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For Professional Use Only

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

Instruction Manual

AmpliSens®

EC REP

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TABLE OF CONTENTS

1. INTENDED USE

AmpliSens® *HPV* HCR screen-titre-FRT PCR kit is an *in vitro* nucleic acid amplification test for detection and quantitation of types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 of *human papillomaviruses* (*HPV*) of high carcinogenic risk (HCR) in clinical material by using real-time hybridization-fluorescence detection.

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 exhibit a high transforming activity and are responsible for over 94 % of cases of cervical dysplasia and *cervix uteri* 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 HEX/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).

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

2. PRINCIPLE OF PCR DETECTION

The method is based on simultaneous real-time 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 internal endogenous 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).

The result of *HPV* DNA amplification is detected in the JOE/Yellow/HEX/TET fluorescence channel in case of two-channel PCR instrument. 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 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 JOE/Yellow; group A7 *HPV*, in the ROX/Orange; and *HPV* types 51 and 56, in the Cy5/Red channel).

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 FAM/Green channel. The DNA

target selected as an endogenous internal control is a human genome fragment. It must be always present in the sample (cervical swab) in sufficient quantities equivalent to the number of cells in the swab (10^3–10^5 genome equivalents). Thus, the use of an endogenous internal control makes it possible not only to monitor test stages (DNA extraction and PCR amplification) but also to assess the adequacy of sampling and storage of clinical material. If epithelial swab was taken incorrectly (the number of epithelial cells is insufficient), the amplification signal of β-globin gene will be underestimated.

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), which are added during amplification. The results of amplification of DNA calibrators are used to construct a calibration curve, on the basis of which the concentration of *HPV* DNA in samples determined. To minimize the effect of variation during material sampling, the quantitative results (*HPV* DNA concentrations) are normalized to the genomic DNA 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 separation of nucleotides and Taq-polymerase by using a wax layer or a chemically modified polymerase (TaqF). Wax melts and reaction components mix only at 95 ºC. Chemically modified polymerase (TaqF) is activated by heating at 95 ºC.

3. CONTENT

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

AmpliSens® *HPV* HCR screen-titre -FRT PCR kit variant screen-titre-FRT 2x, **REF** R-V31-T-2x(RG,iQ,SC)-CE (for use with RG, iQ, SC).

AmpliSens® *HPV* HCR screen-titre -FRT PCR kit variant screen-titre-FRT 4x, **REF** R-V31-T-4x(RG,iQ,Mx)-CE (for use with RG, iQ, Mx).

AmpliSens® *HPV* HCR screen-titre-FRT PCR kit variant screen-titre-FRT 2x includes:

Reagent	***Description***	***Volume, ml***	***Quantity***
PCR-mix-1-FRT *HPV* A9	colorless clear liquid	0.14	6 blue cap tubes
PCR-mix-1-FRT *HPV* A7+	colorless clear liquid	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

AmpliSens® *HPV* HCR screen-titre-FRT PCR kit variant screen-titre-FRT 2x is intended for 216 reactions (108 tests), including controls.

AmpliSens® *HPV* HCR screen-titre-FRT PCR kit variant screen-titre-FRT 4x includes:

Reagent	***Description***	***Volume, ml***	***Quantity***
PCR-mix-1-FRT *HPV* screen-titre	colorless clear liquid	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

AmpliSens® *HPV* HCR screen-titre-FRT PCR kit variant screen-titre-FRT 4x is intended for 108 reactions (including controls).

PCR kit also includes:

1. Compact Disk with:
   - software (Microsoft® Excel format) for data interpretation and result analysis obtaining;
   - template file in Rotor-Gene software format for fast run of experiment;
   - template file in Mx3000P software format for fast run of experiment;
   - amplification program file for Rotor-Gene and iQ iCycler software.
2. Instruction manual (paper document).
3. Guidelines, which contain detailed programming and data processing description.
4. Bulletin, which contain calibration values for determinate lot of AmpliSens® *HPV* HCR screen-titre-FRT PCR kit.

4. ADDITIONAL REQUIREMENTS

- DNA isolation kit.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).

- Sterile pipette tips with aerosol barriers (up to 200 µl).
- Tube racks.
- Vortex mixer.
- Desktop centrifuge with rotor for 2-ml reaction tubes.
- PCR box.
- Refrigerator for 2–8 °C.
- Deep-freezer for ≤ –16 °C.
- Reservoir for disposed tips.
- Two-channel real-time genetic amplification detection system: Rotor-Gene 3000/6000 (Corbett Research, Australia), iCycler iQ (Bio-Rad, USA) with filters for channels FAM and HEX/JOE filters; SmartCycler II (Cepheid, USA) or equivalent for **AmpliSens® *HPV* HCR screen-titre-FRT** PCR kit variant screen-titre-FRT 2x.
- Four-channel real-time genetic amplification detection system: Rotor-Gene 3000/6000 (Corbett Research, Australia), Mx3000P (Stratagene, USA), iCycler iQ5 (Bio-Rad, USA) or equivalent for **AmpliSens® *HPV* HCR screen-titre-FRT** PCR kit variant screen-titre-FRT 4x
- Rotor-Gene: 0.2-ml disposable flat-cap unstrip polypropylene microtubes for PCR (for instance, Axygen, USA) for a 36-well rotor or 0.1 ml microtubes (Corbett Research, Australia) for a 72-well rotor.

  iCycler iQ or iQ5: 0.2-ml disposable domed polypropylene microtubes for PCR (for instance, Axygen, USA), strip domed tubes or a 96-well plate for PCR equipped with heat-sealing optical transparent films (Bio-Rad, USA).

  Mx3000P: 0.2-ml disposable domed strip/unstrip polypropylene microtubes for PCR (for example, Axygen, USA) for a 36-well rotor or a plate for PCR equipped with heat-sealing optical transparent films (Bio-Rad, USA).

  SmartCycler II: disposable polypropylene microtubes for PCR for SmartCycler II intended for 25 µl of reaction mixture (Cepheid, USA). MiniSpin centrifuge and tube rack (Cepheid, USA).

5. GENERAL PRECAUTIONS

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store and handle amplicons away from all other reagents.
- Thaw all components thoroughly at room temperature before starting detection.
- When thawed, mix the components and centrifuge briefly.
- Use protective gloves, laboratory coats protect eye 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 samples and unused reagents in compliance with local authorities requirements.
- Samples should be considered potentially infectious and handled in biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all spills of specimens or reagents using a disinfectant such as 0,5% sodium hypochlorite, or other suitable disinfectant.
- Avoid contact with the skin, eyes, and mucosa. If skin, eyes, or mucosa contact immediately flush with water and seek medical attention
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional; it should begin in the Extraction Area and then move to the Amplification and Detection Area. Do not return samples, equipment, and reagents to the area in which 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 screen-titre-FRT PCR kit variant screen-titre-FRT is intended for the analysis of DNA extracted with DNA extraction kits from:

- *Cervical or urethral scrapes*

6.1. *Cervical or urethral scrapes*

For women: epithelial samples are taken in the same way as for cytological analysis:

Method 1. The sampling kit consists of one or two cervical cytological brushes and a 2-ml tube with 0.5 ml of Transport Medium with Mucolytic **REF** 953-CE.

Place the cervical epithelial swab (endocervix) taken with the first cervical cytobrush and/or the superficial cervical swab (ectocervix) taken with the second cervical cytobrush to the tube with transport medium. The working part of the cytobrush should to be broken off and left in the tube with transport medium.

Method 2. The sampling kit (Digene, USA) consists of a cervical cytobrush and a tube with 1.0 ml of Digene transport medium.

Place the cervical epithelial swab (endocervix) taken with the cervical cytobrush to the tube with Digene transportation medium.

Method 3. The sampling kit consists of a combined gynecological probe for simultaneously taking epithelium from endocervix and ectocervix and a 5-ml tube with 2.0 ml of Transport

Medium with Mucolytic **REF** 953-CE.

Place the cervical epithelial swab (endocervix) and the superficial cervical swab (ectocervix) into the tube with the transport medium. The working part of the probe is to be broken off and left in the tube with the transport medium.

Method 4. The sampling kit consists of a combined gynecological probe for simultaneously taking epithelial samples from endocervix and ectocervix and a jar with transport–fixation medium for fluid cytology purchased from CytoScreen (Italy) or PreservCyt (USA).

Place the cervical epithelial swab (endocervix) and the superficial cervical swab (ectocervix) into the tube with transport–fixation medium. The working part of the probe is to be broken off and left in the tube with the medium.

For men: Place the urethral epithelial swab taken with a universal probe to a 2.0-ml tube with 0.5 ml of Transport Medium with Mucolytic **REF** 953-CE.

Only one freeze–thaw cycle of clinical material is allowed.

7. WORKING CONDITIONS

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

8. PROTOCOL

8.1. DNA Isolation

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

- DNA-sorb-AM, **REF** K1-12-100-CE (for clinical material obtained by first, second or third methods).
- DNA-sorb-B, **REF** K1-2-100-CE (for clinical material obtained by first, second or third methods).
- DNA-sorb-C, **REF** K1-6-50-CE (for biopsy samples).

Extract DNA according to the manufacturer's instruction.

Note that neither Positive Control nor Internal Control is used in the extraction procedure.

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 using *HPV* HCR screen-titre-FRT PCR kit variant screen-titre-FRT 2x.

1. Preparation of the reaction mixture for the required number of samples (Tables 2 and 3). 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 2

Addition of reagents to the tubes

<table>
<tr><th>If 14 samples plus controls are to be analyzed</th><th>If less than 14 samples (for example, N) plus 8 controls are to be analyzed</th></tr>
<tr><td>• 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 mix into each of tubes with PCR-mix-1–FRT HPV A9 (140 µl) and PCR-mix-1-FRT A7+ (140 µl)</td><td>• 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.
• Add 7 *(N+5) of PCR-mix-1-FRT HPV A9 and PCR-mix-1-FRT HPV A7+ to two separate tubes.
• Transfer a half (8*(N+5) µl) of the prepared mix 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 mix to the tube with PCR-mix-1-FRT HPV A7+. (Refer to Table 2 for calculation tips).</td></tr>
<tr><td colspan="2">- Carefully vortex the prepared mixes at the minimum speed and then centrifuge for 1 s. Avoid foaming.</td></tr>
<tr><td>- Prepare 36 PCR tubes.</td><td>- Prepare (N*2 +8) PCR tubes (2 tubes per each clinical samples plus 8 tubes for controls).</td></tr>
</table>

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

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

1. 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 mix 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**
mix 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**
mix 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

Transfer a part of the prepared mixture of PCR-mix-FRT and polymerase (TaqF) to a separate tube and add PCR-mix-1-FRT *HPV* A7+ (green cap).

Number of samples, N	**1**	**2**	**3**	**4**	**5**	**6**	**7**
mix 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**
mix 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. **Add 10 µl of DNA samples** obtained from clinical or control samples at the stage of DNA extraction to the prepared tubes for PCR using tips with aerosol barrier. 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).

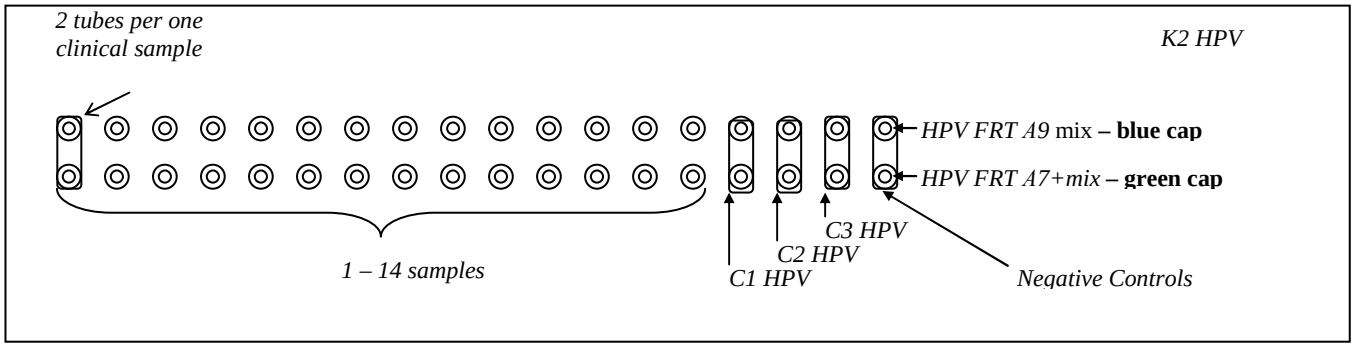
Ensure that the sorbent is not transferred to the PCR reaction mixture while adding DNA samples.

4. Carry out the control amplification reactions:

NCA	- Add **10 µl** of **DNA-buffer** instead of DNA sample into the tube with *HPV* FRT A9 prepared mix and into the tube with *HPV* FRT A7+
HPV DNA calibrators (C1, C2, C3)	- Add 10 µl of each of *HPV* DNA calibrators (**C1 *HPV* 16, 18**, **C2 *HPV* 16, 18**, **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 (**C1 *HPV* 16, 18**, **C2 *HPV* 16, 18**, **C3 *HPV* 16, 18**) to each of three tubes with the prepared *HPV* FRT A7+ mixture.

Scheme 1

Order of tube placement and reagent addition (only if the tubes are to be used)[1].



8.2.2. Amplification

1. Place the tubes in the reaction chamber.

For Rotor-Gene, run one of the two following programs (for details, see Guidelines [2]).

Table 4

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/Green, JOE/Yellow	

The "**AmpliSens-1 RG**" **universal program** for DNA amplification and detection can also be used (see Table 5). This program allows simultaneous performance of any combinations of tests in one instrument (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.

[1] If a PCR plate is used (iCycler iQ), insert samples according to the tubep lacement order (see Guidelines [2]).

Table 5

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/Green, JOE/Yellow, ROX/Orange, Cy5/Red	
	72	15 s		

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

Table 6

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

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

The **"AmpliSens-1 iQ" universal program** for DNA amplification and detection can be also used (see Table 8). This program allows simultaneous performance of any combinations of tests in one instrument (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 7

Amplification program "AmpliSens-1 iQ"

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

For SmartCycler, run the following programs (for details, see Guidelines [2]).

Table 8

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***	

8.2.3. Preparing tubes for PCR using *HPV* HCR screen-titre-FRT PCR kit variant screen-titre-FRT 4x.

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 barriers.

Thus 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. Add the reagents to tubes according to Table 9.

Table 9

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 10). 2. Add **15 µl** of prepared mixture to tubes

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 10

Scheme of reaction mixture preparation for N analyzed 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
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
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
PCR-buffer-FRT and polymerase (TaqF) mix, µl	224	232	240	248	256	264	272	280	288	296

3. **Add 10 µl of DNA samples** obtained from clinical or control samples at the DNA extraction stage to the prepared tubes for PCR using tips with aerosol barrier.

Ensure that the sorbent is not transferred to the PCR reaction mixture while adding DNA samples.

4. Carry out the control amplification reactions:

NCA	-Add **10 µl** of **DNA-buffer**
HPV DNA calibrators (C1, C2, C3)	-Add into three 10 µl of each of *HPV* DNA calibrators (**C1 *HPV***, **C2 *HPV***, **C3 *HPV***)

8.2.4. Amplification

1. Insert the tubes into the reaction chamber.

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

Table 11

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	40 s	FAM/Green, JOE/Yellow, ROX/Orange, Cy5/Red	

The "**AmpliSens-1 RG" universal program** for DNA amplification and detection can also be used (see Table 12). This program allows simultaneous performance of any combinations of tests in one instrument (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 12

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/Green, JOE/Yellow, ROX/Orange, Cy5/Red	
	72	15 s		

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

Table 13

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

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

The "**AmpliSens-1 iQ**" **universal program** for DNA amplification and detection can be also used (see Table 14). This program allows simultaneous performance of any combinations of tests in one instrument (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 14

"AmpliSens-1 iQ" amplification program

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

For Mx3000P instrument, run the following programs (for details, see Guidelines [2]).

Table 15

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 s	–	1
2	65	2 min	–	1
3 (Cycling)	95	20 s	–	5
	64 Touchdown: *1 deg. per cycle*	25 s	–	
	65	55 s	–	
4 (Cycling)	95	20 s	–	40
	60	25 s	–	
	65	55 s	Cy5, FAM, HEX, ROX	

The **universal program** "**AmpliSens-1 Mx**" for amplification and detection can also be used (see Table 16). This program allows simultaneous performance of any combinations of tests in one instrument (for example, combined with tests for STI pathogens DNA detection).

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

Table 16

Amplification program "AmpliSens-1 Mx"

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

9. DATA ANALYSIS

For data processing, see Guidelines [2]

Signal in a tube in the channel is considered to be positive, if corresponding fluorescence accumulation curves cross the threshold line. The signal is characterized by the cycle (threshold Ct), the cycle corresponding to the intersection of the fluorescence curve and the threshold line. The program of automated recording of results of analysis determines the Ct value. The calibration curve is automatically plotted on the basis of these values, and human DNA and *HPV* DNA concentrations are calculated. To obtain the final result, *HPV* DNA concentration is normalized to the number of human genome equivalents according to the formula:

log (*HPV* DNA copies/human DNA copies) × 200000) = log (*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.

The reaction is ***valid*** if

- Negative controls have no signal in all channels (FAM/Green, JOE/Yellow/HEX/TET);
- All calibrators have signals in all channels (FAM/Green, JOE/Yellow/HEX/TET);
- The correlation coefficient for calibration curves for all channels is not less than 0.98.

The result of *HPV* DNA detection of a given sample is considered to be

Negative, if the signal of the Internal Control (IC; FAM/Green channel) is detected in both tubes for the sample and the quantity of human DNA genome equivalents per reaction exceeds 10^3.

Positive, if a signal in the JOE/Yellow/HEX/TET channel 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).

10. TROUBLESHOOTING

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

- If no positive signal in the JOE/Yellow/HEX/TET channel (A9, A7, A5/A6) is detected in any of the two tubes and the signal of the Internal Control (IC; FAM/Green channel) is not detected or number of genome equivalents of human DNA per reaction does not exceed 10^3. In this case, the results of the analysis are considered **invalid** for all samples. It is necessary to repeat the analysis of such samples and to take measures to detect and eliminate the source of contamination.
- Weak positive signal(s) is/are detected in the JOE/Yellow/HEX/TET channel but the signal of the Internal Control (IC) in the FAM/Green channel is not detected and the number of genome equivalents of human DNA per reaction does not exceed 10^3.

If you have any further questions or 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 (except for 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 2–8 ºC when not in use. All components of the **AmpliSens® *HPV* HCR screen-titre-FRT** PCR kit are stable until labeled expiration date. The shelf life of opened reagents is the same as that of unopened reagents, unless otherwise stated.

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 temperature from minus 24 to minus 16 °C when not in use.

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

13. SPECIFICATIONS

13.1. 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 then 2.5×10^4 genome equivalents per 1 ml of sample for *HPV* types 33 and 58.

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 and DNA-sorb-C (manufactured by Federal Budget Institute of Science "Central Research Institute for Epidemiology") are used.

13.2. Specificity

The analytical specificity of AmpliSens® *HPV* HCR screen-titre-FRT PCR kit is ensured by selection of specific primers and stringent reaction conditions. The clinical specificity of AmpliSens® *HPV* HCR screen-titre-FRT PCR kit was confirmed in laboratory clinical trials.

14. REFERENCES

1. Handbook "Sampling, Transportation, and Storage of Clinical Material for PCR Diagnostics", developed by Federal State Institute of Science "Central Research Institute of Epidemiology" of Federal Service for Surveillance on Consumers' Rights Protection and Human Well-Being, Moscow, 2008.
2. Guidelines to AmpliSens® *HPV* HCR screen-titre-FRT PCR kit for detection and quantitation of types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 of *human papillomaviruses* (*HPV*) of high carcinogenic risk (HCR) in clinical 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 accordance with Federal Budget Institute of Science "Central Research Institute for Epidemiology" ISO 13485-Certified Total Quality Management System, each lot of AmpliSens® *HPV* HCR screen-titre-FRT PCR kit is tested against predetermined specifications to ensure consistent product quality.

16. KEY TO SYMBOLS USED

REF	Catalogue number	Σ	Sufficient for
LOT	Batch code		Expiration Date
IVD	*In vitro* diagnostic medical device		Consult instructions for use
VER	Version		Keep away from sunlight
	Temperature limitation	**NCA**	Negative control of amplification
	Manufacturer	**C–**	Negative control of extraction
	Date of manufacture	**C+**	Positive control of amplification
EC REP	Authorised representative in the European Community		
	Caution	**IC**	Internal control

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”