



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

OPTIMIZED REAGENT SET FOR REAL-TIME PCR AMPLIFICATION AND
DETECTION OF 20 HPV GENOTYPES
IN THE FOLLOWING GROUPS:
HIGH-RISK GROUP (16,18,26,31,33,35,39,45,51,52,56,58,59,66,68)
LOW-RISK GROUP (6,11,42,43,44)
INTERNAL CONTROL DETECTED IN EACH SAMPLE.

INSTRUCTION MANUAL (LightCycler 2.0 IVD version)

COMPONENTS

FOR 32 REACTIONS

GenoID REAL-TIME HPV ASSAY[®] HUMAN PAPILOMAVIRUS REAL-TIME PCR KIT FOR LIGHT CYCLER 2.0

R20010

GenoID REAL-TIME HPV ASSAY[®] COLOR COMPENSATION KIT

C20010

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:

- Low-Risk: 6, 11, 42, 43, 44,
- High-Risk: 16, 18, 26, 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 15 high risk and 5 low risk HPV types in the LightCycler 2.0.

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 3 different dyes labeling the type specific molecular beacons: molecular beacons detecting the 14 high risk types are 5'-FAM labeled, low risk types are detected by 5'-TET labeled molecular beacons, while the Internal Control is detected by a 5'-FAM-TexasRed labeled wavelength shifting molecular beacon.

High-risk, low-risk and internal control amplification is detected in separate detection channels, accordingly:

Target	Detector	Detection Channel
High Risk HPV	HR	530 nm
Low Risk HPV	LR	560 nm
Internal Control	HPVRTIC	610 nm

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

Table 1. GenoID REAL-TIME HPV ASSAY® HPV Real-Time PCR KIT-32 REACTIONS - R20010

Label	Cap Color	Name	Final volume
R1	Green	GenoID REAL-TIME HPV ASSAY PCR Buffer TRIS buffer Potassium chloride	367,5 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 (Add R1)	105 µl
R3	Red	GenoID REAL-TIME HPV ASSAY Internal Control DNA Synthetic DNA	100 µl
R4	Violet	GenoID REAL-TIME HPV ASSAY Positive Control Synthetic DNA for HR-HPV, LR-HPV and IC	100µl
R5	White	GenoID REAL-TIME HPV ASSAY Sample Preparation (negative) Control (Add working IC reagent)	1200µl

Table 2. GenoID REAL-TIME HPV ASSAY® LC COLOR COMPENSATION KIT C20010

Label	Cap Color	Name	Final volume
R6	White	GenoID REAL-TIME HPV ASSAY LC Color Compensation Dye Buffer	50 µl
R7	Yellow	GenoID REAL-TIME HPV ASSAY LC Color Compensation Dye for 530 nm Synthetic DNA	50 µl
R8	Orange	GenoID REAL-TIME HPV ASSAY LC Color Compensation Dye for 560 nm Synthetic DNA	50 µl
R9	Blue	GenoID REAL-TIME HPV ASSAY LC Color Compensation Dye for 610 nm Synthetic DNA	50 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 QIAamp MinElute Media Kit (Qiagen, Cat No: 57414) are recommended.
- LightCycler Capillaries (20 µl) (Cat.No: 11 909 339 001) with appropriate LightCycler 2.0 Sample Carousel (20 µl) (Cat.No: 03 603 962 001), LightCycler Centrifuge Adapters (Cat.No: 11 909 312 001), LightCycler Capping Tool (Cat.No: 03 357 317 001).
- 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)
- Sterile polypropylene conical tubes; 15 ml (Corning 430052 or equivalent)
- Centrifuge with rotor for 96 well plates and polypropylene conical tubes (15 ml) (max.6000g)
- Vortex mixer
- Disposable powder-free gloves

Amplification Area

- LightCycler 2.0 Instrument (Cat.No: 03 531 414 201), LightCycler Software 4.05 (Cat.No: 03 604 012 001), LightCycler Capillary Releaser (Cat.No.: 03 603 920 001). This kit is only validated for use on LightCycler 2.0.

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 were validated for use 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 32 tests. For an efficient usage of the kit, specimens and controls should be processed at least in 16-reaction batches.

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.

Note: Before the first experiment with this kit on the LightCycler 2.0 Instrument, carry out the LC color compensation. The resulted color compensation file will be used to evaluate any subsequent GenoID REAL-TIME HPV ASSAY® test results.

LC Color compensation experiment

The GenoID REAL-TIME HPV ASSAY® is designed to detect high-risk, low-risk and internal control amplification in separate channels. In a multicolor reaction, the wavelengths of light emitted by the dyes overlap, causing one channel to detect signals from a dye measured by another channel. This bleed-over of fluorescent signal can result in uninformative data, and can be corrected by applying color compensation. LightCycler algorithms use the data from the color compensation file to compensate for the fluorescence bleed-over. You then see only the specific signals in each channel.

The color compensation is determined by using a color compensation dye set. GenoID REAL-TIME HPV ASSAY® LC COLOR COMPENSATION KIT contains all the necessary reagents to carry out the experiment. Refer to the LightCycler 2.0 Instrument Operator's Manual Software Version 4.05 (Cat.No: 04 717 392 001) Manual B, 7.2 *Using Color Compensation*. The resulted color compensation file has to be created only once, usually before the first run of GenoID REAL-TIME HPV ASSAY®. If you have unsatisfactory color compensation consider repeating the color compensation experiment with the dyes provided in each kit (slight batch differences could exist). This color compensation file is specific for the GenoID REAL-TIME HPV ASSAY KIT®.

1. Thaw: GenoID REAL-TIME HPV ASSAY LC Color Compensation Dye Buffer **(R6)**, GenoID REAL-TIME HPV ASSAY LC Color Compensation Dye for 530 nm **(R7)**, GenoID REAL-TIME HPV ASSAY LC Color Compensation Dye for 560 nm **(R8)**, GenoID REAL-TIME HPV ASSAY LC Color Compensation Dye 610 nm **(R9)**.
2. Prepare the experiment in the following order by adding 22 µl of:

Capillary 1	GenoID REAL-TIME HPV ASSAY LC Color Compensation Dye Buffer (R6)
Capillary 2	GenoID REAL-TIME HPV ASSAY LC Color Compensation Dye for 530 nm (R7)
Capillary 3	GenoID REAL-TIME HPV ASSAY LC Color Compensation Dye for 560 nm (R8)
Capillary 4	GenoID REAL-TIME HPV ASSAY LC Color Compensation Dye for 610 nm (R9) .

DNA ISOLATION

Note: The specimen should be collected in PreservCyt Solution. DNA isolation has to be performed using AmpliLute Liquid Media Kit and equipments (Roche, P/N: 03750540 190).

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 AmpliLute Liquid Media Kit.

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 capillary.

Performing the real-time PCR Reaction

Note: The GenoID REAL-TIME HPV ASSAY® is sufficient for the simultaneous examination of **29 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.

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.**
3. Close the capillaries and centrifuge briefly.
4. To setup a new experiment, select "RUN" in the LightCycler program, then click on the "Template" icon and browse for the previously downloaded and saved template file **GenoID_RTHPV_LightCycler** (available at <http://www.genoid.net/index.php/products-and-services/real-time-pcr-hpv-assays/>). Select, and click „Open“. In the left toolbar select "Samples", fill in the name of the samples, in the following order:

1 = PCR negative control

2 = preparation negative control

3 = positive control (R4)

4-32 = samples

Select "RUN" in the left toolbar, above "Samples". In the new window click on the "Start Run" tab and save the experiment as the new window pops up.

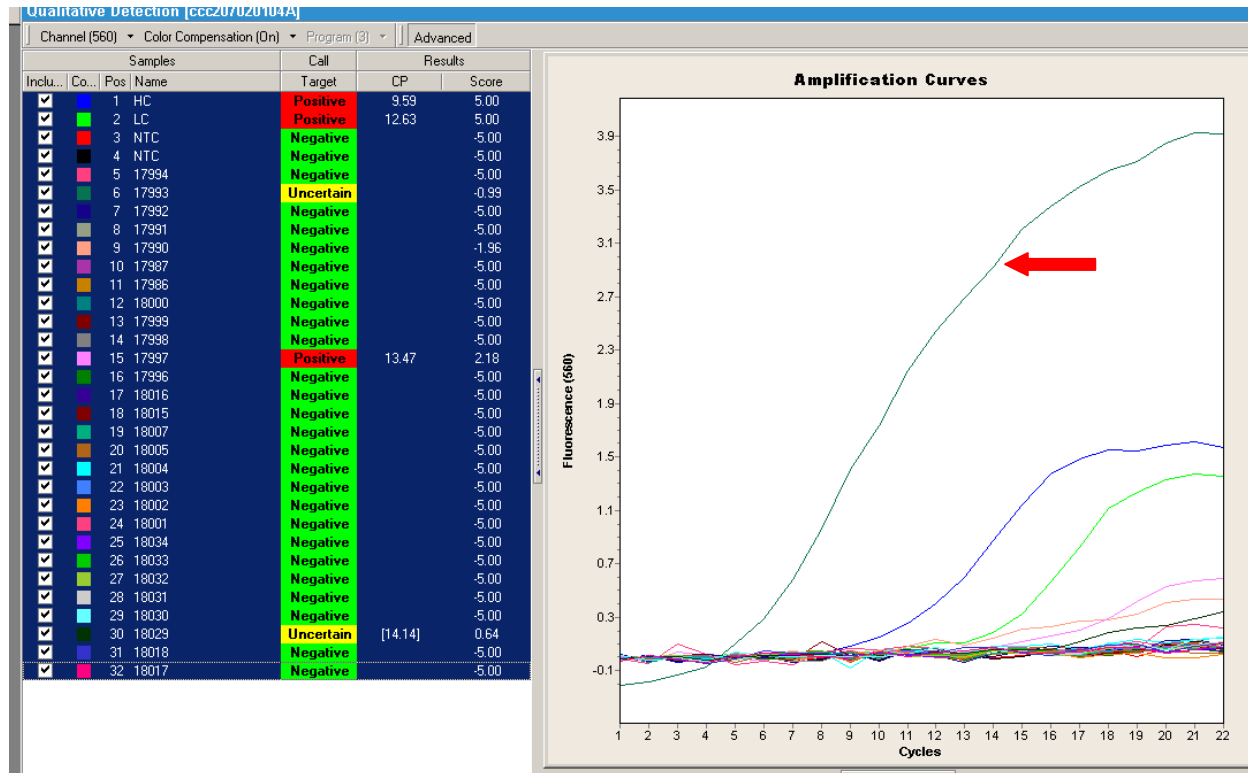
5. Perform the experiment **at stable temperature (21 ± 2°C!!)**.

RESULTS

After the run is completed, evaluate the results. Click 'Analysis' menu and choose 'Qualitative Detection'. A window will appear with the following content: "There are no sample settings for Qualitative Detection analyses". Select "No". **Apply color compensation (prepared in advance).**

The software yields "Positive", "Negative" or "Uncertain" calls as results for the corresponding target (high risk, low risk, or internal control) in each channel (530 nm, 560 nm, 610 nm). However, the software may evaluate sigmoid, positive curves as "Negative" or "Uncertain" (for example the signal marked with the red arrow below).

To analyze the results, select the "Advanced" option, and refer to the "Score". Samples yielding scores above -2.00 are positive (negative between -5 and -2, positive between -2 and +5).



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 HR or LR 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 HR or LR 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

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7. Takács T et al. Molecular beacon based real-time PCR method for detection of 15 high risk and 5 low risk HPV types. J Virol Methods. 2008 Apr;149(1):153-62.

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