AmpliSens® *HPV* HCR genotype-titre-FRT PCR kit

RUO

For Professional Use Only

Instruction Manual

KEY TO SYMBOLS USED

REF	Catalogue number	Σ	Contains sufficient for <n> tests
LOT	Batch code		Use-by Date
RUO	Research Use Only		Consult instructions for use
VER	Version		Keep away from sunlight
	Temperature limit	C–	Negative control of extraction
	Manufacturer	IC	Internal control
	Date of manufacture	C1, C2	DNA-calibrators
	Caution	C+	Positive control of amplification

1. INTENDED USE

AmpliSens® *HPV* HCR genotype-titre-FRT PCR kit is an *in vitro* nucleic acid amplification test for qualitative and quantitative detection and differentiation of DNA of *human papillomaviruses* of high carcinogenic risk (*HPV* HCR) genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 in the biological material (vaginal swab, epithelial scrape from the cervical mucous membrane (ectocervix and endocervix)) using real-time hybridization-fluorescence detection of amplified products.

PCR kit allows detecting separately the *HPV* DNA of 14 genotypes (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68). *HPV* HCR are the main etiological factor in the development of cervical cancer and the previous high grade dysplasia. *HPV* is the cause of 95 % of all cervical cancer. The specified 14 genotypes of *HPV* are the cause of 94 % of HPV-associated cervical cancer.

The material for PCR is the DNA samples extracted from the test material with the use of nucleic acids extraction kits recommended by the manufacturer.

NOTE: For research use only. Not for diagnostic procedures.

2. PRINCIPLE OF PCR DETECTION

Principle of testing is based on simultaneous amplification of DNA fragments of *HPV* genotypes and a DNA fragment of β-globin gene. DNA of β-globin gene is used as an internal endogenous control (IC Glob). The use of an endogenous internal control makes it possible not only to monitor all test stages but also to assess the adequacy of sampling and storage of biological material. An endogenous internal control is a human genome fragment. It must be always present in the sample in sufficient quantities equivalent to the number of cells in the sample (10^3–10^5 cells/ml).

Amplification of DNA *HPV* HCR fragments with the use of specific primers and Taq-polymerase enzyme are performed with the DNA samples obtained at the extraction stage.

In the real-time PCR, the amplified product is detected with the use of fluorescent dyes. These dyes are linked to oligonucleotide probes, which bind specifically to the amplified product during thermocycling. The real-time monitoring of fluorescence intensities during the real-time PCR allows the detection of accumulating product without re-opening the reaction tubes after the PCR run.

AmpliSens® *HPV* HCR genotype-titre-FRT PCR kit uses "hot-start", which greatly reduces the frequency of nonspecifically primed reactions. "Hot-start" is guaranteed by using chemically modified polymerase (TaqF). The chemically modified polymerase (TaqF) is activated by heating at 95 °C for 15 min.

Quantitative analysis of *HPV* HCR DNA is based on the linear dependence between the initial concentration logarithm of DNA target in a test sample and the cycle threshold (*Ct*) (the cycle of beginning of fluorescence signal exponential growth). For the quantitative analysis amplification of DNA from the test samples is carried out simultaneously with DNA-calibrators (samples with the known concentration of the DNA target). Based on the amplification results of DNA-calibrators a calibration line is plotted and it is used for the estimation of concentration of the DNA target in the test samples.

HPV DNA concentration is calculated as the relation between number of *HPV* copies and number of epithelial cells of human membrane mucosa.

The PCR kit contains the system for prevention of contamination by amplicons using the enzyme uracil-DNA-glycosylase (UDG) and deoxyuridine triphosphate (dUTP). The enzyme UDG recognizes and catalyzes the destruction of the DNA containing deoxyuridine, but has no effect on DNA containing deoxythymidine. Deoxyuridine is absent in the authentic DNA, but is always present in amplicons, because dUTP is a part of dNTP mixture in the reagents for the amplification. Due to the deoxyuridine containing contaminating amplicons are sensitive to the destruction by UDG before the DNA-target amplification. So the amplicons cannot be amplified.

The enzyme UDG is thermolabile. It is inactivated by heating at temperature above 50 °C. Therefore, UDG does not destroy the target amplicons which are accumulated during PCR.

HPV HCR genotype detection is carried out in four tubes. The results of amplification of different genotypes of *HPV* HCR DNA and IC Glob DNA for each PCR-mix are registered in the following fluorescence channels:

Table 1

Channel for fluorophore	FAM	JOE	ROX	Cy5
Name of PCR-mix-FL	**DNA-target**			
PCR-mix-FL *HPV* 1	DNA of *HPV* HCR genotype **16**	DNA of *HPV* HCR genotype **31**	DNA of *HPV* HCR genotype **18**	DNA fragment of β-globin gene (IC Glob)
PCR-mix-FL *HPV* 2	DNA of *HPV* HCR genotype **39**	DNA of *HPV* HCR genotype **45**	DNA of *HPV* HCR genotype **59**	DNA fragment of β-globin gene (IC Glob)
PCR-mix-FL *HPV* 3	DNA of *HPV* HCR genotype **33**	DNA of *HPV* HCR genotype **35**	DNA of *HPV* HCR genotype **68**	DNA of *HPV* HCR genotype **56**
PCR-mix-FL *HPV* 4	DNA of *HPV* HCR genotype **58**	DNA of *HPV* HCR genotype **52**	DNA of *HPV* HCR genotype **66**	DNA of *HPV* HCR genotype **51**
Name of PCR-mix-FL	**Target gene**			
PCR-mix-FL *HPV* 1	E6 gene	E6 gene	E7 gene	β-globin gene
PCR-mix-FL *HPV* 2	E7 gene	E6 gene	E6 gene	β-globin gene
PCR-mix-FL *HPV* 3	E6 gene	E6/E7 gene	E6 gene	E1 gene
PCR-mix-FL *HPV* 4	E6 gene	E7 gene	E6 gene	E7 gene

3. CONTENT

AmpliSens® *HPV* HCR genotype-titre-FRT PCR kit is produced in 2 forms:

variant FRT-100 FN, REF H-2261-1-13-CE;

variant FRT-100 FN in bulk[1], REF H-2261-1-13-CE-B.

Variant FRT-100 FN includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-FL *HPV* 1	clear liquid from colorless to blue grey colour	1.2	1 tube
PCR-mix-FL *HPV* 2	clear liquid from colorless to blue grey colour	1.2	1 tube
PCR-mix-FL *HPV* 3	clear liquid from colorless to blue grey colour	1.2	1 tube
PCR-mix-FL *HPV* 4	clear liquid from colorless to blue grey colour	1.2	1 tube
PCR-buffer-H	colorless clear liquid	0.6	4 tubes
C1 *HPV* genotype	colorless clear liquid	0.2	4 tubes
C2 *HPV* genotype	colorless clear liquid	0.2	4 tubes
Negative Control (C–)*	colorless clear liquid	1.2	2 tubes

* must be used in the extraction procedure as Negative Control of Extraction (see **DNA-sorb-AM**, **AmpliSens® MAGNO-sorb-URO**, **AmpliSens® DNA-sorb-D** protocols)

Variant FRT-100 FN is intended for 440 amplification reactions (110 tests), including controls.

AmpliSens® *HPV* HCR genotype-titre software (version 1.0) for automated data processing and Operator manual.

4. ADDITIONAL REQUIREMENTS

For sampling and pretreatment

- Transport medium.
- Cervical sampler.
- Endocervical brush.
- Gynaecological combined probe.
- Disposable tightly closed polypropylene 5.0-ml tubes for sampling.
- Disposable tightly closed polypropylene 1.5 and 5-ml tubes for pretreatment
- Sterile pipette tips (up to 200 µl) and pipette tips with filters (up to 1000 µl).
- Tube racks.
- Desktop centrifuge up to 12,000 g (suitable for Eppendorf tubes).
- Vacuum aspirator with flask for removing supernatant.

For DNA extraction and amplification

- DNA extraction kit.
- Pipettes (adjustable).
- Sterile pipette tips with filters (up to 100 µl).
- Vortex mixer.
- PCR box.
- Real-time instruments (for example, Rotor-Gene Q (QIAGEN, Germany); CFX96 (Bio-Rad, USA)).
- Disposable polypropylene tubes:
  a) screwed or tightly closed 1.5-ml tubes for reaction mixture preparation.
  b) thin-walled 0.2-ml PCR tubes with optical transparent domed or flat caps or strips of eight 0.2-ml tubes with optical transparent caps if a plate-type instrument is used;
  c) thin-walled 0.2-ml PCR tubes with flat caps or strips of four 0.1-ml Rotor-Gene PCR tubes if a rotor-type instrument is used.
- Refrigerator for 2–8 °C.
- Deep-freezer with the range from minus 24 to minus 16 °C.
- Reservoir for used tips.
- Disposable powder-free gloves and a laboratory coat.

[1] In bulk form contains unlabeled tubes. Tubes with identical reagent are packed in one bag with label.

5. GENERAL PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol filters and use a new tip for every procedure.
- Store all extracted positive material (specimens, controls and amplicons) away from all other reagents and add it to the reaction mix in a distantly separated facility.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable protective gloves and laboratory cloths, and protect eyes while samples and reagents handling. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use the PCR kit if the internal packaging was damaged or its appearance was changed.
- Do not use the PCR kit if the transportation and storage conditions according to the Instruction Manual were not observed.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local regulations.
- Samples should be considered potentially infectious and handled in biological cabinet in compliance with appropriate biosafety practices.
- Clean and disinfect all samples or reagents spills using a disinfectant, such as 0.5 % sodium hypochlorite or another suitable disinfectant.
- Avoid inhalation of vapors, samples and reagents contact with the skin, eyes, and mucous membranes. Harmful if swallowed. If these solutions come into contact, rinse the injured area immediately with water and seek medical advice if necessary.
- While observing the conditions of transportation, operation and storage, there are no risks of explosion and ignition.
- Safety Data Sheets (SDS) are available on request.
- The PCR kit is intended for single use for PCR analysis of specified number of samples (see the section “Content”).
- The PCR kit is ready for use in accordance with the Instruction Manual. Use the PCR kit strictly for intended purpose.
- Use of this product should be limited to personnel trained in DNA amplification techniques.
- Workflow in the laboratory must be one-directional, beginning in the Extraction Area and moving to the Amplification and Detection Area. Do not return samples, equipment and reagents in the area where the previous step was performed.

⚠ Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

6. SAMPLING AND HANDLING

AmpliSens® *HPV* HCR genotype-titre-FRT PCR kit is intended for analysis of the DNA extracted with DNA extraction kits from the biological material (vaginal swab, epithelial scrape from the cervical mucous membrane).

Sampling

Vaginal swab. The material should be obtained from the posterolateral vaginal vault by the cotton swab or combined probe into a tube with transport medium (**Transport Medium with Mucolytic Agent**, or **Transport Medium for Swabs** or PreservCyt (Hologic Inc., USA)). Turn the swab while rubbing it against the surface of the lateral vaginal wall. Collect as much of the material as possible by the swab. The minimal presence of impurities such as mucus and blood is allowed. Transfer the swab into a tube with the transport medium. Break off the lower part of the swab and leave it in the tube with transport medium. In case of impossibility of breaking off the lower part of the swab, the biological material should be washed into the tube with the transport media as much as possible. To do this, press the swab to the interior wall of the tube and rotate it 5-10 times in clockwise and counterclockwise order. The use of pair of scissors is unallowable for cutting-off the lower part of the swab!

Tightly cap the tube avoiding an airspace formation and deformation of the interior part of the cap. Mark the tube. If the **Transport Medium with Mucolytic Agent** is used its color can be changed due to the change of pH (then the discharge is acidic).

Store and transport the biological material transferred in the transport medium according to the requirements specified in the instruction manual for the used transport medium. Only one freeze-thawing cycle is allowed.

Epithelial scrape from the cervical mucous membrane. Allow access to the cervix using disposable or nondisposable sterile gynecological speculum. Carry out the sampling using the cervical brush or gynecological combined probe into the tube with transport medium. Before the specimen collection remove the mucous and vaginal discharge from cervix surface by a gauze tampon. The minimal presence of impurities such as mucus and blood is allowed.

Methods of epithelial scraping:

Method 1. One or two cervical cytobrushes and a tube with 0.5 ml of **Transport Medium with Mucolytic Agent** are used. Place the cervical epithelial scrape (endocervix) taken with the first cervical cytobrush and/or the superficial cervical scrape (ectocervix) taken with the second cervical cytobrush to the tube with transport medium.

Method 2. The DNAPAP Cervical Sampler (QIAGEN, Germany) is used. It consists of a cervical cytobrush and a tube with Digene transport medium. Place the cervical epithelial scrape (endocervix) to the tube with transport medium.

Method 3. The combined gynecological probe for simultaneously taking epithelium from endocervix and ectocervix is used. Place the cervical epithelial scrape (endocervix and ectocervix) to the 5-ml tube with previously added **Transport Medium with Mucolytic Agent**.

Method 4. Combined gynecological probe for simultaneously taking epithelial samples from endocervix and ectocervix and a liquid-based cytology vial with PreservCyt transport medium (Hologic Inc., USA) is used. Place the cervical epithelial scrape (endocervix and ectocervix) to the tube with transport medium.

Break off the lower part of the swab and leave it in the tube with transport medium. In case of impossibility of breaking off the lower part of the swab, the biological material should be washed into the tube with the transport media as much as possible. To do this, press the swab to the interior wall of the tube and rotate it 5-10 times in clockwise and contraclockwise order. The use of pair of scissors is unallowable for cutting-off the lower part of the swab!

Tightly cap the tube avoiding an airspace formation and deformation of the interior part of the cap. Mark the tube.

Store and transport the biological material transferred in the transport medium according to the requirements specified in the instruction manual for the used transport medium. Only one freeze-thawing cycle is allowed.

The biological samples can be stored before the PCR-analysis:

- at the temperature from 18 to 25 °C – no more than 7 days;
- at the temperature from 2 to 8 °C – no more than 3 months;
- at the temperature from minus 24 to minus 16 °C – for 1 year. Only one freeze-thawing cycle is allowed;
- in the transport medium for liquid-based cytology at the temperature from 18 to 25 °C – for 1 year.

Pretreatment

The pretreatment is not required for the vaginal swabs and cervical scrapes, collected into Transport Medium with Mucolytic Agent or Digene transport medium.

The pretreatment is required for the cervical scrapes, collected into the transport medium for liquid-based cytology (epithelial cells concentrating).

NOTE: Take an aliquot of cells for the PCR-analysis using only disposable filter tips and disposable tube. It is important to take first an aliquot of cells for the PCR-analysis and then for the liquid-based cytology.

Epithelial cells concentrating

Method 1

1. Take the required number of disposable 5-ml tubes equal to the number of test sample. Mark the tubes. Shake intensively each vial with the sample for liquid-based cytology for cells disintegration. Then gently open and transfer 2-5 ml of cells (depending on density of cells suspension) into the prepared tubes.
2. Leave the tubes in the rack at the temperature from 18 to 25 °C for the night for cells sedimentation and centrifuge on microcentrifuge for **10 min** at **600 g** (for example, **3,000 rpm** for microcentrifuge MiniSpin, Eppendorf Manufacturing Corporation).
3. Remove the supernatant from each tube. Do not disturb the cell pellet. Use a new one 1000-µl filter tip for each sample and pipette.
4. Transfer gently the rest of the cell pellet with supernatant (~1 ml) into a new one 1.5-ml tube using a new one filter tip for each sample. Mark the tubes and centrifuge at **10,000 g** (for example, **12,000 rpm** for microcentrifuge MiniSpin, Eppendorf Manufacturing Corporation) for **2 min**.
5. Remove the supernatant from each tube. Do not disturb the cell pellet. Use a new one 200-µl filter tip for each sample and vacuum aspirator. Leave **100-200 µl of pellet** with supernatant.

Method 2

1. Shake intensively each vial with the sample for liquid-based cytology for cells disintegration and leave for the night for cells sedimentation.
2. Transfer 0.5-1.0 µl of cells from the bottom of the vial into a new one 1.5-ml tube using 1000-µl tip and pipette. Mark the tube.
3. Centrifuge at **10,000 g** (for example, **12,000 rpm** for microcentrifuge MiniSpin, Eppendorf Manufacturing Corporation) for **2 min**.
4. Remove the supernatant from each tube. Do not disturb the cell pellet. Use a new one 200-µl filter tip for each sample and vacuum aspirator. Leave **100-200 µl of pellet** with supernatant.

Interfering substances and limitations of using test material samples

The excessive amount of impurities in biological material such as mucus, blood and pus can lead to the amplification reaction inhibition.

7. WORKING CONDITIONS

AmpliSens® *HPV* HCR genotype-titre-FRT PCR kit should be used at 18–25 °C.

8. PROTOCOL

8.1. DNA extraction

It is recommended to use the following nucleic acid extraction kits:

- **AmpliSens® MAGNO-sorb-URO** for DNA extraction from vaginal swabs and cervical scrapes obtained by the 1st, 2nd and 3rd methods.
- **AmpliSens® DNA-sorb-D** for DNA extraction from cervical scrapes obtained by the 4th method (samples for liquid-based cytology).
- **DNA-sorb-AM** for DNA extraction from vaginal swabs and cervical scrapes obtained by the 1st, 2nd and 3rd methods.

In the extraction procedure it is necessary to carry out the control reaction as follows:

C– – Add **100 µl of Negative Control (C–)** to the tube labelled C– (Negative control of Extraction).

NOTE: Extract DNA according to the manufacturer’s protocol.

NOTE: The volume of the test sample is **100 µl.**
The volume of elution is **100 µl**.

NOTE: **If using AmpliSens® MAGNO-sorb-URO and DNA-sorb-AM kits**, addition of **Internal Control-FL (IC)** is not required.

8.2. Preparing PCR

8.2.1 Preparing tubes for PCR

The total reaction volume is **25 µl**, the volume of the **DNA** sample is **10 µl**.

The type of tubes depends on the PCR instrument used for analysis. Use disposable filter tips for adding reagents, DNA and control samples into tubes.

NOTE: DNA amplification of each biological sample is carried out in 4 tubes with different PCR-mixes-FL *HPV*.

1. Calculate the volume of each reagent required for preparation of 4 reaction mixtures. **10 µl** of **PCR-mix-FL *HPV*** and **5 µl** of **PCR-buffer-H** are required for 1 reaction. Prepare the mixture for the total number of test and control samples (see item 7 for the number of control samples) plus extra volume for several reactions.

NOTE: Components of the reaction mixture should be mixed just before the PCR analysis.

2. Thaw the tube with **PCR-mix-FL *HPV* 1, PCR-mix-FL *HPV* 2, PCR-mix-FL *HPV* 3, PCR-mix-FL *HPV* 4.** Vortex the tubes with all reagents of the kit and then sediment the drops on vortex.
3. To prepare the reaction mixtures with each PCR-mix-FL *HPV* in four tubes. Mix the required quantity of **PCR-mix-FL *HPV*** and **PCR-buffer-H.** Sediment the drops on vortex.

NOTE: The reaction mixtures are to be used within 2 hours after preparation.

4. Take the required number of tubes or strips for amplification of the DNA obtained from test and control samples.

For carrying out the study of N biological samples in **rotor-type** instruments:

a) **in qualitative format** prepare next number of strips (strips of four tubes): **N** (equal to N biological samples) **strips** for test samples + **2 strips** for control samples (4 tubes for the Positive Control of amplification (C+) and 4 tubes for the Negative Control of extraction (C–)). For example, 17 strips are needed for the study of 15 biological samples.

b) **in quantitative format** prepare next number of strips (strips of four tubes): **N** (equal to N biological samples) **strips** for test samples + **3 strips** for control samples (8 tubes for DNA-calibrators C1, C2 and 4 tubes for the Negative Control of extraction (C–)). For example, 18 strips are needed for the study of 15 biological samples.

For carrying out the study of N biological samples in **plate-type** instruments:

a) **in qualitative format** prepare next number of strips (strips of eight tubes) / tubes: **N/2** (1/2 of strip is needed for one biological sample) **strips / N*4** (4 tubes are needed for one biological sample) **tubes** for test samples + **1 strip / 8 tubes** for control samples (4 tubes for the Positive Control of amplification (C+) and 4 tubes for the Negative Control of extraction (C–)). For example, 11.5 strips / 92 tubes are needed for the study of 21 biological samples.

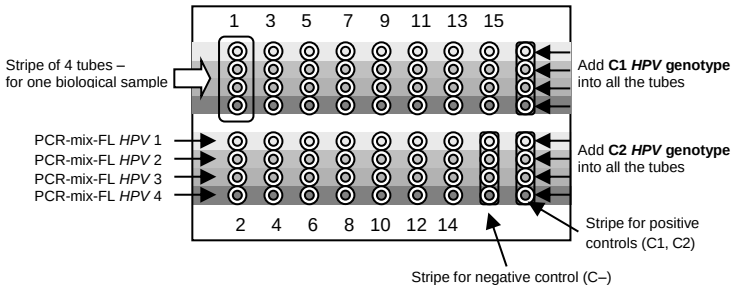
b) **in quantitative format** prepare next number of strips (strips of eight tubes):

N/2 (1/2 of strip is needed for one biological sample) **strips / N*4** (4 tubes are needed for one biological sample) **tubes** for test samples + **1.5 strip / 12 tubes** for control samples (8 tubes for DNA-calibrators C1, C2 and 4 tubes for the Negative Control of extraction (C–)). For example, 12 strips / 96 tubes are needed for the study of 21 biological samples.

5. Transfer **15 µl** of each prepared mixture into corresponding tubes (see schemes below). Utilize the rest of reaction mixture.

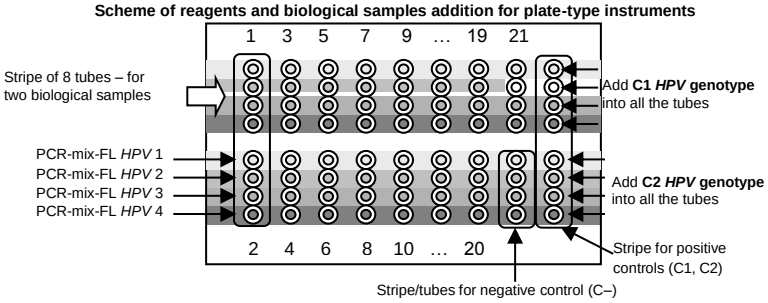
NOTE: For adequate data processing by the software enclosed to PCR kit it is necessary to strictly adhere the following schemes of reaction mixtures and DNA samples addition for the rotor-type and plate-type instruments.

Scheme of reagents and biological samples addition for rotor-type instruments



The numbers of biological samples are specified.

Scheme of reagents and biological samples addition for plate-type instruments



The numbers of biological samples are specified.

6. Add **10 µl** of DNA samples obtained at the DNA extraction stage from test samples into 4 prepared tubes with different reaction mixtures.

NOTE: It is necessary to obey the schemes of samples addition for the rotor-type and plate-type instruments.

NOTE: Avoid transferring sorbent beads together with the DNA sample.

7. Carry out the control amplification reactions:
   For qualitative analysis:

C+ – Add **10 µl** of **C2 *HPV* genotype** into four tubes with different PCR-mixes-FL and labeled C+ (Positive Control of Amplification).

C– – Add **10 µl** of **the sample extracted from the Negative Control (C–) reagent** into four tubes with different PCR-mixes-FL and labeled C– (Negative control of Extraction).

For quantitative analysis:

C1 – Add **10 µl** of **C1 *HPV* genotype** into four tubes with different PCR-mixes-FL and labeled C1 (DNA-calibrator C1).

C2 – Add **10 µl** of **C2 *HPV* genotype** into four tubes with different PCR-mixes-FL and labeled C2 (DNA-calibrator C2).

C– – Add **10 µl** of **the sample extracted from the Negative Control (C–) reagent** into four tubes with different PCR-mixes-FL and labeled C– (Negative control of Extraction).

8.2.2. Amplification

1. Create a temperature profile on your instrument as follows (Table 2, 3)[2]:

Table 2

AmpliSens unified amplification program for rotor-type[3] and plate type[4] instruments

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	50	15 min	–	1
2	95	15 min	–	1
3	95	10 s	–	45
	60	20 s	**FAM, JOE, ROX, Cy5**	

NOTE: Any combination of the tests (including tests with reverse transcription and amplification) can be performed in one instrument simultaneously with the use of the unified amplification program. If several tests in "multiprime" format are carried out simultaneously, the detection is enabled in other used channels except for the specified ones. If in one instrument only the tests for the pathogen DNA detection are carried out simultaneously, the first step of reverse transcription (50 °C – 15 min) can be omitted for time saving.

NOTE: It is recommended not to combine several tests in one run for rotor-type instruments

Table 3

AmpliSens 1 amplification program

Step	Rotor-type instruments[2]			Plate-type instruments[3]		
	Temperature, °C	Time	Cycles	Temperature, °C	Time	Cycles
1	**95**	15 min	1	**95**	15 min	1
2	**95**	5 s	5	**95**	5 s	5
	60	20 s		**60**	20 s	
	72	15 s		**72**	15 s	
3	**95**	5 s	40	**95**	5 s	40
	60	20 s Fluorescence acquiring		**60**	30 s Fluorescence acquiring	
	72	15 s		**72**	15 s	

Fluorescent signal is detected in the channels for the **FAM**, **JOE**, **ROX** and **Cy5** fluorophores.

NOTE: **AmpliSens-1** is an universal program for conducting tests for identifying *human papillomaviruses (HPV* HCR) and detection of STIs and other infections of reproductive system with AmpliSens PCR kits. Therefore, any combination of these tests can be carried out simultaneously in the same instrument.

NOTE: It is recommended not to combine several tests in one run for rotor-type instruments

1. Adjust the fluorescence channel sensitivity according to the *Important Product Information Bulletin*.
2. Insert tubes into the reaction module of the instrument.

NOTE: It is recommended to sediment drops from walls of tubes by short centrifugation (1–3 s) before placing them into the instrument.
Insert empty tubes at the edges of reaction module in case of incomplete filling of plate-type instrument.

3. Run the amplification program with fluorescence detection.
4. Analyze results after the amplification program is completed.

9. DATA ANALYSIS

Analysis of results is performed by the software of the real-time PCR instrument used by measuring fluorescence signal accumulation in four channels:

Table 4

Channel for the fluorophore	FAM	JOE	ROX	Cy5
Name of PCR-mix-FL	Signal registration, indicating the amplification product accumulation			
PCR-mix-FL *HPV* 1	DNA of *HPV* HCR genotype **16**	DNA of *HPV* HCR genotype **31**	DNA of *HPV* HCR genotype **18**	DNA of IC Glob
PCR-mix-FL *HPV* 2	DNA of *HPV* HCR genotype **39**	DNA of *HPV* HCR genotype **45**	DNA of *HPV* HCR genotype **59**	DNA of IC Glob
PCR-mix-FL *HPV* 3	DNA of *HPV* HCR genotype **33**	DNA of *HPV* HCR genotype **35**	DNA of *HPV* HCR genotype **68**	DNA of *HPV* HCR genotype **56**
PCR-mix-FL *HPV* 4	DNA of *HPV* HCR genotype **58**	DNA of *HPV* HCR genotype **52**	DNA of *HPV* HCR genotype **66**	DNA of *HPV* HCR genotype **51**

Results are interpreted by the crossing (or not-crossing) the fluorescence curve with the threshold line set at the specific level that corresponds to the presence (or absence) of a *Ct* value of the DNA sample in the corresponding column of the results grid.

NOTE: Analysis of results is performed using the under mentioned algorithm or the **AmpliSens HPV HCR genotype-titre** software (version 1.0).
Working procedure for the **AmpliSens HPV HCR genotype-titre** software (version 1.0) is described in the operator's manual

Qualitative analysis

Principle of interpretation is the following:

– DNA of *HPV* HCR genotypes 16, 39, 33, 58 is **detected** if the *Ct* value determined in the channel for the FAM fluorophore does not exceed the boundary *Ct* value. Moreover, the fluorescence curve of the sample should cross the threshold line in the area of typical exponential growth of fluorescence.

– DNA of *HPV* HCR genotypes 31, 45, 35, 52 is **detected** if the *Ct* value determined in the channel for the JOE fluorophore does not exceed the boundary *Ct* value. Moreover, the fluorescence curve of the sample should cross the threshold line in the area of typical exponential growth of fluorescence.

– DNA of *HPV* HCR genotypes 18, 59, 68, 66 is **detected** if the *Ct* value determined in the channel for the ROX fluorophore does not exceed the boundary *Ct* value. Moreover, the fluorescence curve of the sample should cross the threshold line in the area of typical exponential growth of fluorescence.

– DNA of *HPV* HCR genotypes 56, 51 is **detected** if the *Ct* value determined in the channel for the Cy5 fluorophore (in the tubes with PCR-mix-FL *HPV* 3 and 4, respectively) does not exceed the boundary *Ct* value. Moreover, the fluorescence curve of the sample should cross the threshold line in the area of typical exponential growth of fluorescence.

– DNA of *HPV* HCR is **not detected** in a sample if the *Ct* value is not determined (fluorescence curve does not cross the threshold line or the *Ct* value exceed the boundary value) in the channels for FAM, JOE, ROX, and also Cy5 (in the tubes with PCR-mix-FL *HPV* 3 and 4) fluorophores, whereas the *Ct* value determined in the channel for the Cy5 fluorophore (in the tubes with PCR-mix-FL *HPV* 1 and 2) does not exceed the boundary value.

– The result is **invalid** if the *Ct* value is not determined (absent) or exceed the boundary value in the channel for FAM, JOE, ROX, and also Cy5 (in the tubes with PCR-mix-FL *HPV* 3 and 4) fluorophores, and the *Ct* value in the channel for the Cy5 fluorophore (in the tubes with PCR-mix-FL *HPV* 1 and 2) is not determined (absent). In such cases, the PCR analysis should be repeated starting from the DNA extraction stage.

NOTE: Boundary *Ct* values are specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

The result of qualitative analysis is considered reliable only if the results obtained for controls of amplification and extraction stages are correct (according to Table 5 and *Important Product Information Bulletin* enclosed to the PCR kit).

Table 5

Results for controls (qualitative analysis)

Control	Stage for control	*Ct* value in the channel for fluorophore			
		FAM	JOE	ROX	Cy5
C–	DNA extraction	Absent in all 4 tubes	Absent in all 4 tubes	Absent in all 4 tubes	Absent in all 4 tubes
C+	PCR	<boundary value in all 4 tubes	<boundary value in all 4 tubes	<boundary value in all 4 tubes	<boundary value in all 4 tubes

[2] The amplification programs (Tables 2, 3) are equivalent for the use.
[3] For example, Rotor-Gene Q (QIAGEN, Germany).
[4] For example, CFX 96 (Bio-Rad, USA).

Quantitative analysis

Based on the obtained *Ct* values and specified concentration values of DNA calibrators (C1 and C2) a calibration line is plotted and the number of copies of each detected genotype of *HPV* HCR as well as human DNA per 1ml of test and control samples is calculated. Obtained values are used for calculation of *HPV* DNA quantity per $1x10^5$ of human cells. Normalized concentration values reflect the number of copies of the pathogen relative to human cells. Moreover the concentration values of human DNA allows to estimate the quality of biological material sampling.

HPV DNA concentration values are calculated according to the formula:

$$\lg\left(\frac{\text{number of } \textit{HPV} \text{ DNA copies in 1 ml}}{\text{number of human DNA copies in 1 ml}} \times 2 * 10^5\right) = \lg(\textit{HPV} \text{ DNA copies per } 10^5 \text{ of human cells})$$

where:

2 is the recalculation coefficient of DNA copies number into cell number.

Normalized concentration values reflect the number of pathogen copies in relation to human cells. In addition, the number of human cells allows to evaluate the quality of biological material sampling.

The number of human cells is calculated as average number of human DNA copies per reaction obtained in the channel for Cy5 fluorophore (in tubes with PCR-mix-FL *HPV* 1 and 2) and divided by 2.

To determine the clinical relevance of the results of quantitative PCR-analysis, lg of total number of DNA copies of detected *HPV* genotypes per 10^5 human cells is to be calculated.

NOTE: Concentration values of DNA-calibrators are specified in the *Important Product Information Bulletin* enclosed to the PCR kit.

NOTE: For the subsequent runs with the given lot of the **AmpliSens® *HPV* HCR genotype-titre-FRT** PCR kit one can use the results of DNA calibrator **C1** obtained in the previous run on this instrument. For that purpose export the results of DNA calibrator **C1** using the software of the instrument. In this case it is necessary to carry out the reaction only with the DNA calibrator **C2.**

Table 6

Results Interpretation for the test samples (quantitative analysis)

Result	Interpretation
Invalid	The *Ct* value in the channel for Cy5 fluorophore in the tubes with PCR-mixes-FL *HPV* 1 and 2 (human DNA) is absent or determined greater than the boundary *Ct* value, while the *Ct* value is not determined (absent) or greater than the boundary *Ct* value in channels for FAM, JOE, ROX fluorophores, as well as Cy5 fluorophore (in the tubes with PCR-mixes-FL *HPV* 3 and 4). It is necessary to repeat the PCR analysis of this sample starting from DNA extraction stage. If IC Glob DNA is absent in the test sample, it is recommended to repeat biological material sampling and PCR-analysis
Insufficient amount of biological material	DNA concentration of IC Glob is less than $1x10^5$ copies/ml (500 cells/reaction) (the value obtained for sample in the channel for Cy5 fluorophore in the tubes with PCR-mixes-FL *HPV* 1 and 2). It is necessary to repeat the PCR analysis of this sample starting from DNA extraction stage. If the amount of biological material is insufficient, it is recommended to repeat biological material sampling and PCR-analysis
HPV HCR DNA is not detected	The *Ct* value for *HPV* HCR DNA is absent and the concentration of IC Glob is greater than $1x10^5$ copies/ml. The result is ***HPV* HCR DNA is not detected**
<3 lg (*HPV* per 10^5 human cells)	Clinically insignificant value
3–5 lg (*HPV* per 10^5 human cells)	Clinically significant value. Dysplasia cannot be excluded; risk of dysplasia development
>5 lg (*HPV* per 10^5 human cells)	Clinically significant, increased value. High probability of dysplasia

The result of qualitative analysis is considered reliable only if the results obtained for controls of amplification and extraction stages are correct (according to Table 7 and *Important Product Information Bulletin* enclosed to the PCR kit).

Table 7

Results for controls (quantitative analysis)

Control	Stage for control	*Ct* value in the channel for fluorophore			
		FAM	JOE	ROX	Cy5
C–	DNA extraction	Absent in all 4 tubes	Absent in all 4 tubes	Absent in all 4 tubes	Absent in all 4 tubes
C1	PCR	**Defined**	**Defined**	**Defined**	**Defined**
C2	PCR	**Defined**	**Defined**	**Defined**	**Defined**

10. TROUBLESHOOTING

Results of analysis are not taken into account in the following cases:

1. For qualitative analysis, the *Ct* value determined for the Positive Control of amplification (C+) in any of the channels is greater than the boundary *Ct* value or absent (see Table 5). The amplification and detection should be repeated for all the samples.
2. The *Ct* value is determined for the Negative Control of Extraction (C–) in channels for FAM and/or JOE and/or ROX and/or Cy5 fluorophores. The contamination of laboratory with amplification fragments or contamination of reagents, test samples is probable at any stage of PCR analysis. Measures for detecting and elimination of contamination source must be taken. The PCR analysis (beginning with the DNA extraction stage) should be repeated for all samples in which specific DNA was detected.
3. For quantitative analysis, the *Ct* value is absent for DNA-calibrators C1 and C2 in any specified channels for the fluorophores (see Table 7). The amplification and detection should be repeated for all the samples.
4. For quantitative analysis, the efficiency E is less than 80 % or greater than 120 % when plotting the calibration curve. Check the correctness of set concentrations of DNA-calibrators in accordance with the bulletin and the correctness of selected level of the threshold line. If set concentrations of DNA-calibrators and the threshold line level are correct but the efficiency does not fit in the required range, then the amplification and detection for all the samples should be repeated.
5. The *Ct* value is determined for the test sample, whereas the area of typical exponential growth of fluorescence is absent (the graphic looks like approximate straight line). It is necessary to check the correctness of selected threshold line level or parameters of base line calculation. If the result has been obtained with the correct level of threshold line (base line), the amplification and detection should be repeated for this sample.

11. TRANSPORTATION

AmpliSens® *HPV* HCR genotype-titre-FRT PCR kit should be transported at 2–8 °C for no longer than 5 days. Transportation at 2-25 °C for no longer than 3 days is allowed.

12. STABILITY AND STORAGE

All components of the **AmpliSens® *HPV* HCR genotype-titre-FRT** PCR kit are to be stored at the temperature from minus 24 to minus 16 °C when not in use. All components of the **AmpliSens® *HPV* HCR genotype-titre-FRT** PCR kit are stable until the expiry date stated on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

NOTE: PCR-mixes-FL *HPV* are to be kept away from light.

13. SPECIFICATIONS

13.1. Analytical sensitivity and linear range

Table 8

Test material	Transport medium	Nucleic acid extraction kit	PCR kit	Micro-organism	Analytical sensitivity, copies/ml[5]	Linear measure-ment range, copies/ml
Vaginal swab, scrape from the cervical mucous membrane (ectocervix and endocervix)	Transport Medium with Mucolytic Agent, Transport Medium for Swabs, Digene transport medium	AmpliSens® MAGNO-sorb-URO. DNA-sorb-AM	variant FRT-100 FN	*HPV* HCR genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	$1x10^3$	$3x10^3$ – $1x10^8$
Scrape from the cervical mucous membrane (ectocervix and endocervix)	Transport medium for liquid-based cytology	AmpliSens® DNA-sorb-D	variant FRT-100 FN	*HPV* HCR genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	$1x10^3$	$3x10^3$ – $1x10^8$

The claimed features are achieved while respecting the rules specified in the section *Sampling and Handling*

13.2. Analytical specificity

The analytical specificity of **AmpliSens® *HPV* HCR genotype-titre-FRT** PCR kit is ensured by the selection of specific primers and probes as well as stringent reaction conditions. The primers and probes have been checked for possible homologies to all sequences published in gene banks by sequence comparison analysis.

The PCR kit detects the DNA fragments of the claimed genotypes of *HPV* HCR. Analytical specificity was proved in studies of DNA of the following microorganisms: *Neisseria gonorrhoeae, Chlamydia trachomatis, Gardnerella vaginalis, Mycoplasma genitalium, Trichomonas vaginalis, Atopobium vaginae, Mycoplasma hominis, Ureaplasma parvum, Streptococcus agalactiae, Streptococcus pyogenes, Candida* spp., *CMV (cytomegalovirus)*, *EBV* (*Epstein-Barr virus*), *VZV (varicella-zoster virus)*, *HSV* I, *HSV* II (*herpes simplex virus* types 1 and 2), *Human herpesvirus 6* (*herpes virus* type 6), DNA of *HPV* relative to the types of low and uncertain risk, particularly, genotypes 6, 11, 67, 70, 84, 81, 82, 62, 72, 73.

While testing the above mentioned microorganisms DNA nonspecific reactions were absent as well as cross-reactions between *HPV* genotypes (when high concentrated samples are used). The specificity of testing was proved by sequencing of amplification fragments being detected.

The information about known interfering substances is specified in the *Interfering substances and limitations of using test material samples*.

13.3. Reproducibility, repeatability and trueness

Reproducibility and repeatability were determined by testing model samples of biological material. Model samples of biological material were prepared by dilution of quality control sample (QCS) containing *HPV* HCR DNA (genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) with three concentration ranges (from $5x10^3$ to $1x10^4$, from $5x10^4$ to $1x10^5$, greater than $5x10^5$ copies/ml) in the biological material which is not contain the *HPV* DNA of any other genotypes and DNA of any other pathogen agents.

Table 9

Reproducibility

HPV genotype	Initial concentration value, copies/ml	Number of repeats	Average concentration value, lg	Standard deviation (SD)	Coefficient of variation (CV), %
16	from $5x10^3$ to $1x10^4$	8	3.56	0.03	0.82
18		8	3.76	0.05	1.20
31		8	3.70	0.08	2.19
33		8	3.91	0.06	1.57
35		8	4.03	0.06	1.45
39		8	3.59	0.10	2.77
45		8	3.78	0.08	2.23
51		8	3.70	0.07	1.78
52		8	3.75	0.11	2.89
56		8	3.82	0.07	1.91
58		8	3.76	0.06	1.52
59		8	3.88	0.05	1.25
66		8	3.70	0.11	2.93
68		8	3.72	0.09	2.34
16	from $5x10^4$ to $1x10^5$	8	5.11	0.07	1.39
18		8	4.89	0.07	1.47
31		8	4.92	0.08	1.71
33		8	4.73	0.10	2.21
35		8	4.57	0.10	2.28
39		8	4.68	0.07	1.55
45		8	4.58	0.10	2.11
51		8	5.06	0.05	0.97
52		8	4.56	0.09	1.99
56		8	4.66	0.17	3.57
58		8	4.62	0.08	1.76
59		8	4.55	0.09	2.06
66		8	4.73	0.07	1.41
68		8	4.89	0.07	1.48

[5] Number of copies of virus DNA in the biological material placed in the specified transport medium and calculated per 1 ml.

HPV genotype	Initial concentration value, copies/ml	Number of repeats	Average concentration value, lg	Standard deviation (SD)	Coefficient of variation (CV), %
16	greater than $5x10^5$	8	7.41	0.08	1.14
18		8	7.37	0.03	0.41
31		8	7.39	0.08	1.02
33		8	7.28	0.06	0.80
35		8	7.16	0.05	0.68
39		8	7.25	0.07	0.92
45		8	7.20	0.06	0.80
51		8	7.29	0.08	1.12
52		8	7.09	0.08	1.09
56		8	7.31	0.06	0.76
58		8	7.24	0.06	0.86
59		8	7.25	0.04	0.53
66		8	7.24	0.05	0.71
68		8	7.28	0.05	0.66

Table 10

Repeatability

HPV genotype	Initial concentration value, copies/ml	Total number of repeats	Average concentration value, lg	Standard deviation (SD)	Coefficient of variation (CV), %
16	from $5x10^3$ to $1x10^4$	16	3.62	0.09	2.43
18		16	3.85	0.11	2.93
31		16	3.74	0.07	1.96
33		16	3.91	0.05	1.34
35		16	4.03	0.06	1.55
39		16	3.62	0.09	2.45
45		16	3.74	0.08	2.23
51		16	3.68	0.09	2.56
52		16	3.77	0.08	2.19
56		16	3.80	0.08	2.07
58		16	3.79	0.05	1.42
59		16	3.84	0.08	2.12
66		16	3.69	0.08	2.30
68		16	3.76	0.10	2.67
16	from $5x10^4$ to $1x10^5$	16	5.22	0.13	2.45
18		16	5.12	0.25	4.81
31		16	5.20	0.30	5.71
33		16	4.90	0.20	4.02
35		16	4.70	0.17	3.54
39		16	4.84	0.18	3.63
45		16	4.71	0.16	3.36
51		16	5.07	0.09	1.77
52		16	4.59	0.11	2.47
56		16	4.73	0.17	3.50
58		16	4.69	0.15	3.10
59		16	4.68	0.16	3.42
66		16	4.85	0.15	3.07
68		16	4.94	0.10	2.07
16	greater than $5x10^5$	16	7.47	0.09	1.24
18		16	7.44	0.09	1.21
31		16	7.50	0.13	1.78
33		16	7.30	0.07	0.92
35		16	7.15	0.05	0.64
39		16	7.31	0.08	1.11
45		16	7.16	0.07	0.97
51		16	7.26	0.09	1.28
52		16	7.08	0.07	1.04
56		16	7.34	0.06	0.78
58		16	7.26	0.10	1.32
59		16	7.03	0.06	0.88
66		16	6.92	0.06	0.94
68		16	7.35	0.04	0.58

The trueness was determined by testing the QCS with the concentration $1x10^5$ copies/ml.

Table 11

Trueness

HPV genotype	Number of repeats	Average value of measurement, lg	Specified value, lg	Bias (B), %
16	32	5.20	5.00	4.00
18	32	5.15	5.00	3.00
31	32	5.09	5.00	1.80
33	32	4.92	5.00	1.60
35	32	4.72	5.00	5.60
39	32	4.87	5.00	2.60
45	32	4.72	5.00	5.60
51	32	5.05	5.00	1.00
52	32	4.70	5.00	6.00
56	32	4.79	5.00	4.20
58	32	4.81	5.00	3.80
59	32	4.80	5.00	4.00
66	32	4.69	5.00	6.20
68	32	4.98	5.00	0.40

14. REFERENCES

1. Doorbar J., Quint W., Bank L., Bravo I.G., Stoler M., Broker T.R., Stanley M.A. The Biology and Life-Cycle of Human Papillomaviruses//Vaccine. 2012. 30S. F55– F70.
2. De Sanjose S. et al. Human papillomavirus genotype attribution in invasive cervical cancer: a retrospective cross-sectional worldwide study//Lancet Oncol. 2010. V11. P.1048–56.

15. QUALITY CONTROL

In compliance with Federal Budget Institute of Science "Central Research Institute for Epidemiology" ISO 13485-Certified Quality Management System, each lot of the **AmpliSens® *HPV* HCR genotype-titre-FRT** PCR kit has been tested against predetermined specifications to ensure consistent product quality.

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
13.07.23 EM	3. Content Footer	REF H-2261-1-13-CE was added

AmpliSens®

Federal Budget Institute of Science "Central Research Institute for Epidemiology"
3A Novogireevskaya Street
Moscow 111123 Russia