Arbeitsanleitung/Manual

MutaPLEX® Blastocystis

real time PCR kit

Für den qualitativen Nachweis
der DNA von Blastocystis hominis
sowie der Subtypengruppe ST1, ST3, ST4 and ST7

For the qualitative detection
of the DNA of Blastocystis hominis
as well as the subtype group ST1, ST3, ST4 and ST7

Gültig ab / Valid from 2019-07-15

REF **KG191896** Σ 96 -20 °C IVD CE

Immundiagnostik AG, Stubenwald-Allee 8a, 64625 Bensheim, Germany
Tel.: +49 6251 70190-0 Fax: + 49 6251 70190-363
e.mail: info@immundiagnostik.com www.immundiagnostik.com

Table of Contents

1 INTENDED USE

The MutaPLEX® Blastocystis real time PCR is an assay for the qualitative in vitro detection of purified DNA of *Blastocystis hominis* and the subtype group ST1, ST3, ST4 and ST7 extracted from biological specimens.

2 BACKGROUND INFORMATION

Blastocystis anaerobic parasites are commonly found in the digestive tract in species of several animal groups. It is the most prevalent protozoon found in human fecal samples and has widespread geographic distribution. The role of *Blastocystis* as a human pathogen remains unclear, but studies have associated *Blastocystis* with acute or chronic digestive disorders. Knowledge about its life cycle is equally limited, and various morphological forms, including the cyst and vacuolar forms that are commonly found in feces, have previously been described. The cyst represents the smallest (2 to 5 µm in diameter) form of the organism and is responsible for the environmental dissemination of the parasite. The results of *in vivo* experiments previously performed with mice have suggested that cysts represent the infectious stage, but the most common form detected during parasitological examination of feces is the vacuolar form (2 to 200 µm in diameter). The vacuolar form also represents the major parasitic stage observed in *in vitro* cultures of *Blastocystis* parasites.

Recent molecular studies revealed high genetic diversity among *Blastocystis* strains, identifying 10 different subtypes (ST1 to ST10) that are defined by the sequence of a 600 bp region of the gene encoding the 18S rRNA of the small ribosomal subunit (SSU rRNA). Subtype distribution differs among hosts such as mammals and birds, but recent observations indicate that numerous subtypes previously considered "zoonotic" are also found in humans. Studies of zookeepers suggest direct transmission of *Blastocystis* from animals to humans, while cyst detection in Scottish and Malaysian sewage evidences waterborne transmission. Subtypes ST1 to ST9 have been recovered from human fecal samples, with ST3 as the predominant ST followed by ST1, ST2 and ST4. Subtypes ST5 to ST9 are rarely found in human feces. Some subtypes have a particular distribution, such as avian subtypes ST6 and ST7, which are more frequently found in Asia and the Middle East. Prevalence levels also differ between areas and are higher in developing countries, reaching 60 % in Indonesian children. *Blastocystis* species are also widely observed in developed countries, including the United States (23 %), France (3 %) and the United Kingdom (3.9 %).

Blastocystosis is generally associated with nonspecific symptoms such as diarrhea and/or abdominal pain. There are reports of acute gastroenteritis and cutaneous disorders in some cases, while other studies have indicated that *Blastocystosis* may be correlated to chronic symptoms. There is evidence that *Blastocystis* species are associated with irritable bowel syndrome. Numerous studies have tackled the

pathogenic ambivalence of *Blastocystis*. A majority of these data focused on parasite factors in an attempt to correlate parasite density and/or subtype with pathogenic power, with few studies addressing host factors. A high prevalence of *Blastocystis* in HIV patients was previously found to be associated with clinical relevance in severely immunocompromised subjects. There are case reports of *Blastocystosis* from other immunocompromised patients, and one study focused on patients suffering from hematological malignancies , but it was limited to symptomatic patients.

3 PRINCIPLE OF THE TEST

The MutaPLEX® Blastocystis real time PCR contains specific primers and dual-labelled probes for the amplification and detection of the DNA of *Blastocystis hominis* and the subtypes ST1, ST3, ST4 and ST7. The presence of nucleic acids is detected by an increase in fluorescence due to hydrolysis of the probes during amplification. The fluorescence signals of the specific probes are measured in the FAM (ST1, ST3, ST4 and ST7) and ROX (*Blastocystis hominis*) channels.

Furthermore, MutaPLEX® Blastocystis real time PCR contains a control DNA, which is added during DNA extraction and detected in the same reaction by a differently labeled probe.

The control DNA allows the detection of PCR inhibition and acts as control for the isolation of the nucleic acid from the clinical specimen. The fluorescence of the control DNA is measured in the HEX channel.

4 PACKAGE CONTENTS

The reagents supplied are sufficient for 96 reactions.

Table 1: Components of the MutaPLEX® Blastocystis real time PCR Kit.

Label	Lid Colour	Content
Reaction mix	yellow	1 x 1536 µl
Positive control	red	1 x 100 µl
Negative control	green	1 x 100 µl
Control DNA	colourless	1 x 480 µl

5 EQUIPMENT AND REAGENTS TO BE SUPPLIED BY USER

- DNA extraction kit (e. g. MutaCLEAN® Universal RNA/DNA, KG1038, or NukEx Complete Mag RNA/DNA, KG1020)
- PCR grade water

- Sterile microtubes
- Pipets (adjustable volume)
- Sterile, DNase/RNase-free disposable pipet tips with aerosol barriers
- Table centrifuge
- Vortex
- Real time PCR instrument
- If using LightCycler® 480 (Roche): colour compensation kit
- Optical PCR reaction tubes or optical PCR reaction plates
- Optional: Liquid handling system for automation

6 TRANSPORT, STORAGE AND STABILITY

The MutaPLEX® Blastocystis real time PCR kit is shipped on dry ice or cool packs. All components must be stored at maximum -20 °C in the dark immediately after receipt. Do not use reagents after the date of expiry printed on the package. Up to 20 freeze and thaw cycles are possible.

For convenience, opened reagents can be stored at 2–8 °C for up to 6 months.

Protect kit components from direct sunlight during the complete test run.

7 WARNINGS AND PRECAUTIONS

- Read the Instructions for use carefully before using the product.
- Use of this product is limited to personnel specially instructed and trained in the techniques of real-time PCR procedures.
- Specimens should always be treated as infectious and/or biohazardous in accordance with safe laboratory procedures.
- Avoid microbial and nuclease (DNase/RNase) contamination of the eluates and the components of the kit.
- Always use DNase/RNase-free disposable pipette tips with aerosol barriers.
- Always wear protective disposable powder-free gloves when handling kit components.
- Use separated and segregated working areas for (1) sample preparation, (2) reaction setup and (3) amplification/detection activities. The workflow in the laboratory should proceed in unidirectional manner. Always wear disposable gloves in each area and change them before entering a different area.
- Dedicate supplies and equipment to the separate working areas and do not move them from one area to another.

- Store positive and/or potentially positive material separated from all other components of the kit.
- Do not open the reaction tubes/plates post amplification, to avoid contamination with amplicons.
- Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organisations.
- Do not autoclave reaction tubes after the PCR, since this will not degrade the amplified nucleic acid and will bear the risk to contaminate the laboratory area.
- Discard sample and assay waste according to your local safety regulations

8 SAMPLE MATERIAL AND PREPARATION

Purified DNA is suitable for downstream processing in real time PCR. For the extraction and purification of DNA from various biological materials, commercial kits are available. The operator needs to evaluate the suitability of the respective DNA extraction kit.

Commercial kits for DNA isolation such as the following are recommended:

- MutaCLEAN® Universal RNA/DNA, KG1038
- NukEx Complete Mag RNA/DNA, KG1020

Important: In addition to the samples always run a water control in your extraction. Treat this water control analogous to a sample.

Comparing the amplification of the control DNA in the sample to the amplification of the internal control in the water control will give insights on possible inhibitions of the real time PCR. Furthermore, possible contaminations during DNA extraction will be detectable.

If the real time PCR is not performed immediately, store extracted DNA according to the instructions given by the DNA extraction kit's manufacturer.

9 CONTROL DNA

A control DNA can be used as extraction control or only as inhibition control. This allows the user to control the DNA extraction procedure and to check for possible real time PCR inhibition.

9.1 Control DNA used as extraction control

Add 5 µl control DNA per extraction (5 µl x (N+1)). Mix well. Perform the DNA isolation according to the manufacturer's instructions. Please follow protocol A.

The control DNA must be added to the lysis buffer of the extraction kit.

9.2 Control DNA used as internal control of the real time PCR

If only inhibition will be checked, please follow protocol B.

10 REAL TIME PCR

10.1 Important points before starting

- Please pay attention to the chapter **„**Warnings and Precautions**"**.
- Before setting up the real time PCR, familiarise yourself with the real time PCR instrument and read the user manual supplied with the instrument.
- The programming of the thermal profile should take place before the PCR set up.
- In every PCR run, a positive control and a negative control should be included.
- Before each use, all reagents - except the enzyme - should be thawed completely at room temperature, thouroughly mixed, and centrifuged very briefly.

10.2 Procedure

If the control DNA is used to control both, the real time PCR and the DNA isolation procedure, please follow protocol A. If the control DNA is solely used to detect possible inhibition of the real time PCR, please follow protocol B.

Protocol A

The control DNA was added during DNA extraction (chapter „Control DNA"). In this case, prepare the master mix according to table 2.

The master mix contains all of the components needed for PCR except the sample. Prepare a volume of master mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 2: Preparation of the master mix (control DNA was added during DNA extraction)

Volume per reaction	Volume master mix
16.0 µl reaction mix	16.0 µl x (N+1)

Protocol B

The control DNA is used for the control of the real time PCR only (see chapter „Control DNA"). In this case, prepare the master mix according to table 3.

The master mix contains all of the components needed for real time PCR except the sample. Prepare a volume of master mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Important: Dilute the control DNA 1:10 in PCR-grade water (e.g. 1 µl control DNA + 9 µl PCR-grade water) before adding it to the master mix.

Table 3: Preparation of the master mix (control DNA is added directly to the master mix)

Volume per reaction	Volume master mix
16.0 µl Reaction Mix	16.0 µl x (N+1)
0.5 µl Control DNA* (diluted 1:10)	0.5 µl x (N+1)*

*The increase in volume caused by adding the control DNA is not taken into account when preparing the PCR assay.

Protocol A and B: real time PCR set up

- Place the number of optical PCR reaction tubes needed into the respective tray of the real time PCR instrument.
- Pipet **16 µl** of the master mix into each optical PCR reaction tube.
- Add **4 µl** of the eluates from the DNA isolation (including the eluate of the water control), the positive control and the negative control to the corresponding optical PCR reaction tube (table 4).
- Close the optical PCR reaction tubes immediately after filling in order to reduce the risk of contamination.

Table 4: Preparation of the real time PCR

Component	Volume
Master mix	16.0 µl
Sample	4.0 µl
Total volume	20.0 µl

10.3 Instrument settings

For the real time PCR use the thermal profiles shown in table 5.

Table 5: real time PCR thermal profile

Description	Time	Temperature	No of cycles
Initial Denaturation	5 min	95 °C	1
Amplification			45
Denaturation	10 s	95 °C	
Annealing and extension	40 s	60 °C	
	Aquisition at the end of this step		

Dependent on the real time PCR instrument used, further instrument settings have to be adjusted according to table 6.

Table 6: Overview of the instrument settings required for the MutaPLEX® Blastocystis real time PCR.

Real time PCR Instrument	Parameter Reaction Mix	Detection Channel	Notes		
LightCycler 480II			Colour Compensation required		
			Melt Factor	**Quant Factor**	**Max Integration Time (s)**
	ST1, 3, 4, 7	465–510	1	10	1
	B. hominis	533–610	1	10	2
	Control DNA	533–580	1	10	2
Stratagene Mx3000P / Mx3005P	ST1, 3, 4, 7	FAM	Gain 8	Reference Dye: None	
	B. hominis	ROX	Gain 1		
	Control DNA	HEX	Gain 1		
Agilent Aria Mx BioRad CFX 96	ST1, 3, 4, 7	FAM		Reference Dye: None	
	B. hominis	ROX			
	Control DNA	HEX			
ABI 7500	ST1, 3, 4, 7	FAM	Option Reference Dye ROX: NO		
	B. hominis	ROX			
	Control DNA	JOE			

Real time PCR Instrument	Parameter Reaction Mix	Detection Channel	Notes		
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	ST1, 3, 4, 7 *B. hominis* Control DNA	Green Orange Yellow	Gain 5 Gain 5 Gain 5		
mic qPCR Cycler	ST1, 3, 4, 7 *B. hominis* Control DNA	Green Orange Yellow	Gain 8 Gain 10 Gain 10		

11 DATA ANALYSIS

The interpretation of the test results is described in table 7.

Table 7: Interpretation of results.

Signal/Ct Values			Interpretation
FAM ST1, 3, 4, 7	ROX B. hominis	HEX Control DNA	
positive	positive	positive or negative*	Positive result, the eluate contains *B. hominis* DNA of the subtypes ST1, ST3, ST4 or ST7.
negative	positive	positive or negative*	Positive result, the eluate contains *B. hominis* DNA.
negative	negative	≤ 34**	Negative result, the eluate contains no *B. hominis* DNA.
negative	negative	negative or > 34**	No diagnostic statement can be made. The real time PCR is either inhibited or errors occurred while DNA extraction.

* A strong positive signal in the FAM and/or ROX channel can inhibit the amplification of the control DNA. In such cases the result for the control DNA can be neglected.

** Depending on the PCR instrument and/or the chosen extraction method, the C_t values might be shifted. The water control can be used as reference. If the HEX Ct value of a sample differs a lot from the water control, partial inhibition has occurred, leading to false negative results in case of weak positive samples.

Figure 1 and figure 2 show examples for positive and negative real time PCR results.

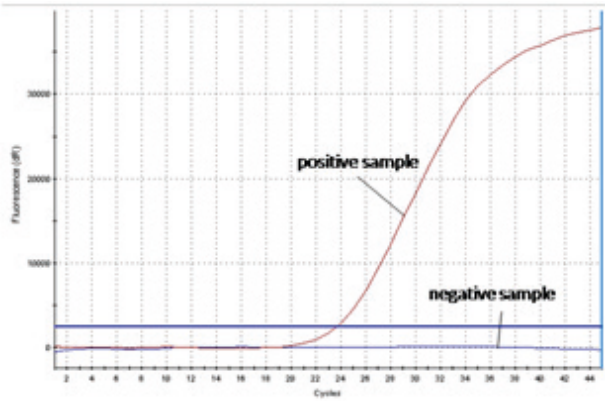


Figure 1: The positive eluate shows specific amplification in the FAM channel, whereas no fluorescence signal is detected in the negative eluate.

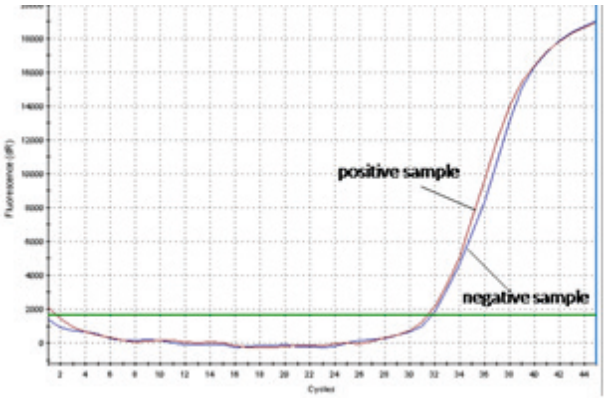


Figure 2: The positive eluate as well as the negative eluate show a signal in the control DNA-specific HEX channel. The amplification signal of the control DNA in the negative eluate shows that the missing signal in the specific FAM channel is not due to PCR inhibition or failure of DNA isolation, but that the eluate is a true negative.

12 ASSAY VALIDATION

Set a threshold as follows:

Negative controls

All negative controls should be below the threshold. If there is a potential contamination (appearance of a curve in the negative control or a cluster of curves in specimens at high C_t – for example above 36), results obtained are not interpretable and the whole run (including extraction) has to be repeated.

Positive controls

All the positive controls must show a positive (i.e. exponential) amplification curve. The positive controls must fall below a C_t of 30.

Internal controls

All internal controls must show a positive (i.e. exponential) amplification curve. The internal control must fall below a C_t of < 34. If the internal control is above C_t 34, this points to a purification problem in DNA-extraction or a strong positive eluate that can inhibit the internal control. In the latter case, the assay is valid. If a water control run is performed, the internal control must fall below a C_t of < 34.

13 LIMITATIONS

- Strict compliance with the Instructions for use is required for optimal results.
- Use of this product is limited to personnel specially instructed and trained in the techniques of real-time PCR and *in vitro* diagnostic procedures.
- Good laboratory practice is essential for proper performance of this assay.
- All reagents should be closely monitored for impurity and contamination. Any suspicious reagents should be discarded.
- This assay must not be used on a biological specimen directly. Appropriate nucleic acid extraction methods have to be conducted prior to using this assay.
- The presence of PCR inhibitors may cause false negative or invalid results.
- Potential mutations within the target regions of the *Blastocysis hominis* genomes covered by the primers and/or probes used in the kit may result in failure to detect the respective DNA.

- As with any diagnostic test, results of the MutaPLEX® Blastocystis real time PCR kit need to be interpreted in consideration of all clinical and laboratory findings.

14 TROUBLESHOOTING

The following troubleshooting guide is included to help you with possible problems that may arise when performing a real time PCR. If you have further questions, please do not hesitate to contact our scientists on info@immundiagnostik.com.

No fluorescence signal in the specific channels of the Positive Control

The selected channel for analysis does not comply with the protocol

Select the channel according to table 6.

Incorrect configuration of the real time PCR

Check your work steps and compare with chapter „Procedure".

The programming of the thermal profile is incorrect

Compare the thermal profile with the protocol (table 5).

Incorrect storage conditions for one or more kit components or kit expired

Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in chapter „Transport, storage and stability".

Weak or no signal of the control DNA and simultaneous absence of a signal in the specific channels.

Real time PCR conditions do not comply with the protocol

Check the real time PCR conditions (see chapter „Real time PCR").

Real time PCR inhibited

Make sure that you use an appropriate isolation method (see chapter „Sample Preparation") and follow the manufacturer's instructions. Make sure that the ethanol-containing washing buffers have been completely removed. An additional centrifugation step at high speed is recommended before elution of the DNA.

DNA loss during isolation process

In case the control DNA was added before extraction, the lack of an amplification signal can indicate that the DNA isolation was not successful. Make sure that you use an appropriate isolation method (commercial kits are recommended) and stick to the manufacturer's protocol.

Incorrect storage conditions for one or more components or kit expired

Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in „Transport, storage and stability".

Detection of a fluorescence signal in the bacteria specific channel of the negative control

Contamination during preparation of the PCR

Repeat the real time PCR in replicates. If the result is negative in the repetition, the contamination occured when the samples were pipetted into the optical PCR reaction tubes. Make sure to pipet the positive control last and close the optical PCR reaction tube immediately after adding the sample. If the same result occurs, one or more of the kit components might be contaminated. Make sure that workspace and instruments are decontaminated regularly. Use a new kit and repeat the real time PCR.

15 KIT PERFORMANCE

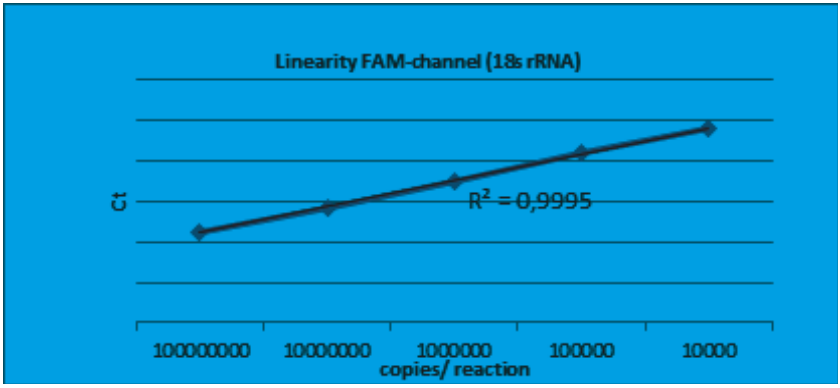
15.1 Analytical sensitivity

The limit of detection (LoD) of MutaPLEX® Blastocystis real time PCR was determined using serial dilutions of synthetic target DNA sequences in a Stratagene Mx3005 real time PCR instrument. The LoD of MutaPLEX® Blastocystis real time PCR for *Blastocystis hominis* is ≤ 10 target copies per reaction each.

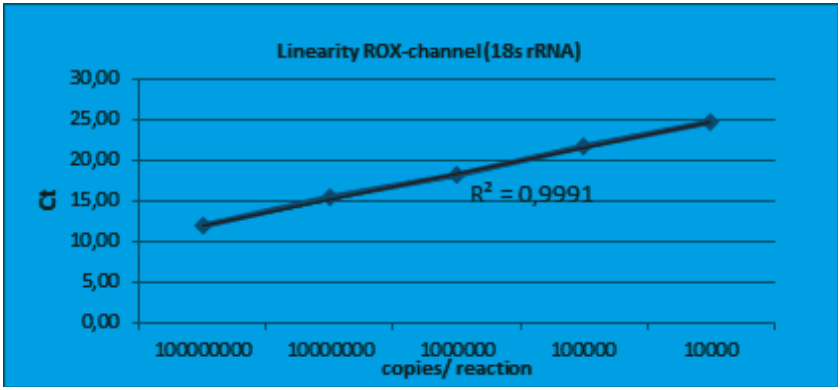
15.2 Linear range

The linear range of the MutaPLEX® Blastocystis real time PCR was evaluated by analysing logarithmic dilution series of synthetic DNA fragments.

Linearity FAM channel (*Blastocystis hominis* ST1, ST3, ST4, ST7)



Linearity ROX channel (*Blastocystis hominis*)



15.3 Analytical specificity

The specificity of the MutaPLEX® Blastocystis real time PCR was evaluated by testing a panel of DNA extracted from bacteria.

The MutaPLEX® Blastocystis real time PCR kit did not cross-react with the DNA from the following bacteria.

Table 11: Determination of the analytical specificity of MutaPLEX® Blastocystis real time PCR.

Strain	Expected result	Result MutaPLEX® Blastocystis
Escherichia coli (ATCC 15597)	negative	negative

Strain	Expected result	Result MutaPLEX® Blastocystis
Enterococcus faecalis (DSMZ 2570)	negative	negative
Shigella sonnei (DSM 5570)	negative	negative
Campylobacter jejuni (DSMZ 4688)	negative	negative
Listeria monocytogenes (DSMZ 15675)	negative	negative
Salmonella enterica (DSMZ 5569)	negative	negative
Citrobacter freundii (Labor Bostel)	negative	negative
Klebsiella spp. (BM01105)	negative	negative
Pseudomonas aeruginosa (ATCC 10145)	negative	negative
Enterobacter cloacae (BM00893)	negative	negative

15.4 Precision

The precision of the MutaPLEX® Blastocystis real time PCR was determined as intra-assay variability, inter-assay variabilitity and inter-lot variability.

Variability data are expressed by standard deviation and coefficient of variation. The data are based on quantification analyses of defined concentrations of *Blastocystis hominis* DNA and on the threshold cycle of the control DNA.

Table 12: Precision of MutaPLEX® Blastocystis real time PCR.

***Blastocystis hominis* ST1, ST3, ST4, ST7**	**copies/µl**	**Standard deviation**	**Coefficient of variation [%]**
Intra-Assay Variability	25	0.19	0.79
Inter-Assay-Variability	25	0.76	3.21
Inter-Lot Variability	25	0.00	0.01

Blastocystis hominis	**copies/µl**	**Standard deviation**	**Coefficient of variation [%]**
Intra-Assay Variability	25	0.17	0.69
Inter-Assay-Variability	25	0.75	3.13
Inter-Lot Variability	25	0.29	1.20

Control DNA	copies/µl	Standard deviation	Coefficient of variation [%]
Intra-Assay Variability	25	0.11	0.40
Inter-Assay-Variability	25	0.05	0.16
Inter-Lot Variability	25	0.40	1.41

16 ABBREVIATIONS AND SYMBOLS

DNA	Deoxyribonucleid acid		Contains sufficient for <n> test
PCR	Polymerase chain reaction		Upper limit of temperature
REACTION MIX	Reaction mix		Manufacturer
CONTROL +	Positive control		Use by YYYY-MM-DD
CONTROL –	Negative control	LOT	Batch
CONTROL DNA IC	Control DNA	CONT	Content
REF	Catalog number		Consult instructions for use
→REF	To be used with	IVD	*In vitro* diagnostic medical device

17 LITERATURE

1. www.cdc.gov/dpdx/blastocystis/index.html.
2. http://www.baemi.de/fileadmin/baemi/der_mikrobiologe_komplett/Mikrobiologe-2004-5__INTERNET-Version.pdf/

Immundiagnostik AG

Stubenwald-Allee 8a
64625 Bensheim, Germany

Tel.: +49 6251 70190-0
Fax: +49 6251 70190-363

info@immundiagnostik.com
www.immundiagnostik.com