ECOLI

eSens

CE

IVD

For Professional Use Only

eSens HPV HR 14 genotype QT PCR kit

REF ES3081A

Instructions for Use

1 INTENDED USE

eSens HPV HR 14 genotype QT PCR kit is an *in vitro* nucleic acid amplification test for the qualitative and quantitative detection and differentiation of *human papillomavirus* DNA of high carcinogenic risk (HPV HR) genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 in biological material (vaginal swab, epithelial scrapings from cervical mucosa (ectocervix and endocervix)) by real-time hybridization-fluorescence detection of amplified products.

eSens HPV HR 14 genotype QT PCR kit can detect HPV DNA of 14 genotypes (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) independently. HPV HR are the main etiological factor in the development of cervical cancer and previous high-grade dysplasia. HPVs are the cause of 95% of all cases of cervical cancer. These 14 HPV genotypes are responsible for 94% of HPV-related cervical cancers.

eSens HPV HR 14 genotype QT PCR kit is used in clinical laboratory diagnostics to study biological material collected from persons suspected of having papillomavirus infection, regardless of the form and presence of manifestations.

The material for PCR is DNA samples extracted from the test material using kits nucleic acid extraction kits recommended by the manufacturer.

NOTE: The results of the PCR analysis are considered in the comprehensive diagnosis of the disease.

2 PRINCIPLE OF PCR DETECTION

The principle of testing is based on simultaneous amplification of DNA fragments of HPV genotypes and a DNA fragment of the β-globin gene, which is used as an internal endogenous control (IC Glob). The use of an endogenous internal control allows not only to monitor all phases of the test but also to assess the adequacy of collection and storage of clinical material. The endogenous internal control is a fragment of the human genome. It must always be present in the sample in sufficient quantities to match the number of cells in the sample (10^3 -10^5 cells/ml).

Amplification of HPV HR DNA fragments using specific primers and Taq-polymerase enzyme is performed with DNA samples obtained in the extraction phase.

In real-time PCR, the amplified product is detected using fluorescent dyes. These dyes are coupled to oligonucleotide probes that bind specifically to the amplified product during thermocycling. Real-time monitoring of fluorescence intensity during real-time PCR allows detection of the accumulating product without the need to reopen the reaction tubes after the PCR is complete.

eSens HPV HR 14 genotype QT PCR kit uses a "hot-start" method that significantly reduces the frequency of non-specific reactions. "Hot-start is guaranteed by the use of chemically modified polymerase (TaqF). The chemically modified polymerase (TaqF) is activated by heating to 95 °C for 15 minutes.

The quantitative analysis of HPV HR DNA is based on a linear relationship between the logarithm of the initial concentration of target DNA in the test sample and the cycle threshold (*Ct*) (the cycle of the beginning of the exponential growth of the fluorescent signal). For quantitative analysis, DNA amplification of the test samples is performed simultaneously with DNA calibrators (samples with known target DNA concentration). Based on the results of the amplification of the DNA calibrators, a calibration line is plotted and used to calculate the concentration of target DNA in the test samples.

The HPV DNA concentration is calculated as the ratio of the HPV copy number to the number of epithelial cells in the human mucosa.

eSens HPV HR 14 genotype QT PCR kit contains a system for preventing amplicon contamination by the enzymes uracil-DNA glycosylase (UDG) and deoxyuridine triphosphate (dUTP). The UDG enzyme recognizes and catalyzes the destruction of deoxyuridine-containing DNA but has no effect on deoxythymidine-containing DNA. Deoxyuridine is absent in authentic DNA but always present in amplicons because dUTP is part of the dNTP mixture in the amplification reagents. Because deoxyuridine is contained in contaminating amplicons, they are susceptible to destruction by UDG prior to DNA-target amplification. Therefore, the amplicons cannot be amplified.

The UDG enzyme is thermolabile. It is inactivated by heating at temperatures above 50 °C. Therefore, UDG does not destroy target amplicons that accumulate during PCR.

HPV HR genotype detection is performed in four tubes. The results of amplification of different HPV HR DNA genotypes and IC Glob DNA for each PCR mixture are recorded in the following fluorescent channels:

Table 1

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

3 CONTENT

eSens HPV HR 14 genotype QT PCR kit (ES3081A) contains:

Reagents	Description	Volume, ml	Quantity
PCR-mix-FL HPV 1	clear liquid from colourless to blue-grey in colour	1.2	1 tube
PCR-mix-FL HPV 2	clear liquid from colourless to blue-grey in colour	1.2	1 tube
PCR-mix-FL HPV 3	clear liquid from colourless to blue-grey in colour	1.2	1 tube
PCR-mix-FL HPV 4	clear liquid from colourless to blue-grey in colour	1.2	1 tube
PCR-buffer-H	colourless clear liquid	0.6	4 tubes
C1 HPV genotype	colourless clear liquid	0.2	4 tubes

C2 HPV genotype	colourless clear liquid	0.2	4 tubes
Negative control (C-)*	colourless clear liquid	1.2	2 tubes

*must be used in the extraction procedure as a negative extraction control

eSens HPV HR 14 genotype QT PCR kit is designed for 440 amplification reactions (110 tests) including controls.

4 ADDITIONAL REQUIREMENTS

For sampling and pre-treatment

- Transport medium.
- Cervical kit and samples.
- Endocervical brush.
- Disposable sealed polypropylene tubes of 5.0 ml for sampling.
- Disposable sealed polypropylene tubes of 1.5 and 5 ml for pre-treatment
- Sterile pipette tips (up to 200 µl) and pipette tips with filters (up to 1000 µl).
- Test tube racks.
- Benchtop centrifuge up to 12,000 g (suitable for Eppendorf tubes).
- Vacuum extractor with flask for supernatant removal.

For DNA extraction and amplification

- DNA extraction kit.
- Pipettes (adjustable).
- Sterile pipette tips with filters (up to 100 µl).
- Vortex, PCR box
- Real-time PCR instruments (e.g. Rotor-Gene Q (QIAGEN, Germany), CFX 96 Touch, CFX 96 Opus (Bio-Rad, USA), QuantStudio 5 (Thermo Fisher Scientific), or equivalent).
- Disposable polypropylene tubes:
- Screw or tightly sealed 1,5 ml tubes for the preparation of the reaction mixture.
- thin-walled 0,2 ml PCR tubes with optically clear domed or flat caps or strips of eight 0,2 ml tubes with optically clear caps if a plate-type instrument is used;
- thin-walled 0,2 ml PCR tubes with a flat cap or 0,1 ml PCR Rotor-Gene tubes of four in a strip if a rotor apparatus is used.
- Refrigerator at 2-8 °C.
- Freezer with a range of -24 to -16 °C.
- Container for used tips.
- Disposable powder-free gloves and lab coat.

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 each procedure.
- Store all extracted positive material (samples, controls and amplicons) separately from all other reagents and add them to the reaction mixture in a remote, separate area.
- Thaw all ingredients thoroughly at room temperature before starting the test.
- After thawing, mix the ingredients and spin briefly.
- Wear disposable protective gloves and lab wipes and protect your eyes when handling samples and reagents. Wash your hands thoroughly afterwards.

- Do not eat, drink, smoke, use cosmetics or handle contact lenses in the laboratory workplace.
- Do not use the PCR kit if the inner packaging has been damaged or its appearance has been altered.
- Do not use the PCR kit if the shipping and storage conditions have not been followed according to the instructions for use.
- Do not use the kit after the expiry date.
- Dispose of all samples and unused reagents in accordance with local regulations.
- Samples should be considered potentially infectious and should be handled in the biological cabinet in accordance with appropriate biosafety procedures.
- Clean and disinfect any spilled samples or reagents with a disinfectant such as 0.5% sodium hypochlorite or other suitable disinfectant.
- Avoid inhalation of vapours, contact of samples and reagents with skin, eyes and mucous membranes. Harmful if swallowed. In case of contact with these solutions, immediately rinse the affected area with water and seek medical attention if necessary.
- There is no risk of explosion and ignition if the conditions of transport, operation and storage are observed.
- Safety Data Sheets (SDS) are available on request.
- The PCR kit is intended for single use for PCR analysis of a number of samples (see section "Contents").
- The PCR kit is ready for use according to the instructions for use. Use the PCR kit only for its intended purpose.
- This product should only be used by personnel trained in DNA amplification techniques.
- The workflow in the laboratory must be unidirectional, starting in the extraction area and moving into the amplification and detection area. Do not return samples, equipment and reagents to 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

eSens HPV HR 14 genotype QT PCR kit is designed for the analysis of DNA extracted with DNA extraction kits from biological material (vaginal swab, epithelial scrapings from cervical mucosa).

Sampling

Vaginal swab. The material should be obtained from the posterolateral vaginal vault using a cotton swab or a combination probe in a tube with transport medium - **Transport Medium with Mucolytic Reagent (952-CE)** or PreservCyt (Hologic Inc., USA). Rotate the swab while rubbing it against the surface of the side wall of the vagina. Use the swab to remove as much material as possible. Minimal presence of contaminants such as mucus and blood is acceptable. Transfer the swab to a tube of transport medium. Break off the lower part of the swab and leave it in the transport medium tube. If it is not possible to break off the lower part of the swab, the biological material should be flushed as far as possible into the transport medium tube. To do this, press the swab against the inner wall of the tube and rotate it clockwise and counterclockwise 5-10 times. It is not permissible to use scissors to cut off the bottom of the swab!

Close the tube tightly to prevent an air gap from forming and deforming the inside of the cap. Label the tube. If a **transport medium with a mucolytic agent (952-CE)** is used, its colour may change due to the change in pH.

Store and transport the biological material carried in the transport medium in accordance with the requirements specified in the Instructions for Use of the transport medium used. Only one freeze-thaw cycle is allowed.

Epithelial scraping from the cervical mucosa. Allow access to the cervix using a disposable or non-disposable sterile gynecological speculum. Perform specimen collection using a cervical brush or gynaecological combination probe into a tube of transport medium. Remove mucus and vaginal discharge from the surface of the cervix with a gauze swab before collecting the specimen. Minimal presence of impurities such as mucus and blood is allowed.

Methods of epithelial scraping:

Method 1. A combined gynaecological probe is used for simultaneous collection of epithelium from the endocervix and ectocervix. Place the cervical epithelial scrapings (endocervix and ectocervix) in a 5 ml tube with pre-added **transport medium with mucolytic agent (952-CE)**. The DNAP Cervical Sampler (QIAGEN, Germany) is used. It consists of a cervical cytobrush and a tube with Digene transport medium. A scrapping of the cervical epithelium (endocervix) is placed in the transport medium tube.

Method 2. The DNAPAP Cervical Sampler (QIAGEN, Germany) is used. It consists of a cervical cytobrush and a tube with Digene transport medium. A scrapping of the cervical epithelium (endocervix) is placed in the transport medium tube.

Method 3. A combined gynaecological probe for simultaneous collection of epithelial samples from the endocervix and ectocervix and a liquid-based cytology vial with PreservCyt transport medium (Hologic Inc., USA) are used. Place the cervical epithelial scrapings (endocervix and ectocervix) into the tube of transport medium.

Break off the bottom of the swab and leave it in the tube of transport medium. If it is not possible to break off the bottom of the swab, the biological material should be flushed into the transport medium tube as much as possible. To do this, press the swab against the inner wall of the tube and rotate it clockwise and counterclockwise 5-10 times. It is not permissible to use scissors to cut off the bottom of the swab!

Close the tube tightly to prevent an air gap from forming and deforming the inside of the cap. Label the tube.

Store and transport the biological material carried in the transport medium in accordance with the requirements specified in the Instructions for Use of the transport medium used. Only one freeze-thaw cycle is allowed.

Biological samples can be stored prior to PCR analysis:

- at a temperature of 18 to 25 °C - no longer than 7 days;
- at a temperature of 2 to 8 °C - no longer than 3 months;
- at a temperature of -24 to -16 °C - for 1 year. Only one freeze-thaw cycle is allowed;
- in a liquid-based cytology transport medium at 18 to 25 °C for 1 year.

Pretreatment

Pretreatment is not required for vaginal swabs and cervical scrapings that are taken in mucolytic agent transport medium or Digene transport medium.

Pretreatment is necessary for cervical scrapings that are taken into the transport medium for liquid cytology (epithelial cell concentration).

NOTE: Collect an aliquot of cells for PCR analysis using only disposable filter tips and a disposable tube. It is important to collect an aliquot of cells first for PCR analysis and then for liquid cytology.

Epithelial cells concentrate

Method 1

1. Take the required number of disposable 5 ml tubes equal to the number of samples to be tested. Label the tubes. Shake each tube of the liquid-based cytology sample vigorously to break down the cells. Then carefully open and transfer 2-5 ml of cells (depending on the density of the cell suspension) into the prepared tubes.
2. Leave the tubes in a rack at 18 to 25 °C overnight to allow sedimentation of the cells and centrifuge them on a microcentrifuge **for 10 min** at **600 g** (e.g. **3000 rpm** for the MiniSpin microcentrifuge, Eppendorf).
3. Remove the supernatant from each tube. Do not disturb the cell pellets. Use a new 1000 µl filter tip for each sample and pipette.
4. Carefully transfer the rest of the cell pellet with the supernatant (~1 ml) into a new 1.5 ml tube, using a new filter tip for each sample. Label the tubes and centrifuge at **10,000 g** (e.g. **12,000 rpm** for a MiniSpin microcentrifuge, Eppendorf) for **2 min.**
5. Remove the supernatant from each tube. Do not disturb the cell pellets. Use a new 200 µl filter tip and vacuum aspirator for each sample. Keep **100-200 µl of the pellet** with the supernatant.

Method 2

1. Shake each sample vial vigorously for liquid-based cytology to break down the cells and leave it overnight for the cells to settle.
2. Transfer 0.5-1.0 ml of cells from the bottom of the vial into a new 1.5 ml tube using a 1000 µl tip and pipette. Label the tube.
3. Centrifuge at **10 000 g** (e.g. **12 000 rpm** for the MiniSpin microcentrifuge, Eppendorf) for **2 min.**
4. Remove the supernatant from each tube. Do not disturb the cell pellets. Use a new 200 µl filter tip and vacuum aspirator for each sample. Keep **100-200 µl of the pellet** with the supernatant.

Interfering substances and restrictions on the use of test material samples

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

7 WORKING CONDITIONS

eSens HPV HR 14 genotype QT PCR kit should be used at 18-25 °C.

8 PROTOCOL

8.1 DNA extraction

Any commercial nucleic acid extraction kit, if IVD-CE validated for the indicated specimen types, could be used.

Ecoli Dx, s.r.o. recommends:

- for manual extraction DNA-sorb-AM (K1-12-100-CE) for DNA extraction from vaginal swabs and cervical scrapings
- for automatic extraction **ePure STD DNA Extraction Kit (E2007**)

  For nucleic acid extraction, follow the manual for the respective extraction kit.

8.2 Preparing PCR

8.2.1 Preparing tubes for PCR

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

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

DNA amplification of each biological sample is performed in 4 tubes with different PCR-FL HPV mixtures.

1. Calculate the volume of each reagent needed to prepare the 4 reaction mixtures. For 1 reaction, **10 µl of PCR-mix-FL HPV** and **5 µl of PCR-buffer-H** are required.

   NOTE: The components of the reaction mixture should be mixed just prior to PCR analysis.

2. Thaw the tube with **PCR-mix-FL HPV 1, PCR-mix-FL HPV 2, PCR-mix-FL HPV 3, PCR-mix-FL HPV 4.** Vortex the tubes with all reagents of the kit and then sediment the droplets on the vortex.
3. Preparation of reaction mixtures with each PCR-FL HPV mixture in four tubes. Mix the desired amount of **PCR-FL HPV mix** and **PCR-buffer-H.**

   NOTE: Reaction mixtures should be used within 2 hours after preparation.

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

   For conducting N studies of biological samples in **rotor-type** instruments:

   a) in qualitative format, prepare an additional number of strips (strips of four tubes): **N** (corresponding to N biological samples) **strips for** test samples + **2 strips for** control samples (4 tubes for positive control of amplification (C+) and 4 tubes for negative control of extraction (C-)). For example, 17 strips are required for a study of 15 biological samples.

   b) in the quantitative format, prepare an additional number of strips (strips of four tubes): **N** (corresponding to N biological samples) **strips for** test samples + **3 strips for** control samples (8 tubes for DNA calibrators C1, C2 and 4 tubes for negative extraction control (C-)). For example, 18 strips are required for a study of 15 biological samples.

   For performing N studies of biological samples in **plate-type** instruments:

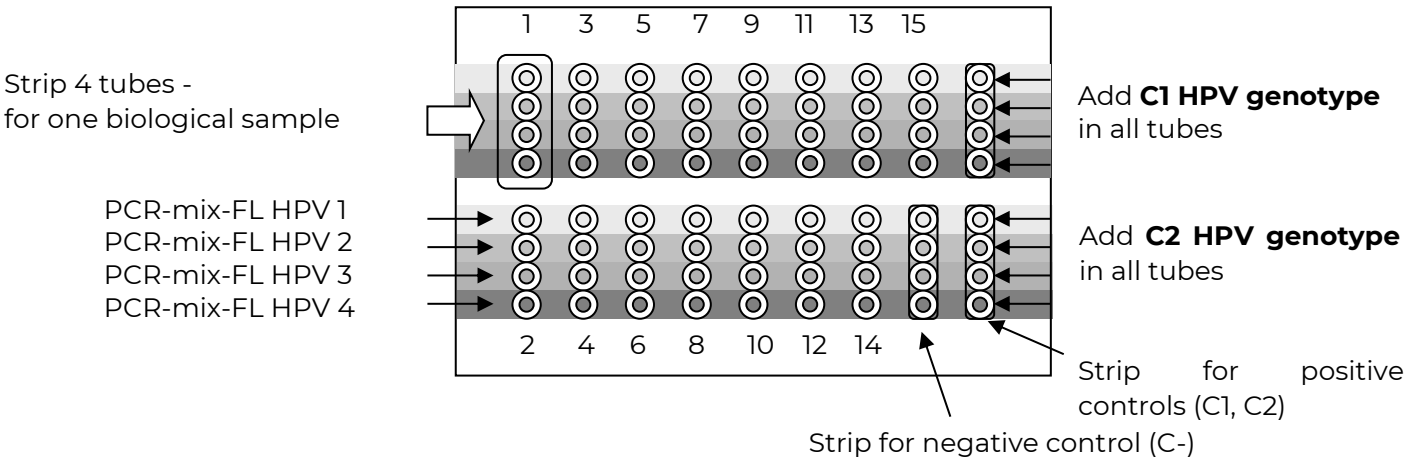
   a) in qualitative format, prepare an additional number of strips (strips of eight tubes) / tubes: **N/2** (1/2 strip needed per biological sample) **strips / N*4** (4 tubes needed per biological sample) **tubes** for test samples + **1 strip / 8 tubes** for control samples (4 tubes for positive control amplification (C+) and 4 tubes for negative control extraction (C-)). For example, 11.5 strips / 92 tubes are required for a study of 21 biological samples.

   b) In the quantitative format, prepare an additional number of strips (strips of eight tubes): **N/2** (1/2 strip needed per biological sample) **strips / N*4** (4 tubes needed per biological sample) **tubes** for test samples + **1.5 strips / 12 tubes** for control samples (8 tubes for DNA calibrators C1, C2 and 4 tubes for negative control extraction (C-)). For example, 12 strips / 96 tubes are required for a study of 21 biological samples.

5. Transfer **15 µl of** each prepared mixture into the appropriate tubes (see diagrams below). Use the rest of the reaction mixture.

For adequate data processing by the software included with the PCR kit, the following reaction mix and DNA sample addition schemes for rotor and plate type instruments must be strictly followed.

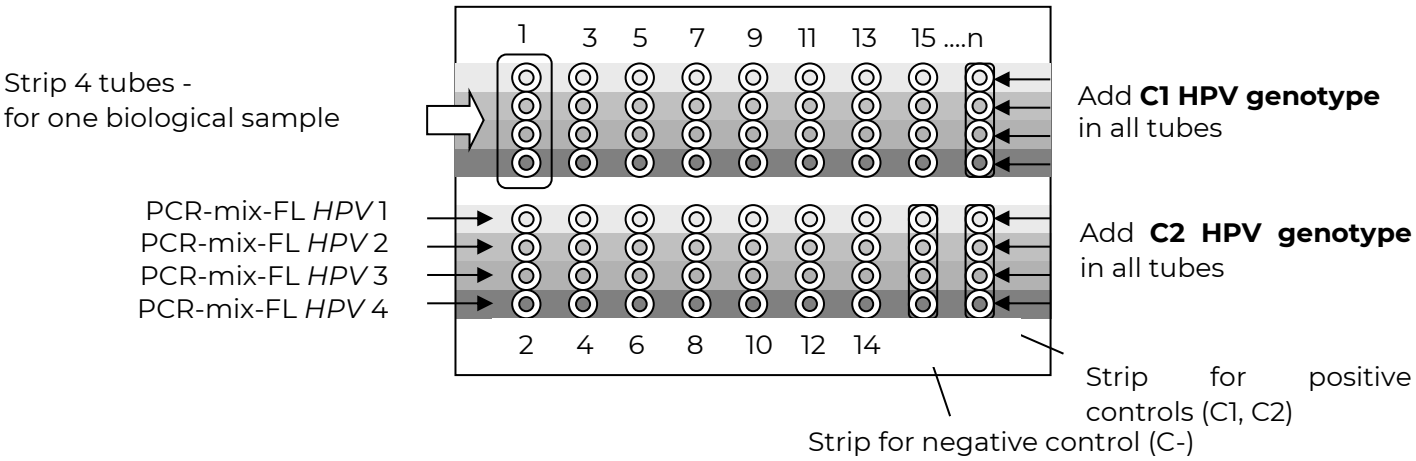
Reagent and biological sample addition scheme for rotor instruments



The numbers of biological samples are given.

Reagent and biological sample addition scheme for plate-based instruments

n = 16 - 21



The numbers of biological samples are given.

6. **Add 10 µl of the DNA samples** obtained in the DNA extraction phase of the test samples to 4 prepared tubes with different reaction mixtures.
   Sample addition schemes for rotor and plate instruments must be followed. Avoid transferring sorption beads with the DNA sample.
7. Perform control amplification reactions:

   For qualitative analysis:

C+	Add 10 µl of the C2 HPV genotype to four tubes with different PCR-FL mixtures and labelled C+ (positive control amplification).
C-	Add 10 µl of the sample extracted from the negative control (C-) to four tubes containing different PCR-FL mixtures and labelled C- (negative control extraction).

For quantitative analysis:

C1	Add 10 µl of the C1 HPV genotype to four tubes with different PCR mixtures - FL and labelled C1 (C1 DNA calibrator).
C2	Add 10 µl of C2 HPV genotype to four tubes with different PCR-FL mixtures and labelled with C2 (C2 DNA calibrator).
C-	Add 10 µl of the sample extracted from the negative control (C-) to four tubes containing different PCR-FL mixtures and labelled C- (negative control extraction).

8.2.2 Amplification

1. Create a temperature profile on your instrument as follows:

Table 2

eSens amplification program

A single amplification program for rotor instruments (For example, Rotor-Gene Q / QIAGEN, Germany.) and wafer instruments (For example, CFX 96 Touch, CFX 96 Opus, QuantStudio 5 or equivalent.).

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

If several tests are performed simultaneously in the "multiprime" format, detection is enabled in the other channels used in addition to those listed. If only the pathogen DNA detection tests are performed simultaneously in one instrument, the first reverse transcription step (50 °C - 15 min) can be omitted to save time.

It is not recommended to combine several tests in one run for rotor instruments.

Table 3

eSens-1 amplification program

	Rotor instruments (For example, Rotor-Gene Q or equivalent.)			Plate type instruments (For example, CFX 96 Touch, CFX 96 Opus, QuantStudio 5 or equivalent.)		
Step	Temperature, °C	Time	Cycles	Temperature, °C	Time	Cycles
1	95	15 minutes	1	95	15 minutes	1
2	95	5 s	5	95	5 s	5
	60	20 s		60	20 s	
	72	15 s		72	15 s	
3	95	5 s	40	95	5 s	40
	60	20 s Fluorescence acquisition		60	30 s Fluorescence acquisition	
	72	15 s		72	15 s	

The fluorescence signal is detected in the **FAM**, **JOE**, **ROX** and **Cy5** fluorophore channels.

NOTE: **eSens-1** is a universal program for performing tests to identify *human papillomaviruses* (HPV HR) and detect sexually transmitted diseases and other infections of the reproductive system using eSens PCR kits. Therefore, any combination of these tests can be performed simultaneously on the same instrument.

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

2. Adjust the sensitivity of the fluorescence channel according to the *information in the datasheet*.
3. Insert the tubes into the reaction module of the instrument.

NOTE:It is recommended to briefly centrifuge the droplets from the tube walls (1-3 s) before inserting them into the instrument. In case of incomplete filling of the plate apparatus, place the empty tubes on the edges of the reaction module.

4. Run the amplification program with fluorescence detection.
5. After completing the amplification program, analyze the results.

9 DATA ANALYSIS

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

Table 4

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

The results are interpreted based on the intersection (or non-intersection) of the fluorescence curve with a threshold line set at a specific level corresponding to the presence (or absence) of the *Ct* value of the DNA sample in the corresponding column of the results table.

NOTE: Analysis of results is performed using the above algorithm or **eSens HPV HR 14 genotype QT excel** (version 1.0).

Qualitative analysis

The principle of interpretation is as follows:

- DNA of HPV HR genotypes 16, 39, 33, 58 **is detected if the** *Ct* value determined in the FAM fluorophore channel does not exceed the *Ct* threshold value. In addition, the fluorescence curve of the sample should exceed the cutoff line in the region of typical exponential fluorescence growth.
- DNA of HPV HR genotypes 31, 45, 35, 52 **is detected if the** *Ct* value determined in the JOE fluorophore channel does not exceed the *Ct* threshold value. In addition, the fluorescence curve of the sample should exceed the cut-off line in the region of typical exponential fluorescence growth.
- DNA of HPV HR genotypes 18, 59, 68, 66 **is detected if the** *Ct* value determined in the ROX fluorophore channel does not exceed the *Ct* threshold. In addition, the fluorescence curve of the sample should exceed the cutoff line in the region of typical exponential fluorescence growth.
- DNA of HPV HR genotypes 56, 51 **is detected if the** *Ct* value determined in the Cy5 fluorophore channel (in tubes with PCR-FL mixture of HPV 3 and 4, respectively) does not exceed the *Ct* threshold value. In addition, the fluorescence curve of the sample should exceed the cut-off line in the region of typical exponential fluorescence growth.
- HPV HR DNA **is not detected** in the sample if the *Ct* value is not determined (fluorescence curve does not cross the threshold line or the *Ct* value exceeds the threshold value) in the channels for the fluorophores FAM, JOE, ROX and also Cy5 (in tubes with PCR-mix-FL HPV 3 and 4), while

the *Ct* value determined in the channel for the fluorophore Cy5 (in tubes with PCR-mix-FL HPV 1 and 2) does not exceed the threshold value.

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

NOTE: The Ct limit values are given in the Technical Sheet supplied with the PCR kit.

The result of the qualitative analysis is considered reliable only if the results obtained in the amplification and extraction checks are correct (according to Table 5 and the Technical Sheet accompanying the PCR kit).

Table 5

Control results (qualitative analysis)

Check	Stages for control	***Ct* value in the fluorophore channel**			
		FAM	**JOE**	**ROX**	**Cy5**
C-	DNA extraction	Missing in all 4 tubes	Missing in all 4 tubes	Missing in all 4 tubes	Missing in all 4 tubes
C+	PCR	< threshold value in all 4 tubes	< threshold value in all 4 tubes	< threshold value in all 4 tubes	< threshold value in all 4 tubes

Quantitative analysis

Based on the obtained *Ct* values and the specified concentration values of the DNA calibrators (C1 and C2), a calibration line is plotted and the number of copies of each detected HPV HCR genotype and human DNA per ml of test and control samples is calculated. The values obtained are used to calculate the amount of HPV DNA per $1x10^5$ human cells. The normalized concentration values reflect the copy number of the pathogen relative to human cells. In addition, the human DNA concentration values provide an estimate of the quality of the biological material collected.

HPV DNA concentration values are calculated according to the formula:

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

where: 2 is the coefficient of conversion of the number of DNA copies to the number of cells.

Normalised concentration values reflect the copy number of the pathogen relative to human cells. In addition, the number of human cells allows to evaluate the quality of the collection of biological material.

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

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

COMMENT: DNA calibrator concentration values are listed in the Technical Sheet included with the PCR kit.

NOTE: For subsequent runs with a given batch of the eSens HPV HR 14 Genotype QT PCR kit, the results of DNA Calibrator C1 obtained in a previous run on this instrument can be used. To do this, export the C1 DNA calibrator results using the instrument software. In this case, you only need to run the reaction with the DNA C2 calibrator.

Table 6

Interpretation of results for test samples (quantitative analysis)

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

The result of the qualitative analysis is considered reliable only if the results obtained in the amplification and extraction checks are correct (according to Table 7 and the Technical Sheet accompanying the PCR kit).

Table 7

Control results (quantitative analysis)

Check	Stages for control	*Ct* value in the fluorophore channel			
		FAM	JOE	ROX	Cy5
C-	DNA extraction	Missing in all 4 tubes	Missing in all 4 tubes	Missing in all 4 tubes	Missing in all 4 tubes
C1	PCR	**Defined**	**Defined**	**Defined**	**Defined**
C2	PCR	**Defined**	**Defined**	**Defined**	**Defined**

10 TROUBLESHOOTING

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

1. For qualitative analysis, the Ct value determined for the positive control of amplification (C+) in any of the channels is greater than the Ct threshold value or is missing (see Table 5). Amplification and detection should be repeated for all samples.
2. The Ct value is determined for the negative control of extraction (C-) in the channels for the fluorophores FAM and/or JOE and/or ROX and/or Cy5. Contamination of the laboratory with amplification fragments or contamination of reagents, test samples is likely at any stage of the PCR analysis. Measures should be taken to detect and remove the source of contamination. PCR analysis (starting with the DNA extraction phase) should be repeated for all samples where specific DNA has been detected.
3. For quantitative analysis, the Ct value for DNA calibrators C1 and C2 in any designated fluorophore channels is missing (see Table 7). Amplification and detection should be repeated for all samples.
4. For quantitative analysis, the efficiency of E in plotting the calibration curve is less than 80 % or greater than 120 %. Check the correctness of the DNA calibrator concentrations and the correctness of the selected threshold level. If the set DNA calibrator concentrations and threshold level are correct but the efficiency does not match the desired range, amplification and detection must be repeated for all samples.
5. The Ct value is determined for the test sample, while the region of typical exponential fluorescence growth is missing (the graph looks like an approximate straight line). It is necessary to check the correctness of the selected threshold level or baseline calculation parameters. If the result was obtained with the correct threshold level (baseline), amplification and detection should be repeated for this sample.

11 TRANSPORTATION

eSens HPV HR 14 genotype QT PCR kit should be stored at 2-8 °C for a maximum of 5 days. Transport at 2-25 °C for a maximum of 3 days is allowed.

12 STABILITY AND STORAGE

All components of the **eSens HPV HR 14 Genotype QT PCR kit should be** stored at -24 to -16 °C when not in use. All **eSens HPV HR 14 Genotype QT PCR kit** components are stable until the expiration date on the label. The shelf life of reagents before and after first use is the same unless otherwise stated.

NOTE: PCR-mixy-FL HPV should be stored away from light.

13 SPECIFICATIONS

13.1 Sensitivity

Table 8

Test material	Transport Medium	Nucleic acid extraction kit	PCR kit	HPV genotype	Analytical sensitivity, copies/ml*	Linear measurement range, copies/ml
Vaginal smear, scraping of the cervical mucosa (ectocervix and endocervix).	Transport medium with mucolytic agent, Digene transport medium, Transport medium for liquid-based cytology	DNA-sorb-AM, ePure STD DNA Extraction Kit	ES3081A	HPV HR genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	$1x10^3$	$3x10^3$ - $1x10^8$

*Number of copies of viral DNA in biological material placed in the specified transport medium and calculated per 1 ml.

These properties are achieved by following the rules given in the section *Sampling and handling*.

13.2 Specificity

The analytical specificity of the **eSens HPV HR 14 genotype QT PCR kit** is ensured by the selection of specific primers and probes and strict reaction conditions. Primers and probes were checked for possible homology to all sequences published in genebanks by sequence comparison analysis.

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

No non-specific DNA reactions or cross-reactions between HPV genotypes (using highly concentrated samples) were detected in the testing of the above microorganisms. The specificity of the testing was demonstrated by sequencing the detected amplification fragments.

The clinical specificity of the **eSens HPV HR 14 genotype QT PCR kit** has been confirmed in clinical laboratory studies.

For information on known interfering substances, see *Interfering substances and restrictions on the use of test material samples*.

13.3 Reproducibility, repeatability and truthfulness

Reproducibility and repeatability were determined by testing model samples of biological material. Model samples of biological material were prepared by dilution of a quality control sample (QCS) containing HPV HCR DNA (genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) in three concentration ranges (from $5x10^3$ to $1x10^4$, from $5x10^4$ to $1x10^5$, more than $5x10^5$ copies/ml) in biological material free of HPV DNA of other genotypes and DNA of other pathogenic agents.

Table 9

Reproducibility

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

HPV genotype	Initial concentration value, copies/ml	Number of repetitions	Average concentration value, lg	Standard deviation (SD)	Coefficient of variation (CV), %
52		8	4.56	0.09	1.99
56		8	4.66	0.17	3.57
58		8	4.62	0.08	1.76
59		8	4.55	0.09	2.06
66		8	4.73	0.07	1.41
68		8	4.89	0.07	1.48
16	larger than $5x10^5$	8	7.41	0.08	1.14
18		8	7.37	0.03	0.41
31		8	7.39	0.08	1.02
33		8	7.28	0.06	0.80
35		8	7.16	0.05	0.68
39		8	7.25	0.07	0.92
45		8	7.20	0.06	0.80
51		8	7.29	0.08	1.12
52		8	7.09	0.08	1.09
56		8	7.31	0.06	0.76
58		8	7.24	0.06	0.86
59		8	7.25	0.04	0.53
66		8	7.24	0.05	0.71
68		8	7.28	0.05	0.66

Table 10

Repeatability

HPV genotype	Initial concentration value, copies/ml	Total number of repetitions	Average concentration value, lg	Standard deviation (SD)	Coefficient of variation (CV), %
16	from $5x10^3$ to $1x10^4$	16	3.62	0.09	2.43
18		16	3.85	0.11	2.93
31		16	3.74	0.07	1.96

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

HPV genotype	Initial concentration value, copies/ml	Total number of repetitions	Average concentration value, lg	Standard deviation (SD)	Coefficient of variation (CV), %
35		16	7.15	0.05	0.64
39		16	7.31	0.08	1.11
45		16	7.16	0.07	0.97
51		16	7.26	0.09	1.28
52		16	7.08	0.07	1.04
56		16	7.34	0.06	0.78
58		16	7.26	0.10	1.32
59		16	7.03	0.06	0.88
66		16	6.92	0.06	0.94
68		16	7.35	0.04	0.58

Accuracy was determined by QCS testing with a concentration of $1x10^5$ copies/ml.

Table 11

Truthfulness

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

13.4 Diagnostic characteristics

The diagnostic properties of the **eSens HPV HR 14 genotype QT PCR kit** were determined according to international requirements for validation of new assays for HPV DNA detection (Meijer CJ, et al. 2009. Guidelines for human papillomavirus DNA test requirements for primary cervical cancer screening in women 30 years and older / Int. J. Cancer 124:516-520.)

Diagnostic sensitivity of the HPV test for the detection of CIN2+ should not be less than 90% of the sensitivity of the Hybrid Capture 2 (HC2) method according to the international requirements for the validation of new HPV DNA tests. This means that the relative sensitivity is at least 90% and samples should be histologically confirmed (at least CIN2). At least 60 samples should be tested using two HPV tests.

Diagnostic specificity of the HPV test for the detection of CIN2+ should not be less than 98% of the specificity of the Hybrid Capture 2 (HC2) method according to the international requirements for the validation of new *HPV* DNA tests. Collection included at least 800 samples obtained from women aged 30 years and older without cytologically/histologically confirmed CIN2.

907 samples (epithelial scrapings from cervical mucosa) were studied to determine the diagnostic sensitivity and specificity of the kit. Of these samples, 100 are histologically confirmed as CIN2+ and the average age of the patients is 35 years (range 30-65 years). And 807 of all samples obtained from the screening study are with cytologically/histologically confirmed absence of CIN2. The mean age of the women is 39 years (range 30 to 65 years). In addition, 300 vaginal swab samples obtained from the screening study were studied.

Table 12

Test results of the eSens HPV HR 14 genotype QT PCR kit compared to the reference test

Type of samples	Results of the eSens HPV HR 14 genotype QT PCR kit		Results of using the reference test	
			Positive	Negative
Cervical mucosal swab (ectocervix and endocervix) with histologically confirmed moderate or severe dysplasia (CIN2+).	100 samples were tested	Positive	100	0
		Negative	0	0
Smear from the cervical mucosa (ectocervix and endocervix) with normal cytology or mild dysplasia.	807 samples were tested	Positive	145	0
		Negative	0	662
Vaginal swab	300 samples were tested	Positive	112	0
		Negative	0	188

Table 13

Diagnostic features of the eSens HPV HR 14 genotype QT PCR kit

Type of samples	Diagnostic sensitivity*[1] , (with 90% confidence level) in the interval, %.	Diagnostic specificity*[2] , (at 90% confidence level) in the interval, %.
scraping from the cervical mucosa (ectocervix and endocervix).	97.0 - 100	99.5 - 100
Vaginal swab	97.5 - 100	98.5 - 100

*[1] Relative sensitivity compared to the reference test used.

*[2] Relative specificity compared to the reference test used.

14 REFERENCES

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

15 QUALITY CONTROL

The production process, including batch release, is carried out in accordance with an established quality management system certified according to ISO 13485.

16 KEY TO SYMBOLS USED

REF	Catalogue number
LOT	Batch code
IVD	*In vitro* diagnostic medical device
VER	Version
	Temperature limit
	Manufacturer
	Date of manufacture
	Notice

	Caution
	Contains sufficient for <n> tests
	Use-by Date
	Consult instructions for use
	Keep away from sunlight
NCA	Negative control of amplification
C-	Negative control of extraction
C+	Positive control of amplification
IC	Internal control
C1, C2	DNA calibrators

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
01_04/2022		
02_08/2024	Chapter 2 - PRINCIPLE OF PCR DETECTION, Table 1	„gen“ was changed to „gene“
	Through the text	REF number was changed: „(H-2261-1-13-CE)“ to „(ES3081A)“.
	Chapter 8.2.1 - Preparing tubes for PCR	Modification of the scheme.
	Chapter 10 - TROUBLESHOOTING	These sections has been modified.
	Chapter 13.4 - Diagnostic characteristics	

Ecoli Dx, s.r.o. , Purkyňova 74/2

110 00 Praha 1, Česká republika
Tel: +420 325 209 912

Mobil: +420 739 802 523