foodproof® *Alicyclobacillus* Detection Kit

Revision A, November 2023

PCR kit for the qualitative detection of *Alicyclobacillus* DNA with simultaneous identification of *Alicyclobacillus acidoterrestris* DNA using real-time PCR instruments.

Product No. KIT230051

Kit for 96 reactions for a maximum of 94 samples

Store the kit at -15 to -25 °C

For food testing purposes.

FOR *IN VITRO* USE ONLY

Table of Contents

1. What This Product Does

1.1 Number of Tests

The kit is designed for 96 reactions with a final reaction volume of 25 µL each. Up to 94 samples (single sample preparation) plus positive and negative control reactions can be analyzed per run.

1.2 Storage and Stability

- Store the kit at -15 to -25 °C through the expiration date printed on the label.
- Once the kit is opened, store the kit components as described in the following Kit Contents table:

1.3 Kit Contents

Vial	Label	Contents / Function / Storage
1 yellow cap	foodproof® *Alicyclobacillus* Master Mix	• 3 x 600 µL • Ready-to-use primer and hydrolysis probe mix specific for *Alicyclobacillus* DNA and the Internal Control (IC). • For amplification and detection of *Alicyclobacillus*-specific sequences. • Store at -15 to -25°C. • **Avoid repeated freezing and thawing!** • **Protect from light!**
2 red cap	foodproof *Alicyclobacillus* Enzyme Solution	• 3 x 32 µL • Contains Taq DNA Polymerase and Uracil-DNA N-Glycosylase (UNG, heat labile) for prevention of carry-over contamination. • Store at -15 to -25°C.
3 white cap	foodproof *Alicyclobacillus* Internal Control	• 3 x 32 µL • Contains a stabilized solution of plasmid DNA and a yellow dye for better visualization. • For use as an internal amplification control. • Store at -15 to -25°C. • After first thawing store at 2 to 8 °C for up to one month.
4 purple cap	foodproof *Alicyclobacillus* Control Template	• 1 x 50 µL • Contains a stabilized solution of plasmid DNA. • For use as a PCR run positive control. • Store at -15 to -25°C. • After first thawing store at 2 to 8 °C for up to one month.
5 colorless cap	H_2O, PCR-grade	• 1 x 1 mL • Nuclease-free, PCR-grade H_2O. • For use as a PCR run negative control. • Store at -15 to -25°C.

1.4 Additional Equipment and Reagents Required

- Real-time PCR cycler suitable for detection of FAM-, VIC/HEX-, and ROX/Texas Red-labeled probes
- Real-time PCR compatible tubes, strips or plates with optical cap or foil applicable for the PCR cycler in use
- foodproof ShortPrep II Kit (Product No. KIT230171)

or

- foodproof StarPrep Two (Product No. KIT230177)
- Nuclease-free, aerosol-resistant pipette tips
- Pipettes
- Sterile reaction tubes for preparing PCR mixes and dilutions

1.5 Applicability Statement

The foodproof *Alicyclobacillus* Detection Kit is intended for the rapid detection of *Alicyclobacillus* DNA, including the simultaneous identification of *Alicyclobacillus acidoterrestris* DNA isolated from enrichment cultures prepared by valid methods and inoculated with all kinds of foods that are potentially contaminated with *Alicyclobacillus* spp.

The kit must not be used in diagnostic procedures.

The performance of the kit described in this Instruction Manual is guaranteed only when it is used with real-time PCR instruments suitable for detection of FAM-, VIC/HEX-, and ROX/Texas Red-labeled probes, e.g., LightCycler® 480 (Roche Diagnostics), ABI 7500 (Applied Biosystems), iCycler iQ5 (BioRad), Mx3000P/Mx3005P (Stratagene) or Rotorgene 6000 (Corbett Life Science).

Note:
A color compensation kit (Color Compensation Set 3; Product No.: KIT230005) is necessary and will be supplied by Hygiena Diagnostics GmbH for users of the LC 480 System. Please contact Hygiena for further information.

2. How to Use this Product

2.1 Before You Begin

2.1.1 Precautions

Detection of *Alicyclobacillus* DNA using the foodproof *Alicyclobacillus* Detection Kit requires DNA amplification by PCR. The detection kit provides all the reagents required for the PCR. However, in order to achieve reliable results, the entire assay procedure must be performed under nuclease-free conditions. Follow the instructions below to avoid nuclease-, carry-over-, or cross-contamination:

- Prepare appropriate aliquots of the kit solutions and keep them separate from other reagents in the laboratory.
- Use nuclease-free labware (*e.g.,* pipettes, pipette tips, reaction vials).
- Wear gloves when performing the assay.
- To avoid cross-contamination of samples and reagents, use fresh aerosol-preventive pipette tips.
- To avoid carry-over contamination, transfer the required solutions for one experiment into a fresh tube, rather than directly pipetting from stock solutions.
- Physically separate the workplaces for DNA preparation, PCR setup, and PCR to minimize the risk of carry-over contamination. Use a PCR hood for all pipetting steps.

Note: Keep the foodproof *Alicyclobacillus* Master Mix (vial 1, yellow cap) away from light.

2.1.2 Sample Material

Use any sample material suitable for PCR in terms of purity, concentration, and absence of inhibitors. For preparation of genomic DNA from raw material or from food enrichments, refer to the corresponding product package inserts of a suitable sample preparation kit (see *"1.4 Additional Equipment and Reagents Required"*).

2.1.2 DNA Extraction

Hygiena Diagnostics GmbH provides sample preparation kits suitable for all kinds of food and raw materials (see *"1.4 Additional Equipment and Reagents Required"*). For more product information, please see www.hygiena.com.

2.1.3 Positive Control

Always run a positive control with the samples. To prepare a positive control, replace the template DNA with the provided control DNA [foodproof *Alicyclobacillus* Control Template (vial 4, purple cap)] or with a positive sample preparation control.

2.1.4 Negative Control

Always run a negative control with the samples. To prepare a negative control, replace the template DNA with H_2O, PCR-grade (vial 5, colorless cap). Include a negative control during sample preparation to monitor reaction purity and cross-contamination. This extraction control can be used as an additional negative control reaction.

2.2 Procedure

2.2.1 Program Setup

Program the PCR instrument before preparing the reaction mixes. The amplification is carried out according to the following temperature-time-program (for details on how to program the experimental protocol, see the operation manual of your real-time PCR cycler):

Pre-incubation	**1** cycle
Step 1:	37 °C for 4 minutes
Step 2:	95 °C for 5 minutes
Amplification	**50** cycles
Step 1:	95 °C for 5 seconds
Step 2*:	60 °C for 60 seconds

* Fluorescence detection in step 2

For some real-time PCR instruments, the type of probe quencher and the usage of a passive reference dye has to be specified. The foodproof *Alicyclobacillus* Detection Kit contains probes with a non-fluorescent ("dark") quencher and no passive reference dye.

For users of the Agilent Mx3005P instrument: Click on 'Instrument → Filter Set Gain Settings' to open the Filter Set Gain Settings dialog box in which the gain settings may be viewed and modified. For FAM, the Filter Set Gain Setting has to be modified to 'x1'.

2.2.2 Preparation of the PCR Mix

Proceed as described below to prepare a 25 µL standard reaction.
Always wear gloves when handling the PCR vessels.

1. Thaw the solutions and, for maximal recovery of contents, briefly spin vials in a microcentrifuge before opening. Mix carefully but thoroughly by pipetting up and down.
2. In a reaction tube (0.5 – 2.0 mL, depending on the number of reactions), prepare the PCR mix by adding the following components in the order mentioned below, then mix gently but thoroughly by pipetting up and down.

The volumes indicated below are based on a single 25 µL standard reaction. Prepare the PCR mix by multiplying the amount in the "Volume" column by the number of reactions (sample and control reactions) to be cycled plus one or two additional reactions to cover pipetting losses.

Component	Volume
foodproof *Alicyclobacillus* Master Mix, (vial 1, yellow cap)	18.0 µL
foodproof *Alicyclobacillus* Enzyme Solution, (vial 2, red cap)	1.0 µL
foodproof *Alicyclobacillus* Internal Control, (vial 3, white cap)	1.0 µL
Total volume	20.0 µL

3. Pipet 20 µL PCR mix into each PCR vessel.
   - For the samples of interest, add 5 µL sample DNA
     (if less than 5 µL, add H_2O to bring volume to 5 µL).
   - For the negative control, add 5 µL H_2O, PCR-grade (vial 5, colorless cap).
   - For the positive control, add 5 µL foodproof *Alicyclobacillus* Control Template (vial 4, purple cap).
4. Seal the PCR vessels accurately with optical caps or sealing foil.
5. Briefly spin the PCR vessels in a suitable centrifuge.
6. Cycle the samples as described above.

2.3 Data Interpretation

The amplification of DNA of *Alicyclobacillus acidoterrestris* is analyzed in the fluorescence channel suitable for FAM-labeled probes detection. The amplification of DNA of all *Alicyclobacillus* species (including *A. acidoterrestris*) is analyzed in the fluorescence channel suitable for the detection of VIC/HEX-labeled probes. The specific amplification of the Internal Control is analyzed in the fluorescence channel suitable for ROX/Texas Red.
Compare the results from channel FAM (*Alicyclobacillus acidoterrestris*), channel VIC/HEX (*Alicyclobacillus* spp.) and channel ROX/Texas Red (Internal Control) for each sample, and interpret the results as described in the table below.

Channel FAM	Channel VIC/HEX	Channel ROX/Texas Red	Result Interpretation
Positive	Positive	Positive or Negative	Positive for *Alicyclobacillus acidoterrestris*
Negative	Positive	Positive or Negative	Positive for *Alicyclobacillus* spp. other than *Alicyclobacillus acidoterrestris*
Negative	Negative	Positive	Negative for *Alicyclobacillus* spp. (including *Alicyclobacillus acidoterrestris*)
Negative	Negative	Negative	Invalid

Note:
A prerequisite for the unambiguous discrimination of *Alicyclobacillus* and *A. acidoterrestris* DNA and Internal Control DNA in this multi-color experiment is a suitable calibration of the PCR instrument for channels FAM, VIC/HEX and ROX/Texas Red. Please refer to the operation manual of your real-time PCR cycler for further information.

3. Troubleshooting

Observation	Possible Reason	Recommendation
No signal increase is observed, even with positive controls.	Incorrect detection channel has been chosen.	• Set Channel settings to FAM, VIC/HEX or ROX/Texas Red.
	Pipetting errors or omitted reagents.	• Check for correct pipetting scheme and reaction setup. Repeat the PCR run. • Always run a positive control along with your samples.
	No data acquisition programmed.	• Check the cycle programs.
No signal increase in channel ROX/Texas Red is observed.	Inhibitory effects of the sample material (e.g., caused by insufficient purification).	• Use the recommended DNA sample preparation kit to purify template DNA. • Dilute samples or pipet a lower amount of sample DNA (*e.g.,* 2.5 µL instead of 5 µL).
Fluorescence intensity is too low.	Inappropriate storage of kit components.	• Store the foodproof *Alicyclobacillus* Master Mix (vial 1, yellow cap) at -15 to -25 °C, protected from light. • Avoid repeated freezing and thawing.
	foodproof *Alicyclobacillus* Master Mix (vial 1, yellow cap) is not homogeneously mixed.	• Mix the foodproof *Alicyclobacillus* Master Mix (vial 1, yellow cap) and the entire PCR-mix thoroughly before pipetting.
	Low initial amount of target DNA.	• Increase the amount of sample DNA. Depending on the chosen DNA isolation method, inhibitory effects may occur.
Negative control samples are positive.	Carry-over contamination.	• Exchange all critical solutions. • Repeat the complete experiment with fresh aliquots of all reagents. • Always handle samples, kit components and consumables in accordance with commonly accepted practices to prevent carry-over contamination. • Add positive controls after sample and negative control reaction vessels have been sealed.
Fluorescence intensity varies.	Insufficient centrifugation of the PCR vessels. Prepared PCR mix is still in the upper part of the vessel.	• Always centrifuge reaction vessels.
	Outer surface of the vessel or the seal is dirty (*e.g.,* by direct skin contact).	• Always wear gloves when handling the vessels and seal.

4. Additional Information on this Product

4.1 How this Product Works

The foodproof *Alicyclobacillus* Detection Kit provides primers and hydrolysis probes (for sequence-specific detection), convenient premixed reagents, and a control template for reliable interpretations of results. To ensure maximum reliability of the kit and to prevent misinterpretation of negative results due to inhibition of the amplification, an Internal Control (IC) is supplied with the kit (vial 3, white cap). The IC has to be added to each reaction. A hydrolysis probe was designed to bind specifically the IC, allowing detection in the ROX/Texas Red channel, whereas the *Alicyclobacillus* DNA is detected in channels FAM (*A. acidoterrestris*) and VIC/HEX (all *Alicyclobacillus* spp.). In case of a negative result due to inhibition of the amplification by the sample DNA of interest, the amplification of the IC is suppressed as well, whereas a negative result for the sample DNA of interest and amplification of the IC clearly indicates the absence of *Alicyclobacillus* DNA in the sample. The foodproof *Alicyclobacillus* Detection Kit minimizes contamination risk and contains all reagents (except for template DNA) needed for the detection of *Alicyclobacillus* DNA. Primers and probes provide specific detection of *Alicyclobacillus* DNA in food samples. The described performance of the kit is guaranteed for use on the real-time PCR instruments listed above only.

4.2 Test Principle

1. Using the kit's sequence-specific primers in a polymerase chain reaction (PCR), the PCR instrument and the supplied reagents amplify fragments of *Alicyclobacillus* spp.-specific sequences.
2. The PCR instrument detects these amplified fragments in real time through fluorescence generated by cleavage of the hybridized probe due to the 5´-nuclease activity of the Taq DNA polymerase. The probe is labeled at the 5´-end with a reporter fluorophore and at the 3´-end with a quencher.
3. During the annealing/elongation phase of each PCR cycle, the probe hybridizes to an internal amplicon sequence and is cleaved by the 5' nuclease activity of the Taq DNA polymerase. This cleavage of the probe separates the reporter dye from the quencher dye, increasing the reporter dye signal.
4. The PCR instrument measures the emitted fluorescence of the reporter dye.

4.3 Prevention of Carry-Over Contamination

The heat-labile Uracil-DNA N-Glycosylase (UNG) is suitable for preventing carry-over contamination between PCR reactions. This technique relies on the incorporation of deoxyuridine triphosphate (dUTP) during all amplification reactions and the pretreatment of all successive PCR mixtures with the heat-labile UNG. The UNG cleaves DNA at any site where a deoxyuridine residue has been incorporated. The resulting abasic sites are hydrolyzed due to the high temperatures during the initial denaturation step and can no longer serve as PCR templates. The heat-labile UNG is inactivated during the initial denaturation step. Native DNA (e.g., the isolated *Alicyclobacillus* genomic DNA) does not contain uracil and is, therefore, not degraded by this procedure. Since dTTP is replaced with dUTP and UNG is included in the foodproof *Alicyclobacillus* Detection Kit, decontamination can be achieved with the provided reagents.

4.4 Background Information

Alicyclobacillus spp. are gram-positive, rod-shaped, thermophilic, acidophilic, spore-forming bacteria. Depending on the different species, growth temperatures range from 20 to 70°C, with optimums from 42 to 60°C. *Alicyclobacillus* spp. can also grow over a wide pH range, generally reported between pH 2.5 and 6.0. The spores are present almost everywhere in the environment and can be brought into the company on contaminated fruit**.** It is one of the microorganisms of concern in the fruit juice industry. The thermophilic and acidophilic characteristics of *Alicyclobacillus* spp. allow resistance to current pasteurization processes, and the ability to produce off-flavors in juice poses potential economic losses for the juice industry. Most studies concerning

Alicyclobacillus spp.-related spoilage is focused on *A. acidoterrestris.* Guaiacol and halophenols were identified as the offensive-smelling agents in many *Alicyclobacillus* spp.-related spoilage (1). Since conventional microbiological methods for the detection and identification of *Alicyclobacillus* are very time-consuming, PCR has been introduced to the food industry as a highly sensitive and specific detection method (2).

4.5 Product Specifications

4.5.1 Specificity

Inclusivity of the foodproof *Alicyclobacillus* Detection Kit has been tested with 38 strains of the genus *Alicyclobacillus*, including the following species: *A. acidiphilus*, *A. acidocaldarius*, *A. acidoterrestris* (7 strains), *A. contaminans*, *A. cycloheptanicus*, *A. disulfidooxidans*, *A. fastidiosus*, *A. herbarius*, *A. hesperidum*, *A. kakegawensis*, *A. macrosporangiidus*, *A. pomorum*, *A. sacchari*, and *A. sendaiensis*.
All strains of *A. acidoterrestris* were detected in channels FAM and VIC/HEX, and all strains of other species than *A. acidoterrestris* were detected in channel VIC/HEX only.
Exclusivity of the foodproof *Alicyclobacillus* Detection Kit has been tested with 40 strains of closely related genera (mainly of the order *Bacillales*). None of them was detected in channel FAM or channel VIC/HEX.

4.5.2 Sensitivity

The foodproof *Alicyclobacillus* Detection Kit detects down to 10^2 - 10^3 CFU/mL in enrichment cultures (depending on the sample preparation kit used).

5. Supplementary Information

5.1 Ordering Information

Hygiena Diagnostics GmbH offers a broad range of reagents and services. For a complete overview and for more information, please visit our website at www.hygiena.com.

5.2 License Notice

The purchase price of this product includes limited, nontransferable rights under U.S. Patent No. 7,687,247 owned by Life Technologies Corporation to use only this amount of the product to practice the claims in said patent solely for activities of the purchaser for bioburden testing, environmental testing, food testing, or testing for genetically modified organisms (GMO) in accordance with the instructions for use accompanying this product. No other rights are conveyed, including no right to use this product for in vitro diagnostic, therapeutic, or prophylactic purposes. Further information on purchasing licenses under the above patent may be obtained by contacting the Licensing Department, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, CA 92008. Email: outlicensing@lifetech.com.

5.3 Trademarks

foodproof® is a registered trademark of Hygiena Diagnostics GmbH. Other brand or product names are trademarks of their respective holders.

5.4 Contact and Support

If you have questions or experience problems with this or any other product of Hygiena Diagnostics GmbH, please contact our Technical Support staff (www.hygiena.com/support). Our scientists commit themselves to providing rapid and effective help. We also want you to contact us if you have suggestions for enhancing our product

performance or using our products in new or specialized ways. Such customer information has repeatedly proven invaluable to us and the worldwide research community.

5.5 References

1. Chang S, Kang D (2004). *Alicyclobacillus* spp. in the fruit juice industry: History, characteristics, and current isolation/detection procedures. *Critical Reviews in Microbiology* 30, 55-74.

2. Scheu PM et al. (1998). Detection of pathogenic and spoilage microorganisms in food with the polymerase chain reaction. *Food Microbiology* 15, 13-31.

5.6 Reference Number

The reference number and original Hygiena Diagnostics GmbH article number: R 302 28

6. Change Index

Version 1, May 2011:
First version of the package insert.

Version 2, March 2017:
License Notice changed.

Revision A, November 2023:
Rebranding and new layout.
R 302 28 20 -> INS-KIT230051-RevA

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