

PrimerDesign™ Ltd

# **Quantification of *Aggregatibacter actinomycetemcomitans* genomes**

**hemoglobin-bindingprotein (hbpA) gene**

**For general laboratory and research use only**

## **Advanced kit**





# Contents

<b>Introduction to <i>Aggregatibacter actinomycetemcomitans</i></b>	<b>3</b>
<b>Specificity</b>	<b>3</b>
<b>Kit Contents</b>	<b>4</b>
<b>Reagents and equipment to be supplied by the user</b>	<b>4</b>
<b>Kit storage</b>	<b>4</b>
<b>Suitable sample material</b>	<b>5</b>
<b>Dynamic range of test</b>	<b>5</b>
<b>Principles of the test</b>	<b>6</b>
<b>Bench side Protocol</b>	<b>8</b>
<b>Amplification Protocol</b>	<b>11</b>
<b>Interpretation of Results</b>	<b>12</b>
<b>Notices and disclaimers</b>	<b>13</b>
<b>Trademarks</b>	<b>13</b>



## Introduction to *Aggregatibacter actinomycetemcomitans*

*Aggregatibacter actinomycetemcomitans* is a non-motile, rod-shaped, Gram-negative bacterium. This bacterium is also a facultative anaerobe which can grow under aerobic or anaerobic conditions. The 43Kbp genome is arranged in a circular configuration and contains up to 60 open reading frames. The outer surface of the cell includes appendages known as fimbriae which are used for host cell adhesion.

*A. actinomycetemcomitans* inhabits the human oral cavity where it adheres to the mucosal tissue of the gum using fimbriae. Here, they are able to create a biofilm with other resident bacterial species that if not treated can cause periodontitis or gingivitis.

The bacterium produces leukotoxin which can be found on the outer bacterial membrane and is used to avoid the host cell immune response. This evasion allows the bacteria to stimulate a chronic inflammatory response that leads to damage of the periodontium which includes connective tissue, ligaments and bone.

The damage to the periodontium that is caused by *A. actinomycetemcomitans* results in redness, bleeding or swelling of the gums which progresses to recession of the gums that can lead to deep pockets forming between the teeth when the attached gingiva is destroyed. Eventually the teeth will become loose and fall out.

## Specificity

The *PrimerDesign™ Quantification Kit for A. actinomycetemcomitans* genomes is designed for the *in vitro* quantification of all clinical isolates. The haemoglobin binding protein gene has previously been identified as a highly specific marker for *A. actinomycetemcomitans* (J. Periodont Res 2008; 43: 352-359). The primers and probe have 100% homology with all reference sequences for *A. actinomycetemcomitans* in the NCBI database.

EF142372.I, EF142371.I, EF142368.I, EF142367.I, EF142366.I, EF142364.I, EF142363.I, AF359451.I, AF359444.I, AF359442.I, EF142443.I, EF142442.I, EF142441.I, EF142440.I, EF142439.I, EF142438.I, EF142437.I, EF142436.I, EF142435.I, EF142434.I, EF142433.I, EF142432.I, EF142431.I, EF142430.I, EF142429.I, EF142428.I, EF142427.I, EF142426.I, EF142425.I, EF142424.I, EF142423.I, EF142422.I, EF142421.I, EF142420.I, EF142419.I, EF142418.I, EF142417.I, EF142416.I, EF142415.I, EF142414.I, EF142413.I, EF142412.I, EF142411.I, EF142410.I, EF142409.I, EF142408.I, EF142407.I, EF142406.I, EF142405.I, EF142404.I, EF142403.I, EF142402.I, EF142401.I, EF142400.I, EF142399.I, EF142398.I, EF142397.I, EF142396.I, EF142395.I, EF142394.I, EF142393.I, EF142392.I, EF142391.I, EF142390.I, EF142389.I, EF142388.I, EF142387.I, EF142386.I, EF142385.I, EF142384.I, EF142383.I, EF142382.I, EF142381.I, EF142380.I, EF142379.I, EF142378.I, EF142377.I, EF142376.I, EF142375.I, EF142374.I, EF142373.I, EF142370.I, EF142369.I, EF142365.I, AF359450.I, AF359449.I, AF359448.I, AF359447.I, AF359446.I, AF359445.I, AF359443.I



## Kit Contents

- Pathogen specific primer/probe mix (150 reactions **BROWN**)
- Pathogen positive control template (for Standard curve **RED**)
- Internal extraction control DNA (150 reactions **BLUE**)
- Internal extraction control primer/probe mix (150 reactions **BROWN**)
- Endogenous ACTB primer/probe mix (150 reactions **BROWN**)
- RNase/DNase free water

## Reagents and equipment to be supplied by the user

- **Real-Time PCR Instrument**
- **Mastermix or Mastermix components**  
This kit is designed to work well with all commercially available Mastermixes. However, we recommend the use of PrimerDesign 2x Precision™ Mastermix.
- **Pipettors and Tips**
- **Vortex and centrifuge**
- **Thin walled 1.5 ml PCR reaction tubes**

## Kit storage

This kit is stable at room temperature but should be stored at -20°C on arrival. Once the lyophilized components have been re-suspended unnecessary repeated freeze/thawing should be avoided. Under these conditions reagents are stable for six months from date of purchase.



## Suitable sample material

All kinds of sample material suited for PCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration, and RNA/DNA integrity (An internal PCR control is supplied to test for non specific PCR inhibitors). Always run at least one negative control with the samples. To prepare a negative-control, replace the template RNA sample with RNase/DNase free water.

## Dynamic range of test

Under optimal PCR conditions PrimerDesign pathogen detection kits have very high priming efficiencies of >95% and can detect between  $1 \times 10^8$  and  $1 \times 10^2$  copies of target template.



# Principles of the test

## Real-Time PCR

A pathogen specific primer and probe mix is provided and the target sequence can be detected through the **FAM** channel.

The primer and probe mix provided exploits the so-called TaqMan<sup>®</sup> principle. During PCR amplification, forward and reverse primers hybridize to the pathogen DNA/cDNA. A fluorogenic probe, is included in the same reaction mixture which consists of an oligonucleotide labeled with a 5`-reporter dye and a downstream, 3`-quencher, During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of real time PCR platforms.

## Positive control

For copy number determination, and as a positive control for the PCR set up, the kit contains positive control template. This can be used to generate a standard curve of pathogen copy number / CT value. Alternatively the positive control can be used at a single dilution for a qualitative analysis of the samples. Each time the kit is used, at least one positive control reaction must be included on the run. A positive result indicates that the primers and probes for quantification of the target pathogen gene are working properly in your particular experimental scenario. If a negative result is obtained the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false positive results. This can be achieved by handling this component in a Post PCR environment.

## Negative control

To confirm absence of contamination, a negative control reaction should be included every time the kit is used. For this reaction, the RNase/DNase free water should be used instead of template. A negative result indicates that the reagents have not become contaminated while setting up the run. If a positive result is obtained the results should be ignored and the test samples repeated. Possible sources or contamination should first be explored and removed.



### Internal DNA extraction control

When performing DNA extraction, it is often advantageous to have an exogenous source of DNA template that is spiked into the lysis buffer. This control DNA is then co purified with the sample DNA and can be detected as a positive control for the extraction process. Successful co-purification and real-time PCR for the control DNA also indicates that PCR inhibitors are not present at a high concentration.

A separate primer and probe mix are supplied with this kit to detect the exogenous DNA using real-time PCR. The primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the control DNA does not interfere with detection of the pathogen target DNA even when present at low copy number. The Internal control is detected through the **VIC** channel and gives a CT value of 26+/-3.

### Endogenous ACTB control

To confirm extraction of a valid biological template a primer and probe mix is included to detect the Actin Beta (ACTB) gene. Detection of ACTB is through the FAM channel and it is **NOT** therefore possible to perform a multiplex for ACTB and the pathogen primers. A poor ACTB signal may indicate that the sample did not contain sufficient human material.

### Carry-over prevention using UNG (optional)

Carry over contamination between PCR reactions can be prevented by including uracil-N-glycosylase (UNG) in the reaction mix. Some commercial mastermix preparations contain UNG or alternatively it can be added as a separate component. UNG can only prevent carry over from PCR reactions that include deoxyuridine triphosphate (dUTP) in the original PCR reaction. PrimerDesign recommend the application of 0.2 U UNG per assay with a 15 minute incubation step at 37°C prior to amplification. The heat-labile UNG is then inactivated during the Taq polymerase activation step (95°C for 10 minutes).



## Bench side Protocol

To minimize the risk of contamination with foreign DNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally this would be a designated PCR lab or PCR cabinet. Barrier tips are recommended for all pipetting steps.

### 1. Pulse-spin each tube in a centrifuge before opening.

This will ensure lyophilised primer and probe mix is in the base of the tube and is not spilt upon opening the tube.

### 2. Reconstitute the kit components according to the table below

To ensure complete reconstitution, vortex each tube thoroughly, allow to stand for 5 minutes and vortex again before use.

Component	Volume
<b>Pre-PCR box</b>	
Primer/Probe mix ( <b>BROWN</b> )	165 µl
Internal extraction control DNA ( <b>BLUE</b> )	600 µl
Internal extraction control primer/probe mix ( <b>BROWN</b> )	165 µl
Endogenous ACTB primer/probe mix ( <b>BROWN</b> )	165 µl
<b>Post-PCR bottle</b>	
Positive Control Template ( <b>RED</b> ) *	500 µl

\* This component contains high copy number template and is a VERY significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.

## DNA extraction

The internal extraction control DNA can be added either to the DNA lysis/extraction buffer or to the DNA sample once it has been resuspended in lysis buffer.

**DO NOT add the internal extraction control DNA directly to the unprocessed biological sample as this can lead to degradation and a loss in signal strength.**

1. Add 4µl of Internal extraction control DNA (**BLUE**) to each sample in DNA lysis/extraction buffer
2. Complete DNA extraction according to the manufactures protocols



## Real-time PCR detection

### 1. Prepare a reaction mix according to the table below

Include sufficient reactions for the standard curve wells (8 samples in duplicate) and also the negative control.

#### Pathogen detection mix

Component	l reaction
2X Precision™ Mastermix	10 µl
Pathogen primer/probe mix ( <b>BROWN</b> )	1.0 µl
Internal extraction control primer/probe mix ( <b>BROWN</b> )	1.0 µl
RNAse/DNAse Free water	3.0 µl
<b>Final volume</b>	<b>15 µl</b>

#### Endogenous ACTB detection mix (Optional)

Component	l reaction
2X Precision™ Mastermix	10 µl
Endogenous ACTB primer/probe mix ( <b>BROWN</b> )	1.0 µl
RNAse/DNAse Free water	4.0 µl
<b>Final volume</b>	<b>15 µl</b>

2. Pipette 15µl of this mix into each well according to your real-time PCR experimental plate set up.

3. Prepare sample DNA templates for each of your samples (suggested concentration 5ng/µl) in RNAse/DNAse free water.

If the concentration of DNA is not known, then dilute your DNA sample reactions 1:20 (10 µl of sample DNA and 190µl of water)

4. Pipette 5µl of diluted template into each well, according to your experimental plate set up.

For negative control wells use 5µl of RNAse/DNAse free water. The final volume in each well is 20µl



## 5. Preparation of standard curve dilution series

- 1) Pipette 900 $\mu$ l of RNase/DNase free water into 7 tubes and label 2-8
- 2) Pipette 100 $\mu$ l of Positive Control Template (**RED**) into tube 2
- 3) Vortex thoroughly
- 4) Change pipette tip and pipette 100 $\mu$ l from tube 2 into tube 3
- 5) Vortex thoroughly

Repeat steps 4 and 5 to complete the dilution series

Standard Curve	Copy Number
Tube 1 Positive control ( <b>RED</b> )	$2 \times 10^7$ per $\mu$ l
Tube 2	$2 \times 10^6$ per $\mu$ l
Tube 3	$2 \times 10^5$ per $\mu$ l
Tube 4	$2 \times 10^4$ per $\mu$ l
Tube 5	$2 \times 10^3$ per $\mu$ l
Tube 6	$2 \times 10^2$ per $\mu$ l
Tube 7	20 per $\mu$ l
Tube 8	2 per $\mu$ l

## 6. Pipette 5 $\mu$ l of standard template into each well, according to your experimental plate set up.

The final volume in each well is 20 $\mu$ l.



# Amplification Protocol

## Amplification conditions using PrimerDesign 2X Precision™ MasterMix.

	Step	Time	Temp
	UNG treatment (if required) **	15 mins	37°C
	Enzyme Activation (if required)***	10 mins	95°C
50 cycles	Denaturation	10s	95°C
	<b>DATA COLLECTION*</b>	60s	60°C

\* Fluorogenic data for the control DNA should be collected during this step through the FAM and VIC channels

\*\* Required if your mastermix includes UNG to prevent PCR carryover contamination

\*\*\* Not all Mastermixes require this enzyme activation step. Follow the manufactures instructions for your mastermix.



## Interpretation of Results

Pathogen	Internal control	Negative control	Positive control	Interpretation
+ve	+ve	-ve	+ve	+ve
+ve	-ve	-ve	+ve	+ve
-ve	+ve	-ve	+ve	-ve
-ve	-ve	-ve	-ve	Exp Fail
+ve	+ve	+ve	+ve	Exp Fail

### Internal PCR control

When used according to the above protocols, assuming a 100% extraction efficiency and that 1:20 of extracted DNA is used in the reaction, a CT value of 26 is expected. However this can vary significantly depending on the extraction efficiency, the quantity of DNA added to the PCR reaction and the individual machine settings. CT values of  $26 \pm 3$  are within the normal range. When amplifying a pathogen sample with high genome copy number, the internal extraction control may not produce an amplification plot. This does not invalidate the test and should be interpreted as a positive experimental result.

### Endogenous ACTB control

The signal obtained from the ACTB primer and probe set will vary according to the amount of biological material present in a given sample.



## Notices and disclaimers

During the warranty period PrimerDesign pathogen detection kits allow precise and reproducible data recovery combined with excellent sensitivity. For data obtained by violation to the general GLP guidelines and the manufacturer's recommendations the right to claim under guarantee is expired.

Black Hole Quencher™, "BHQ", "CAL Fluor", "Quasar" and "Pulsar" are registered trademarks of Biosearch Technologies, Inc., Novato, CA. This technology is protected by U.S. and World-wide patents either issued or in application and is licensed and sold under agreement with Biosearch Technologies, Inc. These products are sold exclusively for R&D use by the purchaser. They may not be used for human or veterinary *in vitro* diagnostic (IVD) applications and they may not be re-sold, distributed or re-packaged without express written authorization from Biosearch.

PCR is a proprietary technology covered by several US and foreign patents. These patents are owned by Roche Molecular Systems Inc. and have been sub-licensed by PE Corporation in certain fields. Depending on your specific application you may need a license from Roche or PE to practice PCR. Additional information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at Roche Molecular Systems, 1145 Atlantic Avenue, Alameda, CA 94501 or Applied Biosystems business group of the Applied Corporation, 850 Lincoln Centre Drive, Foster City, CA 94404. In addition, the 5' nuclease assay and other homogeneous amplification methods used in connection with the PCR process may be covered by U.S. Patents 5,210,015 and 5,487,972, owned by Roche Molecular Systems, Inc. and by U.S. Patent 5,538,848, owned by The Perkin-Elmer Corporation. The purchase of Biosearch Technologies products does not, either expressly or by implication, provide a license to use this or other patented technology. Licensing information can be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404 or the Licensing Department at Roche Molecular Systems Inc., 1145 Atlantic Avenue, Alameda, CA 94501."

## Trademarks

PrimerDesign™ is a trademark of PrimerDesign Ltd.

The PCR process is covered by US Patents 4,683,195, and 4,683,202 and foreign equivalents owned by Hoffmann-La Roche AG.

ABI, ABI PRISM®, GeneAmp® and MicroAmp® are registered trademarks of the Applied Genomics (Applied Biosystems Corporation).

BIOMEK® is a registered trademark of Beckman Instruments, Inc.; iCycler™ is a registered trademark of Bio-Rad Laboratories, Rotor-Gene is a trademark of Corbett Research. LightCycler™ is a registered trademark of the Idaho Technology Inc.

GeneAmp®, TaqMan® and AmpliTaqGold® are registered trademarks of Roche Molecular Systems, Inc.,

The purchase of the PrimerDesign™ reagents cannot be construed as an authorization or implicit license to practice PCR under any patents held by Hoffmann-LaRoche Inc.