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For Professional Use Only

AmpliSens® *Clostridium difficile*-EPh PCR kit

Instruction Manual

AmpliSens®

EC REP

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TABLE OF CONTENTS

1. INTENDED USE

AmpliSens® ***Clostridium difficile*-EPh** PCR kit is an in vitro nucleic acid amplification test for qualitative detection of *Clostridium difficile* DNA in the clinical material (feces) and environmental samples (concentrated water samples) 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

Clostridium difficile DNA detection by the polymerase chain reaction (PCR) is based on the amplification of pathogen DNA specific region using specific *Clostridium difficile* primers. After PCR the amplified product is detected in agarose gel. **AmpliSens**® ***Clostridium difficile*-EPh** 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. Wax melts and reaction components mix only at 95 °C.

3. CONTENT

AmpliSens® ***Clostridium difficile*-EPh** PCR kit is produced in 2 forms:

AmpliSens® *Clostridium difficile*-EPh PCR kit variant 50 R (tubes of 0.5 ml), **REF** B23-50-R0,5-CE.

AmpliSens® *Clostridium difficile*-EPh PCR kit variant 50 R (tubes of 0.2 ml), **REF** B23-50-R0,2-CE.

AmpliSens® ***Clostridium difficile*-EPh** PCR kit variant 50 R includes:

Reagent	***Description***	***Volume, ml***	***Amount***
PCR-mix-1-R *Clostridium difficile* ready-to-use single-dose test tubes (*under wax)*	colorless clear liquid	0.005	55 tubes of 0.5 or 0.2 ml
PCR-mix-2 blue	blue clear liquid	0.6	1 tube
Mineral oil for PCR	colorless viscous liquid	2.0	1 dropper bottle
Positive Control DNA *Clostridium difficile* (C+$_{Clostridium\ difficile}$)	colorless clear liquid	0.1	1 tube
DNA-buffer	colorless clear liquid	0.5	1 tube
Negative Control (C-)*	straw-colored clear liquid	1.6	1 tube

* must be used in the isolation procedure as Negative Control of Extraction (see DNA-

sorb-B, **REF** K1-2-50-CE protocol).

AmpliSens® ***Clostridium difficile*-EPh** PCR kit variant 50 R is intended for 55 reactions, including controls.

4. ADDITIONAL REQUIREMENTS

- DNA extraction kit.
- Agarose gel detection kit.
- Disposable powder-free gloves and laboratory coat.
- Pipettes (adjustable).
- Sterile pipette tips with aerosol barriers (up to 200 µl).
- Vortex mixer.
- PCR box.
- Tube racks.
- Personal thermocyclers (for example, GeneAmp PCR System 2400, GeneAmp PCR System 2700 (Applied Biosystems, USA), Omn-E (ThermoHybaid, Germany), Palm-Cycler (Corbett Research Australia), PTC-100 MiniCycler(MJ Research, USA).
- Disposable polypropylene microtubes for PCR (0.5- or 0.2-ml; for example, Axygen, USA).
- Refrigerator for 2–8 °C.
- Deep-freezer for ≤ –16 °C.
- Waste bin for used tips.

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 disposable 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 a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant, such as 0.5% sodium hypochlorite or another 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 DNA amplification techniques.
- The laboratory process must be one directional, it should begin in the Extraction Area move to the Amplification and Detection Areas. 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

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

AmpliSens® ***Clostridium difficile*-EPh** PCR kit is intended for analysis of DNA extracted with DNA isolation kits from:

- *Concentrated water samples (wastewater, water from water bodies, drinking water).*
- *Feces.*

6.1. *Concentrated water samples: wastewater, water from water bodies, drinking water* (1.0–2.0 ml*).* Additional treatment is not required.

6.2. *Fecal* (0.4–1.0 g) *sample. Feces* are taken from a disposable plastic sachet or a plastic container (Petri dish) placed into a chamber-pot or bedpan; feces of infants are taken from a diaper. Approximately 1.0 g of feces should be transferred to a special sterile container.

Deliver sample to a laboratory within 1 day in a container with an icepack.

6.2.1. *Preparation of 10–20 % fecal suspension* (omit liquid feces).

1. Take a 5 ml tube with a tightly sealing cap and pipette 4 ml of saline solution.
2. Transfer 0.4–1.0 g (0.4 - 0.1 ml) of fecal sample with a spatula into prepared tube. Stir well to ensure homogenous suspension. Add 20 % glycerol and store at – 16 °C for 1 month if necessary.

6.2.2. *Preparation of bacterial fraction of fecal sample.*

1. Centrifuge the tube with prepared suspension or liquid feces at 10,000 g for 5 min.
2. Remove 50 µl of the fecal bacterial fraction (top white-yellowish layer of the pellet) using a tip with aerosol barrier and transfer it to a tube with 800 µl of phosphate buffer. If the pellet or the white-yellowish layer between the pellet and the supernatant are absent, take 100 µl of the sample from the tube bottom or from the layer between the pellet and the supernatant, respectively. Phosphate buffer should contain 137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium monophosphate, and 2 mM potassium diphosphate (pH 7.5 ± 0.2). Store phosphate buffer at 2–8°C for 1 year in a tightly sealed polypropylene tube.
3. Thoroughly resuspend the sample and centrifuge at 10,000 g for 5 min.
4. Discard the supernatant, add 300 µl of phosphate buffer, and resuspend the pellet. Use 100 µl of prepared sample for DNA extraction.

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

7. WORKING CONDITIONS

AmpliSens® *Clostridium difficile*-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-B, **REF** K1-2-50-CE.

Extract DNA in compliance with the manufacturer's protocol.

Add the tube for PCE:
90 µl of Negative Control (C-),
10 µl of Positive Control DNA *Clostridium difficile* (C+).

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 required number of PCR tubes with **PCR-mix-1-R *Clostridium difficile*** for amplification of DNA from clinical and control samples.
2. Add **10 µl** of **PCR-mix-2 blue** to the surface of the wax layer of each tube ensuring that it does not fall under the wax and mix with **PCR-mix-1-R *Clostridium difficile***.
3. Add above **1** drop of **mineral oil for PCR** (~ 25 µl).
4. Using tips with aerosol barrier add **10 µl** of **DNA samples** obtained from clinical or

control samples.

5. Carry out the control amplification reactions:

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

C+ - Add 10 µl of **Positive Control DNA *Clostridium difficile*** to the tube labeled C+ (Positive Control of Amplification).

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 precipitate drops from walls of tubes by short centrifugation (1–3 s) before placing them in the thermocycler.

Table 1

Programming thermocyclers for *Clostridium difficile* DNA amplification

	Thermocyclers with active temperature adjustment						Thermocyclers with block temperature adjustment		
	GeneAmp PCR System 2400 (Perkin Elmer), Omn-E (ThermoHybaid), Terzik (DNA-Technology)			**GeneAmp PCR System 2700 (Applied Biosystems), Palm-Cycler (Corbett Research)**			**PTC-100, MiniCycler (MJ Research)**		
Step	Tempe-rature	Time	Cycles	Tempe-rature	Time	Cycles	Tempe-rature	Time	Cycles
0	**95 °C**	pause		**95 °C**	pause		**95 °C**	pause	
1	**95 °C**	5 min	1	**95 °C**	5 min	1	**95 °C**	5 min	1
2	**95 °C**	10 s	42	**95 °C**	10 s	42	**95 °C**	1 min	42
	63 °C	10 s		**63 °C**	25 s		**63 °C**	1 min	
	72 °C	10 s		**72 °C**	25 s		**72 °C**	1 min	
3	**72 °C**	1 min	1	**72 °C**	1 min	1	**72 °C**	1 min	1
4	**10 °C**	storage		**10 °C**	storage		**10 °C**	storage	

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

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

Analysis of amplification products is performed by separation of DNA fragments in agarose gel.

The amplified samples can be stored for 16 h at room temperature, for 1 week at 2–8 °C, and for a long time at –16 °C (warm up samples to room temperature before electrophoretic run).

9. DATA ANALYSIS

It is recommended to use the following detection agarose kit:

- EPh variant 200, **REF** K5-200-CE.

Analysis of results is based on the presence or absence of specific bands of amplified DNA in agarose gel (1.7%). The lengths of specific amplified DNA fragments are:

- *Clostridium difficile* - 420 bp.

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

9.1. Interpretation of results

Table 2

Results for controls

Control	Which step of test is controlled	Specific bands in the agarose gel 420 bp	Interpretation
C-	DNA isolation	No	OK
PCE	DNA isolation	Yes	OK
NCA	Amplification	No	OK
C+	Amplification	Yes	OK

- The sample is considered to be positive for *Clostridium difficile* DNA if the 420-bp band is present in agarose gel.
- The sample is considered to be negative for *Clostridium difficile* DNA if the 420-bp band is absent.

In addition to the specific bands, fuzzy bands corresponding to primer dimers may appear in lanes below the 100-bp level.

10. TROUBLESHOOTING

Analysis results are not taken into account in the following cases:

- If results of control points do not correspond to those listed above (Table 2), the tests are to be repeated.
- If the 420-bp band is not observed in the lane corresponding to positive control (PCE, C+), the result of analysis is irrelevant. This may be caused by clinical processing errors that led to the loss of DNA or inhibition of PCR. In this case, analysis of this sample should be repeated starting from the DNA extraction stage.
- The appearance of the specific 420-bp band in 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.

11. TRANSPORTATION

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

12. STABILITY AND STORAGE

All components of **AmpliSens® *Clostridium difficile*-EPh** PCR kit are to be stored at 2–8 ºC when not in use. All components of the 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.

13. SPECIFICATIONS

13.1. Sensitivity

Analytical Sensitivity of **AmpliSens® *Clostridium difficile*-EPh** PCR kit is no less than $5x10^3$ genome equivalents per 1 ml of sample (GE/ml).

The claimed analytical features of **AmpliSens® *Clostridium difficile*-EPh** PCR kit are guaranteed only when additional kits of reagents, DNA-sorb-B and EPh (manufactured by Federal Budget Institute of Science “Central Research Institute for Epidemiology”), are used.

13.2. Specificity.

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

14. REFERENCES

1. Manual “Sampling, Transportation, and 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® *Clostridium difficile*-EPh** 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	**IC**	Internal control
	Caution	**PCE**	Positive control of Extraction

List of Changes Made in the Instruction Manual

VER	Location of changes	Essence of changes
08.12.10	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
	8.2.2. Amplification	In the table 1 the time was changed from 2 min to 5 min.
17.06.11 VV	Cover page, text	The name of Institute was changed to Federal Budget Institute of Science “Central Research Institute for Epidemiology”