IVD For *in Vitro* Diagnostic Use

CE

PeriodontScreen Real-TM

Handbook

Real Time PCR test for detection and quantification of *Porphyromonas endodontalis, Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Treponema denticola, Fusobacterium nucleatum, Prevotella intermedia, Tannerella forsythia*

REF T01707-96-S
T01707-96-S/L (for LightCycler-96 instrument only)

Σ **12**

NAME

PeriodontScreen Real-TM

INTRODUCTION

According to the World Health Organization from 80 to 100% of the adult population in different age groups, suffer inflammatory periodontal diseases. Periodontal diseases along with dental caries and its complications are the most common dental diseases. It is now established that in patients older than 40 years, the cause of tooth loss is more periodontal disease than tooth decay.

The etiology of periodontal disease.

One of the main causes of the development of periodontal diseases are specific bacterial agents. Additional risk factors include genetic predisposition of the immune system, poor oral hygiene, smoking, systemic diseases and stress (Fig. 1).

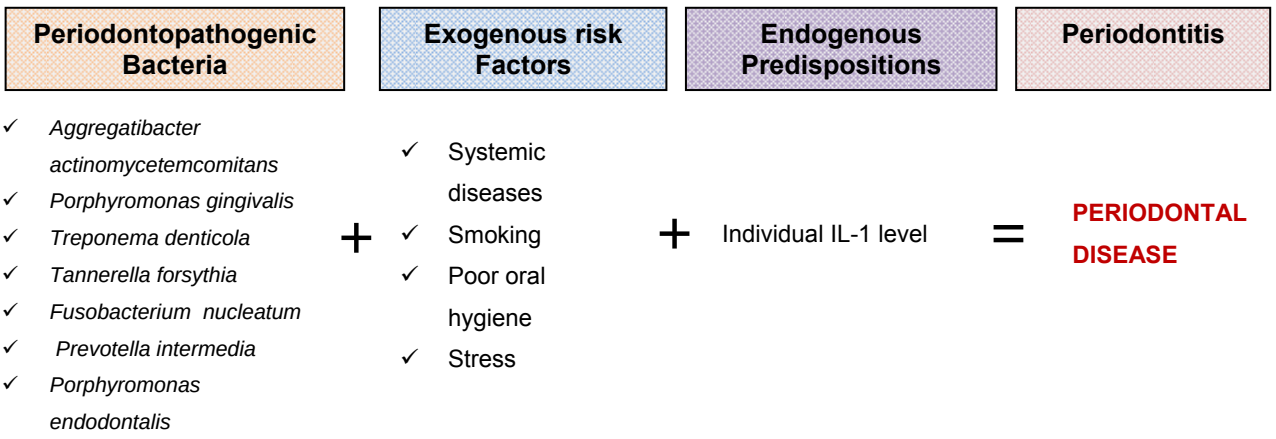


Fig.1 Periodontal disease risk factors

Microbial factor, being one of the most powerful etiologic agents, causes various clinical manifestations of periodontal disease. A significant role in pathogenesis of periodontitis have the composition and types of microorganisms in dental plaque, its volume, time of exposition of the areas of gingival and periodontal tissues. These factors, according to most researchers, explain the development of inflammatory changes in the periodontium with different clinical manifestations.

The infectious nature of periodontal disease

Periodontal diseases are a diverse group of infectious diseases of periodontal tissues with different specific pathogens, which are non-clostridial anaerobic bacteria. According to the WHO classification, they are grouped in the so-called Periodontopathogenic Bacteria (PB) [Fig. 2]. It is now established that this group of pathogens is a major cause of progressive periodontal disease. This leads to the need for specific methods of diagnosis and treatment of identified

bacteria in chronic-progressive-resistant and aggressive periodontal disease. Purely mechanical treatment is generally ineffective, particularly when PB are present in oral cavity and in this case it should be used antibacterial therapy. A necessary condition for choosing the appropriate antimicrobial agent is the knowledge of the bacterial spectrum and the concentration of present microorganisms.

Biological markers of periodontal disease

Periodontopathogenic Bacteria are in divided in high and medium risk of developing the disease.

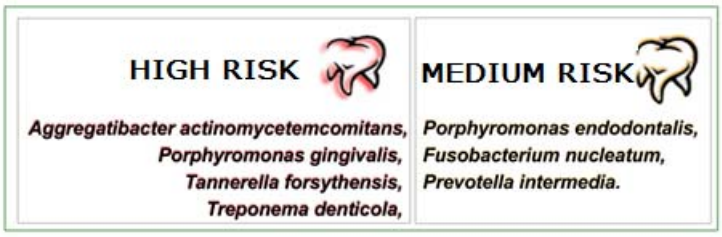


Figure 2. List of high and medium risk Periodontopathogenic Bacteria

All microorganisms detected by **"PeriodontScreen Real-TM"** panel have different pathogenic potential. A positive test result is when the concentration of the desired periodontopathogens is above clinically significant titer.

The physician must evaluate the total potential pathogenic microflora, depending on the type of microorganism and its concentration according to the data presented in the figure below. Identification of microorganisms and their concentration allows not only to choose the treatment regimen, but also to carry out its control.

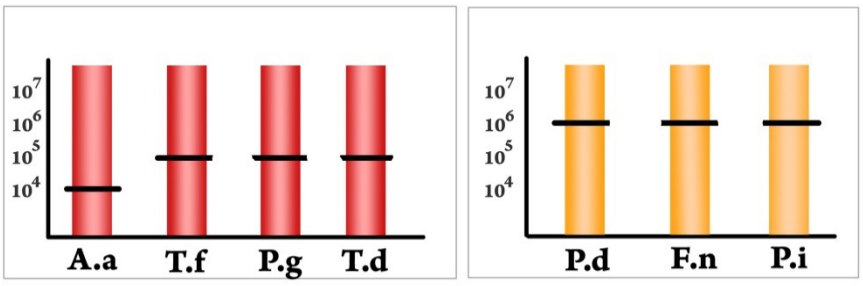


Figure 3. The threshold line "pathogenicity" of microorganisms.

INTENDED USE

PeriodontScreen Real-TM PCR kit is an in vitro nucleic acid amplification test intended for quantitative detection of *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Porphyromonas endodontalis, Treponema denticola, Fusobacterium nucleatum, Prevotella intermedia, Tannerella forsythia*. This kit uses endogenous Internal Control which is present in each reaction tube containing PCR mastermix. The endogenous IC detects human genomic DNA sequence which must always be present in each extracted sample.

This approach allows to monitor not only possible reaction inhibition but also:

- correct collection of clinical specimens;
- effectiveness of sample preparation;
- errors in the analysis (sample not added in the amplification mixture);

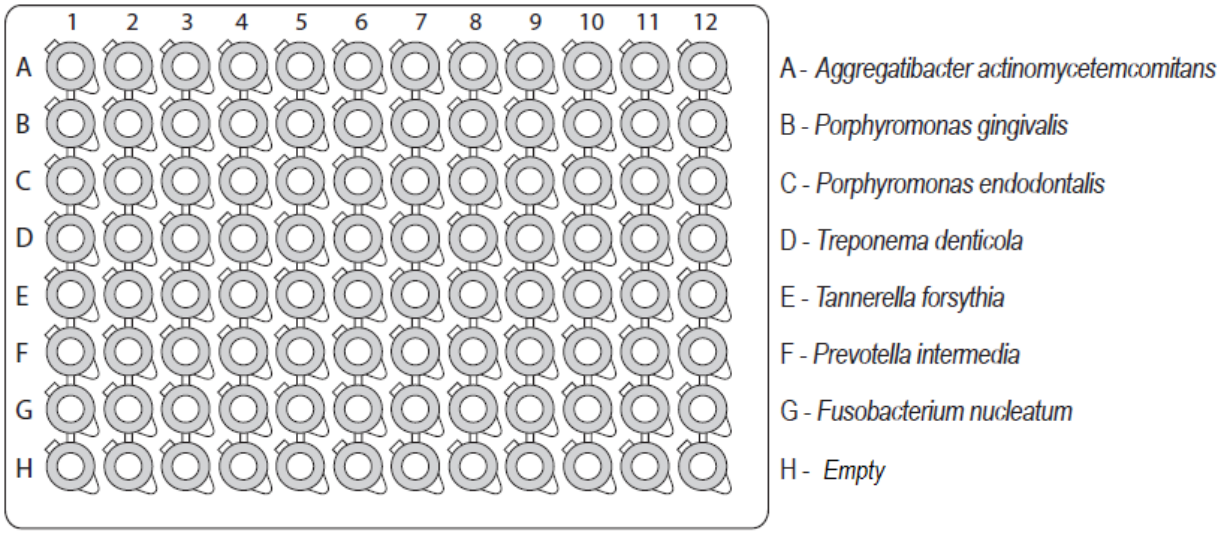
PRINCIPLE OF ASSAY

PeriodontScreen Real-TM is a QUANTITATIVE test that allow the detection by Real Time PCR based on the amplification of the genome specific region using specific primers. In Real Time PCR the amplified product is detected using fluorescent dyes. These dyes are linked to oligonucleotide probes that bind specifically to the amplified product. The real-time monitoring of the fluorescence intensities during the reaction allows the detection of accumulating product without re-opening of the reaction tubes after the PCR run.

MATERIALS PROVIDED

- **12 x 8 strip ready to use** (each PCR tube contains 21 µl)

Amplification Mixes



- Positive Standards (St) Panel, 14 tubes, Lyophilized with known concentrations reported in DataCard:
    - Pos *Aggregatibacter actinomycetemcomitans,* St1 and St2
    - Pos *Porphyromonas gingivalis,* St1 and St2
    - Pos *Porphyromonas endodontalis,* St1 and St2
    - Pos *Treponema denticola,* St1 and St2
    - Pos *Tannerella forsythia,* St1 and St2
    - Pos *Prevotella intermedia,* St1 and St2
    - Pos *Fusobacterium nucleatum,* St1 and St2
- Dilution Sol, 1000 µl;
- Neg Control, 200 µl;
- Taq-Polymerase, 1 x 800 µl.

Contains reagents for 12 tests.

MATERIALS REQUIRED BUT NOT PROVIDED

Zone 1: sample preparation

- DNA extraction kit
- Biological cabinet
- Desktop microcentrifuge for “eppendorf” type tubes
- Dry heat block
- Vortex mixer
- Pipettes
- Sterile pipette tips with filters
- 1,5 ml polypropylene sterile tubes
- Biohazard waste container
- Refrigerator, Freezer

Zone 2: Real Time amplification

- Real Time Thermal cycler
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Vortex mixer
- Desktop centrifuge with rotor for 1,5/2,0 ml tubes
- Freezer, refrigerator
- Tube racks

STORAGE INSTRUCTIONS

PeriodontScreen Real-TM kits must be stored at 2-8°C. The kits can be shipped at 2-8°C and stored as indicated immediately on receipt.

STABILITY

PeriodontScreen Real-TM kits are stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

QUALITY CONTROL

In accordance with Sacace’s ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

WARNINGS AND PRECAUTIONS

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. 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 specimens and unused reagents in accordance with local authorities' regulations.
- Specimens 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 other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

PRODUCT USE LIMITATIONS

Use of this product should be limited to personnel trained in the techniques of DNA amplification (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

SAMPLE COLLECTION, TRANSPORT

PeriodontScreen kit can analyze genomic DNA extracted from:

- *Content of periodontal pockets:* Insert “Sterile paper point #25” in periodontal pocket for 10-20 seconds. Remove the point and place in a test tube with a "RAPID DNA" (Sacace)* or in transport medium;
- *Plaque*: Remove plaque with a sterile universal swab and insert it into the test tube with "RAPID DNA" (Sacace)* or in transport medium. Vigorously agitate swab for 15-20 sec. Snap off shaft at scored line, leaving final section inside tube.
- *Gingival fluid*: Select gingival fluid with sterile paper strips 0.3-0.8 mm or paper wedges number 20 - 40. Put in a test tube with a "RAPID DNA" (Sacace)* or in transport medium.

  *Samples are placed directly in the reagent tube "RAPID DNA" and do not require pretreatment. Delivery of samples to the laboratory should be carried out in a thermos with ice or cold box for maximum 12 hours.

NOTE: perform sample collection during acute phase of infection if possible. Do not brush teeth or wash mouth before sample collection. Sample should be processed within 24h when stored 2-8°C or within 2 weeks when stored at -20°C.

DNA ISOLATION

The following isolation kits are recommended:

⇒ **DNA-Sorb-B** (Sacace, REF K-1-1/B);

⇒ **RAPID DNA** (Sacace, REF K-1-1/R);

Please carry out the DNA extraction according to the manufacturer’s instructions.

PROTOCOL

PeriodontScreen Real-TM kit does not include reagents required for sample preparation and DNA extraction. Blood samples and biological materials must be processed by using the recommended kits or those with similar performances of quality and quantity of extracted DNA. Use of blood samples collected in tubes containing heparin is not recommended.

The analysis of DNA specimens using **PeriodontScreen Real-TM** kit includes the following stages:

1. Preparing the Real Time PCR;
2. Real Time PCR analysis;
3. Data analysis with the software of Real Time PCR instrument;
4. Results analysis and conclusions.

REAGENTS PREPARATION

Before starting any PeriodontScreen Real-TM protocol prepare the Positive controls.

- Choose the requested quantity of lyophilized controls and add 55 µl of Dilution Sol. Close the tubes and incubate all tubes for 10 min at room temperature.
- Votex tubes and centrifuge for 5 sec.

Dissolved reagents must be stored at -20 °C and always protected from light up to 30 days.

REAL TIME PCR CALIBRATION PROCEDURE:

1. Prepare 2 strips with reagents to perform PCR of calibrators.
2. Add **7 µl** of **Taq polymerase** and **7 µl** of resuspended calibrators. Add calibrators 1 (St1) in the 1st strip (from A to G) and calibrators 2 (St2) in the 2nd strip (from A to G), making attention to add St1 and St2 of each pathogen in the corresponding position of amplification mixes (ex. St1 and St2 of *Aggregatibacter actinomycetemcomitans* must be added in the "A" line, St1 and St2 of *Porphyromonas gingivalis* must be added in the "B" line, see previous picture)

Follow the procedure for amplification and detection as described in this manual.

Once an **PeriodontScreen Real-TM** calibration run is accepted and stored, it may be used until the lot in use expires. During this time, all subsequent samples may be tested without further calibration by importing the experiment with Calibration Curve in the experiment with clinical samples using the same **PeriodontScreen Real-TM** lot number.

EXPERIMENTAL PROTOCOL

Total reaction volume: 35 µl

1. Prepare the necessary number of ready-to-use PCR strip tubes.
2. Spin for 3-5 sec the **Taq polymerase**, mix by pipetting and **add 7 µl** to each PCR tube.
3. Add into the corresponding PCR tubes **7 µl** of extracted DNA from sample:
   - **DNA** sample

   Add into the corresponding PCR tubes **7 µl** of controls / calibrators:
   - **C+ Pos Control (St1, St2)**
   - **Negative Control C-**
4. Spin the tubes for 3–5 seconds to collect the drops.
5. Insert the tubes in the Real-time PCR instrument.

Amplification

Create a temperature profile on your instrument as follows:

	Rotor-type Instruments[1]			Plate-type Instruments[2]		
Step	***Temperature, °C***	***Time***	**Repeats**	***Temperature, °C***	***Time***	**Repeats**
Hold	**95**	120 s	1	**80**	120 s	1
Hold	**95**	90 s	1	**95**	90 s	1
Cycling	**95**	15 s	40	**95**	15 s	40
	56	**30 s fluorescence detection**		**60**	30 s **fluorescence detection**	
	72	40 s		**72**	40 s	

1 For example like RotorGene Q (Qiagen) NOTE: If the test is performed on Rotor Gene (Qiagen) instrument the tube caps may be marked and it's recommended to cut the strip into two equal parts (4 tubes), but it is necessary to strictly observe the order of the tubes in the rotor. ATTENTION! In Rotor-Gene software before starting the run be sure that the auto-gain optimization tube is in the position where the reaction mix for Aggregatibacter actinomycetemcomitans is inserted (it is important to have gain optimization in the tube position containing reaction mix for Aggregatibacter actinomycetemcomitans) ;

*2 For example like: SaCycler-96™ (Sacace); CFX-96 / iQ5™ (BioRad); Mx3005P™ (Agilent); ABI® 7500 Real Time PCR (Applied)**;*

Fluorescence is detected in FAM/Green, JOE/Yellow/HEX fluorescence channels.

*** To perform the test with ABI 7500 (Applied) a disposable plate adapter provided with the kit has to be used. Additional adapters can be purchased separately.*

For LightCycler-96™ instrument create the following temperature profile:

	Name	Temperature			Cycles	Acquisition Mode
		Target	Duration	Ramp		
1	Preincubation	80°C	120s	4.4 °C/s	1	None
2	Preincubation	95°C	90s	4.4 °C/s	1	None
3	3 Step Amplification					
		95°C	15 s	4.4 °C/s	10	None
		60°C	30 s	2.2 °C/s		None
		72°C	40 s	4.4 °C/s		None
4	3 Step Amplification					
		95°C	15 s	4.4 °C/s	30	None
		60°C	30 s	2.2 °C/s		**Single**
		72°C	40 s	4.4 °C/s		None
5	Cooling	37	30 s	2.2 °C/s	1	None

Fluorescence is detected in FAM/Green, JOE/Yellow/HEX fluorescence channels.

DATA ANALYSIS

The fluorescent signal intensity is detected in 2 channels as shown in the table below:

FAM / Green	HEX / Yellow
Specific pathogen signal (Positive if Ct < 35)	**Internal Control Signal (Valid if Ct < 35)**

The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; otherwise, the threshold level should be raised. Set the threshold at a level where fluorescence curves are linear and do not cross curves of the negative samples.

Analysis settings for SaCycler-96™ (Sacace Biotechnologies)

	Channel	Threshold	Boundary Ct Value
Internal control	HEX	10	35
Aggregatibacter actinomycetemcomitans	FAM	100	35
Porphyromonas gingivalis	FAM	100	35
Porphyromonas endodontalis	FAM	100	35
Treponema denticola	FAM	100	35
Prevotella intermedia	FAM	100	35
Fusobacterium nucleatum	FAM	100	35
Tannerella forsythia	FAM	100	35

If HEX Ct value is > 35 (or absent) while FAM Ct value is positive the result is considered valid.

If HEX Ct value is > 35 (or absent) and FAM Ct value is > 35 (or absent) result is considered invalid and the sample must be tested again.

Analysis settings for CFX-96™ (Bio-Rad)

	Channel	Threshold	Boundary Ct Value
Internal control	HEX	30	35
Aggregatibacter actinomycetemcomitans	FAM	40	35
Porphyromonas gingivalis	FAM	40	35
Porphyromonas endodontalis	FAM	40	35
Treponema denticola	FAM	40	35
Prevotella intermedia	FAM	40	35
Fusobacterium nucleatum	FAM	40	35
Tannerella forsythia	FAM	40	35

If HEX Ct value is > 35 (or absent) while FAM Ct value is positive the result is considered valid.

If HEX Ct value is > 35 (or absent) and FAM Ct value is > 35 (or absent) result is considered invalid and the sample must be tested again.

Analysis settings for Rotor-Gene Q (Qiagen)

Product	Channel	Dynamic Tube	Slope Correct	NTC Threshold	Ignore First	Threshold	Boundary Ct Value
Internal control	Yellow	on	on	10%	0	0,03	35
Aggregatibacter actinomycetemcomitans	Green	on	on	5%	5	0,03	35
Porphyromonas gingivalis	Green	on	on	5%	5	0,03	35
Porphyromonas endodontalis	Green	on	on	5%	5	0,03	35
Treponema denticola	Green	on	on	5%	5	0,03	35
Prevotella intermedia	Green	on	on	5%	5	0,03	35
Fusobacterium nucleatum	Green	on	on	5%	5	0,03	35
Tannerella forsythia	Green	on	on	5%	5	0,03	35

Analysis settings for LightCycler-96™ (Roche)

	max Cq	Slope	EPF	Efficiency
Internal control (HEX)	25	0,1	0,5	
Aggregatibacter actinomycetemcomitans	25	0,05	0,3	1,99
Porphyromonas gingivalis	25	0,1	0,6	2,07
Porphyromonas endodontalis	25	0,05	0,2	1,81
Treponema denticola	25	0,1	0,3	2,09
Tannerella forsythia	25	0,1	0,6	1,95
Prevotella intermedia	25	0,1	0,6	1,91
Fusobacterium nucleatum	25	0,1	0,6	2,03

QUANTITATIVE RESULTS CLINICAL SIGNIFICANCE

Pathogen	Limit	Clinical significance
A.a *Aggregatibacter actinomycetemcomitar*	10^4	**High risk of developing the disease. May lead to the destruction of bone tissue. Associated with aggressive forms of periodontitis and gingivitis.**
P.g *Porphyromonas gingivalis*	10^5	**High risk of developing the disease. Produce proteases, endotoxins and cytotoxins, damaging the integrity of the gum and bone tissue. Main marker of the disease together with A.a.**
T.f *Tannerella forsythensis*	10^5	**High risk of developing the disease. Produce virulence factors (proteases, lipopolysaccharides). May cause an inhibition of immunity and shift of disease into a chronic form.**
T.d *Treponema denticola*	10^5	**High risk of developing the disease. Promotes adhesion of other pathogens. Often detected at the beginning of rapidly progressive disease.**
P.d *Porphyromonas endodontalis*	10^6	**Medium risk of developing the disease. Producing active enzymes and metabolites. Can inhibit phagocytosis and damage the local immunity.**
F.n *Fusobacterium nucleatum*	10^6	**Medium risk of developing the disease. Detected during acute periodontal diseases, especially gingivitis.**
P.i *Prevotella intermedia*	10^6	**Medium risk of developing the disease. Involved in the formation of biofilms frame, contributing to the adhesion of pathogenic bacteria . At high concentrations have pathogenic properties.**

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KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
	Expiration Date	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**POS**	Positive Control of Amplification
	Consult instructions for use	**IC**	Internal Control
IVD	For *in Vitro* Diagnostic Use		

* SaCycler™ is a registered trademark of Sacace Biotechnologies
* iQ5™ is a registered trademark of Bio-Rad Laboratories
* MX3005P® is a registered trademark of Agilent Technologies
*ABI® is a registered trademark of Applied Biosystems
* LightCycler® 96 is trademark of Roche
* RotorGene Q is trademark of Qiagen

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