foodproof® *Alicyclobacillus* Detection LyoKit

Revision A, May 2024

PCR kit for the qualitative detection of *Alicyclobacillus* spp. and additional differentiation of guaiacol-producing strains using real-time PCR instruments.

Product No. KIT230151 / KIT230152 / KIT230153

Kit for 96 reactions (lyophilized) for a maximum of 94 samples

Store the kit at 2 to 8 °C

For food testing purposes.

FOR *IN VITRO* USE ONLY

Table of Contents

1. Product Overview

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 of Kit/Components

- Store the kit at 2 to 8 °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.

Component	Label	Contents / Function / Storage
foodproof *Alicyclobacillus* Detection LyoKit Microplate, prefilled with 96 reactions (lyophilized)	Aluminum bag containing an 8-tube strip mat • KIT230151 with white low-profile tubes • KIT230152 with clear regular profile tubes • KIT230153 with clear low-profile tubes	• 96 prefilled reactions (lyophilized). • Ready-to-use PCR mix containing primer and hydrolysis probes specific for the detection of *Alicyclobacillus* spp. DNA, the DNA of guaiacol-producing strains, and the Internal Control (IC) as well as *Taq* DNA Polymerase and Uracil-DNA N-Glycosylase (UNG, heat-labile) for the prevention of carry-over contamination. • For amplification and detection of *Alicyclobacillus* spp. and guaiacol-producing strain sequences. • Store at 2 to 8 °C in the aluminum bag (sealed). o **Protect from light and moisture!**
Control Template	Vial 2 (purple cap)	• 1 x 350 µL • Contains a stabilized solution of DNA. • For use as a PCR run positive control. • Store at 2 to 8 °C.
H_2O PCR-grade	Vial 3 (colorless cap)	• 2 x 1 mL • Nuclease-free, PCR-grade H_2O. • For use as a PCR run negative control.
Cap strips	Plastic bag containing 8-cap strips	• 12 x 8-cap strip • For use in real-time PCR after the addition of samples.

1.3 Additional Equipment and Reagents Required

- DNA extraction kits:
  - foodproof StarPrep Two Kit (Product No. KIT230177)
- Nuclease-free, aerosol-resistant pipettor tips
- Pipettors
- Vortex centrifuge Multispin MSC-3000/6000 for PCR strips **with**
  - SR-32, Rotor for MSC-3000/6000
- Alternative: Vortex centrifuge CVP-2 for PCR plates
- Appropriate real-time PCR Cycler
  - KIT230153 is designed to run on Dualo 32® Beverage Instrument

1.4 Applicability Statement

The foodproof *Alicyclobacillus* Detection LyoKit is intended for the rapid screening for the presence of *Alicyclobacillus* spp. in enrichment cultures from juices, juice concentrates and other beverages. DNA of *Alicyclobacillus* spp. is detected in channel HEX, whereas guaiacol-producing *Alicyclobacillus* strains are detected in channel FAM.

The kit must not be used in diagnostic procedures.

- KIT230151 / KIT230152: Real-time PCR cycler suitable for detection of FAM-, HEX- and ROX-labeled probes as well as for using low profile tubes (KIT230151) or regular profile tubes (KIT230152).
    - In cases where the strip tubes don´t fit the instrument, samples must be transferred to appropriate PCR vessels after resuspension of the lyophilized PCR mix.
- KIT230153: Compatible with the Dualo 32® Beverage.

The kit versions KIT230151 (LP) and KIT230152 (RP) described in this instruction manual have been developed for real-time PCR instruments with a FAM, a HEX and a ROX detection channel.

The kit version KIT230153 (DP) has been developed for the Dualo 32 Beverage.

Note: The Color Compensation Set 5 (Product No. KIT230011) is necessary for users of the LightCycler 480 System.

2. How to Use this Product

2.1 Before You Begin

2.1.1 Precautions

Detection of DNA from *Alicyclobacillus* using the foodproof *Alicyclobacillus* Detection LyoKit requires DNA amplification by PCR. The kit provides all 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:

- Keep the kit components separate from other reagents in the laboratory.
- Use nuclease-free labware (*e.g.,* pipettors, pipettor 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.

Keep the foodproof *Alicyclobacillus* lyophilized PCR Mix away from light and moisture.

2.1.2 Sample Material

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

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* Detection Control Template (vial 2, 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 PCR-grade H_2O (vial 3, 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 for Dualo 32 Beverage (KIT230153)

The Dualo 32 Beverage instrument (Product No. MCH230008) can be started from a pre-installed run template: Click on 'New', select the appropriate template, and press 'Select'. After loading the samples, the instrument can be started by clicking on 'Start Run'.

For detailed instructions on how to program and start the PCR run on the Dualo 32 Beverage, please refer to the manual for this instrument.

2.2.2 Program Setup for other cyclers (KIT230151/KIT230152)

The following procedure is optimized for a real-time PCR instrument with a FAM (guaiacol-producing *Alicyclobacillus*), HEX (*Alicyclobacillus* spp.) and ROX (Internal Control) detection channel. Program the PCR instrument before preparing the PCR samples. Use the following real-time PCR protocol for the foodproof *Alicyclobacillus* Detection LyoKit. For details on programming the experimental protocol, see the Instrument Operator's Manual for the real-time PCR cycler used.

Program for KIT230151/ KIT230152**:**

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

Note:

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

2.3 Preparation of the PCR Mix

Proceed as described below to prepare a 25 µL standard reaction. Always wear gloves when handling strips or caps. Use any sample material suitable for PCR in terms of purity, concentration, and absence of inhibitors.

Note: The PCR strips must be stored in the provided aluminum bag with the silica gel pads to avoid liquid absorption.

1. Take the needed number of PCR tube strips out of the aluminum bag. Use scissors or a scalpel to cut the strips apart. Tightly seal the bag afterward and store it under the recommended conditions.
2. Place the PCR tube strips containing the lyophilized reagents in a suitable PCR tube rack. Check that the reagent pellets are at the bottom of the tubes. If not, briefly centrifuge or flick the pellets to the bottom before proceeding.
3. Decap the tube strips cautiously and discard the cap strips.

   Note: Do not leave strips open for extended periods of time. To avoid unwanted liquid absorption, open strips only shortly before filling.

4. Pipet 25 µL sample into each PCR vessel:
   - For samples of interest, add 25 µL sample DNA (if using less volume, add PCR-grade H_2O to achieve 25 µL).
   - For the negative control, add 25 µL PCR-grade H_2O (vial 3, colorless cap).
   - For the positive control, add 25 µL foodproof *Alicyclobacillus* Detection Control Template (vial 2, purple cap).

   Note: To reduce the risk of cross-contamination, it is recommended that only one PCR tube strip be prepared at a time.

5. Seal the vessels accurately and tightly with the colorless cap strips.
6. Mix thoroughly using a vortex centrifuge.

   Note: Hygiena Diagnostics recommends vortex centrifuges Multispin MSC-3000/6000 for PCR strips or vortex centrifuge CVP-2 for PCR plates. Dedicated protocols are available for this centrifuge.

   Note: Alternatively, resuspend the pellet by manual mixing. This may be achieved by cautiously pipetting the sample up and down multiple times during step 4 or flipping the tube strips after sealing while pressing down the cap strip.

7. Spin the PCR tube strips for 30 seconds at 150 – 200 x g in a suitable centrifuge.

   Note: If your centrifuge exceeds 200 x g, do not centrifuge for more than 5 seconds. Avoid centrifugation at forces exceeding 1,000 x g!

8. Place the samples in your PCR cycler and run the program as described above.

   Note: When using the LightCycler 480 instrument, a special adapter (Product No. MIS230005) is necessary.

   Note: For some PCR instruments, the PCR strips should be placed into the cycler block in a balanced order. For example, two strips can be placed in columns 1 and 12.

   For the Dualo 32 Beverage instrument, please ensure that the mount contains at least one strip in rows A and D or single tubes in wells A1, A8, D1 and D8. These positions can be filled with tubes containing reagents or empty tubes. For more detailed information, please refer to the product instructions for the Dualo 32 Beverage.

2.4 Data Interpretation

The amplification of the DNA from guaiacol-producing *Alicyclobacillus* is analyzed in the fluorescence channel suitable for FAM-labeled probe detection. The amplification of the *Alicyclobacillus* spp. specific sequence is analyzed in the fluorescence channel suitable for the detection of HEX-labeled probes. The amplification of the internal amplification control is analyzed in the fluorescence channel suitable for the detection of ROX-labeled probes.

Compare the results from all three detection channels for each sample and interpret the results as described in the table below.

Compare the results from the FAM channel (guaiacol-producing *Alicyclobacillus*), HEX channel (*Alicyclobacillus* spp.) and ROX channel (Internal Control) for each sample, and interpret the results as described in the table below.

FAM	HEX	ROX	Result Interpretation
Positive	Positive*	Positive or Negative	Positive for *Alicyclobacillus* (guaiacol-producing)
Negative	Positive	Positive or Negative	Positive for *Alicyclobacillus* (guaiacol-negative)
Negative	Negative	Positive	Negative for *Alicyclobacillus*
Negative	Negative	Negative	Invalid

***** If the signal in channel FAM is very late (close to the limit of detection), the result in HEX may be interpreted as negative due to the statistical distribution of target DNA. In this case, prolonging the enrichment or using a larger amount of sample DNA might be used to increase the sensitivity.

Note: A prerequisite for the unambiguous discrimination of the target sequences in channels FAM, HEX and ROX in this multi-color experiment is a suitable calibration of the PCR instrument for all used channels. 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, HEX, and ROX.
	Pipetting errors.	• Check for the correct reaction setup. Repeat the PCR run. • Always run a positive control along with your samples.
	White tube strips used for Dualo 32 instruments.	• Use clear tube strips (Product No. KIT230153).
	No data acquisition programmed.	• Check the cycle programs.
No signal increase in channel ROX is observed, with no signal increase in FAM and HEX.	Inhibitory effects of the sample material (e.g., caused by insufficient purification).	• Use the recommended DNA sample preparation kit to generate template DNA. • Dilute samples or pipet a lower amount of sample DNA (e.g., 5 µL instead of 25 µL).
Fluorescence intensity is too low.	Inappropriate storage of kit components.	• Store the foodproof *Alicyclobacillus* lyophilized PCR Mix at 2 to 8 °C, protected from light and moisture.
	Low initial amount of target DNA.	• Increase the amount of sample DNA. Depending on the chosen DNA isolation method, inhibitory effects may occur.
Strong decrease in fluorescence baseline	Resuspension of lyophilized PCR mix not complete	• Always resuspend the lyophilized PCR mix thoroughly.
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 the sample and negative control reaction vessels have been sealed.
Fluorescence intensity varies.	Insufficient centrifugation of the PCR strips. Resuspended PCR mix is still in the upper part of the vessel.	• Always centrifuge PCR strips.
	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.
Pellets are difficult to dissolve.	The lyophilized PCR mix started to rehydrate.	• Always store the lyophilized PCR mix in the aluminum bag with the silica gel pad. • Open each strip shortly before filling.

4. Additional Information on this Product

4.1 How this Product Works

The foodproof *Alicyclobacillus* Detection LyoKit provides all necessary 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 included. A hydrolysis probe was designed to bind specifically the IC, allowing detection in the ROX channel; in contrast, the bacterial DNA is detected in channels FAM (guaiacol-producing *Alicyclobacillus*) and HEX (*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, while a negative result for the sample DNA of interest and amplification of the IC clearly indicates the absence of DNA of *Alicyclobacillus* in the sample. The foodproof *Alicyclobacillus* Detection LyoKit minimizes contamination risk and contains all reagents (except for template DNA) needed for the detection of DNA of the target organisms. Primers and probes provide specific detection in beverage samples. The described performance of the kit is guaranteed for use on the real-time PCR instruments listed in "*Section 6.1 Quality Control*".

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*-related 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 sequence of the amplicon 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 bacterial 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 LyoKit, decontamination can be achieved with the provided reagents.

4.4 Background Information

Alicyclobacillus spp. are gram-positive, rod-shaped, thermophilic and acidophilic, spore-forming bacteria. Depending on the different species, growth temperatures range from 20-70 °C, with optimums from 42-60 °C. *Alicyclobacillus* spp. can also grow over a wide pH range, generally 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. Guaiacol and halophenols were identified as the

offensive-smelling agents in many *Alicyclobacillus* spp. related spoilage organisms (1). As an alternative to time-consuming conventional microbiological methods for the detection and identification of *Alicyclobacillus* spp., PCR has proven to be a highly rapid, sensitive and specific detection method (2). In recent years, apart from the dominant spoiler, *A. acidoterrestris*, new guaiacol-producing species have been identified, such as *A. suci* (3), *A. fastidiosus* (4) or *A. dauci* (5), highlighting the need for detection of guaiacol production capability rather over a broad range of species.

5. References

1. Smit Y, Cameron M, Venter P, Witthuhn RC. (2011) *Alicyclobacillus* spoilage and isolation--a review. *Food Microbiol.* 28(3):331 – 349.
2. Sourri P, Tassou CC, Nychas GE, Panagou EZ. (2022) Fruit Juice Spoilage by *Alicyclobacillus*: Detection and Control Methods-A Comprehensive Review. *Foods.* 11(5):747.
3. Roth K, Rana YS, Daeschel D, Kovac J, Worobo R, Snyder AB. (2021) *Alicyclobacillus mali* sp. nov., *Alicyclobacillus suci* sp. nov. and *Alicyclobacillus fructus* sp. nov., thermoacidophilic sporeforming bacteria isolated from fruit beverages. *Int J Syst Evol Microbiol.* 71(9). doi: 10.1099/ijsem.0.005016.
4. Połaska M, Dekowska A, Sokolowska B. (2021) Isolation and identification of guaiacol producing *Alicyclobacillus fastidiosus* strains from orchards in Poland. *Acta Biochim Pol.* 68(2):301 – 307.
5. Nakano C, Takahashi N, Tanaka N, Okada S. (2015) *Alicyclobacillus dauci* sp. nov., a slightly thermophilic, acidophilic bacterium isolated from a spoiled mixed vegetable and fruit juice product. *Int J Syst Evol Microbiol.* 65(Pt 2):716 – 722.

6. Supplementary Information

6.1 Quality Control

The foodproof *Alicyclobacillus* Detection LyoKit is function-tested using the Dualo 32 Beverage instrument and the Applied Biosystems® 7500 (Thermo Fisher Scientific).

6.2 Ordering Information

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

6.3 License Notice

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6.5 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 team (www.hygiena.com/support). Our scientists commit themselves to providing rapid and effective help. We also ask 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.

6.6 Reference Number

The reference number and original Hygiena Diagnostics GmbH article numbers: F60228-1 (KIT230151), F60228-2 (KIT230152) and F60228-3 (KIT230153).

7. Change Index

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