Operating instructions

The Proto-screen reagent kit is designed for the qualitative detection of DNA pathogens of protozoan infestations (*Lamblia Intestinalis Giardia, Blastocystis hominis, Dientamoeba fragilis, Isospora belli, Cryptosporidium parvum, Entamoeba histolytica*) in clinical samples using polymerase chain reaction (PCR).

Test specimens are fecal samples. Only for *in vitro* tests.

PRINCIPLE OF THE METHOD

Sample analysis includes the following steps:

1. Sample preparation (isolation of DNA from the samples of clinical material);
2. Amplification of specific DNA fragments during PCR reaction with real-time detection.

The reagent kit is intended for use with detection amplifiers for real-time PCR (MiniOpticon, BioRad; DT-96, DNA technology or other amplifiers with similar technical characteristics).

COMPOSITION OF THE REAGENT KIT

Table 1

Kit Component	Purpose	Quantity	
Mixtures for amplification:			
		individual package version	bulk version
Lamb PCR mixture	*Lamblia Intestinalis Giardia* DNA detection mix	50 test tubes (0.2 ml), 10 µl each, sealed with wax	1 test tube (1.5 ml), 520 µl
Bh PCR mixture	*Blastocystis hominis* DNA detection mix	50 test tubes (0.2 ml), 10 µl each, sealed with wax	1 test tube (1.5 ml), 520 µl
AmFr/IsoB PCR mixture	*Dientamoeba fragilis/Isospora belli* DNA detection mix	50 test tubes (0.2 ml), 10 µl each, sealed with wax	1 test tube (1.5 ml), 520 µl
Cp/EnHi PCR mixture	*Cryptosporidium parvum/Entamoeba histolytica* DNA detection mix	50 test tubes (0.2 ml), 10 µl each, sealed with wax	1 test tube (1.5 ml), 520 µl
BK PCR mixture	Mixture for testing the quality of DNA isolation procedure	50 test tubes (0.2 ml), 10 µl each, sealed with wax	1 test tube (1.5 ml), 520 µl
Taq Polymerase Solution		5 test tubes of 520 µl each	
Positive control samples kit (Lamb PCS, Bh PCS, AmFr/IsoB PCS, Cp/ EnHi PCS, BK PCS)		5 test tubes of 30 µl each	
Negative control sample (NCS)		1 test tube (150 µl)	

Each set of reagents is accompanied by an Operating Instruction (1 piece) and a Certificate of the Reagent Kit (1 piece).

Stage 1 - isolation of DNA from the specimen. Reagent kits are used to isolate the DNA from the clinical samples, these kits are recommended for use in clinical laboratory diagnostics for the isolation of DNA from feces. Use of express extraction methods for DNA isolation is allowed only if made with additional purification[1].

Stage 2 - PCR amplification and detection of amplification products in real time using the Proto-screen reagent kit

SAMPLE PREPARATION

1. Collection, transportation and storage of the test material should be carried out in strict accordance with the methodological recommendations "Collection, Transportation, Storage of Clinical Materials for PCR Diagnostics", developed by the Federal State Budget Research Institution Central Research Institute of Epidemiology of the Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing, Moscow, 2012.
Fecal samples are used for the analysis. The container with the material should be delivered to the laboratory and stored until the start of the analysis at 2-8 °C. The time from the collection of the material to the start of the analysis should not exceed 24 hours. If a longer storage period is required, the material should be placed in a freezer and stored at a temperature of no more than minus 18°C.

2. Recommendations for the isolation of DNA from fecal samples

It is desirable to isolate the DNA from a liquid material. If the sample is solid (feces), a small amount of the material (no more than 100 mg) is resuspended in a transport medium or saline solution.

When isolating DNA from a solid material, a small amount of the sample must be transferred directly to a test tube with a lysing solution (an approximate amount of the sample being introduced is shown in the figure - at the end of the dispenser tip).

Caution: Using excess material for isolation may result in PCR inhibition.

CONDUCTING AN ANALYSIS

Total volume of the reaction mixture is 25 µl, including the volume of the DNA sample - 5 µl.

ATTENTION! Disposable tips with an aerosol barrier should be used to add reagents, DNA samples, and control samples to the test tubes!

Preparation for PCR (individual package version)

1. Put the required number of test tubes with the mixture for amplification in the test-tube rack (**n + 2** test tubes of each specificity, where **n** is the number of samples to be analyzed, two additional test tubes are intended for the analysis of PCS and NCS;
2. Add 10 µl of thoroughly mixed Taq polymerase solution to each test tube (without damaging the wax layer), close the test tubes. The test tubes are ready for the introduction of DNA samples, PCS and NCS.
3. Add a drop of mineral oil for PCR (approximately 25 µl) to each test tube, plug the test tubes. The test tubes are ready for the introduction of DNA samples, PCS and NCS.

Preparation for PCR (bulk version)

1. Put the required number of empty 0,2 ml test tubes (**n + 2** test tubes of each specificity, where **n** is the number of samples to be analyzed, two additional test tubes are intended for the analysis of PCS and NCS;
2. Thoroughly mix all the amplification mixtures (Lamb PCR mixture, Bh PCR mixture, AmFr/IsoB PCR mixture, Cp/EnHi PCR mixture, BK PCR mixture), and then precipitate the drops using vortex;
3. Add 10 µl of the appropriate amplification mixture to the prepared test tubes;
4. Add 10 µl of a thoroughly mixed Taq polymerase solution to each test tube.

Conducting PCR (similar for the individual package and bulk versions)

1. Alternately opening the caps of the test tubes, add the following to the test tubes:

- To one of the prepared test tubes - 5 µl of NCS;
- To test tubes intended for the analysis of samples - 5 µl of the analyzed samples;
- To the remaining test tube-5 µl of PCS.

2. Place all the test tubes to the detection amplifier unit;
3. Run the amplification program according to the parameters specified in Table 2.

ATTENTION! For AmFr/IsoB and Cp/EnHi PCR mixtures, registration should be carried out via two fluorescence channels (FAM and HEX)!

Registration of the fluorescent signal via the FAM channel: *Lamblia Intestinalis Giardia, Blastocystis hominis, Dientamoeba fragilis, Cryptosporidium parvum*
Registration of a fluorescent signal via the HEX channel: *Isospora belli, Entamoeba histolytica*

REGISTRATION OF RESULTS

Detection of DNA of *Lamblia Intestinalis Giardia, Blastocystis hominis, Dientamoeba fragilis, Cryptosporidium parvum*, as well as of bacterial DNA (used as an internal quality control of DNA isolation from the fecal samples), should be made using **FAM** channel. HEX channel must be used to detect **DNA** of *Isospora belli* and *Entamoeba histolytica*.

Table 2. Amplification program

Amplification mode for the MiniOpticon, BioRad device		
Temperature	Time	Number of cycles
94°C	5 min	1
94°C	5 sec	40
58°C	6 sec *	
72°C	10 sec	
10°C - storage		
Amplification mode for the DT96 device, DNA technology		
Temperature	Time	Number of cycles
94°C	5 min	1
94°C	10 sec	40
58°C	10 sec *	
72°C	10 sec	
10°C - storage		

* - detection of the fluorescent signal

ANALYSIS AND INTERPRETATION OF THE RESULTS

The analysis of the results is carried out using the software of the device used for conducting PCR with real-time detection. The results are interpreted based on the presence (or absence) of the intersection of the fluorescence curve with the threshold line, which determines the presence (or absence) of the Ct threshold cycle value for this sample. The analyzed sample is considered positive if:

- the graph of the increase in fluorescence has the form of an exponential curve;
- the Ct threshold cycle value was determined for the sample;
- the fluorescence curve crosses the threshold line at the exponential growth site.

Interpretation principle:

1. Registration of the results should be started with the results of the amplification of the positive (PCS) and negative (NCS) control samples.

2. The results of the analysis <u>are not taken into account</u> if:

- During the reaction the detecting amplifier **does not register** an exponential increase in the level of fluorescence in a test tube/s with a positive control sample through the FAM channel; **in this situation, it is necessary to re-examine all the samples using the PCR mixture, which did not pass positive control**;
- An exponential increase in the level of fluorescence **is registered** through the FAM or HEX channel in a test tube with NCS. **It is necessary to take measures to eliminate contamination in the PCR laboratory and re-examine all the samples.**
- In the sample being examined in a test tube with BK there is no registered exponential growth of fluorescence level or Ct threshold cycle value exceeds the permissible value specified in the kit certificate; this indicates a poor quality of DNA extraction; **in this situation, we need to re-analyse this sample, starting from the stage of DNA extraction**. **When the result is repeated, it is concluded that the quality of the collection or storage of clinical material is poor, and the sample should be taken again.**

3. The results of the analysis <u>are taken into account</u> if:

- During the reaction, an exponential increase in the level of fluorescence in a test tube with a positive control sample is registered through the FAM or HEX channel; and the value of the Ct threshold cycles for each of the PCS does not exceed the permissible values specified in the kit certificate;
- During the amplification process, there is no fluorescent signal in the test tube with the NCS through the FAM or HEX channel; for BK PCR mixture the Ct threshold cycle value does not exceed the permissible value specified in the kit certiicate;
- In the test sample, an exponential increase in the level of fluorescence in the test tube with BK is registered and the value of the Ct threshold cycle does not exceed the permissible value specified in the kit certiicate.

4. The sample is **positive** for the presence of DNA of pathogens of protozoan infestations (*Lamblia Intestinalis Giardia, Blastocystis hominis, Dientamoeba fragilis, Isospora belli, Cryptosporidium parvum, Entamoeba histolytica*), if the threshold cycle value is determined for this sample, and the fluorescence curve of this sample must cross the threshold line at the site of exponential growth of fluorescence (table 3).

- The sample is **negative** for the presence of DNA of pathogens of protozoan infestations (*Lamblia Intestinalis Giardia, Blastocystis hominis, Dientamoeba fragilis, Isospora belli, Cryptosporidium parvum, Entamoeba histolytica*), if the threshold cycle value is absent (not determined) for this sample (the fluorescence curve of this sample does not cross the threshold line) (Table 3).
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Table 3. The principle of interpretation of the results of the analysis

	Fluorescence			
	via the FAM channel		**via the HEX channel**	
	The sample is positive for the presence of DNA of pathogens of protozoal infestations	The sample is negative for the presence of DNA of pathogens of protozoal infestations	The sample is positive for the presence of DNA of pathogens of protozoal infestations	The sample is negative for the presence of DNA of pathogens of protozoal infestations
	Lamblia Intestinalis Giardia			
Lamb PCR mixture	+	-		
Lamb PCS	+	+		
NCS	-	-		
BK PCR mixture	+	+		
	Blastocystis hominis			
Bh PCR mixture	+	-		

Bh PCS	+	+		
NCS	-	-		
BK PCR mixture	+	+		
	Dientamoeba fragilis		*Isospora belli*	
AmFr/IsoB PCR mixture	+	-	+	-
AmFr/IsoB PCS	+	+	+	+
NCS	-	-	-	-
BK PCR mixture	+	+		
	Cryptosporidium parvum		*Entamoeba histolytica*	
Cp/EnHi PCR mixture	+	-	+	-
Cp/EnHi PCS	+	+	+	+
NCS	-	-	-	-
BK PCR mixture	+	+		

KIT TRANSPORTATION AND STORAGE CONDITIONS

Shelf life of the reagent kit is 6 months from the date of manufacture.
Conditions of storage of the Proto-screen reagent kit and its individual components are stated on the packaging.
Test tubes with mixtures for amplification should be stored in a dark place at a temperature of 2 to 8°C. The solution of Taq polymerase, PCS and NCS must be stored at a temperature of 2 to 8 °C.