



FOR IN VITRO USE ONLY!

**HIGH RISK HPV
AMPLIFICATION AND GENOTYPING SYSTEM**

OPTIMIZED REAGENT SET FOR PCR AMPLIFICATION AND
GENOTYPING OF 14 HPV GENOTYPES :16,18,31,33,35,39,45,51,52,56,58,59,66,68
INTERNAL CONTROL DETECTED IN EACH SAMPLE.

INSTRUCTIONS MANUAL

VERSION 1.1

GENOID LTD. • 48 RÖPPENTYŰ U. • BUDAPEST, HUNGARY H-1139

PHONE: +36 (1) 465-0124 • FAX: +36 (1) 465-0127

E-MAIL: INFO@GENOID.HU • WWW.GENOID.NET

**COMPONENTS
(FOR 11 SAMPLES)**

FULL SPECTRUM HPV AMPLIFICATION KIT	M20040
FULL SPECTRUM HPV HR GENOTYPING PROBE KIT	P20420
GENERAL DETECTION REAGENT KIT	H20012

STORAGE

FULL SPECTRUM HPV AMPLIFICATION KIT	-18 °C OR BELOW
FULL SPECTRUM HPV HR GENOTYPING PROBE KIT	-18 °C OR BELOW
GENERAL DETECTION REAGENT KIT	2 °C TO 8 °C

General Kit Description

2007. version 3.1

System developed by GenoID.

Human Papillomavirus (HPV) is one of the most common causes of sexually transmitted infections (STI) in the world. Health experts estimate that there are more cases of genital HPV infection than of any other STI in the developed countries. Scientists have identified more than 100 types of HPV, most of which causing minimal disease. About 50 types are spread through sexual contact. Some of HPV types that cause genital infections can also cause cervical cancer. They have been further classified according to the relative risk of causing cancer: 'Low-Risk' and 'High-Risk' genotypes are differentiated thus. The 'Low-Risk' group of genotypes can cause cellular changes (detected by PAP smear) but the infection almost never results in cells with truly malignant phenotype. However, the 'High-Risk' group of genotypes can induce real malignant transformation. Like many STIs, genital HPV infections often do not have visible signs and symptoms and almost half of the women infected with HPV have no obvious symptoms. PAP smear, the classical cytological screening method can be used to detect the cellular changes associated with HPV infections but the procedure lacks both sensitivity and specificity for virus detection and cannot distinguish the 'Low-Risk' and 'High-Risk' genotypes.

Recently new molecular diagnostic techniques gained acceptance for the detection of genital HPV infections, because of their sensitivity and the possibility of genotyping the virus.

Also with the advent of HPV vaccine, the genotyping is more important. Genotyping is used to assess better the relative risk of the infection, helps in the follow-up of the patient and it is used to exclude the presence of the vaccine genotypes to maximize the benefit of the vaccine.

GenoID's Full Spectrum Genital Human Papillomavirus PCR Amplification and Genotyping Kit is designed and optimized to amplify and genotype the following genital HPV genotypes:

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

Most of these genotypes are not detected and identified by any other system available today.

To exclude false negative results the internal control DNA is prepared parallel with the specimen and amplified concurrently in the reaction.

The amplification is based on the L1 region of HPV and designed to detect the non-integrated copies of HPV. The amplification is balanced over the genotypes, which is important to achieve optimal clinical sensitivity.

The amplicons produced by the amplification reactions are detected by solid phased hybridization. The detection of the genotypes and the Internal Controls are carried out in 15 different reaction chambers (wells).

The hybridization procedure is briefly outlined below:

The biotinylated PCR products (achieved using one biotinylated primer during amplification) are captured onto the surface of a microplate well coated with streptavidin. The complementary chain is eluted from the immobilized PCR product and hybridization is carried out in the presence of specific fluorescein labeled probes. The bound probes are reacted with anti-fluorescein-HRPO (horse-radish peroxidase) antibody. The HRPO chromogenic substrate (TMB) yields blue product in case of a positive reaction.

Operational Protocol

Note: It is recommended to use GenoID's Full Spectrum Human Papillomavirus Amplification and Detection System before you genotype your sample using GenoID's High Risk Human Papillomavirus Amplification and Genotyping System. This strategy helps to reduce the genotyping work and the same diluted PCR product can be used, that was previously amplified by GenoID's Full Spectrum Human Papillomavirus Amplification and Detection System for the subsequent genotyping.

The general procedure of the kit is the following:

1. DNA extraction from samples - sample preparation
2. DNA amplification by PCR
3. Detection of PCR amplicons by solid phase hybridization

Attention!

- During the manipulation - because of the sensitivity of PCR - care must be taken to avoid contamination of the samples with each other or with the amplified product.
- Wearing powder-free examination gloves is highly recommended for the PCR set-up, because contamination of the kit components from the hand of the user may lead to degradation of reagents that must be avoided.
- Spin down the reagents before use.
- Use filter tips.
- Separate place and pipettors must be used for
 - mastermix preparation,
 - reaction set-up, and
 - post PCR manipulation.

1. Sample Preparation

The collected specimen should be placed in Cytic PreserveCytR Solution

Recommended sample preparation:

Use ROCHE AmpliLute Liquid Media Extraction Kit or ROCHE High-Pure PCR Template Preparation Kit. (Not provided by GenoID.)

1. Prepare IC Solution by adding 10 µl of **HPV-IC DNA (R3)** to 20 ml of Cytic PreserveCytR Solution (one full vial). Prepare it freshly.
2. Prior to the preparation of the DNA of the specimen centrifuge 1250 µl of specimen (in the original specimen collection Cytic PreserveCytR Solution) for 10 min. at 4000 g and room temperature using Eppendorf tubes and a fixed angle microcentrifuge rotor.
3. Decant the supernatant, do not disturb the pellet.
4. Add 250 µl of IC Solution to the pellet. Vortex and proceed the specimen preparation using ROCHE AmpliLute Liquid Media Extraction Kit or ROCHE High-Pure PCR Template Preparation Kit.

2. Performing the PCR Reaction

The Full Spectrum HPV Amplification Kit is sufficient for the simultaneous examination of **11 samples** and 2 controls (a positive and a negative control respectively).

The following vials are the components of the PCR reaction (reagents are parts of Full Spectrum HPV Amplification Kit M20040):

Vial	Cap Color	Label	Content and usage
R1	Green	Master Mix	980 µl Master Mix
R2	Pink	Control DNA for HPV LR, HR	65 µl HPV positive DNA sample (genotype 6, 16)
R3	Red	HPV-IC DNA	100 µl HPV Internal Control DNA

Other materials and instruments needed (not provided with the kit):

- Thin-walled 0.2ml PCR tubes, or plate (thermocycler compatible,)
- Adjustable pipettors
- Filter Pipette Tips
- GeneAmp® PCR System 9700 (Applied Biosystems) or other compatible thermocycler
- Exclusively AmpliTaq Gold polymerase (Applied Biosystems: Cat.No.: 4311806 (250U))

2 A. PCR Reaction Setup

1. Add **20 µl AmpliTaq Gold enzyme** (Applied Biosystems) into the vial of **“Mastermix” reagent (R1)**. If you would like to prepare less than 11 reactions, both master **Mastermix (R1)** and the enzyme can be scaled down proportionally.
2. Vortex to mix the reagents (this mixture can be stored for ca. 30 days at -20°C).
3. Aliquot **20-20 µl of reaction mixture** into the PCR tubes prepared in advance.
4. Add **5-5 µl template** DNA to the PCR tubes.
5. Set up two reactions for controls: a positive control (using reagent **R2**) and a negative control “no template control” (add 5µl of PCR grade water instead of sample). See [Recommended PCR Plate Design](#) on p6.
6. Close the tubes and place them into the thermocycler, programmed in advance as described herein ([Thermocycling parameters](#) see below).

Carry out the programmed thermocycling.

If you use a thermocycler other than GeneAmp® PCR System 9700, care must be taken to get satisfactory results, prior to the first usage it is recommended to carry out an experiment with a series of dilutions of the positive control.

Recommended PCR Plate Design

0	3	3	3	3	3	0	0	0	0	0	0
0	3	3	3	3	3	0	0	0	0	0	0
1	3	3	3	3	3	0	0	0	0	0	0
2	3	3	3	3	3	0	0	0	0	0	0
3	3	3	3	3	3	0	0	0	0	0	0
3	3	3	3	3	3	0	0	0	0	0	0
3	3	3	3	3	3	0	0	0	0	0	0
3	3	3	3	3	3	0	0	0	0	0	0

0 Empty
 1 PCR negative control
 2 PCR positive control
 3 Samples

This design lets samples be replicated using an 8-channel pipettor directly from the PCR plate to the hybridization plate.

2 B. Thermocycling Parameters

Temperature (°C)	Time	Number of Cycles
95	10 min	1
95	30 sec	10
45 increment +0,3°C/cycle	30 sec	
72	30 sec	
95	30 sec	35
48	30 sec	
72	30 sec	
72	4 min	1
4	∞	

3. Detection of PCR Amplicons by Solid Phase Hybridization

The following bottles and vials are the components of hybridization reaction:

Full Spectrum HPV High Risk Genotyping Probe Kit P20420/1;-/2

Vial	Cap Color	Label	Content and usage
H16	Yellow	HPV-16 Probe	40 µl Hybridization probe for HPV-16
H18	Orange	HPV-18 Probe	40 µl Hybridization probe for HPV-18
H31	Brown	HPV-31 Probe	40 µl Hybridization probe for HPV-31
H33	Bald	HPV-33 Probe	40 µl Hybridization probe for HPV-33
H35	White	HPV-35 Probe	40 µl Hybridization probe for HPV-35
H39	Yellow	HPV-39 Probe	40 µl Hybridization probe for HPV-39
H45	Orange	HPV-45 Probe	40 µl Hybridization probe for HPV-45
H51	Brown	HPV-51 Probe	40 µl Hybridization probe for HPV-51
H52	Bald	HPV-52 Probe	40 µl Hybridization probe for HPV-52
H56	White	HPV-56 Probe	40 µl Hybridization probe for HPV-56
H58	Orange	HPV-58 Probe	40 µl Hybridization probe for HPV-58
H59	Brown	HPV-59 Probe	40 µl Hybridization probe for HPV-59
H66	Bald	HPV-66 Probe	40 µl Hybridization probe for HPV-66
H68	White	HPV-68 Probe	40 µl Hybridization probe for HPV-68
H3	Red	HPV-IC Probe	40 µl Hybridization probe for HPV-IC
H5	Black	HPV16,18,31,33,35,39,45,51,52,56,58,59,66,68 and IC hybridization Positive Control mix	350 µl Hybridization positive control mix for HPV16,18,31,33,35,39,45,51,52,56,58,59,66,68 and IC

Detection Reagent kit H20012

Vial	Cap Color	Label	Content
H6		Binding buffer	10 ml binding buffer
H7		Elution buffer	15 ml elution buffer
H8		Hybridization buffer	15 ml hybridization buffer
H9		Conjugation buffer	25 ml conjugation buffer
H10	Blue	Detection reagent	12,5 µl HRPO conjugated anti flores-cein antibody
H11	Brown	Substrate solution	30 ml ready to use stabilized TMB substrate for HRPO
H12		Washing solution 1	2x50 ml concentrated (10xccc.) washing solution 1
H13		Washing solution 2	60 ml concentrated (10xccc.) washing solution 2
H14		Hybridization plate 24X8 strips	Streptavidin coated plate: 2 "eggcrate" holder frame, 24 (transparent) strip

Additional reagents and equipments required (should be provided by user)

- DNase free PCR-grade water
- 8-channel pipettor (20-200 μ l)
- Adjustable pipettors (1-10 μ l, 20-200 μ l, 100-1000 μ l)
- 1-10 μ l, 20-200 μ l and 1000 μ l pipette tips
- Reagent reservoirs
- Microplate reader (optional) with detection capability at 360 nm,
- Sealing film.

Prepare the following solutions before the procedure:

- Add 50 ml washing solution 1 (H12) to 450 ml distilled water and mix thoroughly.
- Add 60ml washing solution 2 (H13) to 540 ml distilled water and mix thoroughly.
- Incubate the hybridization buffer (H8) at 55 °C until use.

The detections of 14 type of HPV-HR, from one sample are carried out in fifteen separate wells (Fourteen genotypes and IC). Hybridization controls need fifteen wells (fourteen genotypes and hybridization negative control). For one sample you will need four strips, for one more sample two more strips, (the hybridization controls will be the same). Altogether the fourteen hybridization reactions of 11 samples take two whole plates.

1. Dilute each PCR product 10 times (25µl/225µl) with DNase free water and mix well.
2. Wash the hybridization wells once with the diluted **washing solution 1 (H12)**: Pipette 200-200 µl **washing solution 1 (H12)** into each well using an 8-channel pipettor, then remove the washing solution with a strong downward motion and **dump** the plate upside down against an absorbent paper to remove the rest of the washing solution.
3. Dispense 40-40 µl **binding buffer (H6)** into each well using an 8-channel pipettor.
4. Add 10 µl of the 10 times diluted PCR product and the controls to the wells using the following order (see [Recommended Hybridization Plate Design](#) on p9). For each four strip:

well 1: Hybridization NTC: 10 µl DNase-free distilled water,

well 2: PCR NTC: 10 µl 10 times diluted PCR negative control (from the amplification reaction,)

well 3: PCR PC: 10 µl 10 times diluted PCR positive control (from the amplification reaction), this sample should be hybridized with **H16**

well 4: Hybridization PC: 10 µl **hybridization positive control (H5)**, fourteen times for each genotype

well 5: 10 µl 10 times diluted PCR products of samples.

The same order of the samples are replicated on the other three half plates (order of the half plate is the following: LR, HR, general HPV, internal control). Incubate the plates for 10 minutes at room temperature with shaking.

5. Dispense 50-50 µl **elution buffer (H7)** into each well and incubate for 2-3 minutes at room temperature with shaking (don't remove the binding buffer and PCR product mix before this step).
6. Get ride of the liquid from the wells with a strong downward motion and dump the plate upside down against a fresh absorbent paper and wash **3 times** with 200-200 µl **washing solution 1 (H12)**.
7. Add 10 µl hybridization probe for each HR-HPV (**H16 - H68**), into **fourteen separated tubes** containing 1ml prewarmed (55°C) **hybridization buffer (H8)**. Mix well and dispense 50-50 µl hybridization solution into the wells according to the sample distribution.
8. Incubate for 20 minutes at **55°C**.
9. Get ride of the liquid from the wells with a strong downward motion and dump the plate upside down against a fresh absorbent paper and wash the wells 6 times with 200 µl **washing solution 2 (H13)**.
10. Add 4 µl **detection reagent (H10)** in 12 ml **conjugation buffer (H9)**. Mix well. Dispense 50-50 µl solutions into each well and incubate for 10 minutes at room temperature with shaking.

11. Get rid of the liquid from the wells with a strong downward motion and dump the plate upside down against a fresh absorbent paper and wash **10 times** with 200 µl **washing solution 1 (H12)**.

12. Dispense 140 µl **substrate solution (H11)** into the wells and incubate for 15 minutes at room temperature with shaking.

13. Read the microplate by a microplate reader at 370 nm or 655 nm, keep the records.

Interpretation by simple visual inspection is also possible. Wells with visible blue color are considered as positive reactions.

Recommended Hybridization Plate Design for one genotyping reaction

1	4	4	5															
2	4	5	5															
3	4	5	5															
4	4	5	5															
4	4	5	5															
4	4	5	5															
4	4	5	5															
4	4	5	5															


1 Hybridization negative control - -detection with H16

2 PCR negative control – detection with H16

3 PCR positive control – detection with H16

4 Hybridization positive controls (H5) – detection with H16-H66

5 Samples – Fourteen times for each sample for each genotype, and once for IC detection for each sample

 These fifteen wells are repeating, according to the sample number.

Interpretation of Results

Microplate Reader Protocol

Record the absorbance readings (optical density values of the reactions at 370 nm or 655 nm.) Determine the positive cut-off values for each probe: the positive cut-off value is 130% of the OD value of the PCR negative control for the corresponding probe. Determine the negative cut-off values: the negative cut-off value is 110% of the OD value of the PCR negative control for the corresponding probe.

For a valid assay, the ratio of the PCR negative controls and corresponding hybridization negative controls should be below the number of two for each ratios and the values of the hybridization positive controls and PCR positive controls should be at least greater than 2 times of the positive cut-off value.

If the assay is valid and the OD value of the sample shows a value greater than the positive cut-off of the given probe, the reaction is considered as positive. The samples show values between the positive cut-off value and the negative cut-of value for a given probe are equivocal. If the repeated reaction has the same value, the reaction could be interpreted as weak positive.

For other details refer to the *Results Table*.

Visual Inspection Protocol

If you intend to interpret the result by simple visual inspection, you should take into consideration its possible lower sensitivity. A skilled investigator, however, could reliably interpret the results.

For a valid assay the hybridization negative controls and the PCR negative controls should be transparent and colorless. The hybridization positive controls and the PCR positive controls should develop intense color. If the assay is valid, even a developed faint blue color could be considered as positive reaction.

Result Table

Features		Results
HPV-Type-X	IC	
-	-	Repeat the procedure.
-	+	Negative result.
+	-	Positive result for identified HR HPV genotype , strong amplification.
+	+	Positive result for identified HR HPV genotype, medium or weak amplification.

Troubleshooting

No signal in the sample reactions.

Examine the control samples; if both the internal control and the positive controls produced satisfactory signals (and they are valid), then there could be a problem with the template DNA (purity, concentration).

Solution: Repeat sample preparation and PCR reaction.

No signals can be detected in either of the control reactions.

The kits are quality controlled; if they are stored properly we guarantee they will work satisfactory prior to expiration.

Signal is detected in the PCR negative control.

It is a sign of contamination during the set-up of the PCR. In this case results obtained for the assayed samples are equivocal, they must be repeated. The source of contamination could be the pipettes, pipette tips, PCR tubes and the examiner as well.

Solution: Use filter pipette tips. Re-examine all samples with cleaned pipettes, after revising the former reaction circumstances and correcting the problems which were eventually revealed.

No signal detected in the positive control.

It can be caused by pipetting error, or contamination and degradation of control DNA.

Solution: It is wise to judge by eye the pipetted amounts while pipetting, since in case of small volumes, measuring errors may occur. If the control is not amplified after repeating the reaction, the DNA has been degraded.

LITERATURE

zur Hausen H. (2002) Papillomaviruses and Cancer: from Basic Studies to Clinical Application. *Nat Rev Cancer* 2(5):342-50.

Jenkins D. (2001) Diagnosing Human Papillomaviruses: Recent Advances. *Curr Opin Infect Dis* 14(1):53-62.

Human papillomavirus DNA testing as an adjunct to cytology in cervical screening programs.

Lorincz AT, Richart RM. *Arch Pathol Lab Med*. 2003 Aug;127(8):959-68.

Cost-effectiveness analysis based on the atypical squamous cells of undetermined significance/low-grade squamous intraepithelial lesion Triage Study (ALTS). Kulasingam SL, Kim JJ, Lawrence WF, Mandelblatt JS, Myers ER, Schiffman M, Solomon D, Goldie SJ; ALTS Group. *J Natl Cancer Inst*. 2006 Jan 18;98(2):92-100.

The clinical relevance of human papillomavirus testing: relationship between analytical and clinical sensitivity. Snijders PJ, van den Brule AJ, Meijer CJ. *J Pathol*. 2003 Sep;201(1):1-6.

TERMS OF USE

© 2004-2006 GenoID® Ltd. All rights reserved.

By opening and using this Full Spectrum HPV kit ("Kit") you ("user," "Licensee") acknowledge that you have read this "Terms of Use" document ("Agreement"), understand it, and agree to be bound by its terms and conditions. You further agree that this Agreement is the complete and exclusive statement of the Agreement between you and GenoID and supersedes any proposal or prior Agreement, oral or written, any other communications between you and GenoID relating to the subject matter of this Agreement.

The user ("licensee") is granted a single license by the licensor (GenoID) for the use of the Kit.

Licensee agrees not to duplicate or copy or imitate any part of the Kit, reverse engineer, resell, redistribute, or in any way modify the Kit (or its copy) unless granted express permission by GenoID.

This Kit is protected by copyright law and international treaties. Unauthorised reproduction or distribution of this Kit 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.

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.

Licensee may not sub-license, assign or transfer the license or the Kit, in whole or in part, except as expressly provided in this Agreement. Any attempt otherwise to sub-license, assign or transfer any of the rights, duties or obligations hereunder is void.

This Agreement will be governed by the laws of Hungary.

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

The trademarks, logos and service marks (collectively, the "trademarks") displayed in this Agreement or other GenoID documents are registered and unregistered trademarks owned or licensed by GenoID (or by third parties).

Nothing in this Agreement or other GenoID documents should be construed as granting, by implication or otherwise, any license or right without the express written permission of GenoID in addition, the entire content of all GenoID documents, including all images or texts, is copyrighted. The authorised user of GenoID's products may reproduce the kit insert (instruction manual) for the purpose of reference only.

The Kit is provided "AS IS" without warranty of any kind, either expressed or implied, including, but not limited to the implied warranties of merchantability, fitness for a particular purpose and non-infringement. The entire risk as to the quality and performance of the Kit is with the Licensee, should the Kit prove to be defective.

GenoID makes no further warranties or guarantees nor explicit nor implied.

GenoID makes no representation or warranty as to the accuracy of any information contained in this Agreement or any document provided by GenoID electronically or in print, including the kit inserts ("Documents"). GenoID further assumes no liability or responsibility for any errors or omissions in the content of the Documents. Although every attempt is made by GenoID to ensure the accuracy of the Documents, GenoID shall in no event be liable for any damages of any kind (whether direct, consequential or punitive) arising out of the use of the Documents.

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 realised to and in countries in which the product is protected by intellectual property rights of third parties.

The PCR process is covered by patents issued and applicable in certain countries. GenoID does not support the unauthorised or unlicensed use of PCR process. The end-user (Licensee) of this product may need license to perform PCR. The license may be purchased or can be obtained via other methods.

In no event will GenoID (or their respective officers, employees, consultants, attorneys or agents), be liable for any special, indirect, incidental, or consequential damages (including, but not limited to, lost profits, lost data or information, business interruption, loss of business reputation or goodwill, or downtime costs) which the Licensee or others may incur or experience, directly or indirectly arising out of or relating to the Kit, Documents, this Agreement, or the termination of this Agreement, even if GenoID has been advised of the possibility of such damages and notwithstanding any failure of essential purpose.

Patent pending in Europe and other countries.