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

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RealLine HPV HCR Genotype (Str-Format)	**VBD8479**	**96 Tests**
valid from	**September 2019**	

BIORON Diagnostics GmbH
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RealLine HPV HCR Genotype Str-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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RealLine HPV HCR Genotype Str-Format

Table of content:

RealLine HPV HCR Genotype Str-Format

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 extraction kits:
RealLine DNA-Express (REF VBC8899)
RealLine DNA-Extraction 3 (REF VBC8889)
RealLine Extraction 100 (REF VBC8896)

RealLine HPV HCR Genotype kit is intended for the analysis of clinical materials: epithelial cell swabs. The assay is based on the real-time polymerase chain reaction (PCR) method with fluorescent detection of the amplified product.

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), based on plasmid DNA with integrated DNA fragments of human papilloma viruses, including HPV DNA types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59	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	

RealLine HPV HCR Genotype Str-Format

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 analyze 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).

Table 1

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

The **efficacy of DNA extraction** from the samples is controlled by isolating an **Internal Control IC** from clinical samples together with DNA of infectious agent. The subsequent detection occurs in MM 1, 2, 3) by the **Cy5** (*Red*) channel.

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 analyzed samples. The detection occurs in channel **Cy5** (*Red*).

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 the reference kit.

RealLine HPV HCR Genotype Str-Format

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.

7. ADDITIONAL MATERIALS AND DEVICES REQUIRED BUT NOT SUPPLIED

- real time PCR system, as described in p.1
- DNA-Extraction Kit: **RealLine DNA Express, RealLine DNA-Extraction 3 or Realine Extraction 100**
- 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

RealLine HPV HCR Genotype Str-Format

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 **RealLine DNA-Express, 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 – 8) °C for 24 hours

After initial opening shelf life of Positive Control sample at (2 – 8) °C is 1 month C or in 50 µl aliquots at minus (18 - 60) °C for up to 3 months.

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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 razor or scalpel.

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

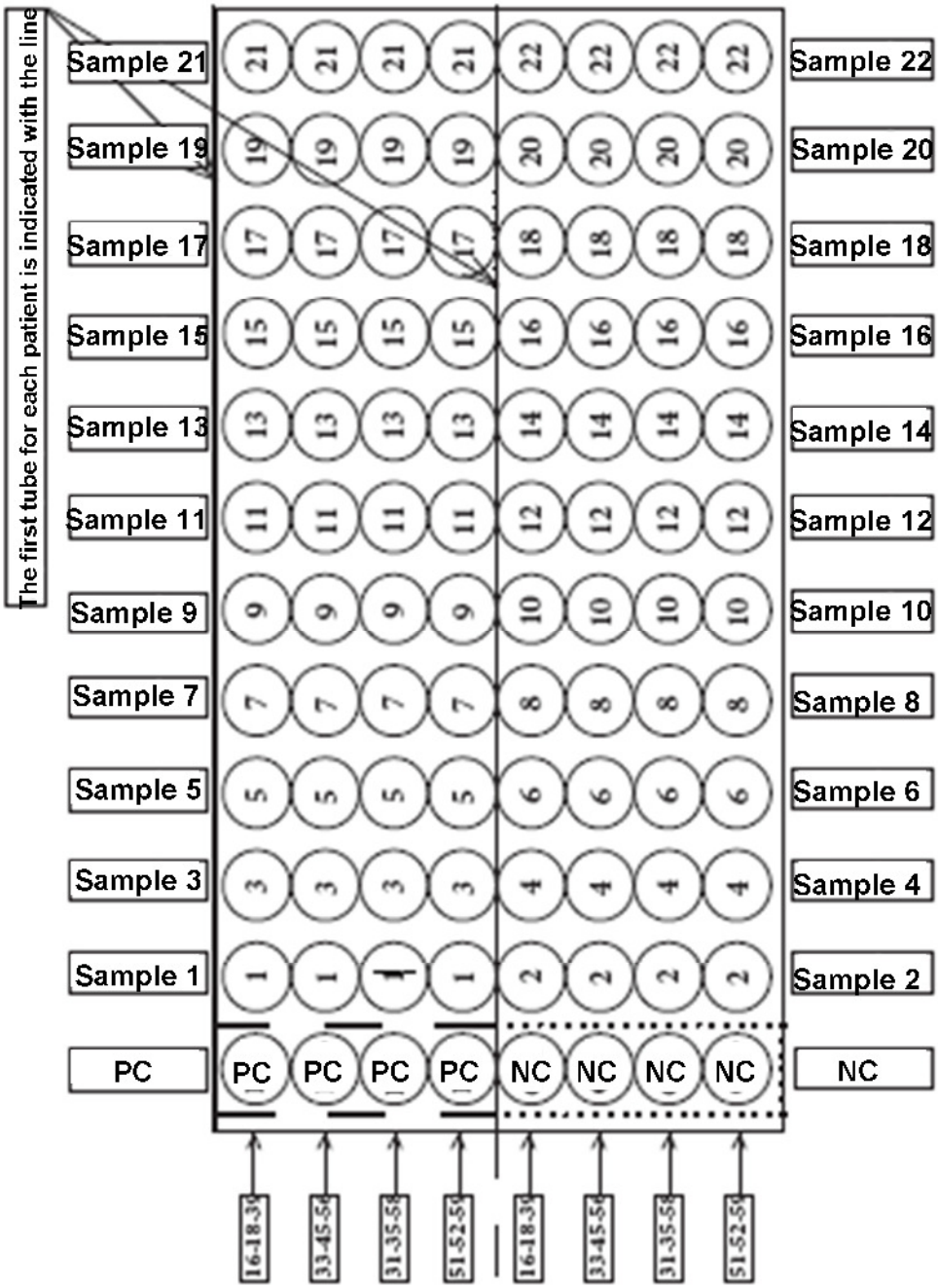
- Half of the strip, containing RMM1, RMM2, RMM3, RMM4).
- The tube with RMM1 is marked with a line.

Cut the tubes together with the covering film.

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.

RealLine HPV HCR Genotype Str-Format

Recommended order of tubes in the cycler



RealLine HPV HCR Genotype Str-Format

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 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.

9.4 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			

9.5 Select the amplification detection channels.

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

9.6 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.

9.7 Run the program.

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.

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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**. No **FAM, HEX** and **ROX** fluorescent increase should appear (*no HPV DNA amplification*) up to **Ct 35** for HPV types **31, 33, 35, 39, 45, 51, 52, 56, 58, 59** and up to **Ct 40** for HPV types **16 and 18**.

- For **NC** in **RMM 4** no **Cy5** fluorescent increase should appear (*no human □-actin gene DNA amplification*) up to **Ct 32**.

If **Ct** value for NC through **FAM, ROX** or **HEX** channels for HPV types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 **is less than or equal to 35** or for HPV types 16 and 18 **is less than or equal to 40** or **Ct** value through **Cy5** in RMM 4 **is less than or equal to 32**, it indicates the presence of contamination (see p. 9.7).

10.3 Analysis of the specimen validation results.

10.3.1. For each sample in **RMM 4** an increase in human β-actin DNA amplification signal (channel **Cy5**) should be detected.

10.3.2. The specimen is considered **valid** if the **Ct value** for this specimen via **Cy5** in RMM 4 **is less than or equal to 32**.

10.3.3 For specimens found to be **invalid** (**Ct** through the **Cy5** channel in RMM 4 is **above 32**), a repeated collection of specimens, DNA extraction and PCR is required.

Attention! *For subsequent analysis, only the specimens recognized as valid at this stage can be used.*
Specimen validation allows evaluating the accuracy of the specimen collection and storage. If the epithelial swab specimen was obtained incorrectly (insufficient number of cells in the specimen) or stored in conditions that did not ensure the stability of the DNA, the amplification signal of β-actin gene DNA would be too low, and the specimen would be found invalid. Negative result of HPV DNA detection in this case could be erroneous.

RealLine HPV HCR Genotype Str-Format

10.4 Analysis of results of HPV HCR detection.

10.4.1. For each specimen in **RMM 1-3** an increase of the amplification signal of IC DNA (channel **Cy5**) should be detected and IC **Ct** determined.

10.4.2. Calculate (IC **Ct**)$_{av}$ as an average IC **Ct** of all analysed specimens (including PC and NC) independently for each of RMM 1-3. IC **Ct** values that differ by more than 2 from the (IC **Ct**)$_{av}$ should be rejected. Recalculate the (IC **Ct**)$_{av}$ for the remaining values after the screening.

10.5 The sample is considered **negative** (not containing HPV DNA of the corresponding type), if **Ct** value via **FAM, HEX** and **ROX** channels for this sample is **above 35** or is not determined.

If 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 *HPV DNA*, when **Ct** value via **FAM HEX** and **ROX** channels for this sample (*in any of RMM 1, 2 , 3, 4 tubes*) is **less than or equals to 35** for HPV types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 or is **less than or equals to 40** for HPV types 16 and 18**.** Correspondence of results with HPV types is shown in the table:.

	Positive detection signal in the channel			
Tube	**FAM (*Green*)**	**JOE/HEX (*Yellow*)**	**ROX (*Orange*)**	**Cy5 *(Red)***
MM1	**type 16**	type 39	**type 18**	IC
MM2	type 33	type 56	type 45	IC
MM3	type 31	type 58	type 35	IC
MM4	type 52	type 59	type 51	**human β-actin DNA**

Purple: cut-off is **Ct 40** for HPV types 16 and 18
Pink: cut-off is Ct 35 for types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59
Light Blue: IC: cut-off is Ct 28 – 31 dependent on the cyler
Dark Blue: cut-off is Ct 32 for human beta-actin

Note: Analyzed samples may contain HPV DNA of one or several types in any combinations.

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, all samples of this run that were identified as positive have to be repeated. Samples, that have shown negative results in this run, should be considered as 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 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 C or in 50 µl aliquots at minus (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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