RMM 4 the program should detect:

- increase of the human β-actin gene DNA amplification signal (channel **Cy5**) and determine the threshold cycle of **β - Actin Ct**;
- increase of the HPV DNA amplification signal (channels **FAM, HEX, ROX**) and determine the **PC Ct** value for each channel.

10.2 For NC in each of RMM 1-3 the program should detect

- the increase of the amplification signal of IC DNA (channel **Cy5**) and determine the threshold cycle, IC **Ct**. Neither FAM nor HEX nor ROX fluorescence increase should appear or, Ct for HR HPV in the tubes with NC should **not be less than 40.**
- **For NC in RMM4**, the program should detect no increase in the amplification signal of human β-actin gene or, Ct should **not be less than 32** (channel Cy5). Neither FAM nor HEX nor ROX fluorescence increase should appear or, Ct should not be less than 40. See chapter 9.9 if the Ct value for NC:
  - is less than or equal to 40 in the FAM, HEX, or ROX channel in any of RMM1–4 or
  - is less than or equal to 32 in the Cy5 channel in RMM4.

10.3 For each test sample in RMM4, the program should detect the increase in the amplification signal of human β-actin gene (Cy5 channel) and determine β-actin Ct. The test sample is considered valid if the Ct in the Cy5 channel in RMM4 is less than or equal to 32. For the test samples that were found not valid (Ct in the Cy5 channel in RMM4 is above 32), a repeated sampling is required. The validation is performed in order to evaluate sampling accuracy and storage conditions. In case the sampling was performed incorrectly (insufficient number of epithelial cells) or the storage conditions didn't provide preservation of DNA, the amplification signal of human β-actin gene will be low, and the test sample will be found not valid. In this case, the obtained negative result of HR HPV DNA detection **may be false.**

10.4 For each test sample in RMM1–3, the program should detect the increase in the amplification signal of IC DNA (Cy5 channel) and determine IC Ct in each tube.

10.5 Calculate $(IC\ Ct)_{av}$ as an average IC Ct of all samples (including PC and NC) for RMM1, RMM2, and RMM3 separately. IC Ct values that differ by more than 2 from $(IC\ Ct)_{av}$ should be ignored. Recalculate $(IC\ Ct)_{av}$ for the remaining values.

10.6 The sample is considered positive, i.e. containing HR HPV DNA, if the Ct **is less than or equal to 40** in the FAM, HEX, or ROX channel in any of RMM1–3. The correspondence between HR HPV types and detection channels is shown in Table 2.

Specimens may contain HR HPV DNA of one or several types in any combination.

Table 2

	Positive detection signal in the channel			
Tube	**FAM**	**JOE/HEX**	**ROX**	**Cy5**
RMM1	**type 16**	type 39	**type 18**	IC
RMM2	type 33	type 56	type 45	IC
RMM3	type 31	type 58	type 35	IC
RMM4	type 52	type 59	type 51	**human β-actin DNA**

10.7 The sample is **considered negative** if the Ct is **above 40 or not determined** in the FAM, HEX, and ROX channels in RMM1, RMM2, and RMM3.

If IC Ct value for such sample differs from $(IC\ Ct)_{av}$ value by more than 2, the result is considered **equivoca**l. A repeated analysis of the sample, starting from the DNA extraction step is required.

10.8 If the Ct value for NC:

- is **less than or equal to 40** in the FAM, HEX, or ROX channel in any of RMM1–4 or
- is **less than or equal to 32** in the Cy5 channel in RMM4,

it indicates the presence of contamination. In this case, all positive results in this PCR test run obtained for the HPV type that contaminated the reaction are considered **equivocal.** Actions are required to identify and eliminate the source of contamination. Repeat the analysis of all samples of this run that were determined positive. Samples that showed negative results in this run should be considered **negative.**

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11. STORAGE AND TRANSPORTATION

- Store and transport the assay kit at (2 - 8) °C in the manufacturer's packing for the entire shelf life. The shelf life of the kit is 12 months from the manufacture date.
- Transport at (2 - 8) °C. Transportation up to 25°C no more than 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 (-18 - -60) °C for up to 3 months.
  Ready Master Mix (RMM): 3 months at (2 – 8) °C.

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**
- **Cy5** to **500**

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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