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

CE IVD

For Professional Use Only

Instruction Manual

KEY TO SYMBOLS USED

REF	Catalogue number	⚠	Caution
LOT	Batch code	Σ	Contains sufficient for <n> tests
IVD	*In vitro* diagnostic medical device		Use-by Date
VER	Version		Consult instructions for use
	Temperature limit		Keep away from sunlight
	Manufacturer	**NCA**	Negative control of amplification
	Date of manufacture	**C–**	Negative control of extraction
EC REP	Authorized representative in the European Community	**C1, C2, C3**	DNA-calibrators
		IC	Internal control

1. INTENDED USE

AmpliSens® *HPV* HCR screen-titre-FRT PCR kit is an *in vitro* nucleic acid amplification test for qualitative and quantitative detection of high carcinogenic risk (HCR) *human papillomaviruses* (*HPV*) types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 DNA in the biological material (cervical and urethral scrapes) using real-time hybridization-fluorescence detection of amplified products.

AmpliSens® *HPV* HCR screen-titre-FRT PCR kit is able to detect (without genotyping) DNA of *HPV* of two main phylogenetic groups, A7 and A9, which include 10 types (16, 18, 31, 33, 35, 39, 45, 52, 58, and 59), as well as DNA of *HPV* type 51 (group A5) and *HPV* type 56 (group A6). These types have a high transforming activity and are the cause of more than 94 % of high-grade cervical dysplasia and cervical cancer.

AmpliSens® *HPV* HCR screen-titre-FRT PCR kit is adapted for two-channel devices, for example, Rotor-Gene 3000/6000 (Corbett Research, Australia), iCycler iQ (Bio-Rad, USA) with filters for FAM and JOE channels, SmartCycler II (Cepheid, USA), or for four-channel devices, for example, Rotor-Gene 3000/6000 (Corbett Research, Australia), Mx3000P (Stratagene, USA), iCycler iQ5 (Bio-Rad, USA).

NOTE: The results of PCR analysis are taken into account in complex diagnostics of disease.

2. PRINCIPLE OF PCR DETECTION

High carcinogenic risk (HCR) *human papillomaviruses* (*HPV*) types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 DNA detection by the polymerase chain reaction (PCR) is based on the amplification of the pathogen genome specific region using specific primers. 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.

The method is based on simultaneous amplification (multiplex PCR) and real-time detection of DNA fragments of *HPV* genes E1-E2 and a DNA fragment of β-globin gene, which is used as an endogenous internal control. PCR analysis for the presence of DNA of 12 *HPV* types is carried out in two tubes (PCR kit variant screen-titre-FRT 2x) or in a single tube (PCR kit variant screen-titre-FRT 4x).

In case of using the two-channel PCR instruments, the result of *HPV* DNA amplification is detected in the channel for the JOE fluorophore. The genotypes belonging to phylogenetic group A9 (16, 31, 33, 35, 52, and 58) are detected in one tube, whereas the genotypes belonging to phylogenetic group A7 (18, 39, 45, and 59) as well as genotypes 51 and 56 are detected in the other tube.

In case of using the four-channel PCR instruments, the result of amplification of DNA of each *HPV* phylogenetic group is detected in separate fluorescent channels (group A9 *HPV*, in the channel for the JOE fluorophore; group A7 *HPV*, in the channel for the ROX fluorophore; and *HPV* types 51 and 56, in the channel for the Cy5 fluorophore).

NOTE: Detection of phylogenetic groups in different tubes is not considered to be a virus genotyping because each group consists of different *HPV*-genotypes.

The result of Internal Control amplification is detected in the channel for the FAM fluorophore. The DNA-target selected as an endogenous internal control is the fragment of human genome and must be present in a sample (cervical scrape) in sufficient quantity equivalent to that of cells in the sample (10^3-10^5 human DNA copies or more than 500 cells). Therefore, the use of an endogenous internal control makes it possible not only to monitor test stages (DNA extraction and amplification) but also to assess the adequacy of sampling and storage of biological material. In case of incorrect sampling of epithelial scrape (insufficient amount of epithelial cells), amplification signal of β-globin gene will be too low.

Quantitative analysis of *HPV* DNA is based on the linear dependence between the cycle threshold (*Ct*) and the initial concentration of *HPV* DNA. Quantitative analysis is performed in the presence of DNA calibrators (samples with a known concentration of *HPV* DNA). Based on the amplification results of DNA-calibrators a calibration line is plotted and it is used for the estimation of concentration of the *HPV* DNA in the test samples. To minimize the effect of variation during material sampling, the quantitative results are normalized to the genomic DNA quantity (*HPV* DNA concentrations per epithelial cells quantity).

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

Quantitative approach to the diagnostics of papillomaviruses infection

It is known that the *HPV* DNA load correlates with the severity and prognosis of *HPV* infection. In case of correct and uniform sampling of the biological material, the viral load less than 10^5 DNA copies of *HPV* HCR in a scrape (Snijders, 2003) or 10^3 DNA copies per 10^5 of human cells is considered *clinically insignificant*, because it does not occur normally at high grade dysplasia and cervical cancer and it is associated with the minimal risk of their development. In case of proved fact of persistent infection (*HPV* is detected more than 1 year) the quantity of the virus greater than 10^5 DNA copies per 10^5 of human cells is considered *increased HPV load* and associated with an increased risk of high grade dysplasia development, it is more frequent in cervical cancer. Finally, the viral load monitoring provides some information. It has been argued that the reduction of *HPV* DNA quantity more than ten times can be a marker of transient infection. The viral load increase after 3, 6 and 9 months after performed treatment testify about the chance of a recurrence.

The results of amplification are registered in the following fluorescence channels:

Table 1

Channel for fluorophore	FAM	JOE	ROX	Cy5
PCR-mix-1-FRT	**DNA-target**			
HPV A9	DNA fragment of β-globin gene (IC Glob)	*HPV* HCR DNA (16, 31, 33, 35, 52, 58 genotypes)	—	—
HPV A7+	DNA fragment of β-globin gene (IC Glob)	*HPV* HCR DNA (18, 39, 45, 51, 56, 59 genotypes)	—	—
HPV screen-titre	DNA fragment of β-globin gene (IC Glob)	*HPV* HCR DNA (16, 31, 33, 35, 52, 58 genotypes)	*HPV* HCR DNA (18, 39, 45, 59 genotypes)	*HPV* HCR DNA (51, 56 genotypes)
PCR-mix-1-FRT	**Target gene**			
HPV A9	β–globin gene	E1 gene (for 16, 31, 33, 35, 52, 58 genotypes) / E2 gene (for 16, 33 genotypes)	—	—
HPV A7+	β–globin gene	E2 gene (for 18, 39, 45, 59 genotypes) / E7 gene (for 51 genotype) / E1 gene (for 56 genotype)	—	—
HPV screen-titre	β–globin gene	E1-E2 gene	E2 gene	E7 gene (for 51 genotype) / E1 gene (for 56 genotype)

3. CONTENT

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

variant screen-titre-FRT 2x, REF R-V31-T-2x(RG,iQ,SC)-CE;

variant screen-titre-FRT 4x, REF R-V31-T-4x(RG,iQ,Mx)-CE.

Variant screen-titre-FRT 2x includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-1-FRT *HPV* A9	clear liquid from colorless to light lilac colour	0.14	6 blue cap tubes
PCR-mix-1-FRT *HPV* A7+	clear liquid from colorless to light lilac colour	0.14	6 green cap tubes
PCR-buffer-FRT	colorless clear liquid	0.30	6 tubes
Polymerase (TaqF)	colorless clear liquid	0.02	6 tubes
DNA calibrator C1 *HPV* 16, 18	colorless clear liquid	0.04	3 tubes
DNA calibrator C2 *HPV* 16, 18	colorless clear liquid	0.04	3 tubes
DNA calibrator C3 *HPV* 16, 18	colorless clear liquid	0.04	3 tubes
DNA-buffer	colorless clear liquid	0.5	1 tube
Negative Control (C–)	colorless clear liquid	1.2	1 tube

Variant screen-titre-FRT 2x is intended for 216 reactions (108 tests), including controls.

Variant screen-titre-FRT 4x includes:

Reagent	Description	Volume, ml	Quantity
PCR-mix-1-FRT *HPV* screen-titre	clear liquid from colorless to light lilac colour	0.28	3 tubes
PCR-buffer-FRT	colorless clear liquid	0.30	3 tubes
Polymerase (TaqF)	colorless clear liquid	0.02	3 tubes
DNA calibrator C1 *HPV*	colorless clear liquid	0.04	3 tubes
DNA calibrator C2 *HPV*	colorless clear liquid	0.04	3 tubes
DNA calibrator C3 *HPV*	colorless clear liquid	0.04	3 tubes
DNA-buffer	colorless clear liquid	0.5	1 tube
Negative Control (C–)	colorless clear liquid	1.2	1 tube

Variant screen-titre-FRT 4x is intended for 108 reactions (including controls).

PCR kit also includes:

Compact Disk with:

- software (Microsoft® Excel format) for data interpretation and result analysis obtaining;
- template files for fast run of experiment.

4. ADDITIONAL REQUIREMENTS

- DNA extraction kit.
- Disposable powder-free gloves and a laboratory coat.
- Pipettes (adjustable).
- Sterile pipette tips with aerosol filters (up to 200 µl).
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with rotor for 2-ml reaction tubes.
- PCR box.
- Two-channel real-time instruments (for example, Rotor-Gene 3000/6000 (Corbett Research, Australia), iCycler iQ (Bio-Rad, USA) with filters for channels FAM and JOE channels; SmartCycler II (Cepheid, USA) for PCR kit variant screen-titre-FRT 2x.
- Four-channel real-time instruments (for example, Rotor-Gene 3000/6000 (Corbett Research, Australia), Mx3000P (Stratagene, USA), iCycler iQ5 (Bio-Rad, USA) for PCR kit variant screen-titre-FRT 4x.
- For Rotor-Gene instrument: 0.2-ml disposable polypropylene nonstriped PCR tubes with flat caps for a 36-well rotor or 0.1 ml tubes for a 72-well rotor.
- For iCycler iQ or iCycler iQ5 instruments: 0.2-ml disposable polypropylene striped or nonstriped PCR tubes with domed caps or a 96-well plate for PCR equipped with heat-sealing optical transparent films.
- For Mx3000P instrument: 0.2-ml disposable polypropylene striped or nonstriped PCR tubes with domed caps or a plate for PCR equipped with heat-sealing optical transparent films..
- For SmartCycler II instrument: disposable polypropylene tubes for PCR for SmartCycler II intended for 25 µl of reaction mixture (Cepheid, USA). MiniSpin centrifuge and tube rack (Cepheid, USA).
- Refrigerator for 2–8 °C.
- Deep-freezer at the temperature from minus 24 to minus 16 °C.
- Reservoir for used tips.

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 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.
- Safety Data Sheets (SDS) are available on request.
- 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

NOTE: Obtaining samples of biological materials for the PCR-analysis, transportation and storage are described in the manufacturer's handbook [1]. It is recommended that this handbook is read before starting work.

AmpliSens® *HPV* HCR screen-titre-FRT PCR kit is intended for the analysis of DNA extracted with DNA extraction kits from the biological material (cervical or urethral scrapes).

Female: samples of epithelial cells should be obtained as for cytological examination.
Method 1. The sampling kit with one/two cervical cytobrushes and 2-ml tube with 0.5 ml of **Transport Medium with Mucolytic Agent** REF 953-CE 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. Break the lower part of the cytobrush and leave it in the tube with transport medium.
Method 2. The Digene cervical sampler (USA), which contains cervical cytobrush and a tube with 1.0 ml of Digene transport medium, is used.
Place the cervical epithelial scrape (endocervix) taken with the cervical cytobrush to the tube with Digene transportation medium.
Method 3. The sampling kit, which contains the combined gynecological probe for simultaneously taking epithelium from endocervix and ectocervix and 5-ml tube with 2.0 ml of **Transport Medium with Mucolytic Agent** REF 952-CE, is used.

Place the endocervix and ectocervix into the tube with transport medium. Break the lower part of the probe and leave it in the tube with transport medium.
Method 4. The sampling kit, which contains the combined gynecological probe for simultaneously taking epithelial samples from endocervix and ectocervix and a liquid-based cytology vial with CytoScreen (Italy) or PreservCyt (USA) transport medium, is used.
Place the endocervix and ectocervix into the tube with transport medium. Break the lower part of the probe and leave it in the vial with transport medium.

Male: Obtain urethral epithelial scrape by universal probe, place it into the 2.0 ml tube with 0.5 ml of **Transport Medium with Mucolytic Agent** REF 953-CE.

Storage conditions:
- at the temperature from 18 to 25 °C – no more than 5 days;
- at the temperature from 2 to 8 °C – no more than 20 days;
- 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 room temperature – for 1 year.

7. WORKING CONDITIONS

AmpliSens® *HPV* HCR screen-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:

- **DNA-sorb-AM**, REF K1-12-100-CE (for biological material obtained by the 1st, 2nd and 3rd methods);
- **DNA-sorb-B**, REF K1-2-100-CE (for biological material obtained by the 1st, 2nd and 3rd methods);
- **DNA-sorb-C**, REF K1-6-50-CE (for biopsy samples);
- **AmpliSens® DNA-sorb-D** REF K8-2331-100-CE (for samples for liquid-based cytology).

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 the DNA according to the manufacturer's protocols.

NOTE: **If using the DNA-sorb-AM kit** take into account that in case of previously mixing the whole volume of sorbent with the whole volume of **Lysis Solution**, 320 µl of this mixture should be used for adding into the 1.5 ml sterile disposable tubes.

Variant screen-titre-FRT 2x

8.2. Preparing PCR

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

8.2.1. Preparing tubes for PCR

1. Prepare the reaction mixture for the required number of samples (Tables 1 and 2). During calculations, it should be taken into account that every run requires at least four control samples (negative control and three calibrators). In addition, it is necessary to take reagents for one extra reaction. One amplification reaction requires:
   - **7.0 µl** of **PCR-mix-1-FRT *HPV* A9** or **PCR-mix-1-FRT *HPV* A7+**;
   - **7.5 µl** of **PCR-buffer-FRT**;
   - **0.5 µl** of **polymerase (TaqF)**.

Table 1

Addition of reagents to the tubes

If 14 samples plus controls are to be analyzed	*If less than 14 samples (for example, N) plus 8 controls are to be analyzed*
• Take one tube of each of the following reagents: **polymerase (TaqF)**, **PCR-buffer-FRT**, **PCR-mix-1-FRT *HPV* A9** and **PCR-mix-1-FRT *HPV* A7+**. • Transfer the whole volume (20 µl) of **polymerase (TaqF)** to the tube with **PCR-buffer-FRT** (300 µl). Carefully vortex the tube at the minimum speed and then centrifuge on vortex for 1 s. Avoid foaming. • Add **160 µl** of prepared mixture into each of tubes with **PCR-mix-1-FRT *HPV* A9 (140 µl)** and **PCR-mix-1-FRT A7+ (140 µl)**	• Mix in a separate tube **15*(N+5) µl** of **PCR-buffer-FRT** and **1.0*(N+5) µl** of **polymerase (TaqF)**. Carefully vortex the tube at the minimum speed and then centrifuge for 1 s. Avoid foaming. • Add **7 *(N+5)** of **PCR-mix-1-FRT *HPV* A9** and **PCR-mix-1-FRT *HPV* A7+** into two separate tubes. • Transfer a half (8*(N+5) µl) of the prepared mixture of **PCR-buffer-FRT** with **polymerase (TaqF)** to the tube that contains **PCR-mix-1-FRT *HPV* A9**; transfer the other half (8*(N+5) µl) of the prepared mixture to the tube with **PCR-mix-1-FRT *HPV* A7+**. (Refer to Table 2 for calculation tips).
• Carefully vortex the prepared mixtures at the minimum speed and then centrifuge for 1 s. Avoid foaming.	
• Prepare 36 PCR tubes.	• Prepare (N*2 +8) PCR tubes (2 tubes per each clinical samples plus 8 tubes for controls).

The mixture of **polymerase (TaqF)** and **PCR-buffer-FRT** can be stored at 2–8 ºC for 3 months.

NOTE: The mixture of **polymerase (TaqF)**, **PCR-buffer-FRT**, and **PCR-mix-1-FRT *HPV* A9** as well as the mixture of **polymerase (TaqF)**, **PCR-buffer-FRT**, and **PCR-mix-1-FRT *HPV* A7+** should be used within 2 h after preparation.

Table 2

Scheme of reaction mixture preparation for N test samples, negative control, and three calibrators

– Mix in a separate tube.

Number of samples, N	**1**	**2**	**3**	**4**	**5**	**6**	**7**
PCR-buffer-FRT, µl	90	105	120	135	150	165	180
Polymerase (TaqF), µl	6	7	8	9	10	11	12
Number of samples, N	**8**	**9**	**10**	**11**	**12**	**13**	**14**
PCR-buffer-FRT, µl	195	210	225	240	255	270	Whole tube
Polymerase (TaqF), µl	13	14	15	16	17	18	Whole tube

– Mix in a separate tube the part of prepared mixture of **PCR-buffer-FRT** and **polymerase (TaqF)** with **PCR-mix-1-FRT *HPV* A9** (blue cap).

Number of samples, N	**1**	**2**	**3**	**4**	**5**	**6**	**7**
mixture of PCR-buffer-FRT and polymerase (TaqF), µl	48	56	64	72	80	88	96
PCR-mix-1-FRT A9, µl (blue cap)	42	49	56	63	70	77	84
Number of samples, N	**8**	**9**	**10**	**11**	**12**	**13**	**14**
mixture of PCR-buffer-FRT and polymerase (TaqF), µl	104	112	120	128	136	142	150
PCR-mix-1-FRT A9, µl (blue cap)	91	98	105	112	119	126	Whole tube

– Mix in a separate tube the part of prepared mixture of **PCR-buffer-FRT** and **polymerase (TaqF)** with **PCR-mix-1-FRT *HPV* A7+** (green cap).

Number of samples, N	**1**	**2**	**3**	**4**	**5**	**6**	**7**
mixture of PCR-buffer-FRT and polymerase (TaqF), µl	48	56	64	72	80	88	96
PCR-mix-1-FRT A7+, µl (green cap)	42	49	56	63	70	77	84
Number of samples, N	**8**	**9**	**10**	**11**	**12**	**13**	**14**
mixture of PCR-buffer-FRT and polymerase (TaqF), µl	104	112	120	128	136	142	150
PCR-mix-1-FRT A7+, µl (green cap)	91	98	105	112	119	126	Whole tube

2. Add **15 µl** of the prepared mixture of *HPV* FRT A9 (per each tube) to half of the tubes and **15 µl** of the prepared mixture of *HPV* FRT A7+ (per each tube) to the other half of tubes.

3. Using tips with aerosol filter, add **10 µl** of **DNA samples** obtained at the stage of DNA extraction to the prepared tubes for PCR. Add DNA samples first to the tubes with the prepared mixture of *HPV* FRT A9 and then to the tubes with the prepared mixture of *HPV* FRT A7+ (Scheme 1).

NOTE: Avoid transferring the sorbent together with the DNA samples into the reaction mixture.

4. Carry out the control amplification reactions:

NCA – Add **10 µl** of **DNA-buffer** into two tubes, labeled NCA (Negative Control of Amplification): into the tube with prepared *HPV* FRT A9 mixture and then into the tube with *HPV* FRT A7+ mixture.

***HPV* DNA calibrators (C1, C2, C3)** – Add **10 µl** of each of *HPV* DNA calibrators (**DNA calibrator C1 *HPV* 16, 18**, **DNA calibrator C2 *HPV* 16, 18**, **DNA calibrator C3 *HPV* 16, 18**) to each of three tubes with the prepared *HPV* FRT A9 mixture and **10 µl** of each of *HPV* DNA calibrators to each of three tubes with the prepared *HPV* FRT A7+ mixture.

C– – Add **10 µl** of the sample extracted from the **Negative Control (C–) reagent** to the pair of tubes labeled C– (Negative Control of Extraction).

Scheme 1

Order of tube placement and reagent addition (only if the tubes are to be used)

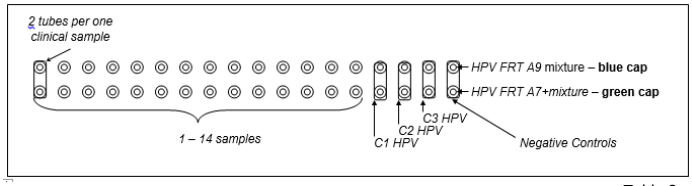


Table 3

DNA amplification program for HCR *HPV* types 16, 18, 31, 33, 35; 39, 45, 51, 52, 56, 58, and 59

Step	Temperature, °C	Time	Fluorescence detection	Cycles
Hold	**95**	**15 min**	**–**	**1**
Hold2	**65**	**2 min**	**–**	**1**
Cycling	**95**	**20 s**	**–**	**5**
	64 Touchdown: *1 deg. per cycle* / Lower temperature of each step on 1 ºC	**25 s**	**–**	
	65	**55 s**	**–**	
Cycling2	**95**	**15 s**	**–**	**40**
	60	**25 s**	**–**	
	65	**40 s**	**FAM, JOE**	

NOTE: The "**AmpliSens-1 RG**" **universal program** for DNA amplification and detection can also be used (see Table 4). Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program (for example, combined with tests for STI pathogen DNA detection).

The analytical performance characteristics of the reagent kit do not change when the universal amplification program is used.

Table 4

Amplification program "AmpliSens-1 RG"

Step	Temperature, °C	Time	Fluorescence detection	Cycles
Hold	**95**	**15 min**	**–**	**1**
Cycling	**95**	**5 s**	**–**	**5**
	60	**20 s**	**–**	
	72	**15 s**	**–**	
Cycling2	**95**	**5 s**	**–**	**40**
	60	**20 s**	**FAM, JOE, ROX, Cy5**	
	72	**15 s**	**–**	

Note – The ROX and Cy5 channels are enabled when required if the "multiprime" format tests are performed.
For iCycler iQ, run one of the following two programs (for details, see Guidelines [2]).

Table 5

DNA amplification program for HCR *HPV* types 16, 18, 31, 33, 35; 39, 45, 51, 52, 56, 58, and 59

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	**95**	**15 min**	**–**	**1**
2	**65**	**2 min**	**–**	**1**
3	**95**	**20 s**	**–**	**5**
	64 Temp -: *1.0 for a cycle*	**25 s**	**–**	
	65	**55 s**	**–**	
4	**95**	**20 s**	**–**	**42**
	60	**25 s**	**–**	
	65	**55 s**	**FAM, JOE**	

NOTE: The **"AmpliSens-1 iQ" universal program** for DNA amplification and detection can also be used (see Table 6). Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program (for example, combined with tests for STI pathogen DNA detection).

The analytical performance characteristics of the reagent kit do not change when the universal amplification program is used.

Table 6

Amplification program "AmpliSens-1 iQ"

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	**95**	**15 min**	**–**	**1**
2	**95**	**5 s**	**–**	**5**
	60	**20 s**	**–**	
	72	**15 s**	**–**	
3	**95**	**5 s**	**–**	**40**
	60	**30 s**	**FAM, JOE, ROX, Cy5**	
	72	**15 s**	**–**	

Note – The ROX and Cy5 channels are enabled when required if the "multiprime" format tests are performed.
For SmartCycler, run the following programs (for details, see Guidelines [2]).

Table 7

Amplification program for HCR *HPV* 16, 18, 31, 33, 35; 39, 45, 51, 52, 56, 58, 59 types DNA

Step	Temperature, °C	Time	Fluorescence detection	Cycles
Stage 1. Hold	**95**	**900 s**	**–**	**1**
Stage 2. Hold	**65**	**120 s**	**–**	**1**
Stage 3. 3-Temperature Cycle	**95**	**20 s**	**–**	**5**
	63	**30 s**	**–**	
	65	**60 s**	**–**	
Stage 4. 3-Temperature Cycle	**95**	**25 s**	**–**	**42**
	60	**30 s**	**–**	
	65	**60 s**	**Switched on**	

1. Adjust the fluorescence channel sensitivity according to the *Important Product Information Bulletin* and Guidelines [2].
2. Insert tubes into the reaction module of the device.
3. Run the amplification program with fluorescence detection.
4. Analyze results after the amplification program is completed.

Variant screen-titre-FRT 4x

8.3. Preparing PCR

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

8.3.1. Preparing tubes for PCR

1. At first, prepare the mixture of **PCR-buffer-FRT** and **polymerase (TaqF)**. Transfer the contents of one tube with **polymerase (TaqF) (0.02 ml)** to the tube with **PCR-buffer-FRT (0.3 ml).** Carefully vortex the tube. Avoid foaming. Mark each tube with the mixture preparation date. To prepare the mixture, use only sterile tips with aerosol filters.

NOTE: The prepared mixture is intended for 40 reactions. The mixture can be stored at 2–8 °C for 3 months. It can be used when necessary.

2. Take the required number of tubes for amplification for the clinical and control samples. The type of tubes depends on the PCR instrument used for analysis.
3. Add the reagents into the tubes according to Table 9.

Table 8

Methods of reagent addition to the tubes

1st method	2nd method
1. Add **7 µl of PCR-mix-1-FRT *HPV* screen-titre** to tubes 2. Add **8 µl** of the prepared mixture of **PCR-buffer-FRT and polymerase (TaqF)**	1. Prepare the reaction mixture for needed number of reaction – mix in a separate tube **PCR-mix-1-FRT *HPV* screen-titre** and prepared mixture of **PCR-buffer FRT** and **polymerase (TaqF)**. The following quantities of reagents are required per one reaction: - **7 µl of PCR-mix-1-FRT *HPV* screen-titre** - **8 µl** of the mixture of **PCR-buffer-FRT and polymerase (TaqF)** During calculations, it should be taken into account that every run requires at least four control samples (negative control and three calibrators). In addition, it is necessary to take reagents for one extra reaction (see Table 9). 2. Add **15 µl** of prepared mixture to tubes

NOTE: The mixture of **polymerase (TaqF)**, **PCR-buffer-FRT**, and **PCR-mix-1-FRT *HPV* screen-titre** should be used within 2 hours after preparation.

Table 9

Scheme of reaction mixture preparation for N test samples, negative control, and three DNA calibrators

Number of samples, N	3	4	5	6	7	8	9	10	11	12
PCR-mix-1-FRT *HPV* screen-titre, µl	56	63	70	77	84	91	98	105	112	119
mixture of PCR-buffer-FRT and polymerase (TaqF) mix, µl	64	72	80	88	96	104	112	120	128	136
Number of samples, N	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
PCR-mix-1-FRT *HPV* screen-titre, µl	126	133	140	147	154	161	168	175	182	189
mixture of PCR-buffer-FRT and polymerase (TaqF) mix, µl	144	152	160	168	176	184	192	200	208	216
Number of samples, N	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**
PCR-mix-1-FRT *HPV* screen-titre, µl	196	203	210	217	224	231	238	245	252	259
mixture of PCR-buffer-FRT and polymerase (TaqF) mix, µl	224	232	240	248	256	264	272	280	288	296

4. Using tips with aerosol filter, add 10 µl of DNA samples obtained at the DNA extraction stage to the prepared tubes for PCR.

NOTE: Avoid transferring the sorbent together with the DNA samples into the reaction mixture.

5. Carry out the control amplification reactions:

NCA – Add **10 µl** of **DNA-buffer** into the tube labeled NCA (Negative Control of Amplification)

HPV* DNA calibrators (C1, C2, C3)** – Add 10 µl of each of *HPV* DNA calibrators (**DNA calibrator C1 *HPV, **DNA calibrator C2 *HPV***, **DNA calibrator C3 *HPV***) into three tubes.

C– – Add **10 µl** of the sample extracted from the **Negative Control (C–) reagent** to the pair of tubes labeled C– (Negative Control of Extraction).

8.3.2. Amplification

1. Create a temperature profile on your instrument as follows:
For the Rotor-Gene instrument, run one of the following two programs (for details, see Guidelines [2]).

Table 10

Amplification program for HCR *HPV* 16, 18, 31, 33, 35; 39, 45, 51, 52, 56, 58, 59 types DNA

Step	Temperature, °C	Time	Fluorescence detection	Cycles
Hold	95	15 min	–	1
Hold2	65	2 min	–	1
Cycling	95	20 s	–	5
	64 Touchdown: *1 deg. per cycle* / Lower temperature of each step on 1 ºC	25 s	–	
	65	55 s	–	
Cycling2	95	15 s	–	40
	60	25 s	–	
	65	25 s	FAM, JOE, ROX, Cy5	

NOTE: The "**AmpliSens-1 RG**" **universal program** for DNA amplification and detection can also be used (see Table 11). Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program (for example, combined with tests for STI pathogen DNA detection).

The analytical performance characteristics of the reagent kit do not change when the universal amplification program is used.

Table 11

Amplification program "AmpliSens-1 RG"

Step	Temperature, °C	Time	Fluorescence detection	Cycles
Hold	95	15 min	–	1
Cycling	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Cycling2	95	5 s	–	40
	60	20 s	FAM, JOE, ROX, Cy5	
	72	15 s	–	

Note – The ROX and Cy5 channels are enabled when required if the "multiprime" format tests are performed.

For iCycler iQ5 instrument, run one of the following two programs (for details, see Guidelines [2]).

Table 12

DNA amplification program for HCR *HPV* types 16, 18, 31, 33, 35; 39, 45, 51, 52, 56, 58, and 59

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	95	15 min	–	1
2	95	15 s	–	6
	65 Temp -: *1.0 for a cycle*	55 s	–	
	65	25 s	–	
3	95	15 s	–	41
	60	55 s	Real-time	
	65	25 s	–	

NOTE: The "**AmpliSens-1 iQ**" **universal program** for DNA amplification and detection can also be used (see Table 13). Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program (for example, combined with tests for STI pathogen DNA detection).

The analytical performance characteristics of the reagent kit do not change when the universal amplification program is used.

Table 13

"AmpliSens-1 iQ" amplification program

Step	Temperature, °C	Time	Fluorescence detection	Cycles
1	95	15 min	–	1
2	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
3	95	5 s	–	40
	60	30 s	FAM, JOE, ROX, Cy5	
	72	15 s	–	

Note – The ROX and Cy5 channels are enabled when required if the "multiprime" format tests are performed.
For Mx3000P instrument, run the following programs (for details, see Guidelines [2]).

Table 14

DNA amplification program for HCR *HPV* types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59

Step	Temperature, °C	Time	Fluorescence detection	Cycles
Segment 1	95	15 min	–	1
Segment 2	65	2 min	–	1
Segment 3 (Cycling)	95	20 s	–	5
	64 Touchdown: *1 deg. per cycle*	25 s	–	
	65	55 s	–	
Segment 4 (Cycling)	95	20 s	–	40
	60	25 s	–	
	65	55 s	Cy5, FAM, JOE, ROX	

NOTE: The "**AmpliSens-1 Mx**" **universal program** for DNA amplification and detection can also be used (see Table 15). Any combination of the tests can be performed in one instrument simultaneously with the use of the unified amplification program (for example, combined with tests for STI pathogen DNA detection).

The analytical performance characteristics of the reagent kit do not change when the universal amplification program is used.

Table 15

Amplification program "AmpliSens-1 Mx"

Step	Temperature, °C	Time	Fluorescence detection	Cycles
Segment 1.	95	15 min	–	1
Segment 2. (Cycling)	95	5 s	–	5
	60	20 s	–	
	72	15 s	–	
Segment 3. (Cycling)	95	5 s	–	40
	60	30 s	FAM, JOE, ROX, Cy5	
	72	15 s	–	

Note – The ROX and Cy5 channels are enabled when required if the "multiprime" format tests are performed.

9. DATA ANALYSIS

Signal in a tube in the channel is considered to be positive, if corresponding fluorescence accumulation curve cross the threshold line. The signal is characterized by the threshold cycle, the cycle corresponding to the crossing of the fluorescence curve and the threshold line. The *Ct* values are analyzed by the software of automated recording of results of analysis. Based on the obtained *Ct* values a calibration line is automatically plotted and human DNA and *HPV* DNA concentrations are calculated. To obtain the final result, *HPV* DNA concentration is normalized to the number of human cells according to the formula:

lg (*HPV* DNA copies/human DNA copies) × 200,000) = lg (*HPV* on 100,000 cells)

Each lot of the reagent kit is provided with calibrator concentrations. These values are specified in the *Important Product Information Bulletin* and must be entered into corresponding cells of the software for automated analysis of results.

Variant screen-titre-FRT 2x
The run is ***valid*** if
- Negative controls have no signal in all the channels for the fluorophores (FAM, JOE);
- All calibrators have signals in all the channels for the fluorophores (FAM, JOE);
- The correlation coefficient for calibration curves for all the channels is no less than 0.98.

NOTE: If the run is invalid all obtained results are considered to be unreliable. The PCR run should be repeated.

The result of *HPV* DNA detection of a given sample is considered to be
Negative, if the signal of the Internal Control (IC; channel for the FAM fluorophore) is detected in both tubes for the sample and the quantity of human DNA cells per reaction exceeds 500.
Positive, if a signal in the channel for the JOE fluorophore is detected at least in one of the two tubes. Result:
- One or several types belonging to the phylogenetic group A9 (if the signal is detected in the tube with the *HPV* FRT A9 mixture);
- One or several types belonging to the phylogenetic group A7 or types 51/56 (if the signal is detected in the tube with the *HPV* FRT A7+ mixture).

Variant screen-titre-FRT 4x
The run is ***valid*** if
- Negative controls have no signal in all the channels for the fluorophores (FAM, JOE, ROX, Cy5);
- All calibrators have signals in all the channels for the fluorophores (FAM, JOE, ROX, Cy5);
- The correlation coefficient for calibration curves for all the channels is no less than 0.98.

NOTE: If the run is invalid all obtained results are considered to be unreliable. The PCR run should be repeated.

The result of *HPV* DNA detection of a given sample is considered to be
Negative, if the signal of the Internal Control (IC; channel for the FAM fluorophore) is detected for the sample and the quantity of human DNA cells per reaction exceeds 500.
Positive, if:
- a signal in the channel for the JOE fluorophore is detected. Result: one or several types belonging to the phylogenetic group A9 (16, 31, 33, 35, 52, 58);
- a signal in the channel for the ROX fluorophore is detected. Result: one or several types belonging to the phylogenetic group A7 (18, 39, 45, 59);
- a signal in the channel for the Cy5 fluorophore is detected. Result: 51 and/or 56 type(s) (the phylogenetic branches A5 and A6);

10. TROUBLESHOOTING

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

Variant screen-titre-FRT 2x
The result is **invalid** in the following cases:
- If no positive signal in the channel for the JOE fluorophore (A9, A7, A5/A6) is detected in any of the two tubes and the signal of the Internal Control (IC; the channel for the FAM fluorophore) is not detected or number of human DNA cells per reaction does not exceed 500.
- Weak positive signal(s) is/are detected in the channel for the JOE fluorophore but the signal of the Internal Control (IC) in the channel for the FAM fluorophore is not detected and the number of genome equivalents of human DNA per reaction does not exceed 10^3. In case of invalid result, the analysis for this sample should be repeated from the DNA extraction stage or from the material sampling.

Variant screen-titre-FRT 4x
The result is **invalid** if no positive signals in the channels for the JOE, ROX, Cy5 fluorophores (A9, A7, A5/A6) are detected and the signal of the Internal Control (IC; the channel for the FAM fluorophore) is not detected or number of human DNA cells per reaction does not exceed 500. In case of invalid result, the analysis for this sample should be repeated from the DNA extraction stage or from the material sampling.
If you have any further questions or if you encounter problems, please contact our Authorized representative in the European Community.

11. TRANSPORTATION

AmpliSens® *HPV* HCR screen-titre-FRT PCR kit should be transported at 2–8 ºC for no longer than 5 days.

12. STABILITY AND STORAGE

All components of the **AmpliSens® *HPV* HCR screen-titre-FRT** PCR kit are to be stored at 2–8 ºC when not in use (except for PCR-mix-1-FRT *HPV* A9, PCR-mix-1-FRT *HPV* A7+, PCR-mix-1-FRT *HPV* screen-titre, and polymerase (TaqF)). All components of the **AmpliSens® *HPV* HCR screen-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-mix-1-FRT *HPV* A9, PCR-mix-1-FRT *HPV* A7+, PCR-mix-1-FRT *HPV* screen-titre, and polymerase (TaqF) are to be stored at the temperature from minus 24 to minus 16 °C.

NOTE: PCR-mix-1-FRT *HPV* A9, PCR-mix-1-FRT *HPV* A7+ and PCR-mix-1-FRT *HPV* screen-titre are to be kept away from light.

13. SPECIFICATIONS

13.1. Analytical sensitivity

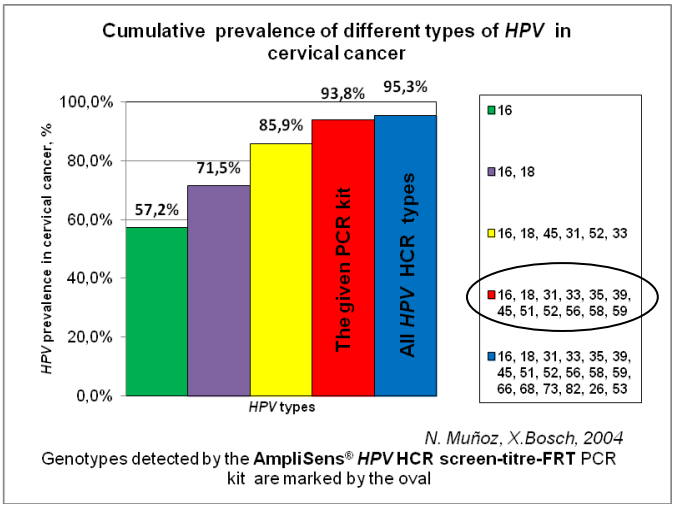
The analytical sensitivity of **AmpliSens® *HPV* HCR screen-titre-FRT** PCR kit is no less then $5x10^3$ genome equivalents per 1 ml of sample for *HPV* types 16, 18, 31, 35, 39, 45, 51, 52, 56 and 59 and no less than 2.5×10^4 genome equivalents per 1 ml of sample for *HPV* types 33 and 58.

NOTE: The claimed analytical features of **AmpliSens® *HPV* HCR screen-titre-FRT** PCR kit are guaranteed only when additional reagent kits DNA-sorb-AM, DNA-sorb-B, DNA-sorb-C and AmpliSens® DNA-sorb-D (manufactured by Federal Budget Institute of Science "Central Research Institute for Epidemiology") are used.

13.2. Analytical specificity

The analytical specificity of **AmpliSens® *HPV* HCR screen-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 clinical specificity of **AmpliSens® *HPV* HCR screen-titre-FRT** PCR kit was confirmed in laboratory clinical trials.

13.3. Diagnostic sensitivity



Genotypes detected by the **AmpliSens® *HPV* HCR screen-titre-FRT** PCR kit are marked by the oval

All currently determined genotypes of *HPV* of high carcinogenic risk (*18 genotypes*) are the cause of 95 % of all cervical cancer. Detection of small number of genotypes (from 1 to 6) lead to the insufficient diagnostic sensitivity of the test (86 %). The prevailing 12 genotypes of *HPV* HCR detected by the given PCR kit are sufficient for the achievement of high diagnostic sensitivity (94 %) at maintenance of suitable test format – in two tubes for PCR (PCR kit variant screen-titre-FRT 2x) or in one tube for PCR (PCR kit variant screen-titre-FRT 4x).

13.4. Diagnostic specificity

The diagnostic specificity of *HPV*-test is defined as the ability not to detect the cases of latent *HPV*-infection, i. e. without clinical features of cervical dysplasia. In case of *HPV*-test the diagnostic specificity is defined by detection of high-risk oncogenic *HPV* genotypes only and by the introduction of the quantitative threshold of clinically significance of infection.

14. REFERENCES

1. Handbook "Sampling, Transportation, and Storage of Biological Material for PCR diagnostics", developed by Federal Budget Institute of Science "Central Research Institute for Epidemiology" of Federal Service for Surveillance on Consumers' Rights Protection and Human Well-Being.
2. Guidelines to **AmpliSens® *HPV* HCR screen-titre-FRT** PCR kit for qualitative and quantitative detection of high carcinogenic risk (HCR) *human papillomaviruses* (*HPV*) types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 DNA in the biological material by the polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection developed by Federal Budget Institute of Science "Central Research Institute for Epidemiology".

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 screen-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
16.12.10	Content	The names of DNA calibrators were corrected (e.g., C1 *HPV* 16, 18 instead of K1 HPV 16, 18) and given in full (e.g. DNA calibrator C1 *HPV* 16, 18)
		New sections "Working Conditions" and "Transportation" were added
		The "Explanation of Symbols" section was renamed to "Key to Symbols Used"
	Stability and Storage	The information about the shelf life of open reagents was added
		Information that PCR-mixes-1-FRT are to be stored away from light was added
	Key to Symbols Used	The explanation of symbols was corrected
	Text	"Titre" was changed to "titre" in the name of the PCR kit
22.06.11 RT	Cover page, text	The name of Institute was changed to Federal Budget Institute of Science "Central Research Institute for Epidemiology"
16.08.16 ME	Text	Corrections according to the template
	2. Principle of PCR detection	Information about quantitative approach to the diagnostics of papillomaviruses infection was added
	8.1. DNA extraction	AmpliSens® DNA-sorb-D nucleic acid extraction kit is recommended. Information about the control of extraction was added
	9. Data analysis, 10. Troubleshooting	Information about data analysis and troubleshooting for variant screen-titre-FRT 4x was added
	13. Specifications	The subsections with diagnostic sensitivity and diagnostic specificity was added
13.02.18 ME	Through the text	"Genome equivalents" were changed to "cells"
	9. Data analysis	The quantity of the human cells per reaction was changed from $5*10^3$ to 500
	Text	The reference number of AmpliSens® DNA-sorb-D nucleic acid extraction kit was changed
12.09.18 EM	3. Content	The colour of the reagents was specified
16.11.18 EM	5. General precautions	The section was corrected according to the template
	Text	"Clinical material" was changed on "biological material"
26.11.18 EM	3. Content	Information about the content of Compact Disk was changed
21.04.20 EM	Through the text	The text formatting was changed
	Footer	The phrase "Not for use in the Russian Federation" was added
	2. Principle of PCR detection	The table with targets was added
25.03.21 EM	—	The name, address and contact information for Authorized representative in the European Community was changed

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