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For Professional Use Only

AmpliSens® *HPV* HCR genotype-EPh PCR kit

Instruction Manual

AmpliSens®

EC REP

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1. INTENDED USE

AmpliSens® *HPV* HCR genotype-EPh PCR kit is an in vitro nucleic acid amplification test for qualitative detection and differentiation of high carcinogenic risk (HCR) *Human Papillomavirus* (*HPV*) types 16, 31, 33, 35, 18, 39, 45, 59 and 52, 56, 58, 66 in the clinical material (cervical or urethral swabs) by using electrophoretic detection of the amplified products in agarose gel.

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

2. PRINCIPLE OF PCR DETECTION

Human papillomavirus of high carcinogenic risk detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen DNA specific region using special *HPV* HCR primers. After PCR, the amplified product is detected in agarose gel. AmpliSens® *HPV* HCR genotype-EPh PCR kit is based on simultaneous amplification in one tube (multiplex-PCR) of four types of *human papillomavirus* DNA and β-globin gene, which is used as an endogenous Internal Control. PCR test for detection of DNA of twelve *HPV* types is performed in three tubes. Since all amplified products differ in length, the genotype of the virus can be identified. The DNA target selected as an endogenous Internal Control is a human genome fragment. It must be present in the sample in a sufficient quantity equivalent to that of cells in the sample (not less than 10^3–10^5 genomes). If the number of epithelial cells in the sample is insufficient or the amount of mucus is too high, the band corresponding to the Internal Control will be absent in agarose gel. AmpliSens® *HPV* HCR genotype-EPh PCR kit uses “hot-start”, which greatly reduces the frequency of nonspecifically primed reactions. “Hot-start” is guaranteed by using chemically modified polymerase (TaqF). Chemically modified polymerase (TaqF) is activated by heating at 95 ºC for 15 min.

3. CONTENT

AmpliSens® *HPV* HCR genotype-EPh PCR kit is produced in 1 form:

AmpliSens® *HPV* HCR genotype-EPh PCR kit variant 50 F, **REF** V25-50-F-CE.

AmpliSens® *HPV* HCR genotype-EPh PCR kit variant 50 F includes:

Reagent	*Description*	*Volume, ml*	*Amount*
PCR-mix-1 *HPV* 16/35	colorless clear liquid	0.3	1 tube
PCR-mix-1 *HPV* 18/59	colorless clear liquid	0.3	1 tube
PCR-mix-1 *HPV* 52/66	colorless clear liquid	0.3	1 tube
2.5x PCR-buffer red	red clear liquid	0.6	3 tubes
Polymerase (TaqF)	colorless clear liquid	0.09	1 tube
Mineral oil for PCR	colorless viscous liquid	8.0	1 vial
Positive Control DNA human ($C+_h$)	colorless clear liquid	0.2	1 tube
DNA-buffer	colorless clear liquid	0.5	1 tube
Negative Control (C–)*	colorless clear liquid	1.2	1 tube

* must be used in the isolation procedure as Negative Control of Extraction (see DNA-sorb-AM **REF** K1-12-50-CE, DNA-sorb-B, **REF** K1-2-50-CE, DNA-sorb-C, **REF** K1-6-50-CE Protocols).

Panel of Positive Control samples of *HPV* HCR DNA includes:

Reagent	*Description*	*Volume (ml)*	*Amount*
Positive Control DNA *HPV* type 16 ($C+_{HPV\ 16}$)	colorless clear liquid	0.15	1 tube
Positive Control DNA *HPV* type 31 ($C+_{HPV\ 31}$)	colorless clear liquid	0.15	1 tube
Positive Control DNA *HPV* type 33 ($C+_{HPV\ 33}$)	colorless clear liquid	0.15	1 tube
Positive Control DNA *HPV* type 35 ($C+_{HPV\ 35}$)	colorless clear liquid	0.15	1 tube
Positive Control DNA *HPV* type 18 ($C+_{HPV\ 18}$)	colorless clear liquid	0.15	1 tube
Positive Control DNA *HPV* type 45 ($C+_{HPV\ 45}$)	colorless clear liquid	0.15	1 tube
Positive Control DNA *HPV* type 39 ($C+_{HPV\ 39}$)	colorless clear liquid	0.15	1 tube
Positive Control DNA *HPV* type 59 ($C+_{HPV\ 59}$)	colorless clear liquid	0.15	1 tube
Positive Control DNA *HPV* type 52 ($C+_{HPV\ 52}$)	colorless clear liquid	0.15	1 tube
Positive Control DNA *HPV* type 56 ($C+_{HPV\ 56}$)	colorless clear liquid	0.15	1 tube
Positive Control DNA *HPV* type 58 ($C+_{HPV\ 58}$)	colorless clear liquid	0.15	1 tube
Positive Control DNA *HPV* type 66 ($C+_{HPV\ 66}$)	colorless clear liquid	0.15	1 tube

AmpliSens® *HPV* HCR genotype-EPh PCR kit variant 50 F is intended for 165 reactions (55

tests), including controls.

4. ADDITIONAL REQUIREMENTS

- DNA isolation kit.
- Agarose gel detection kit.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile DNase-free pipette tips with aerosol barriers (up to 200 µl).
- Vortex mixer.
- Desktop microcentrifuge with a rotor for 2-ml reaction tubes (RCF max. 16,000 x g).
- PCR box or Biological cabinet.
- Vacuum aspirator with flask for removing supernatant.
- Tube racks.
- 1.5-ml sterile polypropylene tubes.
- Refrigerator for 2–8 °C.
- Deep-freezer for ≤ –16 °C.
- Waste bin for used tips.
- Permanent pen for labeling.
- Thermostatic bath or dry block for tubes with controlled temperature for 25–100 °C.
- Personal thermocyclers (for example, Terzik (DNA-Technology, Russia), Gradient Palm Cycler (Corbett Research, Australia), MaxyGene (Axygen Scientific, 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 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 samples and unused reagents in compliance with local authorities' requirements.
- Samples should be considered potentially infectious and handled in biological cabinet in compliance with appropriate biosafety practices.

- Clean and disinfect all sample or reagent spills with 0.5% sodium hypochlorite solutions or another suitable disinfectant.
- Avoid contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes 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 DNA amplification techniques.
- The laboratory process must be one-directional; it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment, and reagents to the area where you carried out the previous step.

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

6. SAMPLING AND HANDLING

Obtaining samples of biological materials for PCR-analysis, transportation and storage are described in manufacturer's handbook [2]. It is recommended that this handbook is read before starting work.

AmpliSens® *HPV* HCR genotype-EPh PCR kit is intended for analysis of DNA extracted with DNA isolation kits from cervical or urethral swabs.

Clinical material:

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 cytobrushes 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 genotype-EPh 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 K1-12-50-CE (for clinical material obtained by methods 1, 2, and 3).
- DNA-sorb-B REF K1-2-50-CE (for clinical material obtained by methods 1, 2, and 3).
- DNA-sorb-C REF K1-6-50-CE (for mucous biopsy material).

Extract DNA in compliance with the manufacturer's protocol.

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 with **PCR-mix-1 *HPV* 16/35** for N reactions:

   5∗(N+1) µl of PCR-mix-1 *HPV* 16/35

   10∗(N+1) µl of 2.5x PCR-buffer red

   0.5∗(N+1) µl of polymerase (TaqF)

When calculating the reaction mixture volume, additional reactions should be included: six controls (one negative and five positive) and one extra reaction.

2. Mix by vortexing the tube with the reaction mixture. Pipette 15 µl of the reaction mixture to PCR tubes.
3. Add above 1 drop of **mineral oil for PCR**.
4. Prepare reaction mixtures with **PCR-mix-1 *HPV* 18/59** and **PCR-mix-1 *HPV* 52/66** as described above. Pipette reaction mixture 16–35 to the blue tubes, reaction mixture 18–59

to the pink tubes, and reaction mixture 52–66 to the green tubes.

5. Arrange tubes in three rows in a PCR tube rack.
6. Using tips with aerosol barrier, add **10 µl DNA samples** obtained from clinical or control samples. DNA of the same clinical sample should be added to the corresponding tubes of all three rows.
7. Carry out the control amplification reactions:

NCA	- Add 10 µl of **DNA-buffer** to the tube for Negative Control of Amplification (NCA).
$C+_{h}$	- Add 10 µl of **Positive Control DNA human** to the tube for Positive Control of human DNA
$C+_{HPV\ 16}$, $C+_{HPV\ 31}$, $C+_{HPV\ 33}$, $C+_{HPV\ 35}$ (reaction mixture 16-35)	- Add 10 µl of **Positive Control DNA HPV type 16,** 10 µl of **Positive Control DNA *HPV* type 31,** 10 µl of **Positive Control DNA *HPV* type 33,** 10 µl of **Positive Control DNA *HPV* type 35** to four blue tubes for Positive Controls of *HPV*.
$C+_{HPV\ 18}$, $C+_{HPV\ 39}$, $C+_{HPV\ 45}$, $C+_{HPV\ 59}$ (reaction mixture 18-59)	- Add 10 µl of **Positive Control DNA *HPV* type 18,** 10 µl of **Positive Control DNA *HPV* type 39,** 10 µl of **Positive Control DNA *HPV* type 45,** 10 µl of **Positive Control DNA *HPV* type 59** to four pink tubes for Positive Controls of *HPV*.
$C+_{HPV\ 52}$, $C+_{HPV\ 56}$, $C+_{HPV\ 58}$, $C+_{HPV\ 66}$ (reaction mixture 52-66)	- Add 10 µl of **Positive Control DNA *HPV* type 52,** 10 µl of **Positive Control DNA *HPV* type 56,** 10 µl of **Positive Control DNA *HPV* type 58,** 10 µl of **Positive Control DNA *HPV* type 66** to four green tubes for Positive Controls of *HPV*.

8.2.2 Amplification

Run the following program in the thermocycler (see Table 1). When the temperature reaches 95 °C (pause mode), insert tubes into the cells of the thermocycler and press the button to continue.

It is recommended to sediment drops from walls of tubes by short centrifugation (1–3 s) before placing them in the thermocycler.

Table 1.

Programming thermocyclers for amplification of DNA of *HPV* HCR types 16, 31, 33, 35; 18, 39, 45, 59 and 52, 56, 58, 66

	Thermocyclers with active temperature adjustment:			Thermocyclers with block temperature adjustment:		
	GeneAmp PCR System 2700 (Applied Biosystems), Terzik (DNA-Techology)			**PTC-100 (MJ Research), Gradient Palm Cycler (Corbett Research), MaxyGene (Axygen)**		
Step	Tempe-rature	Time	Cycles	Tempe-rature	Time	Cycles
1	**95 °C**	**15 min**	1	**95 °C**	**15 min**	1
2	**95 °C**	30 s	42	**95 °C**	30 s	42
	63 °C	30 s		**63 °C**	40 s	
	72 °C	40 s		**72 °C**	50 s	
3	**72 °C**	1 min	1	**72 °C**	1 min	1
4	**10 °C**	storage		**10 °C**	storage	

Amplification in thermocyclers with block temperature adjustment lasts for 2 h 30 min; in thermocyclers with active temperature adjustment, for 1 h 50 min.

After the reaction is finished, PCR tubes must be collected and transferred to the room for PCR product analysis.

The amplification products are analyzed by separation of DNA fragments in agarose gel.
The amplified samples can be stored at room temperature for 16 h and at 2–8 °C for 1 week (be sure to warm the samples to room temperature before running electrophoresis).

9. DATA ANALYSIS

It is recommended to use the following detection agarose kit:

- EPh variant genotype-300, **REF** K6-300-CE.

Analysis of results is based on the presence or absence of specific bands of amplified DNA in high resolution agarose gel (3%). The length of the gel should be no less than 5 cm (when using “SE-2” camera, insert 3 racks instead of 6). Ensure that the leading dye (cresol red) has migrated two thirds of the gel length (3.5 cm).

Prepare **control samples of electrophoresis** in detection area:

$CPh+_{16\text{-}35}$ - mix in one clean tube the content of four blue tubes containing amplified products of control panel samples $C+_{HPV\ 16}$, $C+_{HPV\ 31}$, $C+_{HPV\ 33}$, $C+_{HPV\ 35}$

$CPh+_{18\text{-}59}$ - mix in one clean tube the content of four pink tubes containing amplified products of control panel samples $C+_{HPV\ 18}$, $C+_{HPV\ 39}$, $C+_{HPV\ 45}$, $C+_{HPV\ 59}$

$CPh+_{52\text{-}66}$ - mix in one clean tube the content of four green tubes containing

amplified products of control panel samples $C+_{HPV\ 52}$, $C+_{HPV\ 56}$, $C+_{HPV\ 58}$, $C+_{HPV\ 66}$

The tube with PCR-mix-1 *HPV* 16/35 contains the primers for amplifying *HPV* types 16, 31, 33, 35 DNA; the tube with PCR-mix-1 *HPV* 18/59 contains primers for amplifying *HPV* types 18, 39, 45, 59 DNA; and the tube with PCR-mix-1 *HPV* 52/66 contains primers for amplifying *HPV* types 52, 56, 58, 66 DNA. Each tube contains primers for amplifying the human genome (β-globin gene) DNA fragment.

Table 2.

The length of specific amplified DNA fragments

PCR-mix-1 R *HPV* 16/35			**PCR-mix-1 R *HPV* 18/59**			**PCR-mix-1 R *HPV* 52/66**		
Virus genotype	**The length of specific amplified DNA fragment**	**The length of internal control amplified fragment**	**Virus genotype**	**The length of specific amplified DNA fragment**	**The length of internal control amplified fragment**	**Virus genotype**	**The length of specific amplified DNA fragment**	**The length of internal control amplified fragment**
HPV 16	325 bp	723 bp	*HPV* 18	425 bp	723 bp	*HPV* 52	360 bp	723 bp
HPV 31	520 bp		*HPV* 45	475 bp		*HPV* 56	325 bp	
HPV 33	227 bp		*HPV* 39	340 bp		*HPV* 58	240 bp	
HPV 35	280 bp		*HPV* 59	395 bp		*HPV* 66	304 bp	

Put on a protective mask or use a glass filter while watching and photographing the gel.

9.1. Interpretation of results

Table 3.

Results for controls

Control	**Controlled step**	**Specific bands in agarose gel**				**Interpretation**
		723 bp	**325 bp, 520 bp, 227 bp, 280 bp**	**425 bp, 475 bp, 340 bp, 395 bp**	**360 bp, 325 bp, 240 bp, 304 bp**	
C-	DNA isolation	No	No	No	No	OK
NCA	Amplification	No	No	No	No	OK
$C+_h$	Amplification	Yes	No	No	No	OK
$CPh+_{16-35}$	Electrophoresis	No	Yes	No	No	OK
$CPh+_{18-59}$	Electrophoresis	No	No	Yes	No	OK
$CPh+_{52-66}$	Electrophoresis	No	No	No	Yes	OK

- The sample is considered to be positive if one or more specific bands are present in agarose gel:
  – 325, 520, 227 or 280 bp – if amplified with reaction mixture 16-35 (blue tubes);
  – 425, 475, 340 or 455 bp – if amplified with reaction mixture 18-59 (pink tubes);
  – 360, 325, 240 or 304 bp – if amplified with reaction mixture 52-66 (green tubes);
  – and the band of Internal Control (**723 bp) –** in all tubes regardless of their color.
- The sample is considered to be negative if only the 723-bp Internal Control band is present.

In addition to the specific bands, fuzzy bands corresponding to primer dimers may appear in

lanes below the 100-bp level.

Line the amplified bands of clinical samples with corresponding bands of CPh+ in order to identify virus genotype (Appendix 1).

10. TROUBLESHOOTING

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

- If results of analysis of control points do not correspond to those listed above (Table 3), the tests should be repeated. Discard any reagents that may be suspect.
- If the Internal Control band (723 bp) is absent in one of three lanes corresponding to the sample, the result of sample analysis is invalid. PCR amplification should be repeated.
- If the Internal Control band (723 bp) is not observed in all three lanes corresponding to the sample, the result of sample analysis is invalid and analysis of this sample must be repeated from the DNA extraction stage. If a similar result is obtained for the second time, sampling should be repeated.
- The appearance of nonspecific bands of different molecular weight in lanes may be caused by the lack of “hot start” or an inappropriate temperature regime in the thermocycler. In this case, the results of analysis are invalid.
- The appearance of specific bands in the lanes corresponding to negative controls (NCA, C–) suggests contamination of reagents or samples. In such cases, the results of analysis are considered to be invalid. Analysis of all samples must be repeated and measures to detect and eliminate the source of contamination must be taken.

If you have any further questions or encounter problems, please contact our Authorized Representative in the European Community.

11. TRANSPORTATION

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

12. STABILITY AND STORAGE

All components of **AmpliSens® *HPV* HCR genotype-EPh** PCR kit (except for polymerase (TaqF) are to be stored at 2–8 ºC when not in use. All components of **AmpliSens® *HPV* HCR genotype-EPh** PCR kit are stable until the expiration date on the label. The shelf life of reagents before and after the first use is the same, unless otherwise stated.

Polymerase (TaqF) is to be stored at temperature from minus 24 to minus 16 ºC when not in use.

13. SPECIFICATIONS

13.1. Sensitivity

Analytical Sensitivity of **AmpliSens® *HPV* HCR genotype-EPh** PCR kit is no less than

$5x10^3$ genome equivalents per 1 ml of sample (GE/ml).

The claimed analytical features of AmpliSens® *HPV* HCR genotype-EPh PCR kit are guaranteed only when additional reagent kits DNA-sorb-AM or DNA-sorb-B or DNA-sorb-C and EPh are used.

13.2. Specificity

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

14. REFERENCES

1. Wieland U, Pfister H. Molecular diagnosis of persistent human papilloma virus infections.Intervirology. 1996; 39(3):145-57.
2. Handbook “Sampling, transportation, storage of clinical material for PCR diagnostics”, developed by Federal Budget Institute of Science “Central Research Institute for Epidemiology”, Moscow, 2008.

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 **AmpliSens® *HPV* HCR genotype-EPh** PCR kit is tested against predetermined specifications to ensure consistent product quality.

16. KEY TO SYMBOLS USED

REF	Catalogue number		Sufficient for
LOT	Batch code		Expiration Date
IVD	*In vitro* diagnostic medical device		Consult instructions for use
VER	Version	**NCA**	Negative control of amplification
	Temperature limitation	**C–**	Negative control of extraction
	Manufacturer	**C+$_{h}$**	Positive Control DNA human
	Date of manufacture	**IC**	Internal control
EC REP	Authorised representative in the European Community	**CPh+$_{16-35}$** **CPh+$_{18-59}$** **CPh+$_{52-66}$**	Control samples of electrophoresis
	Caution		

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
09.12.10	Cover page	The phrase “For Professional Use Only” was added
	Intended use	The phrase “The results of PCR analysis are taken into account in complex diagnostics of disease” was added.
	Content	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
	Key to Symbols Used	The explanation of symbols was corrected
22.06.11 VV	Cover page, text	The name of Institute was changed to Federal Budget Institute of Science “Central Research Institute for Epidemiology”