



GenoID REAL-TIME HPV KIT

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

GROUP A: HPV16, 18,

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

INTERNAL CONTROL DETECTED IN EACH SAMPLE.

INSTRUCTION MANUAL

(For use with the ABIStepOnePlus Real-Time PCR System)

for research use only!

FOR 96 REACTIONS

COMPONENTS

GenoID REAL-TIME HPV KIT[®] FOR ABIStepOnePlus **R20040**

STORAGE

SHIP AND STORE AT -20°C

INTENDED USE

The GenoID REAL-TIME HPV KIT (HPV 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 high risk genital HPV DNA types:

- HPV 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 KIT 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 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 ^[7]. There are 3 different dyes labeling the type specific molecular beacons: molecular beacons detecting the 12 high risk types are 5'-JOE labeled, HPV 16 and 18 are detected by 5'-FAM labeled molecular beacons, while the Internal Control is detected by a 5'-ROX labeled molecular beacon.

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

| Target | Detector | Detection Channel |
|--|----------|-------------------|
| HPV 16, 18 | 16 18 | FAM |
| HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 | HR | JOE |
| Internal Control | HPVRTIC | ROX |

The kit also contains a Positive Control, which represents approximately 7000 DNA copies/ reaction. Note, that the assay has a general analytical sensitivity of 70-700 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 KIT® HPV Real-Time PCR KIT for ABIStepOnePlus contains sufficient reagents for 96 tests.

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

STORAGE

The components of the GenoID REAL-TIME HPV KIT 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 KIT 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),
- 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 StepOnePlus Real-Time PCR System (P/N: 4376592), StepOne Software Version2.0 (P/N: 4386000), AB MicroAmp Fast Optical 96-Well Reaction Plate (0.1 ml) (P/N: 4346906), MicroAmp Optical Adhesive Film (P/N: 4311971). This kit is only validated for use on ABI StepOnePlus.

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 KIT®. 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 KIT® 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 KIT® 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 40 µl GenoID REAL-TIME HPV KIT 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 KIT PCR **Mastermix** according to the following pipetting scheme (final volume/ reaction: 15 µl):

| Reagent | Volume (µl) |
|---|-------------|
| GenoID REAL-TIME HPV KIT PCR Buffer (R1) | 10.5 µl |
| GenoID REAL-TIME HPV KIT 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 GenoID REAL-TIME HPV KIT® HPV Real-Time PCR KIT for ABIStepOnePlus 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 ABIStepOnePlus 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 ABIStepOnePlus Software Version2.0.
5. Select **New Experiment** and choose:

From Template: Browse and open the downloaded **GenoID_RTHPV_S1+** Template file (available at <http://www.genoid.net/index.php/products-and-services/real-time-pcr-hpv-assays/>).

6. After opening the Template file, go to **File menu** and **Save** your experiment file.
7. Place the plate into the instrument tray in the right position!

8. Start the experiment by clicking the “Start” button.

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_Step1Plus_RTHPV_ROX** Template file contains the optimized threshold and base line values for the automatic analysis. Samples with Ct values below 10 are detection artifacts, Ct values above 10 indicate positive samples.

1. After the run has completed click on the Analyze button (**larger green triangle** in the tool bar) for analyzing the data.
2. Samples above threshold value are confirmed as positive for the given target (16 18, HR and IC).
3. 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.
2. Walboomers JM et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol.* 1999 Sep;189(1):12-9.
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.
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.
6. Naucler P et al. Human papillomavirus and Papanicolaou tests to screen for cervical cancer. *N Engl J Med.* 2007 Oct 18;357(16):1589-97.
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.

TERMS OF USE (INCLUDING COPYRIGHT INFORMATION, DISCLAIMER, LIMITED WARRANTY)

1. GENERAL INFORMATION

These Terms of Use are applicable to GenoID products for in vitro use. By opening and using these product you ("Customer,") acknowledge that you have read this "Terms of Use" ("Agreement"), understand it, and agree to be bound by its terms and conditions.

2. COPYRIGHT, TRADEMARK AND LICENSE TO USE GENOID'S PRODUCTS

Should you agree to be bound by this Agreement, and your order is valid and price of GenoID's product is fully paid, GenoID grants you a single-use license for such products on a non-transferable, non-exclusive basis.

GenoID's products are protected by copyright law and international treaties. Unauthorized reproduction or distribution of the products may result in severe civil and criminal penalties, and will be prosecuted to the maximum extent possible under law. Please note that it is GenoID's policy to enforce its intellectual property rights to the fullest extent of the law.

Use of GenoID's products for commercial purposes without the prior written consent of GenoID may constitute patent infringement.

Nothing in this document should be construed as granting, by implication or otherwise, any license to use products manufactured or sold by GenoID without the express written permission of GenoID. Duplicating or copying or imitating any part of the products, reverse engineer, repackaging, resell, redistribute, or in any way modify the products is prohibited. Patent pending in Europe and other countries.

The trademarks, logos and service marks (collectively, the "trademarks") displayed in this document are registered and unregistered trademarks owned or licensed by GenoID (or by third parties). Nothing in this document should be construed as granting, by implication or otherwise, any license or right in or to the trademarks without the express written permission of GenoID. In addition, the entire content of GenoID documents, including all images or texts, is copyrighted. Any reproduction or other use of the contents of this document is prohibited.

Certain GenoID products may in certain countries be protected by intellectual property rights of third parties. Sales of a GenoID product are not to be realized to and in countries in which the product is protected by intellectual property rights of third parties.

The polymerase chain reaction (PCR) process is covered by patents issued and applicable in certain countries.

GenoID does not support the unauthorized or unlicensed use of PCR process. The end-user (Licensee, Customer) of this product in certain countries must have a license to perform PCR.

3. RETURN OF GENOID'S PRODUCTS

Upon your receipt of goods, you shall inspect the goods and notify us of any claims for shortages, defects or damages. If you fail to so notify us within three days after you receive the goods, the goods shall conclusively be deemed to conform to these Terms of Sale and the relevant product descriptions and to have been irrevocably accepted by you. Authorization for all product returns must be approved by GenoID in writing.

Not all items will be authorized for return, due to temperature and packing requirements. Items authorized for return must arrive at our facilities in a state satisfactory for resale to be eligible for product credit. Shipping charges will not be credited. Goods may not be returned for credit after 20 days after your receipt of the goods. At our discretion, we may issue a product credit or refund for the product value and shipping charges. Any product credit not used within four months of the date of issue shall expire.

4. USE OF GENOID PRODUCTS

GenoID products are intended for in vitro diagnostic use or for research purposes. GenoID products may contain hazardous substances. Please refer to the package insert of the products for details.

The GenoID products should be used by trained personnel. GenoID does not assume any liability for damages resulting from wrong handling, from use outside of the intended use or from not following the instructions in the kit inserts.

5. DISCLAIMER OF LIABILITY

We warrant to our Customer that our goods shall conform substantially to the description of such goods as provided in the kit inserts and literature accompanying the goods until their respective expiration dates. This warranty is exclusive, and we make no other warranty, express or implied, including any implied warranty of merchantability or fitness for any particular purpose. Our warranty shall not be effective if we determine, in our sole discretion, that you have altered or misused the goods or have failed to use or store them in accordance with instructions furnished by us.

Our sole and exclusive liability and your exclusive remedy with respect to goods proved to our satisfaction (applying analytical methods reasonably selected by us) to be defective or nonconforming shall be the replacement of such goods free of charge, upon the return of such goods in accordance with our instructions, although at our discretion we may provide a credit or refund.

In no event shall we be liable under any legal theory (including but not limited to contract, negligence, strict liability in tort or warranty of any kind) for any indirect, special, incidental, consequential or exemplary damages (including but not limited to lost profits), even if we had notice of the possibility of such damages.

GenoID's products can be ordered exclusively via the GenoID Online Store or by fax directly from GenoID or from an authorized distributor.

Therefore GenoID's products ordered, received from other sources may result in infringement of GenoID's or third party's intellectual property rights, and also may result in obtaining an improper and possibly hazardous and unsafe product. GenoID does not assume any liability for damages arising from any kind of unlawful commercial activity. Not contradicting to the foregoing, GenoID's liability shall not exceed the actual amount paid to GenoID for GenoID's products.

6. GOVERNING LAW AND JURISDICTION

This Agreement will be governed by the laws of Hungary. For the settlement of disputes arising out of or in relation to this Agreement or the breach, termination, validity or interpretation thereof, GenoID and the Customer shall submit themselves to the exclusive award of the Permanent Court of Arbitration (Budapest) organized alongside the Hungarian Chamber of Commerce and Industry, with the understanding that the Arbitration court proceeds in accordance with its own Rules of Procedure.

7. COMPLIANCE WITH APPLICABLE LAWS

The Customer shall comply with all applicable laws affecting the purchase and use of GenoID's products, agrees to maintain all registrations with governmental bodies, commercial registries, chambers of commerce, or other offices which may be required under law in order to properly conduct commercial business, and use GenoID's products.

8. MISCELLANEOUS

GenoID is not responsible for typographic errors.

Should any part of this Agreement be declared void or unenforceable by a court of competent jurisdiction, the remaining terms shall remain in full force and effect. Failure of GenoID to enforce any of its rights in this Agreement shall not be considered a waiver of its rights, including but not limited to its rights to respond to subsequent breaches. © 2002-2006 GenoID Ltd. All rights reserved.