AB ANALITICA
ADVANCED BIOMEDICINE

code RQ-99
code RQ-99R

CE IVD

REALQUALITY
RQ-HPV HR/LR Multiplex

Kit for detection of 20 high-risk and
2 low-risk genotypes of the
Human Papillomavirus (HPV)
by Real Time PCR

User Manual

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1 PRODUCT INFORMATION

1.1 Intended use

The REALQUALITY RQ-HPV HR/LR Multiplex kit is an IVD for detection of the DNA of 14 high-risk, 6 possibly high-risk and 2 low-risk genotypes of the Human Papillomavirus (HPV) and genotyping of HPV 6, HPV 11, HPV 16 and HPV 18.
The test is based on Real Time PCR on DNA extracted from human clinical samples.
This *in vitro* diagnostic test for detection of Human Papillomavirus (HPV) is an auxiliary device for diagnosis and monitoring of HPV infections.
It is recommended to use this kit as indicated in the instructions herein.

This manual is related to the following products:

REALQUALITY RQ-HPV HR/LR Multiplex
Kit for detection of 20 high-risk and 2 low-risk genotypes of the Human Papillomavirus (HPV) by Real-Time PCR.

This product is in accordance with Directive 98/79/EC (Annex III) on *in vitro* diagnostic medical devices (CE IVD marking).

Contains all reagents needed for Real-Time PCR.

Code	Product	PKG
RQ-99-XM*	REALQUALITY RQ-HPV HR/LR Multiplex	25 tests
RQ-99-4M		50 tests
RQ-99-6M		100 tests
RQ-99-4A	REALQUALITY RQ-HPV HR/LR Multiplex (To use on automatic GENEQUALITY X120 platform)	50 tests
RQ-99-6A		100 tests

* sample code

Validated on:
- □ Applied Biosystems 7500 Fast/7500 Fast Dx Real Time PCR System (ABI 7500 Fast/Fast Dx – *Applied Biosystems*)
- □ Dx Real Time System (Bio-Rad Dx – *Bio-Rad*)
- □ CFX96 Real Time PCR Detection System (Bio-Rad CFX96 – *Bio-Rad*)
- □ CFX96 Real Time PCR Detection System-IVD (Bio-Rad CFX96-IVD – *Bio-Rad*)
- □ Rotor-Gene Q MDx (RG-Q MDx – *QIAGEN*)

Code	Product	PKG
RQ-99R-XM*	REALQUALITY RQ-HPV HR/LR Multiplex	25 tests
RQ-99R-4M		50 tests
RQ-99R-6M		100 tests

* sample code

Validated on:
- □ LightCycler® 480 Real Time PCR System version II (LC 480 II – *Roche*)

2 KIT CONTENT

2.1 Code RQ-99

BOX RG

STORAGE AT -30°C/-20°C

DESCRIPTION	LABEL	CAP COLOR	RQ-99-XM (25 tests)	RQ-99-4M (50 tests)	RQ-99-6M (100 tests)	RQ-99-4A (50 tests)	RQ-99-6A (100 tests)
Mastermix containing the amplification reagents	HPV RX-1 Real time mix	Red	1 × 540 µL	2 × 540 µL	4 × 540 µL	2 × 660 µL	4 × 660 µL
Mastermix containing the amplification reagents	HPV RX-2 Real time mix	Green	1 × 540 µL	2 × 540 µL	4 × 540 µL	2 × 660 µL	4 × 660 µL
Amplification negative control	aNTC	-	-	-	-	1 × 200 µL	1 × 200 µL

BOX PC

STORAGE AT -30°C/-20°C

DESCRIPTION	LABEL	CAP COLOR	RQ-99-XM (25 test)	RQ-99-4M (50 tests)	RQ-99-6M (100 tests)	RQ-99-4A (50 tests)	RQ-99-6A (100 tests)
Positive Control HPV 16/18/33/BG (DNA containing part of the genome of HPV 16, HPV 18, HPV 33 and the β globin gene - BG)	PC HPV RX-1	Red	1 × 50 µL	1 × 50 µL	2 × 50 µL	3 × 70 µL	6 × 70 µL
Positive Control HPV 6/11/26 /BG (DNA containing part of the genome of HPV 6, HPV 11, HPV 26 and the β-globin gene)	PC HPV RX-2	Green	1 × 50 µL	1 × 50 µL	2 × 50 µL	3 × 70 µL	6 × 70 µL

2.2 Code RQ-99R

BOX RG

STORAGE AT -30°C/-20°C

DESCRIPTION	LABEL	CAP COLOR	RQ-99R-XM (25 tests)	RQ-99R-4M (50 tests)	RQ-99R-6M (100 tests)
Mastermix containing the amplification reagents	HPV RX-1 Real time mix	Red	1 × 540 µL	2 × 540 µL	4 × 540 µL
Mastermix containing the amplification reagents	HPV RX-2 Real time mix	Green	1 × 540 µL	2 × 540 µL	4 × 540 µL
Mix containing an additional reagent for LC 480 II amplification instrument	LC Real time mix	White	1 × 40 µL	1 × 40 µL	2 × 40 µL

BOX PC

STORAGE AT -30°C/-20°C

DESCRIPTION	LABEL	CAP COLOR	RQ-99R-XM (25 tests)	RQ-99R-4M (50 tests)	RQ-99R-6M (100 tests)
Positive Control HPV 16/18/33/BG (DNA containing part of the genome of HPV 16, HPV 18, HPV 33 and the β-globin gene - BG)	PC HPV RX-1	Red	1 × 50 µL	1 × 50 µL	2 × 50 µL
Positive Control HPV 6/11/26/BG (DNA containing part of the genome of HPV 6, HPV 11, HPV 26 and the β-globin gene)	PC HPV RX-2	Green	1 × 50 µL	1 × 50 µL	2 × 50 µL

3 STORAGE AND STABILITY OF REAGENTS

Each part of the kit must be must be stored at the conditions indicated on the label of each box:

Box RG	Store at -30°C/-20°C
Box PC	Store at -30°C/-20°C

If stored at the recommended temperatures, the kit components are stable until the expiration date on the box.

4 PRECAUTIONS FOR USE

- The kit must be used only as an IVD and be handled by qualified technicians who are trained in techniques of molecular biology applied to diagnostics;
- Before using the kit read the user manual carefully and completely;
- Keep the kit protected from heat;
- Please pay particular attention to the expiration date on the label of each box. Do not use any part of the kit after the expiration date;
- The reagents present in the kit must be considered an indivisible unit. Do not use them separately or in combination with reagents from other kits or lots;
- The HPV RX-1 Real time mix, HPV RX-2 Real time mix and LC Real time mix reagents must be thawed at room temperature before use. Homogenize the solutions by inverting the tubes several times (do not vortex!). Then centrifuge briefly;
- The positive controls must be thawed at room temperature before use. Homogenize the solution, then centrifuge briefly;
- Work quickly, particularly if preparing the reactions at room temperature. If possible, work on ice or on a cooling block. Take care, as far as possible, to work away from direct light.

In case of any doubt concerning storage conditions or box integrity, please contact the technical support team at AB ANALITICA before use:

customersupport@abanalitica.it

Please observe the following precautions for the nucleic acid amplification:

- Use filter tips;
- Store biological samples, extracted DNA, the positive controls and all amplification products separate from the HPV RX-1 Real time mix, HPV RX-2 Real time mix, the LC Real time mix and aNTC;
- Setup pre- and post-PCR areas. Do not share instruments or consumables (pipettes, tips, tubes, etc.) between those areas;
- Change gloves frequently;
- Clean the bench surfaces with 10 % sodium hypochlorite, then wash with distilled water.
- To clean the GENEQUALITY X120 platform, refer to the system user manual.

5 SAFETY RULES

5.1 General safety rules

- Wear disposable gloves when handling reagents and clinical samples. Wash hands after the procedure;
- Do not pipet by mouth;
- No known diagnostic method can ensure the absence of infective agents. Therefore, consider all clinical samples to be potentially infectious and handle them accordingly;
- All devices that come into contact with clinical samples must be considered contaminated and disposed of as such. In case of accidental spilling of samples, clean up with 10 % sodium hypochlorite. The material used to clean must be disposed of in special containers for contaminated products;
- Decontaminate and then dispose of clinical samples, materials and contaminated products.

5.2 Safety rules concerning the kit

The risks for use of this kit are related to the single components.
Dangerous components: **none**.
The Safety Information Sheet (SIS) of this device is available upon request.

6 REQUIRED, BUT NOT SUPPLIED MATERIALS

6.1 Reagents

- Reagents for DNA extraction;
- DNAse- and RNAse-free sterile water;
- REALQUALITY LC 480 COLOR COMPENSATION KIT, code RQ-SCLC48 (required, when using the kit with code RQ-99R).

6.2 Instruments

- GENEQUALITY X120 instrument (*AB ANALITICA*) (code 08-20)*;
- Laminar flow cabinets;
- Micropipettes (range: 0.5-10 µL; 2-20 µL; 10-100 µL; 200-1000 µL);
- Microcentrifuge (max. 12-14,000 rpm);
- Plate centrifuge (optional);
- Real Time PCR instrument.

This kit has been validated on:

- Applied Biosystems 7500 Fast/7500 Fast Dx Real Time PCR System (ABI 7500 Fast/7500 Fast Dx – *Applied Biosystems*)
- Dx Real Time System (Bio-Rad Dx – *Bio-Rad*)
- CFX96 Real Time PCR Detection System (Bio-Rad CFX96 – *Bio-Rad*)
- CFX96 Real Time PCR Detection System-IVD (Bio-Rad CFX96-IVD – *Bio-Rad*)
- Rotor-Gene Q MDx (RG-Q MDx – *QIAGEN*)
- LightCycler® 480 Real-Time PCR System version II (LC 480 II – *Roche*)

6.3 Disposables

- Talc-free disposable gloves;
- Disposable sterile filter tips (range: 0.5 10 µL; 2-20 µL; 10-100 µL; 200-1000 µL);
- Tips with filter 300 µL, code 20-49008-0104*;
- Tips with filter 50 µL, code 20-49010-0104*;
- Waste bags, code 20-199202*;
- Micro-tubes 2 mL PP non capillaries, code 20-72664*;
- Neutral caps for micro-tubes 2 mL PP (non capillaries), code 20-65716*;
- Instrument specific consumables for Real Time PCR:
  - transparent plates with adhesive optical films for ABI 7500 Fast/7500 Fast Dx instrument
  - white plates with optical caps for Bio-Rad CFX96/Bio-Rad CFX96-IVD instrument
  - white plates with optical adhesive films for Bio-Rad Dx and LC 480 II instruments
  - tube strips of 0.1 mL and caps for RG-Q MDx instrument

*materials provided exclusively for use on the GENEQUALITY X120 platform.

For further information, please contact the technical support team at AB ANALITICA.

7 INTRODUCTION

Human Papillomavirus (HPV) infection is the most common and widespread of sexually transmitted diseases. The different types of HPV are generally distinguished in low-risk and high-risk cancer genotypes. The latter (in particular HPV 16 and 18) are recognized as the necessary cause of cervical cancer and, in general, among the most important carcinogenic viruses for the human species. The transforming role, in fact, does not end in the genesis of cervicocarcinoma, but is strongly correlated to other neoplasms of the female and male genital sphere (vulva, vagina, anus, penis) and extragenital neoplasms (oral cavity, pharynx, larynx). The carcinogenic potentials can be ascribed to two viral oncoproteins (E6 and E7) which, after the viral genome has been integrated into the host genome, determine an uncontrolled proliferative stimulus. This leads to a decreased capability in controlling the cellular surveillance mechanisms, with consequent accumulation of genetic abnormalities, increased genomic instability and the appearance of aneuploidy.
During the process of viral integration, part of the HPV DNA can be lost. However, the process never involves the E6 and E7 genes, thus using a screening system targeting those genes exclude the possibility of false negative results due to viral integration events.

8 TEST PRINCIPLE

The PCR (Polymerase Chain Reaction) technique was the first method to amplify *in vitro* a specific part of DNA (target sequence) by a thermostable DNA polymerase.
This technique proved to be a valuable and versatile instrument of molecular biology. It allowed a much more efficient research of new genes and their expression and has revolutionized, among others, the fields of laboratory diagnostics and forensic medicine.

The Real Time PCR technology represents an advancement in the basic PCR technique, since it is possible to measure the number of DNA target molecules present in the sample during the exponential phase of the reaction.
The detection of the amplicons is based on the use of labelled probes or fluorescent intercalating agents able to bind to double helix DNA molecules produced during each amplification cycle. The fluorescence is registered in real time by using a thermal cycler with an optical detector.

The main advantage of the Real Time PCR compared to conventional techniques of DNA amplification is the possibility to perform a semi-automated amplification. This means that extra steps necessary to visualize the amplification product can be avoided and the risk of contamination by post-PCR manipulation is reduced.

9 PRODUCT DESCRIPTION

The REALQUALITY RQ-HPV HR/LR Multiplex kit, code RQ-99 and RQ-99R, allows the detection of 14 high-risk genotypes (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68), 6 possibly high-risk genotypes (HPV 26, 53, 67, 70, 73, 82) and 2 low-risk genotypes (HPV 6 and 11) of Human Papillomavirus in two reactions. In addition, the kit is able to specifically identify HPV 6, HPV 11, HPV 16 and HPV 18. The used technique is Real time PCR.
The target regions and the used fluorophores are shown below:

Genotype	Target region	Fluorophore
HPV 16	- *E7* gene	JOE
HPV 18	- *E7* gene	Cy5
HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 (HPV RX-1)	- *E6* and *E7* genes	FAM
HPV 6	- *E6* and *E7* genes	JOE
HPV 11	- *E7* gene	Cy5
HPV 26, 53, 67, 70, 73, 82 (HPV RX-2)	- *E6* and *E7* genes	FAM

The positive controls supplied with this kit contain DNA fragments that correspond to the target regions of HPV 16, 18 and 33 and the β-globin gene (PC HPV RX-1) and to the target regions of genotypes HPV 6, 11 and 26 and the β-globin gene (PC HPV RX-2). As such, these controls are not harmful for the user.

The kit also allows to evaluate the presence of reaction inhibitors in the extracted DNA and to monitor the extraction process by amplification of the *β-globin* gene (BG - internal control) in multiplex with the target pathogens. This valid tool helps the operator to recognize false negative results. The amplification of the BG gene is detected with the ROX fluorophore.

The kit includes two ready-to-use PCR master mixes that contains all reagents needed for the reaction as well as the dUTP/UNG system that allows to prevent contaminations from previous amplifications, being able to remove uracil residues embedded in single or double stranded DNA molecules.

10 SAMPLE COLLECTION, MANIPULATION AND PRETREATMENT

This kit has been validated on DNA extracted from cervical, vaginal, urethral, buccal and anal swabs, from urethral, vaginal and foreskin biopsy tissue, from vaginal secretion and from formalin-fixed and paraffin-embedded (FFPE) histology samples.

10.1 Cytological samples

Regarding the swabs, the collection is performed using a swab or a special sterile brush (cyto-brush).
The collected cells are diluted in a suitable medium and stored according to the manufacturer's instructions.
If the sample cellularity is very low, before proceeding with DNA extraction, it may be necessary to concentrate the sample by repeated centrifugation steps. PBS or physiological solution may be used for resuspension of the sample.
One or more steps of centrifugation and subsequent resuspension of the sample can also be useful to eliminate the possible presence of mucus, red blood cells or other interfering materials.

10.2 Histological samples

Histological samples include fresh, frozen or formalin-fixed and paraffin-embedded biopsies.
The biopsy is taken as routine.
The biopsy can be treated as fresh, within minutes from collection, or rapidly frozen in liquid nitrogen and subsequently stored at −80°C until the moment of mechanical disintegration, using a sterile scalpel, and the following digestion with Proteinase K.
In case the biopsy has to be fixed and then included in paraffin, it is recommended to use formalin buffered at pH 7, with sodium or potassium salts, according to Lilie's formula, at 10%. Biopsies fixed in non-buffered formalin, in Bouin, in Holland or in acid-based fixatives (e.g. osmic acid) are not suitable for DNA extraction, since these substances form cross-links in the tissue, making it indigestible.
In the case of a fresh or frozen histological sample (up to about 50 mg), proceed rapidly with the mechanical disintegration of the tissue using a sterile scalpel. This operation must be carried out on a watch glass. The disaggregated tissue will be transferred to a test tube and digested with Proteinase K.
In the case of formalin-fixed and paraffin-embedded sample, it is necessary to perform paraffin removal before proceeding with sample digestion.
To increase the yield of the target DNA, it will be up to the investigator to limit the extraction to the preferred tissue part.

11 PROTOCOL

11.1 DNA extraction

This device has been validated for use on DNA extracted with the most common manual and automated extraction systems, including:

- GENEQUALITY X120 - GENEQUALITY X120 Pathogen Kit (AB ANALITICA).

For further information on device validation and compatibility with other extraction methods, Appendix 1 is available; please, contact the technical support team at AB ANALITICA.

11.2 Programming of the Real Time PCR instrument

11.2.1 Thermal profile and fluorescence reading

Set up the following thermal profile in the instrument:

Step	Repeats	Time	°C
1	1	02:00	50.0
2	1	10:00	95.0
3	45	00:15	95.0
		01:00	60.0*

* Fluorescence detection step

For the detection of the different fluorophores, each instrument uses the channels indicated below:

ABI 7500 Fast*#

ABI 7500 Fast Dx*#

	Target	Reporter Dye
Reaction 1 RX-1	HPV 16	JOE
	HPV 18	Cy5
	HPV RX-1	FAM
	BG (internal control)	ROX
Reaction 2 RX-2	HPV 6	JOE
	HPV 11	Cy5
	HPV RX-2	FAM
	BG (internal control)	ROX

Bio-Rad Dx#

Bio-Rad CFX96#

Bio-Rad CFX96-IVD#

	Target	Fluorophore
Reaction 1 RX-1	HPV 16	HEX
	HPV 18	Cy5
	HPV RX-1	FAM
	BG (internal control)	ROX
Reaction 2 RX-2	HPV 6	HEX
	HPV 11	Cy5
	HPV RX-2	FAM
	BG (internal control)	ROX

LC 480 II

	Target	Filter Combination
Reaction 1 RX-1	HPV 16	533 - 580
	HPV 18	618 - 660
	HPV RX-1	465 - 510
	BG (internal control)	533 - 640
Reaction 2 RX-2	HPV 6	533 - 580
	HPV 11	618 - 660
	HPV RX-2	465 - 510
	BG (internal control)	533 - 640

RG-Q MDx

	Target	Channel
Reaction 1 RX-1	HPV 16	Yellow
	HPV 18	Red
	HPV RX-1	Green
	BG (internal control)	Orange
Reaction 2 RX-2	HPV 6	Yellow
	HPV 11	Red
	HPV RX-2	Green
	BG (internal control)	Orange

*Select None as passive reference for all wells in use.
#The use of the product with the AB Genius Report software involves setting the name of the targets/detectors listed in the respective user manual

Set the final reaction volume where required.

Attention: For a more detailed protocol about PCR runs setup on the different Real Time PCR instruments, Appendix 2 is available. Please contact the technical support at AB ANALITICA and specify the Real Time PCR instrument of interest.

11.3 Preparation of the Real Time PCR plate (manual protocol)

11.3.1 Preparation of the reaction mix for code RQ-99

Once thawed, mix the HPV RX-1 Real time mix and HPV RX-2 Real time mix reagents and the relative positive controls several times (do not use vortex!), then subject them to brief centrifugation.
Quickly prepare the reaction (Rx) at room temperature or work on ice or on a cooling block. Take care, as far as possible, to work away from direct light.
Aliquot the following volumes in a sufficient number of tubes/wells for analysis of the samples and their controls:

RX-1	
Reagents	**1 Rx**
HPV RX-1 Real time mix	20 µL
Extracted DNA or PC HPV RX-1 or Negative control	5 µL
Final volume	**25 µL**

RX-2	
Reagents	**1 Rx**
HPV RX-2 Real time mix	20 µL
Extracted DNA or PC HPV RX-2 or Negative control	5 µL
Final volume	**25 µL**

Make sure no air bubbles remain in the wells and centrifuge at 4000 rpm for about 1 minute.
Load the samples on the Real Time PCR instrument and make sure the plate/tubes are placed correctly and start the amplification cycle.

HPV RX-1 Real time mix and HPV RX-2 Real time mix reagents are sensitive to changes in physical state: it is recommended not to subject them to more than three (3) freeze/thaw cycles. If performing runs with low numbers of samples, it is recommended to aliquot the reagents beforehand.
HPV RX-1 Real time mix and HPV RX-2 Real time mix contain fluorescent molecules: it is recommended to store these reagents away from light.
Positive controls are sensitive to changes in physical state: it is recommended not to subject them to more than three (3) freeze/thaw cycles If performing runs with low numbers of samples, it is recommended to aliquot the reagents beforehand.

11.3.2 Preparation of the reaction mix for the code RQ-99R

Thaw sufficient aliquots of HPV RX-1 Real time mix, HPV RX-2 Real time mix and LC Real time mix for the number of tests you want to perform.
After thawing, homogenize the mixes by inverting the tubes several times. Do not vortex! Centrifuge briefly.
Work rapidly. If possible, work on ice or a cooling block and in an area protected from direct light.
Add 6.75 µL of LC Real time mix to each tube of thawed HPV RX-1 Real time mix and HPV RX-2 Real time mix, taking care to signal the reconstitution of the latter, signing with an "R" the cap of the tubes themselves.
In the following, the reconstituted HPV RX-1 Real time mix and HPV RX-2 Real time mix reagents will be referred to as HPV RX-1 Real time mix R and HPV RX-2 Real time mix R.
Homogenize the HPV RX-1 Real time mix R and HPV RX-2 Real time mix R reagents (do not use vortex!) and the relative positive controls, previously thawed, by inverting the tubes several times. Centrifuge briefly.
Aliquot the following volumes in a sufficient number of tubes/wells for analysis of the samples and their controls:

RX-1	
Reagents	**1 Rx**
HPV RX-1 Real time mix R	20 µL
Extracted DNA or PC HPV RX-1 or Negative control	5 µL
Final volume	**25 µL**

RX-2	
Reagents	**1 Rx**
HPV RX-2 Real time mix R	20 µL
Extracted DNA or PC HPV RX-2 or Negative control	5 µL
Final volume	**25 µL**

Make sure no air bubbles remain in the wells and centrifuge at 4000 rpm for about 1 minute.
Transfer the plate on the thermal cycler being careful to position it correctly and start the amplification cycle.

HPV RX-1 Real time mix and HPV RX-2 Real time mix reagents are sensitive to changes in physical state: it is recommended not to subject them to more than three (3) freeze/thaw cycles. If performing runs with low numbers of samples, it is recommended to aliquot the reagents beforehand.
The LC Real time mix reagent is sensitive to changes in physical state: it is recommended not to subject it to more than four (4) freeze/thaw cycles. If performing runs with low numbers of samples, it is recommended to aliquot the reagent beforehand.
HPV RX-1 Real time mix and HPV RX-2 Real time mix contain fluorescent molecules: it is recommended to store these reagents away from light.
HPV RX-1 Real time mix R and HPV RX-2 Real time mix R reagents must be stored at -30°C/-20°C, away from direct light. It is recommended not to subject them to more than two (2) freeze/thaw cycles.
Positive controls are sensitive to changes in physical state: it is recommended not to subject them to more than three (3) freeze/thaw cycles If performing runs with low numbers of samples, it is recommended to aliquot the reagents beforehand.

11.3.3 Real Time instrument setup

Set up samples and controls in the instrument software, indicating their respective positions, and identify each one with its own name.

11.4 Preparation of the Real Time plate on GENEQUALITY X120 platform

11.4.1 Setup of the GENEQUALITY X120 instrument

The GENEQUALITY X120 system is able to perform nucleic acid extraction and Real time PCR plate setup in a single run; alternatively, the two phases can be performed independently.
In the software of GENEQUALITY X120 instrument, in the *PCR Preparation Parameters* window, identify the type of the assays that will be analyzed.
Finally, define for each assay which samples are in analysis.
The software of the GENEQUALITY X120 system automatically generates the Real-Time PCR plate layout.

11.4.2 Preparation of the reaction mix

During the preparation phase, before loading, thaw the reagents at room temperature, taking care as far as possible, to work away from direct light.
Once thawed, mix by inverting the HPV RX-1 Real time mix and HPV RX-2 Real time mix several times (do not use vortex!), then subject them to short centrifugation.
The master mix volume to be loaded on the GENEQUALITY X120 instrument is automatically calculated by the instrument software based on the number of samples to be analyzed.
Transfer into 2 mL tubes the volumes of HPV RX-1 Real time mix and HPV RX-2 Real time mix indicated in the *PCR Preparation Parameters* window.
Once thawed, mix the PCs using the vortex or inverting the tubes several times, then subject them to short centrifugation.
Attention: avoid the formation of bubbles or foam in the mastermix/positive control tubes that could cause plate loading errors.

In the loading phase of the Real-Time PCR reagents, place:

- The aliquots of HPV RX-1 Real time mix and HPV RX-2 Real time mix in the cooling block of the instrument in the position indicated by the software;
- The PC HPV RX-1 and PC HPV RX-2 tubes in the PCR reagents rack;
- The aNTC tube (amplification negative control) in the PCR reagents rack.

For the preparation of the Real-Time PCR plate, the GENEQUALITY X120 instrument will move for each reaction (Rx) the following volumes:

RX-1	
Reagents	**1 Rx**
HPV RX-1 Real time mix	20 µL
Extracted DNA or PC HPV RX-1 or Negative control	5 µL
Final volume	**25 µL**

RX-2	
Reagents	**1 Rx**
HPV RX-2 Real time mix	20 µL
Extracted DNA or PC HPV RX-2 or Negative control	5 µL
Final volume	**25 µL**

Once the run setup is complete, close the plate with optical caps (see paragraph 6.3 Disposables).
Make sure there are no bubbles in the bottom of the wells and centrifuge the plate at 4000 rpm for about 1 minute.

Attention:
HPV RX-1 Real time mix and HPV RX-2 Real time mix reagents are sensitive to physical state changes: it is recommended not to subject them to more than three (3) freeze/thaw cycles. If performing runs with low numbers of samples, it is recommended to aliquot the reagent beforehand.
HPV RX-1 Real time mix and HPV RX-2 Real time mix contain fluorescent molecules: it is recommended to store these reagents away from light.
Positive controls cannot be used for more than three (3) runs on the GENEQUALITY X120 instrument.

11.4.3 Real Time instrument setup

Once the plate preparation has been completed, in the *Run* window export the configuration of the PCR setup of interest and select the Real-Time PCR instrument to be used.
Insert the plate in the thermo cycler paying attention to position it correctly.

Configure the Real-Time PCR instrument using the plate layout file exported from the GENEQUALITY X120, and then save the run file.

For more information, refer to the GENEQUALITY X120 instrument user manual.

12 ANALYSIS OF RESULTS

At the end of the reaction, visualize the graph in linear scale.
Analyze separately graphs and amplification results of each target, by selecting the appropriate target, and interpret them as indicated below.

Attention:
The LC 480 II instrument requires the generation of a Color Compensation file before proceeding with the analysis run with the REALQUALITY RQ-HPV HR/LR Multiplex kit (see Paragraph 6 REQUIRED, BUT NOT SUPPLIED MATERIALS).

12.1 Validation of analytical run

Before interpreting the result obtained for the samples, verify the PCR run according to the tables below.

HPV RX-1 Real time mix:

	RESULT	INTERPRETATION
Positive control PC HPV RX-1	Amplification signal simultaneously present in FAM, JOE, Cy5 and ROX*	Control and PCR worked correctly
	No amplification signal in FAM and/or JOE and/or Cy5 and/or ROX	Problems with amplification of HPV and/or BG DNA: repeat the analysis
Negative control	Amplification signal in FAM and/or JOE and/or Cy5 and/or ROX*	Contamination: repeat the analysis
	No amplification signal in any channel	Control and PCR worked correctly

* The amplification signal must be determined by a rapid and regular increase of the fluorescence values and not by peak phenomena or gradual increase of the background signal (irregular or high background).

HPV RX-2 Real time mix:

	RESULT	INTERPRETATION
Positive control PC HPV RX-2	Amplification signal simultaneously present in FAM, JOE, Cy5 and ROX*	Control and PCR worked correctly
	No amplification signal in FAM and/or JOE and/or Cy5 and/or ROX	Problems with amplification of HPV and/or BG DNA: repeat the analysis
Negative control	Amplification signal in FAM and/or JOE and/or Cy5 and/or ROX*	Contamination: repeat the analysis
	No amplification signal in any channel	Control and PCR worked correctly

* The amplification signal must be determined by a rapid and regular increase of the fluorescence values and not by peak phenomena or gradual increase of the background signal (irregular or high background).

The analytical run is considered validated with a suitable result for all controls.

12.2 Interpretation of results

If the controls show the expected results, please proceed to result interpretation as shown below.

12.2.1 Internal control

The amplification of BG gene (internal control) allows to evaluate the presence of PCR reaction inhibitors in the extract and to monitor the extraction process. It is also useful for recognizing any false negative results.

Result	Interpretation	
BG	**HPV negative sample**	**HPV positive sample**
Amplification signal with Ct ≤ 34	Suitable sample	Suitable sample
Amplification signal with Ct > 34	Unsuitable sample	Suitable sample*

*The assay was standardized to favor the amplification reaction of the pathogenic target, therefore it is possible, in positive samples, to have a delayed Ct in the β-globin gene amplification signal.

A sample is negative for HPV only when the amplification curve of the internal control has a Ct lower than or equal to 34.

12.2.2 Pathogenic target

The evaluation of the different fluorescence channels allows to define the presence or absence of the pathogen in the tested sample.

	RX-1		
	JOE/HEX/533-580/Yellow	Cy5/618-660/Red	FAM/465-510/Green
	Interpretation	**Interpretation**	**Interpretation**
Amplification signal[#]	Positive for HPV 16	Positive for HPV 18	Positive for one or more of the following genotypes: HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68
No amplification signal	Negative for HPV 16[§]	Negative for HPV 18[§]	Negative for HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68[§]

	RX-2		
	JOE/HEX/533-580/Yellow	Cy5/618-660/Red	FAM/465-510/Green
	Interpretation	**Interpretation**	**Interpretation**
Amplification signal[#]	Positive for HPV 6	Positive for HPV 11	Positive for one or more of the following genotypes: HPV 26, 53, 67, 70, 73, 82
No amplification signal	Negative for HPV 6[§]	Negative for HPV 11[§]	Negative for HPV 26, 53, 67, 70, 73, 82[§]

[#]The amplification signal must be determined by a rapid and regular increase in fluorescence values and not by peak phenomena or gradual increase in the background signal (irregular or high background).
[§]Negative or below the limit of analytical sensitivity of the product (see paragraph 15.2 Analytical sensitivity).

13 TROUBLESHOOTING

No amplification signal for positive control and samples

- *The instrument was not programmed correctly*

► Repeat the amplification taking care of the instrument programming. Pay particular attention to the thermal profile, the selected fluorophores and that the positions of the samples in the instrument setup correspond to the actual positions of samples/controls.

- *The reaction mix did not work properly*

► Make sure that HPV RX-1 Real time mix and HPV RX-2 Real time mix are correctly stored at -30°C/-20°C and, if the RQ-99R code is used, also the LC Real time mix, HPV RX-1 Real time mix R and HPV RX-2 Real time mix R. Avoid unnecessary freeze/thaw cycles. Keep them away from direct light.

► Do not use the product past the expiration date reported on the label.

- *For the automatic setup, the reaction mix was not positioned correctly in the cooling block*

► Make sure to position correctly the reaction mix as indicated by the GENEQUALITY X120 instrument software

Very weak amplification signal for the positive control

- *The positive controls were not stored correctly and have degraded*

► Make sure the positive controls have been stored at -30°C/-20°C and make sure they do not undergo more than three (3) freeze/thaw cycles.

► Do not use the product after the expiration date reported on the label.

Amplification signal of β-globin is very delayed or absent in the extracted sample (negative for HPV)

- *The extracted DNA is not suited for PCR and the reaction was inhibited*

► Make sure that the nucleic acids are correctly extracted.

► If an extraction method uses washing steps with solutions containing ethanol, make sure no ethanol residue remains in the DNA extract.

► Use one of the extraction systems listed in paragraph 11.1 or in Appendix 1.

► If present, make sure to remove the magnetic beads present in the eluate.

- *The clinical sample is not suited for analysis*

► Make sure clinical samples are correctly stored and pretreated before performing the analysis.

In case of any further problems, please contact the technical support team at AB ANALITICA:

customersupport@abanalitica.it

fax (+39) 049-8709510

tel. (+39) 049-761698

14 DEVICE LIMITATIONS

The kit can have reduced performance if:

- The clinical sample is not suitable for this analysis;
- The DNA is not suitable for PCR due to the presence of PCR inhibitors or the use of an inappropriate extraction method;
- The kit was not stored correctly.

Moreover, the presence of possible mutations/polymorphisms in the region of probe and/or primers annealing could compromise the identification of pathogenic DNA.
The product is an auxiliary tool for the diagnosis and monitoring of HPV infections and therefore requires that the obtained results are interpreted taking into consideration all clinical data and other laboratory tests regarding the patient.
Finally, as for any other diagnostic device, even if it has been reduced to a minimum, there is a residual risk to obtain invalid/false positive/false negative results; this risk cannot be eliminated or further reduced.

15 DEVICE PERFORMANCE

15.1 Analytical specificity

The specificity of the REALQUALITY RQ-HPV HR/LR Multiplex kit is guaranteed by an accurate and specific selection of primers and probe and by the use of stringent amplification conditions.
Alignment of primers and probes in the most important databases showed no non-specific pairing.
In order to analyse possible cross-reactions of this assay, samples positive for potentially cross-reactive pathogens as well as HPV genotypes that are not assayed by this device were tested with this IVD. None of the tested pathogens gave a positive result.

15.2 Analytical sensitivity: detection limit

For the determination of the analytical sensitivity in regard to the HPV genotypes that are detected by this device, serial dilutions of target plasmid DNA at various concentrations, depending on the genotype, were tested. Overall the plasmid concentrations ranged from 0.1 to 5 viral genome copies / µL. Five microliters (5 µL) of each dilution were amplified in eight replicates per run and in multiplex with the internal control.
The results were then analysed using a probit analysis.
The limits of analytical sensitivity for the device REALQUALITY RQ-HPV HR/LR Multiplex are listed in the following table (p = 0.05):

Target	copies/rx*	copies/mL§
HPV 16	3.44	155.25
HPV 18	4.05	182.25
HPV 31	12.54	564.75
HPV 33	3.62	162.00
HPV 35	18.30	823.50
HPV 39	5.90	265.50
HPV 45	5.24	236.25
HPV 51	8.85	398.25
HPV 52	4.55	204.75
HPV 56	7.23	326.25
HPV 58	6.31	283.50
HPV 59	5.15	231.75
HPV 66	6.25	281.25
HPV 68a	5.80	261.00
HPV 6	3.90	175.50
HPV 11	10.18	459.00
HPV 26	4.41	198.00
HPV 53	7.03	315.00
HPV 67	4.92	220.50
HPV 70	7.01	315.00
HPV 73	10.80	486.00
HPV 82	11.39	513.00

* rx (reaction): using 5 µL of DNA extract per reaction.
§ Values obtained considering an extraction system using 400 µL of initial sample and 90 µL of elution volume.

The device REALQUALITY RQ-HPV HR/LR Multiplex was also tested on the samples of the “2013 WHO LabNet Proficiency Panel”. All samples of the panel were correctly identified.

15.3 Reproducibility

In order to determine the intra-assay variability (variability in one analysis session among replicates of the same sample) monoinfected samples of each assayed HPV genotype were tested in eight replicates in one run. The intra-assay coefficients of variation (CV) in regard to the cycle threshold (Ct) were calculated. The coefficients of variation for each HPV genotype are listed below:

Target	Intra-assay CV (%)
HPV 16	0.30
HPV 18	0.17
HPV 31	0.63
HPV 33	0.48
HPV 35	1.03
HPV 39	0.40
HPV 45	1.28
HPV 51	0.89
HPV 52	0.76
HPV 56	0.42
HPV 58	0.71
HPV 59	0.67
HPV 66	0.84
HPV 68a	0.71
HPV 68b	0.63
HPV 6	0.61
HPV 11	0.30
HPV 26	0.64
HPV 53	0.97
HPV 67	0.43
HPV 70	0.47
HPV 73	0.47
HPV 82	0.78

In order to determine the inter-assay variability (variability over different analysis sessions of replicates of the same sample), monoinfected samples of each assayed HPV genotype were tested in three consecutive runs. The coefficient of variation was calculated from the Ct of the samples for each HPV genotype.
The inter-assay coefficients of variation for each HPV genotype are listed below:

Target	Inter-assay CV (%)
HPV 16	0.06
HPV 18	0.15
HPV 31	1.48
HPV 33	0.59
HPV 35	0.43
HPV 39	0.93
HPV 45	0.92
HPV 51	0.86
HPV 52	0.90
HPV 56	0.17
HPV 58	0.39
HPV 59	0.62
HPV 66	0.77
HPV 68a	0.16
HPV 68b	0.45
HPV 6	1.36
HPV 11	1.88
HPV 26	0.61

Target	Inter-assay CV (%)
HPV 53	0.84
HPV 67	0.83
HPV 70	0.42
HPV 73	0.97
HPV 82	0.53

15.4 Diagnostic specificity

A statistically significant number of samples negative for HPV or positive for HPV strains that are not assayed by this system were tested simultaneously with REALQUALITY RQ-HPV HR/LR Multiplex and another CE IVD device. From the obtained results the diagnostic specificity was calculated. The diagnostic specificity of this device is 99.1 % for reaction 1 and 99.2 % for reaction 2.

15.5 Diagnostic sensitivity

A statistically significant number of samples positive for the HPV genotypes assayed with this device were tested simultaneously with REALQUALITY RQ-HPV HR/LR Multiplex and another CE IVD device. From the obtained results the diagnostic sensitivity was calculated. The diagnostic sensitivity of this device is 99.5 % for reaction 1 and 97.8 % for reaction 2.

15.6 Accuracy

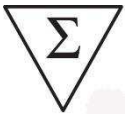
The accuracy was calculated as the ratio of the number of correct test results to the total number of executed tests. The accuracy of REALQUALITY RQ-HPV HR/LR Multiplex is 99.3 % for reaction 1 and 98.6 % for reaction 2.

16 BIBLIOGRAPHY

Consensus Guidelines, Bethesda 2001.

Human papillomavirus laboratory manual. First edition, (WHO/IVB/10.12), 2009.

17 SYMBOLS

SYMBOLS	TYTLE OF THE SYMBOL
	CE mark
	Medical device for *in vitro* diagnostics
	Refer to instructions for use
	Protect from direct light
	Temperature range
	Expiration date
	Manufacturer
	Lot number
	Product code
	Sufficient for “N” tests

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