Manual

MutaPLEX® EU/BAC/BIF

real time PCR kit

For quantitative in vitro detection of DNA of Eubacterium rectale group, Bacteroides species and Bifidobacterium species in clinical specimens

Distribuito in ITALIA da
Li StarFish S.r.l.
Via Cavour, 35
20063 Cernusco S/N (MI)
telefono 02-92150794
fax 02-92157285
info@listarfish.it
www.listarfish.it

Valid from 2018-07-20

REF **KG1912-96**
KG1912-384

Σ 96/384

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1 INTENDED USE

The MutaPLEX® EU/BAC/BIF real time PCR Kit is an assay for the quantitative detection of DNA of the three species *Bacteroides*, *Eubacterium* and *Bifidobacterium* in clinical specimens (stool samples) using real time PCR microplate systems. Using the quantitative MutaPLEX® EU/BAC/BIF real time PCR, the DNA content of the respective bacteria can be determined as copy number per reaction.

2 PATHOGEN INFORMATION

***Bacteroides* species** are gram-negative, strictly anaerobic, pleomorph, non-spore-forming bacteria. Bacteroides species are the most substantial portion of the mammalian gastrointestinal flora [1]. Approximately 10^{10}–10^{11} bacteria per gram stool have been reported. Their main role is the processing sugars to fermentation products which are beneficial to humans.

***Eubacterium rectale* group**, are gram-positive, strictly anaerobic, non-spore-forming bacteria. The group consists of some *Eubacteria* species and some *Roseburia* species and is part of *Clostridium* cluster XIVa, a cluster of firmincutes, the second largest phylogenic group in the human gut microbiome.

***Bifidobacterium* species**, is a genus of gram-positive, often branches anaerobic bacteria. These species are the third major group in the human gastrointestinal flora and are specialized on the fermentation of milk and plant oligosaccharides [2].

The balance in the composition of the gut flora of these three groups may give indications on the human health. A decreased number of *Bacteroides* species while an increasing number of the *E. rectale* group was detected in patients with obesity [3, 4].

3 PRINCIPLE OF THE TEST

The MutaPLEX® EU/BAC/BIF real time PCR kit contains specific primers and dual-labeled probes for the amplification and detection of DNA of *Bacteroides* species, *Eubacterium rectale* group and *Bifidobacterium* species in clinical specimens. The procedure includes the detection of bacteria like *Bacteroides fragilis*, *B. caccae*, *B. intestinalis*, *B. ovatus*, *B. vulgatus* as well as *Eubacterium rectale*, *E. hallii*, *Roseburia hominis*, *R. intestinalis*, *R. faecis* and *Bifidobacterium adolescens*, *B. bifidum*, *B. infantis* and more.

The presence of nucleic acid is detected by an increase in fluorescence due to hydrolysis of the probes during amplification. The emitted fluorescence is measured in the FAM channel (*Eubacterium rectale* group) the Rox channel (*Bifidobacterium species*) and the Cy5 channel (*Bacteroides species*).

Furthermore, the MutaPLEX® EU/BAC/BIF real time PCR kit contains a control DNA, which is added during DNA extraction and detected in the same reaction by a differently labelled probe.

The control DNA allows the detection of PCR inhibition and acts as control, that the nucleic acid was isolated from the clinical specimen. The fluorescence of the control DNA is measured in the VIC®/HEX/JOE/TET channel.

4 PACKAGE CONTENTS

The reagents supplied are sufficient for 96 (KG1912-96) or 384 (KG1912-384) reactions, respectively.

Table 1: Components of the MutaPLEX® EU/BAC/BIF real time PCR kit

Label	Lid colour	Content	
		KG1912-96	KG1912-384
Reaction Mix	yellow	1x 1536 µl	4x 1536 µl
Positive Control	red	1x 100 µl	1x 100 µl
Negative Control	green	1x 100 µl	1x 100 µl
Control DNA	colourless	1x 480 µl	4x 480 µl
Standard 1	black	1x 100 µl	–*
Standard 2	purple	1x 100 µl	–*
Standard 3	orange	1x 100 µl	–*

Note: the application of the standard curve is needed once per each lot.

Label	Lid colour	Content
Standard 1	black	1x 100 µl
Standard 2	purple	1x 100 µl
Standard 3	orange	1x 100 µl

5 EQUIPMENT AND REAGENTS TO BE SUPPLIED BY USER

- Nucleic acid isolation kit (e. g. MutaCLEAN® Universal RNA/DNA, KG1038)
- PCR grade water
- Sterile microtubes
- Pipets (adjustable volume)
- Sterile pipet tips with filter
- Table centrifuge

- Vortex
- Real time PCR instrument
- Optical PCR reaction tubes with lid or reaction plates with foil
- Optional: Liquid handling system for automation
- Optional: colour compensation kit

* Immundiagnostik AG recommends the use of Ultra Pure Water (Water Type 1; ISO 3696), which is free of undissolved and colloidal ions and organic molecules (free of particles > 0.2 µm) with an electrical conductivity of 0.055 µS/cm at 25 °C (≥ 18.2 MΩ cm).

6 TRANSPORT, STORAGE AND STABILITY

The MutaPLEX® EU/BAC/BIF 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 IMPORTANT NOTES

- The MutaPLEX® EU/BAC/BIF real time PCR must be performed by qualified personnel only.
- Good Laboratory Practice (GLP) has to be applied.
- Clinical samples must always be regarded as potentially infectious material and all equipment used has to be treated as potentially contaminated.

8 GENERAL PRECAUTIONS

- Stick to the protocol described in the instructions for use.
- Set up different laboratory areas for the preparation of samples and for the set up of the PCR in order to avoid contaminations.
- Pipets, tubes and other materials must not circulate between those different laboratory areas.
- Always use filter tips.
- Regulary decontaminate equipment and benches with ethanol-free decontaminant.

- Do not combine MutaPLEX® EU/BAC/BIF real time PCR kit components of different lot numbers.

9 SAMPLE MATERIAL

Starting material for the MutaPLEX® EU/BAC/BIF real time PCR is nucleic acid isolated from clinical specimens (stool samples).

10 SAMPLE PREPARATION

The MutaPLEX® EU/BAC/BIF real time PCR is suitable for the detection of the DNA of *Bacteroides* species, *Bifidobacterium* species and the *Eubacterium rectale* group in clinical specimens (stool samples) isolated with suitable isolation methods.

Commercial kits for DNA isolation such as MutaCLEAN® Universal RNA/DNA (KG1038) are recommended.

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 samples 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 nucleic acid extraction will be detectable.

Please note chapter 11 "Control DNA".

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

11 CONTROL DNA

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

DNA isolation from clinical specimens

Control DNA used as extraction control

MutaPLEX® EU/BAC/BIF real time PCR control DNA is added to the DNA extraction.

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

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

12 REAL TIME PCR

12.1 Important points before starting

- Please pay attention to chapter 7 "Important notes".
- 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, one positive control and one negative control should be included.
- Before each use, all reagents should be thawed completely at room temperature, thouroughly mixed, and centrifuged very briefly.
- For quantification of *Bacteroides* species, *Bifidobacterium* species and the *Eubacterium rectale* group-positive DNA in clinical samples, a standard curve using standards 1, 2 and 3 must be applied. The standard curve needs to be saved separately on the real time PCR instrument. It can be imported and used in subsequent runs with kits of the same lot.

  Note: the application of the standard curve needed once per each lot.

12.2 Procedure

The control DNA was added during DNA extraction (see chapter 'Control DNA'). 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 µl Reaction Mix	16 µl x (N+1)

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 or each well of the optical reaction plate.

- 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 tubes (table 3).
- Add 4 µl of standard 1, 2 and 3, respectively, to the corresponding optical PCR reaction tube or the respective well of the optical reaction plate (table 3)
- Close the optical PCR reaction tubes or optical reaction plates immediately after filling in order to reduce the risk of contamination.

Table 3: Preparation of the real time PCR

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

12.3 Instrument settings

For the real time PCR use the thermal profile shown in table 4.

Table 4: real time PCR thermal profile

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

The MutaPLEX® EU/BAC/BIF real time PCR is designated for the LightCycler® 480 II real time PCR instrument. Instrument settings have to be adjusted according to table 5.

Table 5: Overview of the instrument settings required for the MutaPLEX® EU/BAC/BIF real time PCR.

<table>
<tr><th>Real time PCR Instrument</th><th>Parameter</th><th>Detection Channel</th><th>Notes</th></tr>
<tr><td rowspan="4">LightCycler 480II</td><td>Eubacterium rectale group</td><td>FAM (465–510)</td><td rowspan="4">Colour compensation kit required
<table><tr><th>Melt factor</th><th>Quant factor</th><th>Max integration time [s]</th></tr><tr><td>1</td><td>10</td><td>1</td></tr><tr><td>1</td><td>10</td><td>2</td></tr><tr><td>1</td><td>10</td><td>2</td></tr><tr><td>1</td><td>10</td><td>3</td></tr></table></td></tr>
<tr><td>Bifidobacterium species</td><td>ROX (533–610)</td></tr>
<tr><td>Control DNA</td><td>HEX (533–580)</td></tr>
<tr><td>Bacteroides species</td><td>Cy5 (618–660)</td></tr>
</table>

Note: For standards 1, 2 and 3, the total copy number per reaction needs to be entered in the setup file of the LightCycler® 480 II instrument. 4 µl of each standard DNA are used, resulting in the following concentrations:

- Standard 1: 1 x 10^6 copies/reaction
- Standard 2: 1 x 10^4 copies/reaction
- Standard 3: 1 x 10^2 copies/reaction

13 ASSAY VALIDATION

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 CT – for example above 36), results obtained are not interpretable and the whole run (including extraction) has to be repeated.

Positive control

The positive control must show a positive (exponential) amplification curve. The positive control must fall below a CT of 30.

Extraction controls

All extraction controls must show a positive (exponential) amplification curve. The extraction control must fall below a CT of 35. If the extraction control is above CT 35, this points to a purification problem or a strong positive sample that can inhibit the EC. In the latter case, the assay is valid. If a water control run is performed, the EC must fall below a CT of 33.

Postive Control and the Negative Control do not contain control DNA. Therefore, no amplification signal is detected.

Standards 1, 2, and 3

All standards must show a positive (exponential) amplification curve.

Standard 1, 2, and 3 must show a CT as described in the certificate of analysis.

14 DATA ANALYSIS

The *Eubacterium rectale* group-specific amplification is measured in the FAM channel, the *Bifidobacterium* species-specific amplification is measured in the ROX channel, and the *Bacteroides* species-specific amplification in the Cy5 channel.

The amplification of the control DNA is measured in the VIC®/HEX/JOE/TET channel.

The following results can occur:

A signal in the FAM, ROX and/or Cy5 channel is detected:

The result is positive, the sample contains bacterial DNA.

In this case, detection of a signal of the control DNA in the VIC®/HEX/JOE/TET channel is inessential, as high concentrations of bacterial DNA may reduce or completely inhibit amplification of the control DNA.

No signal in the FAM, ROX and/or Cy5 channel, but a signal in the VIC®/HEX/JOE/TET channel is detected:

The result is negative, the sample does not contain bacterial DNA.

The signal of the control DNA excludes the possibilities of DNA isolation failure and/or real time PCR inhibition. If the CT value of a sample differs significantly from the CT value of the water control, a partial inhibition occured, which can lead to negative results in weak positive samples (see chapter 'Troubleshooting').

Neither in the FAM, ROX and/or Cy5 channel nor in the VIC®/HEX/JOE/TET channel a signal is detected:

A diagnostic statement cannot be made.

The DNA isolation was not successful or an inhibition of the PCR has occurred.

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

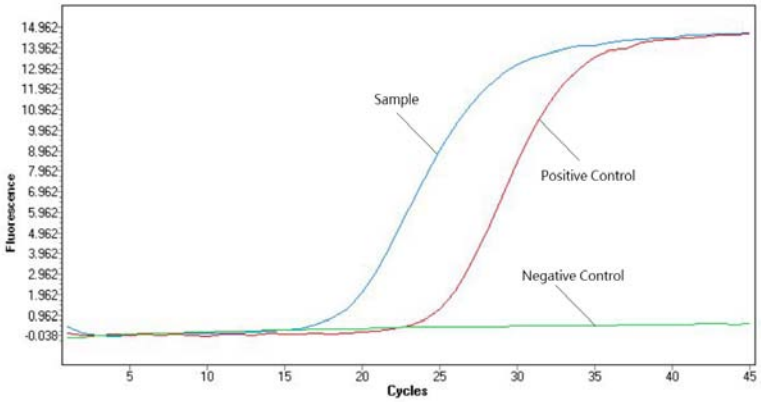

Figure 1: The positive sample shows bacteria-specific amplification in the FAM, ROX, Cy5 channel, whereas no fluorescence signal is detected in the negative control.

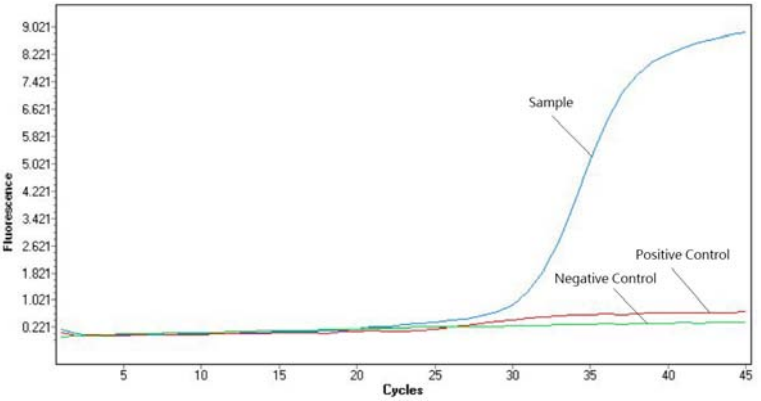

Figure 2: The sample shows a signal in the control DNA specific VIC®/HEX/JOE/TET channel. The postive control and the negative control do not contain control DNA. Therefore, no amplification signal is detected.

15 QUANTIFICATION OF SAMPLES

For quantification of *Bacteroides* species, *Bifidobacterium* species and the *Eubacterium rectale* group-positive DNA in clinical samples, a standard curve using standards 1, 2, and 3 must be applied. The standard curve needs to be saved separately on the real time PCR instrument. It can be imported and used in subsequent runs with kits of the same lot.

Note: the application of the standard curve needed once per each lot.

Using the quantitative MutaPLEX® EU/BAC/BIF real time PCR, the DNA content of the respective bacteria are determined in copy number per reaction. Using correction factor K, the translation of copies/reaction to bacteria/g feces is made. The correction factor considers the dilution of the DNA extraction (dependent on the respective DNA extraction kit), the dilution of the PCR and the number of target sequences in the whole genome of *Bacteroides* species, *Bifidobacterium* species and the *Eubacterium rectale* group. For translation of the result of the PCR into bacterial load of the sample, the following formula is used:

$$n_{\text{Bacteroides}}\left[\frac{\text{cells}}{\text{g}_{\text{feces}}}\right] = n_{\text{LC480}}\left[\frac{\text{copies}}{\text{reaction}}\right] * K_{\text{Bacteroides}}\left[\frac{1}{\text{g}}\right]$$

$$n_{\text{Eubacterium}}\left[\frac{\text{cells}}{\text{g}_{\text{feces}}}\right] = n_{\text{LC480}}\left[\frac{\text{copies}}{\text{reaction}}\right] * K_{\text{Eubacterium}}\left[\frac{1}{\text{g}}\right]$$

$$n_{\text{Bifidobacterium}}\left[\frac{\text{cells}}{\text{g}_{\text{feces}}}\right] = n_{\text{LC480}}\left[\frac{\text{copies}}{\text{reaction}}\right] * K_{\text{Bifidobacterium}}\left[\frac{1}{\text{g}}\right]$$

n_{LC480}	Calculated copy number per reaction by PCR Instrument, based on the CT value and the standard curve.

The calculation of the correction factor K requires the following parameters:

m_{sample}[g]	Mass of the stool sample
b_{buffer}	Dilution factor of the transport and storage buffer
$c_{extraction}$	Correction factor for the extraction
V_{eluate}[µl]	Total Eluate volume
a_{eluate}	Dilution factor of the eluate
$\frac{1}{4[\mu l]}$	Amount of µl used in the PCR reaction
$\frac{1}{\text{target copies}}$	Copies of target gene per genome

The target genes are 3 times present in *Bifidobacterium* species, 5 times in *Eubacterium* species and 6 times in *Bacteroides* species.

$$K_{\text{Bacteroides}}\left[\frac{1}{\text{g}}\right] = \frac{1}{m_{\text{sample}}[\text{g}]} * b_{\text{buffer}} * c_{\text{extraction}} * V_{\text{eluate}}[\mu l] * a_{\text{eluate}} * \frac{1}{4[\mu l]} * \frac{1}{\text{target copies}}$$

$$K_{\text{Eubacterium}}\left[\frac{1}{\text{g}}\right] = \frac{1}{m_{\text{sample}}[\text{g}]} * b_{\text{buffer}} * c_{\text{extraction}} * V_{\text{eluate}}[\mu l] * a_{\text{eluate}} * \frac{1}{4[\mu l]} * \frac{1}{\text{target copies}}$$

$$K_{\text{Bifidobacterium}}\left[\frac{1}{\text{g}}\right] = \frac{1}{m_{\text{sample}}[\text{g}]} * b_{\text{buffer}} * c_{\text{extraction}} * V_{\text{eluate}}[\mu l] * a_{\text{eluate}} * \frac{1}{4[\mu l]} * \frac{1}{\text{target copies}}$$

Table 6: Example for the calculation of correction factor K for *Bacteroides* species

	Description	Factor
$\frac{1}{m_{\text{sample}}[\text{g}]}$	200 mg stool sample in 1 ml buffer	x 5
b_{buffer}	250 µl stool buffer (incl. factor for density)	x 2.552
$c_{\text{extraction}}$	Correction factor for extraction	x 50
$V_{\text{eluate}}[\mu\text{l}]$	Volume eluate	x 100
a_{eluate}	Dilution of the eluate	x 10
$\frac{1}{4[\mu\text{l}]}$	4 µl sample volume for PCR	x 1/4
$\frac{1}{\text{target copies}}$	6 copies of target gene per genome	x 1/6
$K_{\text{Bacteroides}}\left[\frac{1}{\text{g}}\right]$	Correction factor for quantification	2.66 x 10^4

Table 7: Example for the calculation of correction factor K for *Eubacterium* species

	Description	Factor
$\frac{1}{m_{\text{sample}}[\text{g}]}$	200 mg stool sample in 1 ml buffer	x 5
b_{buffer}	250 µl stool buffer (incl. factor for density)	x 2.552
$c_{\text{extraction}}$	Correction factor for extraction	x 50
$V_{\text{eluate}}[\mu\text{l}]$	Volume eluate	x 100
a_{eluate}	Dilution of the eluate	x 10
$\frac{1}{4[\mu\text{l}]}$	4 µl sample volume for PCR	x 1/4
$\frac{1}{\text{target copies}}$	5 copies of target gene per genome	x 1/5
$K_{\text{Eubacterium}}\left[\frac{1}{\text{g}}\right]$	Correction factor for quantification	3.19 x 10^4

Table 8: Example for the calculation of correction factor K for *Bifidobacterium* species

	Description	Factor
$\frac{1}{m_{\text{sample}}[\text{g}]}$	200 mg stool sample in 1 ml buffer	x 5
b_{buffer}	250 µl stool buffer (incl. factor for density)	x 2.552
$c_{\text{extraction}}$	Correction factor for extraction	x 50
$V_{\text{eluate}}[\mu\text{l}]$	Volume eluate	x 100
a_{eluate}	Dilution of the eluate	x 10
$\frac{1}{4[\mu\text{l}]}$	4 µl sample volume for PCR	x 1/4
$\frac{1}{\text{target copies}}$	3 copies of target gene per genome	x 1/3
$K_{\text{Bifidobacterium}}\left[\frac{1}{\text{g}}\right]$	Correction factor for quantification	5.31 x 10^4

16 LIMITATIONS OF THE METHOD

The results must always be considered in relation to the clinical symptoms. Therapeutical consequences should be made in consideration of clinical data.

17 TROUBLESHOOTING

The following troubleshooting guide is included to help you with possible problems that may arise when performing a real time PCR.

No fluorescence signal in the FAM, ROX, or Cy5 channel of the positive controls

The selected channel for analysis does not comply with the protocol

Select the FAM channel for analysis of the *Eubacterium rectale* group-specific amplification, the ROX channel for analysis of the *Bifidobacterium* species-specific amplification, and the Cy5 channel for analysis of the *Bacteroides* species-specific amplification.

Select the VIC®/HEX/JOE/TET channel for the amplification of the control DNA.

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 4).

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 bacteria-specific FAM, ROX or Cy5 channel

real time PCR conditions do not comply with the protocol

Check the real time PCR conditions (chapter 12).

real time PCR inhibited

Make sure that you use an appropriate isolation method (see "Sample preparation") and follow the manufacturer's instructions. Make sure that the ethanol-containing washing buffer of the isolation kit has 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 chapter "Transport, Storage and Stability".

Detection of a fluorescence signal in the FAM, ROX or Cy5 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 work space and instruments are decontaminated regularly. Use a new kit and repeat the real time PCR.

18 ABBREVIATIONS AND SYMBOLS

DNA	Deoxyribonucleid acid	REF	Catalog number
PCR	Polymerase chain reaction	→REF	To be used with
REACTION MIX	Reaction mix		Contains sufficient for <n> test
CONTROL +	Positive control		Upper limit of temperature
CONTROL –	Negative control		Manufacturer
CONTROL DNA IC	Control DNA		Use by
STANDARD 1	Standard 1	LOT	Lot number
STANDARD 2	Standard 2	CONT	Content
STANDARD 3	Standard 3		Consult instructions for use
		IVD	*In vitro* diagnostic medical device

19 LITERATURE

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