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Instructions for use
HPV-Risk assay

for use with the
CFX96 Touch™ Real time PCR detection system

Version 1

IVD For in vitro diagnostic use

CE

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Contents

Intended Use

The HPV-Risk assay is an in vitro real-time PCR-based assay for the qualitative detection of human papillomavirus (HPV) DNA of the following 15 (probably) high-risk HPV genotypes, i.e., 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 67, and 68.

Samples that may be tested with HPV-Risk assay include DNA isolated from specimens that are collected in the following ways:

- Cervical specimens collected using a brush/broom-type collection device (collected by a physician)
- Vaginal specimens collected using a brush-broom or lavage device (self-collected)

Indications for use:

- As a primary test in screening of women for the risk of cervical (pre)cancer to determine the need for referral to colposcopy or other follow-up procedures
- As a follow-up test for women with Pap test results with atypical squamous cells of undetermined significance (ASC-US) or low-grade squamous intra-epithelial neoplasia (lsil) to determine the need for referral to colposcopy of other follow-up procedures

This product is intended to be used by professional users, such as technicians and laboratorians, who are trained in in vitro diagnostics procedures, molecular biological techniques, and the CFX96 Touch real-time PCR detection system.

Summary and Explanation

Human papillomaviruses (HPV) belong to the family of Papillomaviridae and are small double-stranded DNA viruses. The circular genome is approximately 7.9 kilo bases in size. More than 100 types of HPV have been identified, of which certain HPV types, known as high-risk HPV (hrHPV) like HPV 16 and 18, are associated with the induction of mucosal lesions that can progress to malignancy. Cervical cancer and its precursor lesions (cervical intraepithelial neoplasia, CIN) are the most well-known complications of a persistent infection with a high-risk type of HPV [1-3].

The viral genome contains early (E) and late (L) genes, which encode proteins necessary for early and late stages of the HPV life cycle, respectively. The E6 and E7 gene products of hrHPV types have carcinogenic properties and are necessary for malignant transformation of the host cell [4]. Malignant progression is often associated with viral integration into the genome of the host cell [5]. Integration results in interruption of the viral genome in a region that may extend from the E1 to the L1 open reading frame [6]. This may have consequences for PCR-mediated amplification of viral DNA in these regions. As not only the initiation but also the maintenance of the transformed phenotype depends on continuous expression of the viral oncoproteins [7,8], the viral E6/E7 region is invariably retained in integrated viral genomes in cervical cancers [6]. The HPV-Risk assay targets a conserved region within the E7 gene. The assay has been clinically validated according to the international guidelines for HPV detection assays and in other studies [9,10,14,15].

Principle of the procedure

The HPV-Risk assay is a multiplex, real-time PCR-based assay directed against the E7 gene of 15 (probably) hrHPV types that uses fluorescent probes for the detection of one or more accumulating PCR products. During each PCR cycle the fluorescent signal increases in a logarithmic manner resulting in an amplification curve. As soon as the amplification curve of the target comes above its threshold, the sample is considered positive for that target. The multiplex format allows the simultaneous detection of four different fluorescent dyes per reaction, with each fluorescent dye representing different targets. The four different targets are: 1. HPV 16, 2. HPV 18, 3. the 13 other hrHPV types as a pool and 4. the human β-globin gene. The HPV-Risk assay separately detects HPV 16, HPV 18, and the pool of 13 other hrHPV genotypes. The human β-globin gene is used as the sample control determining both the quality of the sample DNA and the presence of potential inhibitory substances.

Materials Provided

HPV-Risk kit content

Number of reactions		72
HPV-Risk assay master mix (1 tube)	Transparent color	1080 µL
HPV-Risk assay Positive Control (1 tube)	Transparent color	100 µL
HPV-Risk assay Negative Control (1 tube)	Transparent color	100 µL

Materials required but not provided

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Consumables and reagents for preparation for clinical specimens

- Standard DNA extraction kits, such as QIAamp® DSP virus spin kit (QIAGEN, cat.no. 61704), QIAsymphony® DSP Virus/Pathogen Midi Kit (QIAGEN, cat. no. 937055) and NucleoMag 96 Tissue kit (Macherey-Nagel, cat.no. 744300)
- Hologic PreservCyt® Solution (for self-collected samples storage)
- PBS for handling cervical specimens in PreservCyt collection medium
- AL buffer (QIAGEN, cat.no. 19075) for pretreatment of cervical samples collected in SurePath and CellSolutions collection medium

Consumables and reagents for the CFX96 Touch™ real-rime PCR detection system

- Hard-Shell® Thin-Wall 96-White-Well skirted PCR plates (Bio-Rad) or equivalent
- Mircoseal® 'B' adhesive Seals (Bio-Rad) or equivalent

Equipment

- Adjustable pipets (1–10 µl; 10–100 µl) and filter tips
- Disposable gloves
- Benchtop centrifuge with a speed >10,000 rpm
- Vortex mixer
- Desktop plate centrifuge

Equipment for real-time PCR

- Bio-Rad® CFX96 Touch™ real time PCR detection system (with CFX Manager software version 1.0 or higher or CFX Maestro qPCR analysis software 1.1 and higher)
- HPV-Risk assay run profile

Calibration

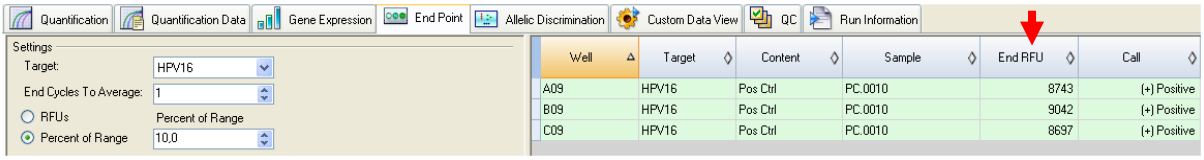
The HPV-Risk assay must be calibrated for the CFX96 Touch™ real time PCR detection system because of machine-to-machine variation in fluorescence detection. The calibration will ensure correct threshold settings for the four reporter dyes, e.g. FAM, VIC, Texas Red, and CY5. The calibration has to be performed only once for the installation of the HPV-Risk assay profile.

The calibration is performed as follows:

1. Add 15 µl of the ready-to-use HPV-Risk assay Master mix to 3 separate wells in a Hard-Shell® Thin-Wall 96-White-Well skirted PCR plate.
2. Add 5 µl of the HPV-Risk assay Positive Control to the same wells.
3. Seal the plate with a Mircoseal® 'B' adhesive Seal using the adhesive seal applicator.
   NOTE: *Do not touch the top of the seal with your hands.*
4. Briefly centrifuge plate to collect the solution on the bottom of the wells and to remove air bubbles. Air bubbles will interfere with fluorescence detection.
5. Place the reaction plate in the CFX96 Touch™ real time PCR detection system and run the PCR program as described in section "*Procedure: HPV-Risk assay on the CFX96*" on page 10.
6. When the run is completed enter for each reporter dye separately the 'Baseline Begin' and the 'Baseline End' values as described in section "*Acquiring Cq values – Threshold, Baseline start and Baseline end settings*" on page 11.

The calibrated threshold value for each reporter dye is than determined as follows:

7. For each reporter dye the 'End RFU value' of the 3 HPV-Risk Positive Controls can be read after the PCR run.
   - Go to the tab 'End point' (see figure below).
   - Select the wells containing the 3 HPV-Risk Positive Controls.
   - For each reported dye set 'End cycles to average' to 1.
   - The 'End RFU' values can be read from the table in the column 'End RFU'.



8. Use the following formulas to determine the threshold value for the corresponding reporter dye.

Threshold FAM (HPV16): $\frac{(\text{END RFU}_1 + \text{END RFU}_2 + \text{END RFU}_3)}{3} \times 0.056$

Threshold VIC (HPV Other): $\frac{(\text{END RFU}_1 + \text{END RFU}_2 + \text{END RFU}_3)}{3} \times 0.499$

Threshold Texas Red (β-globin): $\frac{(\text{END RFU}_1 + \text{END RFU}_2 + \text{END RFU}_3)}{3} \times 0.066$

Threshold CY5 (HPV18): $\frac{(\text{END RFU}_1 + \text{END RFU}_2 + \text{END RFU}_3)}{3} \times 0.106$

Create new HPV-Risk run profile

1. To create a new HPV-Risk run profile, choose user-defined as run type.
2. Select “Create New…” and enter the PCR program settings as described below and in Table 2:
   - Sample volume is 20 µl

Table 2. PCR program

Step	Temperature	Duration	Ramp rate**	Cycles
Enzyme activation	95°C	2 minutes	-	1
Denaturation	95°C	5 sec	1.6°C/second	40***
Amplification and data acquisition*	60°C	30 sec	1.6°C/second	

*) The fluorescence must be measured at the end of each amplification step.

**) Ramp rate is by default set to maximum in the software. For ‘Denaturation’ to the ‘Amplification and data acquisition’ step and for the ‘Amplification and data acquisition‘ to the ‘Denaturation’ step the ramp rate needs must be set to 1.6 °C/second.

***) In the software this is indicated in step 4 as ‘ GOTO 2, 39 more times’.

3. Save the new run profile as “HPV-risk CFX96 v1”.

Warnings and Precautions

For in vitro diagnostic use only.

Safety information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs).
HPV-Risk assay positive and Negative Controls contain sodium azide as a preservative (0.01%). Sodium azide may react with lead and copper plumbing to form explosive metal azides. On disposal through the sink, flush drains with large amounts of cold water to prevent azide build-up.

General precautions

Use of PCR tests require good laboratory practices, including maintenance of equipment, that are dedicated to molecular biology and is compliant with applicable regulations and relevant standards.
Always pay attention to the following:

- Wear protective disposable powder-free gloves, a laboratory coat, and eye protection when handling specimens.
- Prevent microbial and nuclease (DNase) contamination of the specimen and the kit. DNase may cause degradation of the DNA template.
- Sample preparation and PCR set up should be performed in a dedicated area separated from the area in which PCR amplification will be performed. To minimize the risk for PCR reaction contamination the use of a PCR-cabinet with UV-radiation is strongly recommended during handling.
- The working area needs to be kept clean especially from PCR contaminants. Cleaning can be performed with 0.1 N HCl and/or UV-radiation, or by a user preferred and proven method.
- Avoid DNA or PCR product carryover contamination, which could result in a false-positive signal.
- Use extreme caution to prevent contamination of the mixes with the synthetic materials that are contained in the PCR reagents.
- Always use DNase-free disposable pipet tips with aerosol barriers.
- Reagents of HPV-Risk assay are optimally diluted. Do not dilute reagents further as this may result in a loss of performance.
- All reagents supplied in the HPV-Risk assay kit are intended to be used solely with the other reagents supplied in the same kit. Do not substitute any reagent from one kit with the same reagent from another HPV-Risk assay kit, even from the same batch, as this may affect performance.
- Refer to the Bio-Rad CFX96 Touch™ real time PCR detection system user manual for additional warnings, precautions, and procedures.
- Alteration of incubation times and temperatures may result in erroneous or discordant data.
- Do not use components of the kit that have passed their expiration date, or that have been incorrectly stored.
- Minimize the exposure of components to light; reaction mixes may be altered due to exposure.
- Discard sample and assay waste according to your local safety procedures.

Reagent storage and handling

HPV-Risk assay is shipped on dry ice. If any component of the HPV-Risk assay is not frozen upon arrival, the outer packaging has been opened during transit please contact the local distributor.
Upon receipt store immediately at –30 to –15°C in a constant-temperature freezer and protected from light. It is advised to store the kit in a laboratory that is free of PCR amplicons.
When stored under the specified storage conditions, HPV-Risk assay is stable until the expiration date stated on the box label. Once opened, reagents can be stored in their original packaging at –30 to –15°C. Repeated thawing and freezing should be avoided. Do not exceed a maximum of 5 freeze–thaw cycles. Before use, thaw the reagents completely. Mix by gently vortexing and centrifuge the tubes before opening.

Sample handling

Cervical specimens

The HPV-Risk assay is for use with genomic DNA samples extracted from cervical specimens (scrapes). Validated collection media for cervical specimens (scrapes) are PreservCyt, CellSolutions®, Pathtezt®, and SurePath® collection medium. Optimal storage temperature of the clinical samples is 2–8°C upon arrival at the lab.
Cervical samples collected in PreservCyt can be stored up to 210 days after sample collection at 18-25°C, up to two and half years at 2-8°C, and up to 2 years at ≤-20°C. Cervical samples collected in SurePath can be stored up to 10 weeks after sample collection at 2-30°C, up to two and half years at 2-8°C, and up to 210 days at ≤-20°C.

Self-collected vaginal brush specimens

The HPV-Risk assay is for use with genomic DNA samples extracted from self-collected vaginal brush and self-collected cervico-vaginal lavage specimens. Self-collected vaginal brush specimens can be collected and shipped dry and upon arrival in the laboratory, stored in PreservCyt. Self-collected cervico-vaginal lavage specimens are collected and shipped in saline (0.9% w/v NaCl) and, upon arrival in the laboratory, stored in PreservCyt. Self-samples in PreservCyt can be stored up to 210 days after sample collection at 18-25°C, up to two and half years at 2-8°C, and up to 2 years at ≤-20°C.

Genomic DNA samples

Once genomic DNA is extracted, it can be stored at 2–8°C for short-term storage (≤2 days) or at –30 to –15°C for up to 12 months.

Sample preparation

DNA extraction

Standard DNA extraction kits (e.g., column- and magnetic bead-based kits, such as QIAamp® DSP virus spin kit, QIAsymphony® DSP Virus/Pathogen Kit, and NucleoMag 96 Tissue kit (Macherey-Nagel)) are compatible with this assay.

Clinical specimens in PreservCyt or PathTezt collection medium

For cervical specimens (scrapes) suspended in PreservCyt or PathTezt collection medium, the fraction of DNA to be used as input in the PCR represents 0.125% of the 20 ml PreservCyt or PathTezt cervical scrape sample. This corresponds with 25 µL of the original sample. Since at maximum only 5 µL of extracted DNA can be used as input in the PCR, DNA extraction procedures should be executed such that 5 µL DNA extract corresponds with 25 µL cervical specimen (scrape) sample to ensure that the correct fraction of the cervical sample is used in the PCR. Equivalent media should be processed similarly.

IMPORTANT: *PreservCyt medium can interfere with the DNA extraction process. To overcome this the PreservCyt sample can be 1) diluted (1:1) with lysis buffer that is compatible with the DNA extraction kit prior to starting DNA extraction, or 2) pellet the sample by centrifugation and remove the PreservCyt by pipetting. The pellet is resuspended in appropriate volume of PBS or lysis buffer that is compatible with the DNA extraction kit prior to starting DNA extraction. Equivalent media should be processed similarly.*

Clinical specimens in SurePath or CellSolutions collection medium

For cervical specimens (scrapes) suspended in SurePath or CellSolutions collection medium, the fraction of DNA to be used as input in the PCR represents 0.25% of the 10 ml SurePath or CellSolutions cervical scrape sample. This corresponds with 25 µL of the original sample. Since at maximum only 5 µL of extracted DNA can be used as input in the PCR, sample volume and DNA elution volume should be selected such that 5 µL DNA extract corresponds with 25 µL cervical specimen (scrape) sample to ensure that the correct fraction of the cervical sample is used in the PCR.

IMPORTANT*: Clinical specimens collected in SurePath and CellSolutions medium must be pretreated prior to use to overcome formaldehyde-induced crosslinking using the described below.*

Pretreatment clinical specimens collected in SurePath and CellSolutions medium:

1. Dilute the cervical specimens in SurePath or CellSolutions 1:1 with AL buffer (QIAGEN) and mix thoroughly.
2. Incubate at 90°C for 20 minutes followed by equilibration to room temperature before proceeding with DNA extraction.

Equivalent media containing formaldehyde should be processed similarly.

Self-collected specimens

For self-collected vaginal brush specimens suspended in Hologic PreservCyt Solution, DNA extraction procedures should be executed, such that 5 µL DNA extract used as input in the PCR represents 0.5% of

the vaginal sample. For example, the vaginal self-sample will be suspended in 3 ml PreservCyt Solution then 5 µL input DNA corresponds with 15 µL of the self-sample suspension.
IMPORTANT: PreservCyt medium can interfere with the DNA extraction process. To overcome this the PreservCyt sample can be 1) diluted (1:1) with lysis buffer that is compatible with the DNA extraction kit prior to starting DNA extraction, or 2) pellet the sample by centrifugation and remove the PreservCyt by pipetting. The pellet is resuspended in appropriate volume of PBS or lysis buffer that is compatible with the DNA extraction kit prior to starting DNA extraction.

For self-collected cervico-vaginal lavage specimens, the fraction of DNA to be used as input in the PCR represents 0.5% of the lavage self-sample. Thus, in the case of a total lavage volume of 3 ml, DNA extraction procedures should be executed such that 5 µL input DNA corresponds with 15 µL of the original lavage self-sample.

Procedure: HPV-Risk assay on the CFX96

HPV-Risk assay reaction setup for CFX96

Before you start:

- To minimize the risk for PCR contamination, it is strongly recommended that a PCR-cabinet with UV-irradiation capability is used.
- Dispensing of the HPV-Risk assay master mix must be performed in an area separate from the area where the DNA extraction is performed.
- Clean the bench area, pipets, and tube rack prior to use with a DNA-degrading solution to prevent template or nuclease contamination

In a designated DNA- and amplicon-free area:

1. Thaw the HPV-Risk assay master mix, Positive Control and Negative Control completely, and protect the HPV-Risk assay master mix from light whenever possible
   NOTE: *Do not exceed 30 minutes for the thawing step, to avoid any material degradation.*
2. Briefly vortex and spin down prior to pipetting.
3. Dispense 15 µl of ready-to-use HPV-Risk assay Master mix into the appropriate wells of a Hard-Shell® Thin-Wall 96-White-Well skirted PCR plate.
4. Return the vial with remaining HPV-Risk assay master mix to the freezer to avoid any material degradation.
5. Transfer the PCR plate to designated amplicon-free area.

In a designated amplicon-free area:

6. Homogenize the Positive Control and the Negative Control by briefly vortexing and centrifuge briefly prior to pipetting.
7. Add 5 µl of the HPV-Risk assay Negative Control (NC) to the 'Negative Control' well.
8. Add 5 µl of the HPV-Risk assay Positive Control (PC) to the appropriate well.
9. Seal the plate with a Mircoseal® 'B' adhesive Seal using the adhesive seal applicator.
   NOTE: *Do not touch the top of the seal with your hands.*

10. Briefly centrifuge plate to collect the solution on the bottom of the wells and to remove air bubbles. Air bubbles may interfere with fluorescence detection.
11. Return the HPV-Risk assay Positive and Negative Controls to the freezer to avoid any material degradation.
12. Place the reaction plate in the Touch™ Real time PCR Detection System.

CFX96 machine set up

13. Start the Bio-Rad CFX Manager software or CFX Maestro qPCR analysis software.
14. Choose user-defined as run type.
15. Go to 'Select Existing' and choose the protocol "HPV-risk CFX96 v1" (created according to section – Create new protocol) on page 6.
16. Go to the 'Plate' folder.
17. Under the 'Express Load' option, choose 'Quickplate_96 wells_All Channels.pltd'.
18. Go to 'Edit selected', select in the 'Settings' drop-down menu 'Plate type' and set to 'BR White'.
19. Go to 'Select Fluorophores…' and select the fluorescent dyes as described in Table 3.
20. The target names can be added in the 'Experiment settings…'. The Positive Control is always on position 1 and the Negative Control on position 2. Appoint in the 'Sample Type' section the Positive Control as Positive Control and the Negative Control as Negative Control.
21. Once the plate layout has been edited, click OK to save layout.

Table 3. Target and fluorescent dye settings

Target	Reporter dye
β-globin	Texas Red
HPV 16	FAM
HPV 18	Cy5
HPV Other *	VIC

*) HPV Other comprises the pool of 13 non-16/18 HPV types.

22. Click *Start run.*
23. Select location to save file, and name the run.
24. Click OK, and the run will begin.

Acquiring Cq values - Threshold, Baseline start and Baseline end settings

25. The following settings apply to Bio-Rad CFX manager software version 1.0 (or higher) and CFX Maestro qPCR analysis software version 1.1 (or higher).
26. Go to 'Settings', select 'Baseline setting', and then select 'Baseline Subtracted Curve Fit'.
27. For each reporter dye the 'Baseline Cycles' and the 'Threshold' must be entered separately.
28. Go to 'Settings' and select 'Baseline Threshold'. Enter for each target the Threshold, Baseline Begin, and Baseline End settings as indicated in Table 4.

Table 4. Threshold and Baseline Begin and Baseline End cycle setting for the four targets β-globin, HPV16, HPV18, and HPV Other.

Target	Threshold	Baseline Begin	Baseline End
β-Globin	See section 'Calibration'	3 – 12*	18**
HPV 16		3 – 12*	18**
HPV 18		3 – 12*	18**
HPV Other		3 – 12*	18**

*) Select a value from 3 to 12 on the basis of specifications indicated below in section "Interpretation of results – A. Check amplification curves".

**) Adjust to a lower value in case the amplification curve increases already exponentially before cycle 18.

Interpretation of results

Analysis of the data must be performed as indicated below.

A. Check amplification curves

Check the fluorescent data for each reaction for each dye for typical amplification curves (i.e., logarithmic increase of fluorescence). If the amplification curve(s) is/are not satisfactory, modify baseline settings as follows:

- For the **Baseline start** a value from in between 3 to 12 should be filled such that the start cycle value is within a flat, linear part of the amplification curve and not within an irregular part. Sometimes irregular (i.e. outlying high and/or low fluorescent signals that may alternate) curve patterns can be observed in the earliest cycles. Selection of a Baseline start cycle value within such irregular area should be avoided since it may negatively influence baseline level calculations.
- The **Baseline end** value is set at 18. In rare circumstances the amplification curve of a target increases already exponentially before cycle 18. In that case it is recommended to choose a **Baseline end** value that corresponds with two cycles just before the exponential increase is seen.

B. Validation criteria HPV-Risk controls

28. All targets in the HPV-Risk assay Positive Control should give Cq values that are lower than 35. If this is not the case and baseline and threshold settings are correct, the experiment should be repeated.
29. None of the targets in the HPV-Risk assay Negative Control should give a signal above the threshold till the end of the PCR run (i.e., cycle 40; 'N/A' assay outcome). If a signal is seen before cycle 40, and baseline and threshold settings are correct, the experiment should be repeated.

C. Interpretation of sample results

30. The result for a sample should be interpreted as indicated below.

Table 5. Interpretation of results

	Cq value HPV target(s)	Cq value β-globin	Interpretation
1.	<36	Any	HPV-positive
2.	>36 or no signal*	<33	HPV-negative
3.	>36 or no signal	>33	Invalid

*) designated as N/A by software

1. HPV positive

when Cq value(s) of HPV16 and/or HPV18 and/or Other HPV is (are) below 36 (irrespective of Cq value of β-globin). The reporter dye indicates the type(s) present.

2. HPV negative

when Cq value for β-globin is below 33 and Cq values for HPV16, HPV18, and Other HPV are above 36 or show no signal (i.e., N/A).

3. Invalid

when Cq value of β-globin is >33 and Cq values of HPV16, 18, and Other HPV are >36 or show no signal (i.e., N/A).

Troubleshooting

This troubleshooting guide may be helpful in solving any problems that may arise. If the problem persists or in case of questions or other information contact your local distributor or Self-screen at info@self-screen.nl.

Comments and suggestions	
Sample is scored invalid: the amplification of β-globin is too low or absent	
a) Pipetting error or omitted reagents	Check pipetting scheme and the reaction setup. Repeat the PCR run.
b) Check the DNA eluate	Always check if there are no remains of buffer or beads in the DNA eluate. Repeat DNA extraction.
c) Inhibitory effects	Always check if there are no remains of buffer or beads in the DNA eluate. Repeat DNA extraction.
d) PCR program	Check if PCR program settings are set correctly according to Table 2.
e) Acquiring Cq values	1) Check if Cq threshold value for each separate detection channel is set according to the value determined after 'Calibration'. 2) Check if Baseline start and Baseline end values are set correctly.
f) Quality clinical specimen	Check the amount of cells in the clinical specimen by eye. Repeat DNA extraction.
Positive Control is scored invalid: the amplification is too low or absent for one or more of the targets	
a) Pipetting error or omitted reagents	Check pipetting scheme and the reaction setup. Repeat the PCR run.
b) Partial degradation	Store kit contents at –30 to –15°C. Avoid repeated freezing and thawing of the kit components to a maximum of five cycles.

Comments and suggestions	
c) PCR reagents partially degraded	Store kit contents at –30 to –15°C and keep the reaction mixes protected from light. Avoid repeated freezing and thawing of the kit components to a maximum of five cycles.
d) Expiry date	Check the expiry date of the used kit.
e) Time-delay between pipetting samples and start of the run	PCR reactions mixes can be stored at 2–8°C for 30 minutes in the dark between dispensing samples into the PCR reactions and starting the run in the machine.
f) PCR program	Check if PCR program settings are set correctly according to Table 2.
g) Acquiring Cq values	1) Check if Cq threshold value for each separate detection channel is set according to the value determined after 'Calibration'. 2) Check if Baseline start and Baseline end values are set correctly.
Negative Control is invalid	
a) Pipetting error	Check pipetting scheme and the reaction setup. Repeat the PCR run.
b) Cross-contamination	Replace all critical reagents. Always handle samples, kit components, and consumables in the designated areas and in accordance with commonly accepted practices to prevent carryover contamination.
c) Reagent contamination	Replace all critical reagents. Always handle samples, kit components, and consumables in the designated areas and in accordance with commonly accepted practices to prevent carryover contamination.
d) Time-delay between pipetting samples and start of the run	PCR reactions mixes can be stored at 2–8°C for 30 minutes in the dark between dispensing samples into the PCR reactions and starting the run in the machine.
e) Probe degradation	Keep reaction mixes protected from light.
f) PCR program	Check if PCR program settings are set correctly according to Table 2.
g) Acquiring Cq values	1) Check if Cq threshold value for each separate detection channel is set according to the value determined after 'Calibration'. 2) Check if Baseline start and Baseline end values are set correctly.

Limitations

- For the indicated intended use the test should be performed on cervical scrape specimens or self-collected (cervico-)vaginal specimens. However, the HPV-Risk assay has also been evaluated for use with DNA extracted from formalin-fixed paraffin-embedded (FFPE) biopsy specimens.
- Use of PCR tests requires good laboratory practices, including maintenance of equipment, that are dedicated to molecular biology and is compliant with applicable regulations and relevant standards.

- The HPV-Risk assay is to be used by laboratory professionals trained in the use of the CFX96 Touch™ real time PCR detection system.
- Any diagnostic results that are generated must be interpreted in conjunction with other clinical or laboratory findings.
- Strict compliance with the Instructions for Use (handbook) is required for optimal HPV-Risk assay results.
- Attention should be paid to expiration dates printed on the box and labels of all components. Do not use expired components.
- All reagents supplied in the HPV-Risk assay are intended to be used solely with the other reagents supplied in the same kit. This may otherwise affect performance.
- Any off-label use of this product and/or modification of the components will void Self-screen B.V.'s liability.
- It is the user's responsibility to validate system performance for any procedures used in their laboratory that are not covered by the performance studies.

Performance Characteristics

Limit of Detection (LOD)

The limit of detection (LOD) was determined using plasmids containing the genome of an HPV genotype. Serial 5-fold dilutions series of the 15 targeted HPV types (i.e. 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 67, and 68) were prepared in a background of 100 ng human DNA, and tested in 4-fold. For β-globin, the LoD was assessed on a 3-fold serial dilution series of human placenta DNA in 4-fold. See Table 6.

Table 6. Limit of detection (LOD) of the HPV-Risk assay of 15 HPV types and β-globin gene

Target	LOD (copies per PCR)
HPV 16	255
HPV 18, 45	463
HPV 33, 35, 39, 51, 56, 59, 67	2,547
HPV 31, 58	4,630
HPV 66, 68	25,469
HPV52	46,307
β-globin	17 genome equivalents

Analytical specificity *

Analytical specificity was determined against plasmid DNAs of non-targeted HPV genomes (i.e., HPV 6, 11, 26, 40, 42, 43, 53, 61, and 70) at a concentration of at least 46,000 copies/test and against the 3 most potentially pathogenic vaginal microorganisms Chlamydia trachomatis, Neisseria gonorrhoeae, and Candida albicans at a concentration of at least 10,000 copies/test. The test did not show any cross-reactivity with the non-targeted HPV types 6, 11, 26, 40, 42, 43, 53, and 61, or the micro-organisms. Only for HPV 70, a positive signal was observed in the 'HPV Other' channel (i.e., the channel that detects the pool of 13 non-16/18 HPV types), which after further diluting could be detected at >17,000 copies/test. HPV 70 is considered probably carcinogenic on the basis of epidemiological, phylogenetic, and functional studies [11-13].

Interfering substances *

Traces of EDTA (0.5M), HCl (1N), Silica beads (1 µL), Blood (1 µL), Ureum (40 g/100 ml), and lysis buffer inhibited the performance of the test. ETOH 96% (1 µL) and DMSO 4 % (v/v) had no inhibitory effect on the performance of the test. Inhibition is monitored by the sample control (β-globin target).

** Performance characteristics are indicated for HPV-Risk assay test version ABI7500. Equivalence analysis demonstrated similar performance for HPV-Risk assay for the CFX96.*

Clinical Performance

Clinical performance on cervical specimens (scrapes) *

The clinical sensitivity and specificity of the test for cervical intraepithelial neoplasia grade 2 or higher (CIN2+) in cervical specimens (scrapes) were validated by a non-inferiority analysis relative to high-risk HPV GP5+/6+ PCR following international guidelines for HPV test requirements for cervical cancer screening [9]. The clinical sensitivity for CIN2+ was 97.1% (67/69) and the clinical specificity for CIN2+ was 93.9% (774/824). The clinical sensitivity and specificity were non-inferior to that the reference assay GP5+/6+-PCR [10], indicating a very good clinical performance.
The clinical performances of the HPV-Risk assay have also successfully been validated in the VALGENT frameworks. In the VALGENT 3 study the relative clinical sensitivity and specificity values for CIN3+ against the reference assay hybrid capture 2 (HC2, QIAGEN) on cervical scrapes collected in PreservCyt collection medium were of 1.00 (95%CI: 0.95 to 1.05) and 1.02 (95%CI: 1.01-1.04), respectively [14]. In the VALGENT 4 study the relative clinical sensitivity and specificity values for CIN3+ against the reference assay GP5+/6+-PCR on cervical scrapes collected in SurePath collection medium were of 1.01 (95%CI: 0.97 to 1.06) and 1.04 (95%CI: 1.02-1.06), respectively [15]. Performance of the HPV-Risk compared to the reference assays was non-inferior with all P-values ≤0.006.

For women with ASC-US or LSIL cytology, the clinical sensitivity and specificity values for CIN2+ were 97.4% (37/38; 95%CI 83.5–99.6) and 59.8% (52/87; 95%CI: 49.2–69.5), respectively [14].

Reproducibility clinical specimens (scrapes) *

The intra-laboratory reproducibility and inter-laboratory agreement of the test were validated according to the international guidelines for HPV test requirements for cervical cancer screening [9]. The intra-laboratory reproducibility on cervical specimens (scrapes) over time was 99.5% (544/547) with a kappa value of 0.99 and the inter-laboratory agreement was 99.2% (527/531) with a kappa value of 0.98, indicating very good agreement [10].

Clinical performance on self-collected (cervico-)vaginal specimens (scrapes) *

The performance of the HPV-Risk assay on self-collected (cervico-)vaginal specimens has been validated for two different sampling methods: 1) self-collected lavage specimens, and 2) self-collected brush specimens. For self-collected lavage specimens, the agreement with the reference assay GP5+/6+ PCR was 96.7% (59/61) with a CIN2+ sensitivity of 91.4% (21/23) [10]. For self-collected brush specimens, the agreement with GP5+/6+ PCR was 92.9% (104/112) with a CIN2+ sensitivity of 93.9% (31/34) [10].

Equivalence performance study CFX96 Touch™ Real time PCR Detection system

Bridging studies of the HPV-Risk assay version CFX96 with the HPV-Risk assay version ABI7500 showed >99.5% concordance in assay findings, i.e., 100% on cervical scraping specimens of women with CIN2+ (30/30), 99.44% on cervical scraping specimens of women without evidence of CIN2+(178/179), and 100% when evaluating intra- and inter-laboratory agreement (60/60 and 11/11, respectively).

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Symbols

The following symbols may appear on the packaging and labeling:

Symbol	Symbol definition
	Use by
	In vitro diagnostic medical device
	CE-IVD marked symbol
	Contains reagents sufficient for <N> reactions
	Catalog number
	Lot number
	Material number
	Components
	Contains
	Number
Rn	R is for revision of the Instructions for Use (Handbook) and n is the revision number
	Global Trade Item Number
	Temperature limitation
	Manufacturer
	Protect from light
	Consult instructions for use
	Caution

Contact information

For technical assistance and more information, please contact Self-screen or our local distributors.

Manufacturer: Self-screen B.V., Plesmanlaan 125, 1066CX, Amsterdam, The Netherlands.
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Revision History

Revision	Changes
1	First version
2	In section ‘Create new HPV-Risk run profile’ (p. 6) ramp rate is indicated for the different temperature changes in the PCR program.