



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

OPTIMIZED REAGENT SET FOR REAL-TIME PCR AMPLIFICATION AND  
DETECTION OF 14 HIGH RISK AND 2 LOW RISK HPV GENOTYPES  
IN THE FOLLOWING GROUPS:

GROUP A: HPV6, 11,

GROUP B: HPV16, 18,

GROUP C: HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68

INTERNAL CONTROL DETECTED IN EACH SAMPLE.

## INSTRUCTION MANUAL

(For use with the ABI7900HT Fast Real-Time PCR System 4 channels)

**for research use only**

**FOR 96 REACTIONS**

**COMPONENTS**

GenoID REAL-TIME HPV ASSAY<sup>®</sup> HUMAN PAPILLOMAVIRUS REAL-TIME PCR KIT FOR ABI7900HT 4 channels

**R20040**

**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 low risk genital HPV DNA types:

- HPV 6, 11,  
And high risk genital HPV DNA types:
- 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 <sup>[1]</sup>. 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 <sup>[2,3]</sup>. 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 <sup>[4]</sup>. 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) <sup>[5]</sup>. 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 <sup>[6]</sup>.

## 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 2 low and 14 high risk HPV types in the ABI 7900HT Fast 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 <sup>[7]</sup>. There are 4 different dyes labeling the type specific molecular beacons: molecular beacons detecting the 12 high-risk types are 5'-TET labeled, HPV 16 and 18 are detected by 5'-JOE labeled molecular beacons, the low-risk type HPV6 and 11 are 5'-TexasRed-(T)<sub>6</sub>-FAM (wavelength shifted) labelled, while the Internal Control is detected by a 5'-FAM labeled molecular beacon.

HPV 6 11, HPV 16 18, high-risk and internal control amplification is detected in separate detection channels, accordingly:

Target	Detector	Detection Channel
HPV 6; 11	6 11	WS
HPV 16, 18	16 18	JOE
HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	HR	TET
Internal Control	HPVRTIC	FAM

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 DNA copies/ reaction.

A template file with the optimal PCR thermal profile, detection and analysis conditions is available at <http://genoid.net/EngCD/products/downloads/>.

## REAGENTS AND MATERIALS PROVIDED

The GenoID REAL-TIME HPV ASSAY® HPV Real-Time PCR KIT for ABI7900HT 4 channels contains sufficient reagents for 96 tests.

Label	Cap Color	Name	Final volume
R1	Green	<b>GenoID REAL-TIME HPV ASSAY PCR Buffer</b> TRIS buffer pH=8.0 Potassium chloride	1050 µl
R2	Yellow	<b>GenoID REAL-TIME HPV ASSAY PCR PPN</b> <0,001% dATP, dCTP, dGTP, dTTP <0,001% each of forward and reverse primers and probes <0,001% fluorescein, rhodamine	300 µl
R3	Red	<b>GenoID REAL-TIME HPV ASSAY Internal Control DNA</b> Synthetic DNA	100 µl
R4	Violet	<b>GenoID REAL-TIME HPV ASSAY Positive Control DNA</b> Synthetic DNA for HPV 6 11 16 18, HR-HPV and IC detection	100µl

## 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.
- AB MicroAmp Fast Optical 96-Well Reaction Plate (0.1 ml) (P/N: 4346906) with appropriate AB MicroAmp Optical Adhesive Film (P/N: 4311971), ABI 96 Well Fast Block Modules (P/N: 4351405)
- 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 1.5 ml Eppendorf tubes (max.6000g)
- Vortex mixer
- Disposable powder- free gloves

### Amplification Area

- ABI 7900HT Fast Real-Time PCR System (P/N: 4330966), 7900HT Version2.3 Sequence Detection Systems (P/N: 4366253), AB MicroAmp Fast Optical 96-Well Reaction Plate (0.1 ml) (P/N: 4346906), MicroAmp Optical Adhesive Film (P/N: 4311971), ABI 96 Well Fast Block Modules (P/N: 4351405). This kit is only validated for use on ABI 7900HT.
- Dye calibration for HPV6 11 wavelength shifted dye is needed before use. For performing you have to ask local ABI support.

## 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 REACTION 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 ( <b>R1</b> )	10.5 µl
GenoID REAL-TIME HPV ASSAY PCR PPN ( <b>R2</b> )	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 GenoID REAL-TIME HPV ASSAY® HPV Real-Time PCR KIT for ABI7900HT contains sufficient reagents for the simultaneous examination of **93 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. Seal the plate with the appropriate sealing film (please refer to "Additionally required materials" section). Centrifuge briefly and keep the plate at + 4°C until starting the experiment.
4. Open 7900HT Sequence Detection System Version2.3 software (**SDS**).
5. Select **New Document** and set the followings:

For **Assay type**: Standard Curve (AQ)

For **Container**: 96 Wells Clear Plate

For **Template**: Browse and open the downloaded **GenoID\_RTHPV\_ABI7900HT** Template file (available at <http://genoid.net/EngCD/products/downloads/>).

6. After opening the Template file, set up the followings:

For **Table settings**: click on the **Table setting icon** to open **Table setting dialog box**.

In the **Table setting dialog box** choose **Table setting bar**, click on **Show None** button and sign **Ct** among the specifications. Click on **Apply** button and close the dialog box.

7. Go to **Instrument Bar** and select **Real-Time tab**.

8. Connect to the instrument clicking on the "**Connect**" button.

9. Click "**Open/Close**" to rotate instrument tray to the OUT position.

10. Place the plate into the instrument tray in the right position!

11. Load the plate by clicking the "**Open/Close**" button.

12. Start the experiment by clicking the "**Start**" button.

13. After starting the experiment, **Save** the experiment by entering a file name for the plate document.

### Recommended Plate Design

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	4	4	4	4	4	4	4	4	4	4	4
B	2	4	4	4	4	4	4	4	4	4	4	4
D	3	4	4	4	4	4	4	4	4	4	4	4
E	4	4	4	4	4	4	4	4	4	4	4	4
G	4	4	4	4	4	4	4	4	4	4	4	4
H	4	4	4	4	4	4	4	4	4	4	4	4
I	4	4	4	4	4	4	4	4	4	4	4	4
J	4	4	4	4	4	4	4	4	4	4	4	4

1 = PCR negative control

2 = preparation negative control

3 = positive control (R4)

4 = samples

### DATA ANALYSIS

Note: The criteria to consider the PCR successful and valid are: positive control yields positive signal in the recommended range, preparation negative control is negative for 16 18 and HR detectors and positive for IC detector, and the PCR negative control is negative for all three detectors. The **Genoid\_RTHPV\_ABI7900HT** Template file contains the optimized threshold (=5) and base line (=5-11) values for the automatic analysis. Samples with Ct values below 10 are detection artifacts, Ct values above 10 indicate positive samples and the term "**undetermined**" for Ct values denotes negative results. Data may be analyzed without the Template file on the  $\Delta$ RN/cycle or RN/cycle plot

1. After the run has completed click on the Analyze button (**larger green triangle** in the tool bar) for analyzing the data.
2. Open **Results** window.
3. Choose  **$\Delta$ RN/cycle** for Plot.
4. Samples with **10  $\leq$  Ct value** are confirmed as positive for the given target (16 18, HR and IC).
5. In the **Detector** tab you can select for 16 18 (HPV 16, 18), HR (High Risk) and IC (Internal control) detector to visualize the amplification plot of the sample for the given target.

**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 16 18 and/or HR signals. When samples yield strong 16 18 or HR signals, the IC signal might disappear, because the strong 16 18 or HR 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 16 18 or HR signal 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 16 18 or HR signal 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

1. Parkin DM et al. Global cancer statistics, 2002. *CA Cancer J Clin.* 2005 Mar-Apr;55(2):74-108.
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3. Bosch FX et al. The causal relation between human papillomavirus and cervical cancer. *J Clin Pathol.* 2002 Apr;55(4):244-65.
4. Muñoz N et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med.* 2003 Feb 6;348(6):518-27.
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