

GenoID REAL-TIME HPV ASSAY HUMAN PAPILLOMAVIRUS REAL-TIME PCR SYSTEM

OPTIMIZED REAGENT SET FOR REAL-TIME PCR AMPLIFICATION AND
DETECTION OF 14 HPV GENOTYPES
OF THE FOLLOWING HIGH-RISK GROUPS:
Group A: 16, 18,
Group B: 31, 33, 45, 52, 58,
Group C: 35,39,51,56,59,66,68
AND INTERNAL CONTROL DETECTED IN EACH SAMPLE.

INSTRUCTION MANUAL

(For use with the Corbett Life Science Rotor-Gene 6600 (6-Plex) Real-Time PCR System)

72-well rotor

VERSION 1.0

for research use only

FOR 72 REACTIONS

COMPONENTS

GenoID REAL-TIME HPV ASSAY[®] HUMAN PAPILLOMAVIRUS REAL-TIME PCR KIT
FOR Rotor-Gene 6600 (6-Plex) 72-well rotor
R20030

STORAGE

SHIP AND STORE AT -20°C

INTENDED USE

The GenoID REAL-TIME HPV ASSAY (Human papillomavirus Real-Time PCR Kit) is a qualitative real-time PCR based in vitro test for the detection of HPV DNA in cervical cells collected in PreservCyt® Solution. The assay is designed and optimized to detect the following genital HPV DNA types:

- High-Risk: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68.

PATHOGEN INFORMATION

Cervical cancer is the second most common cancer in women worldwide, with an estimated 493,000 new cases and 274,000 deaths in 2002 ^[1]. It has been established that certain types of HPVs are associated with cervical cancer and it is estimated that HPV DNA is present in over 99% of these cancers. These HPV types are considered the cause of invasive cervical cancer ^[2,3]. More than 100 HPV types were identified, and approximately 40 types are considered genital HPVs. These were classified into two groups according to their relative risk of causing cancer: 'Low-Risk' and 'High-Risk' types ^[4]. Low- risk types can cause genital warts or benign low-grade abnormalities in cervical cells, but are not associated with cervical cancer. However, the 'High-Risk' genotypes induce cervical dysplasias and invasive cervical cancer. Thus HPV detection and typing methods have been proposed to enhance screening and detection efficiency. These methods, besides detecting the presence of the virus, allow typing and patient follow-up. When compared to Pap testing (Papanicolaou test), HPV testing has greater sensitivity for the detection of cervical intraepithelial neoplasia (CIN) ^[5]. Moreover, the combination of Pap test and HPV testing reduced the incidence of grade 2 or 3 cervical intraepithelial neoplasia or cancer detected by subsequent screening examinations ^[6].

PRODUCT DESCRIPTION

The GenoID REAL-TIME HPV ASSAY is a ready-to-use molecular beacon- based one-step multiplex real-time PCR system for the detection of 14 high risk HPV types in the Corbett Life Science Rotor-Gene 6600 (6-Plex) Real-Time PCR System.

The system is designed for the specific amplification of a ~150 bp. region of the L1 gene. The amplicon is detected by measuring the fluorescence of the labeled molecular beacons. An Internal Control (IC) added before sample DNA extraction is also detected by a specific molecular beacon to ensure proper DNA isolation procedure and PCR ^[7]. There are 4 different dyes labeling the type specific molecular beacons: molecular beacons detecting HPV 16 and 18 are 5'-Marina Blue labeled, HPV types 31, 33, 45, 52 are detected by 5'-FAM labeled molecular beacons, HPV types 35, 39, 51, 56, 58, 59, 66 and 68 are detected by 5'-TET labeled molecular beacons, while the Internal Control is detected by a 5'-FAM-TEXASRED labeled wavelength shifting molecular beacon. The amplification of the high-risk groups and the internal control is detected in separate detection channels, accordingly:

Target	Detection Channel
HPV 16, 18	Blue
HPV 31, 33, 45, 52, 58	Green
HPV 35, 39, 51, 56, 59, 66, 68	Yellow
Internal control (HPVRTIC)	Orange

The kit also contains a Positive Control, which represents approximately 7000 DNA copies/ reaction. Note, that the assay has a general analytical sensitivity of 100-700 DNA copies/ reaction.

A template file with the optimal PCR thermal profile, detection and analysis conditions is available at GenoID's Product website. All product dedicated template files can be found at <http://www.genoid.net/index.php/products-and-services/real-time-pcr-hpv-assays/>.

REAGENTS AND MATERIALS PROVIDED

The GenoID REAL-TIME HPV ASSAY® HPV Real-Time PCR KIT for Rotor-Gene 6600 contains sufficient reagents for 72 tests.

Label	Cap Color	Name	Final volume
R1	Green	GenoID REAL-TIME HPV ASSAY PCR Buffer TRIS buffer Potassium chloride	787,5 µl
R2	Yellow	GenoID REAL-TIME HPV ASSAY PCR PPN <0,001% dATP, dCTP, dGTP, dTTP <0,001% each of upstream and downstream primers and probes <0,001% fluorescein, rhodamine	225 µl
R3	Red	GenoID REAL-TIME HPV ASSAY Internal Control DNA Synthetic DNA	100 µl
R4	Violet	GenoID REAL-TIME HPV ASSAY Calibrator Synthetic DNA for, HPV16 18, HR HPV and IC	100 µl

STORAGE:

The components of the GenoID REAL-TIME HPV ASSAY should be stored at -20 °C, and are stable until the expiry date stated on the label.

Repeated thawing and freezing (>2x) should be avoided, as this may reduce the sensitivity. Prepare and freeze aliquots if reagents are to be used intermittently.

Working GenoID REAL-TIME HPV ASSAY PCR Mastermix must be prepared freshly.

ADDITIONALLY REQUIRED MATERIALS AND INSTRUMENTS

Reagent Preparation Area

- ABI AmpliTaq Gold DNA polymerase (Applied Biosystems, P/N: 4311820)
- Adjustable pipettes with aerosol barrier or positive displacement, DNA and DNase-free tips with filters
- Disposable powder-free gloves

Specimen and Control Preparation Area

- DNA isolation kit: AmpliLute Liquid Media Kit and equipments (Roche, P/N: 03750540 190), or QIAmp MinElute Media Kit (Qiagen, Cat No: 57414) are recommended.
- Corbett strip tubes and caps (0.1 ml) (P/N: 3001-002), Corbett 72-High Profile Rotor (P/N: 6001-001), 72-Well Rotor Locking Ring (P/N: 6001-002).
- Adjustable pipettes with aerosol barrier or positive displacement, DNA and DNase-free tips with filters
- Sterile, disposable serological pipettes (10 ml)
- Pipetting Aid
- PreservCyt Solution (Cytic Corp., REF 70097-003)
- Eppendorf tubes; 1.5 ml
- Centrifuge with rotor for 96 well plates and Eppendorf tubes (1.5 ml) (max.6000g)
- Vortex mixer
- Disposable powder-free gloves

Amplification Area

- Corbett Life Science Rotor-Gene 6600 (6-Plex) Real-Time PCR System, Corbett strip tubes and caps (0.1 ml) (P/N: 3001-002), Corbett 72-High Profile Rotor (P/N: 6001-001), 72-Well Rotor Locking Ring (P/N: 6001-002). This kit is only validated for use on RotorGene6600.

WARNINGS AND PRECAUTIONS

- Follow strictly the present protocol to obtain optimal PCR results.
- This test is for use with human cervical cells collected in PreservCyt Solution.
- Wear protective disposable powder-free gloves, laboratory coats and eye protection when handling specimens and kit reagents. Wash hands thoroughly after handling specimens and test reagents.
- Avoid microbial and DNA contamination of reagents when removing aliquots from reagent bottles. The use of sterile disposable pipettes, DNA-free and DNase-free pipette tips with filters are recommended.
- Do not remove seals from the plates after amplification to minimize product contamination. Avoid sample contamination with other samples or amplified products.
- When thawed, mix the reagents and centrifuge briefly.
- Do not pool reagents from different lots or from different bottles of the same lot.
- Dispose of unused reagents and waste in accordance with country and local regulations.
- Do not use a kit after its expiration date.
- Material Safety Data Sheets (MSDS) are available on request.
- Apply unidirectional workflow for reagent preparation, sample and control preparation, using dedicated equipment in dedicated areas. The amplification area must be separated from other parts of the laboratory. Change protective clothing and gloves before leaving that area.
- Handle samples as potentially infectious material. Clean thoroughly and disinfect surfaces and equipments after work.

SPECIMEN COLLECTION, TRANSPORT AND STORAGE

Specimen collection

Only specimens collected in PreservCyt Solution may be used with the GenoID REAL-TIME HPV ASSAY®. Collect samples according to the manufacturer's instructions. Do not process samples having inadequate quality or insufficient volume. Too frequent sampling could result in false negative tests.

Specimen transport and storage

Specimens collected in PreservCyt Solution can be transported at 2-30 °C. Specimens collected in PreservCyt Solution may be stored at room temperature for up to 21 days or at 2-8 °C for up to 12 weeks.

PROTOCOL

Each GenoID REAL-TIME HPV ASSAY® contains sufficient reagents for 96 tests. The workflow includes two steps: 1. specimen preparation/DNA isolation, 2. real-time PCR. DNA isolation and GenoID REAL-TIME HPV ASSAY® can be completed in a single workday.

DNA ISOLATION

Note: The specimen should be collected in PreservCyt Solution. DNA isolation has to be performed using one of the recommended kits. (AmpliLute Liquid Media Kit and equipments (Roche, P/N: 03750540 190), or QIAmp MinElute Media Kit (Qiagen, Cat No: 57414))

1. Prepare **working IC reagent** by adding 20 µl GenoID REAL-TIME HPV ASSAY Internal Control DNA (**R3**) reagent to 20 ml PreservCyt® Solution (one vial). Prepare it freshly.
2. Mix the sample in the original container prior to the procedure. Transfer 1,25 ml sample to a sterile 1.5 ml Eppendorf tube. Centrifuge the sample at 4000 g, for 10 min., at room temperature using a fixed angle rotor.
3. Discard the supernatant, do not disturb the pellet.
4. Add 250 µl **working IC reagent** to the pellet and vortex thoroughly.
5. Use 250 µl of **working IC reagent** as **preparation negative control**.
6. Proceed with sample and control preparation using one of the recommended kits.

PCR SETUP

Note: Apply unidirectional workflow and use dedicated areas and equipments. Only AmpliTaq Gold DNA polymerase (5 U/µl, Applied Biosystems, Part.No: 4311820) is validated for the test.

1. Prepare GenoID REAL-TIME HPV ASSAY PCR **Mastermix** according to the following pipetting scheme (final volume/ reaction: 15 µl):

Reagent	Volume (µl)
GenoID REAL-TIME HPV ASSAY PCR Buffer (R1)	10.5 µl
GenoID REAL-TIME HPV ASSAY PCR PPN (R2)	3 µl
AmpliTaq Gold Polymerase 5U/µl	1.5 µl

2. Pipette 15 µl **Mastermix** into each well.

PERFORMING THE REAL-TIME PCR

Note: **The experiment must be performed at 20-24°C. If you wish to perform two experiments in a row, allow your equipment 1 hour to chill in switched off position.** The ambient temperature influences the performances of the RotorGene. Experiments are only valid if performed in the indicated temperature range.

The GenoID REAL-TIME HPV ASSAY® HPV Real-Time PCR KIT for Rotor-Gene 6600 (6-Plex) 72-well rotor contains sufficient reagents for the simultaneous examination of **69 samples** and 3 controls: one **positive control (R4) containing** mixed high-risk, low-risk and IC DNA, one **preparation negative control** containing IC DNA and one **PCR negative control containing only distilled water**.

Note: The amplification area must be separated from other parts of the laboratory and the **Mastermix** preparing area. Change protective clothing and gloves before leaving the amplification area.

Switch on ABI7900HT Real-Time PCR at least 20 minutes before starting the experiment.

1. Add **7 µl template** DNA to the wells containing the **Mastermix**.
2. Set up 3 control reactions: one **positive control (R4) containing** mixed high-risk, low-risk and IC DNA, one **preparation negative control** containing IC DNA and one **PCR negative control containing distilled water,,** according to the **Recommended plate design**.
3. Close the tubes with the appropriate caps (please refer to "Additionally required materials" section) and place the rotor into the RotorGene.
4. Open Corbett Rotor-Gene 6000 software.
5. Select **Hydrolysis Probes**, click **New** and set the followings:

For **rotor**: 72 well rotor, then click "**locking ring attached**", then click **Next**

Type **operator name**, set reaction volume to **22**, then click **Next**.

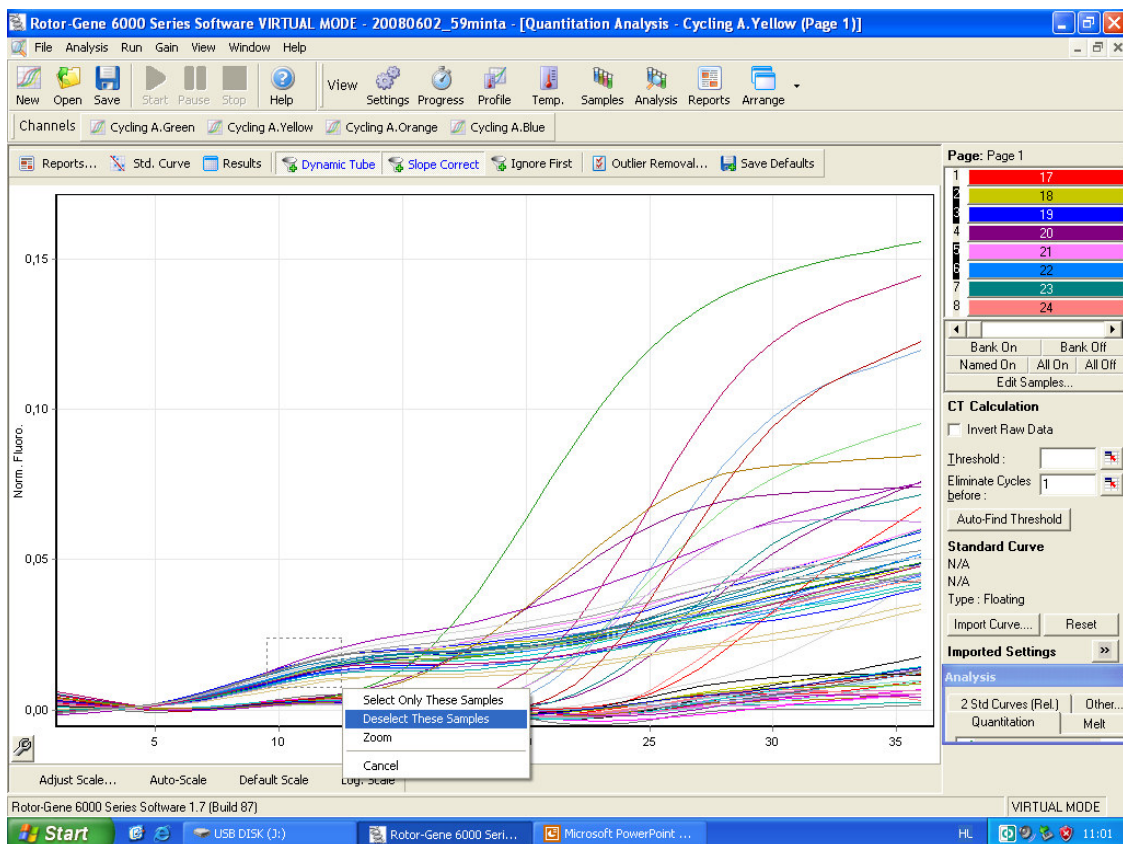
Click **Edit profile**, then click on the **Open** icon and browse/open the downloaded **GenoID_RTHPV_RotorGene4ch** Template file (available at <http://www.genoid.net/index.php/products-and-services/real-time-pcr-hpv-assays/>).

- After opening the Template file, click **Gain optimization**, **Optimize all** and press **OK** for the different channels. Then select "**Perform optimization before the first acquisition**", then **Close**, and click **Next**. After clicking on **Start run**, name your experiment and complete the sample list.

The order of samples is: 1 = PCR negative control, 2 = preparation negative control, 3 = positive control (R4) 4-72 = samples

DATA ANALYSIS

- After the run has completed click on the Analyze icon to analyze the data.
- In the Analysis window select the **Blue** channel, click **Show**, then in the **Quantitation analysis** window select "linear scale" and set the threshold to 0,02. Go to the **Quantitation results** window, samples showing values in the Ct column are positive for either HPV16 or HPV18.
- In the Analysis window select the **Green** channel, click **Show**, then in the **Quantitation analysis** window select "linear scale" and set the threshold to 0,02. Go to the **Quantitation results** window, samples showing values in the Ct column are positive for HPV31, 33, 45, 52, or 58.
- In the Analysis window select the **Yellow** channel, click **Show**, then in the **Quantitation analysis** window select "linear scale" and mark the separate group of (negative) signals detached from the baseline by a left mouse click (see image below), then in the new window click on "**Deselect these samples**". Set the threshold to 0,03. Go to the **Quantitation results** window, samples showing values in the Ct column are positive for HPV35, 39, 51, 56, 59, 66, or 68.



5. In the Analysis window select the **Orange** channel, click **Show**, then in the **Quantitation analysis** window select "linear scale" and set the threshold to 0,04. Go to the **Quantitation results** window, samples showing values in the Ct column are positive for the internal control.

WE RECOMMEND SENDING US (rthpv@genoid.hu) THE RESULT FILE OF THE FIRST EXPERIMENT FOR CONFIRMATION! Contact us in case of any questions or problems related to your results!

TROUBLESHOOTING

A. Problems possibly occurring in a valid reaction:

No IC signal in the samples, but samples present strong HR and/or LR signals. When samples yield strong HR or LR signals, the IC signal might disappear, because the strong HR or LR reaction inhibits the IC reaction.

B. No IC signal in the preparation negative control

a. Incorrect configuration of the working IC solution. Check your work steps by means of the pipetting scheme.

b. An error occurred during DNA isolation, especially if there are no IC signals in any samples either. Please review and follow strictly the instructions of the manufacturer during DNA isolation, and repeat the DNA isolation step.

C. No signal of the positive control

The storage conditions did not comply with the instructions given and the positive control is degraded. Please check the storage conditions and use a new kit, if necessary.

D. Positive signal in Blue, Green or Yellow channel in PCR negative control

A contamination occurred during preparation of the PCR. Repeat the PCR with new reagents in replicates. Pipette samples with extreme care, and make sure that work space and instruments are regularly decontaminated.

E. Positive signal in Blue, Green or Yellow channel in preparation negative control

A contamination occurred during DNA isolation. Repeat the extraction and PCR of the samples to be tested using new reagents. Strictly follow the instructions of the manufacturer during DNA isolation.

F. No signal in any detector

Please repeat the experiment, follow the exact instructions of the manufacturers.

REFERENCES

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5. Mayrand MH et al. Human papillomavirus DNA versus Papanicolaou screening tests for cervical cancer. *N Engl J Med.* 2007 Oct 18;357(16):1579-88.
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