



4e For everyone™ Detection Kit B

Alicyclobacillus sp. Screening

USER GUIDE

Detection of Alicyclobacillus species

For research use only

Catalog #2401-18

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Section 1

4e For everyone™ Detection Kit B Alicyclobacillus sp. Screening

Alicyclobacillus are motile gram positive rod-shaped bacteria which are common in soil and water. The bacteria are thermophilic and acidophilic, and can commonly be found on fruit at orchards, especially apples and oranges. Due to their high tolerance for heat - *Alicyclobacillus* endospores can resist temperatures of 90 °C and higher - and low pH, *Alicyclobacillus* is a spoiler of fruit juices, as spores brought in with fruit survive and are activated during pasteurization.

Currently 18 different species of *Alicyclobacillus* are described. *A. acidoterrestris* is the most important spoiler. Guajacol, which is one of the metabolites produced by *Alicyclobacillus*, is not harmful when consumed, but its detection threshold is so low that even small amounts cause product spoilage. Contaminated fruit juice has an unpleasant phenolic smell and taste.

The detection of all *Alicyclobacillus* species in one single test provides a simple and easy “go-no go” screen for presence in your sample.

Section 2

Introduction to the 4e For everyone™ Detection Kit Technology

Today, the use of PCR is accepted as the standard method for detecting nucleic acids from numerous microorganisms in a diversity of food and beverages, both functional species as well as spoilers. Real time PCR is one of the most powerful, specific and reliable methods for the quantitative detection and identification of microorganisms at an early process stage to prevent spoilage and to maintain overall product quality.

The 4e For everyone™ Detection Kit system is based on DNA amplification and detection of microorganisms by real time PCR. The specific PCR reagents, primers and probes, come in a ready-made dried format in the PCR tubes for unrivalled ease of use and temperature stability.

All PCR tests use the FAM channel (494/518 nm) for detection of the target microorganisms and the HEX channel (535/556 nm) for an internal control reaction. This allows 4e For everyone™ Detection Kits to prevent false negatives due to sample inhibition, allowing you to be truly confident about negative results.

A typical workflow includes the following four steps:



Data analysis is optimized for use with the Chai Dual Channel Open qPCR™ real time PCR thermocycler (www.chaibio.com).

In order to achieve lowest detection limits, we recommend sample enrichment in our PCR certified FastOrange® enrichment media (<https://pika-weihenstephan.de/en/products/fastorange-media/>).

Section 3

Kit Components

The 4e For everyone™ Detection Kit B Alicyclobacillus sp. Screening kit contains sufficient reagents for 48 reactions.

Kit material for DNA isolation and analysis	Amount	Storage
Washing buffer A (yellow cap)	2 x 10.0 mL	2-8 °C
Lysis buffer A (blue cap), contains glass beads which form a clearly visible sediment	1 x 10.0 mL	
Rehydration buffer B (white cap)	1 x 5.0 mL	
Positive control DNA (red cap)	1 x 50 µL	
PCR tubes (strips of 8) with BHOmix	6	
2 x Master mix (green cap)	1 x 850 µL	
Cap strips (strips of 8) for covering the PCR reaction tubes	6	2-8 °C or ambient

Table 1: Materials supplied

Section 4

Shelf Life and Storage

Once received, the kit must be stored at 2-8 °C. Reagents stored at this temperature can be used until the expiration date indicated on the package label.

Section 5

Materials Required but Not Supplied

Equipment	Supplies
Real time PCR thermocycler for 0.1 mL tubes with detection channels for FAM (520 nm emission) and HEX (550 nm emission)	1.5 ml reaction tubes, 2 per sample plus 1 per run
Benchtop microcentrifuge for 1.5 mL reaction tubes, 10,000 rpm (RCF: 10,000 x g) minimum	Pipette tips with filters
Centrifuge for 8-tube strips 0.1 mL or adaptor for benchtop microcentrifuge	Gloves, powder free
Reaction tube mixer (Vortexer)	
Microliter pipettes for DNA extraction 100-1,000 µL variable volume 200 µL fixed volume (optional)	Ideally use different pipettes for DNA extraction and PCR set-up
Microliter pipettes for PCR set-up 100-1,000 µL variable volume 25 µL fixed volume 5 µL fixed volume, or variable equivalent	

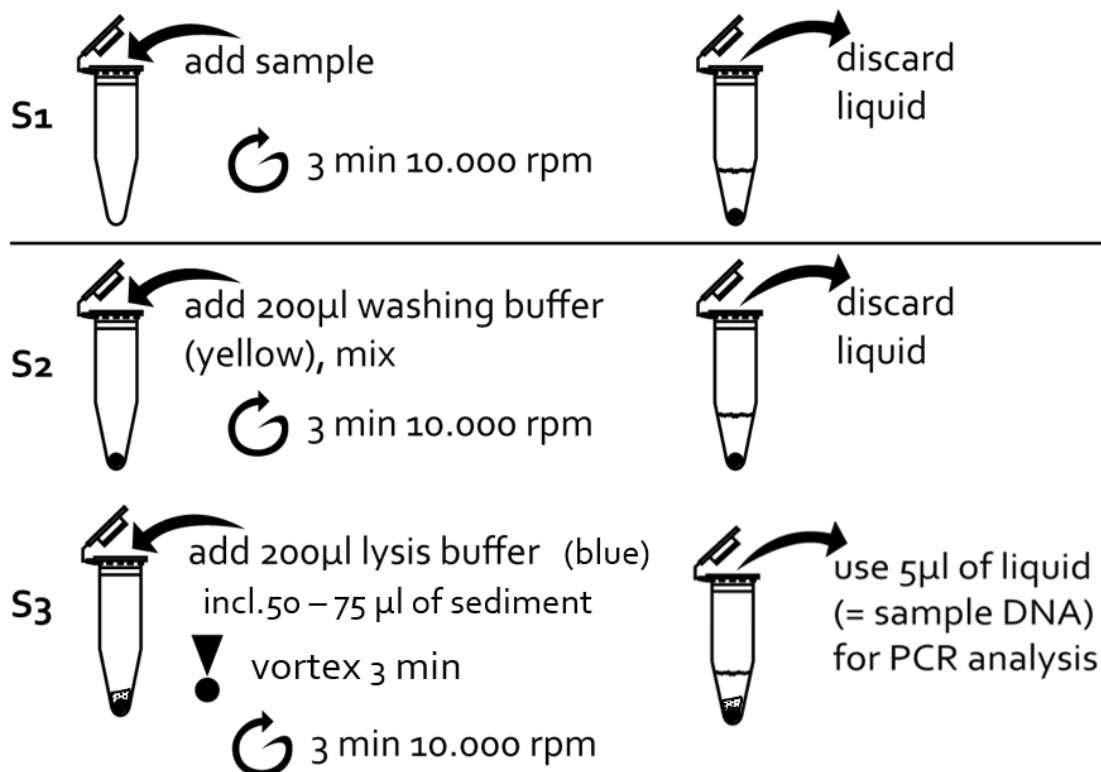
Table 2: Additional materials required

Section 6

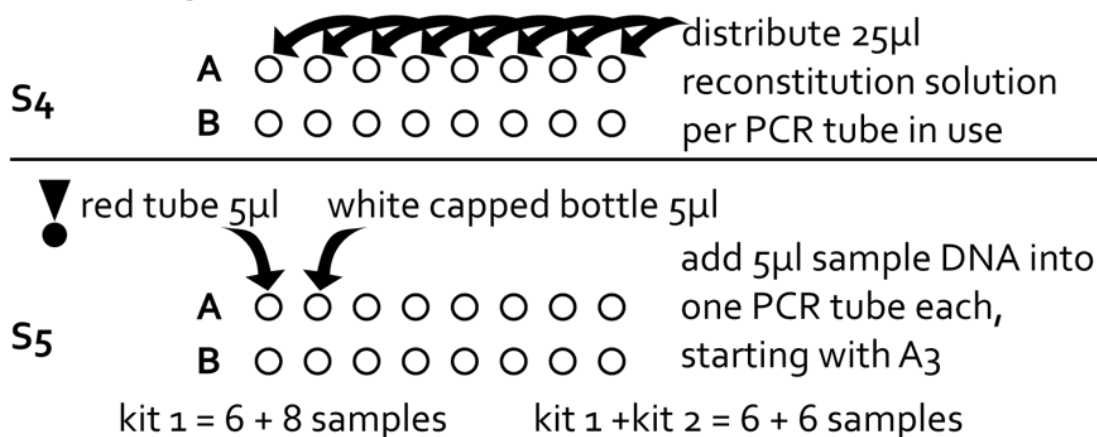
Overview

Sample Prep

Detailed instructions at www.pika-weihenstephan.com/4e



PCR analysis (use table on the back of this box)



Section 7

Detailed Instructions



Warning! Read the manual and the Safety Data Sheets before starting the analysis. Safety Data Sheets are available in the download area from www.pika-weihenstephan.com. All handling steps should be performed under sterile conditions. Wear appropriate protective clothing and powder free gloves. The use of filter tips is recommended.

Sample Preparation

1. Transfer the samples into a 1.5 mL reaction tube:
 - a) *Liquid samples:*
 - Clear samples (rinse water, filtered juice, enrichment without visible growth, etc):
 - o Standard volume, sample after enrichment:
 - Use 1.0 - 1.5 mL. Proceed to step 2.
 - o Larger volume, sample without enrichment: use larger volume, up to 50 mL:
 - Centrifuge sample 5 min at 4,000 - 5,000 rpm (RCF: 2,700-3,000 x g)
 - Slowly decant the liquid, leave about 1 ml in the cone of the tube
 - Mix liquid in the cone with a pipette tip and transfer it completely into a 1.5 mL reaction tube
 - Proceed to step 2.
 - Turbid samples, if turbidity caused by microbial growth (previously enriched sample, yellow color in FastOrange®, or contaminated product):
 - o Use 50 µL. Proceed to step 2.
 - Turbid sample, if turbidity is caused by other cell material:
 - o Use 50 - 200 µL to reach a pellet size of app. 2 mm in diameter after centrifugation (see fig. 1). Proceed to step 2.
 - b) *Colonies:* Single colonies as well as a couple of different colonies together can be processed as one sample. For molds, it is recommended to pick some cell material from the outer edge of the colony to get freshly grown mycelium as a sample.
 - o First transfer 200 µL Washing buffer A into a 1.5 mL reaction tube
 - o Add cell material from all colonies to be analyzed into the liquid. Proceed to step 2.
2. Centrifuge in a mini centrifuge for 3 min at >10,000 rpm (RCF: 8,500 x g) or alternatively in a larger centrifuge for 10 min at 4,000 – 5,000 rpm (RCF: 2,700 – 3,000 x g)
3. Control the pellet sizes, which is the cell material, of your samples. The pellet size should not exceed 2 mm in diameter (see fig. 1).
 - o If necessary, remove part of the pellet together with the liquid phase in step 4.
4. Remove the liquid phase carefully and discard



Fig. 1: recommended pellet sizes
Left: maximum pellet size for turbidity from microbes
Right: maximum pellet size for other turbid samples

5. Wash the pellet as follows:
 - Add 200 μ L Washing buffer A to the pellet
 - Resuspend pellet by vortexing or pipetting up and down, and repeat steps 2. - 4.
 - Extended washing might be necessary for samples known to likely be inhibited:
 - Repeat the whole washing procedure as above, and/or
 - Use a higher volume of washing buffer - up to 1,000 μ L per wash
6. **Using a 1,000 μ L pipette tip**, add 200 μ L of Lysis buffer A to the pellet – do **NOT** use a 100 or 200 μ L tip as it will be clogged by the glass beads
 - Attention! Take care that 50-75 μ L of the transferred volume consists of glass beads / sediment, ref. to Fig. 2
7. Vortex 3 min at max. speed
8. Centrifuge again as in step 2.
 - Attention! Do **NOT** discard liquid now as it contains your sample DNA!
 - The pellet contains cell debris and other waste particles, which were separated from the DNA
9. Use the liquid phase for PCR, take care **NOT** to touch the pellet or the sediment in the bottom of the tube when pipetting
10. Transfer 100 μ L of the liquid phase from 8. into a new 1.5 mL reaction tube
11. Store at 2-8 $^{\circ}$ C for same day PCR analysis; for long-term storage, freeze at -18 to -20 $^{\circ}$ C



Fig. 2: recommended volume of beads per Lysis buffer in the pipette tip

DNA Analysis by real time PCR

All reaction components for PCR except the 2-fold concentrated Master mix are provided in a dried form in the PCR tubes. Each tube contains BHOmix which delivers primers and probes for the detection of the target species plus the reagents for the internal positive control (IPC).

The number of PCR tubes and cap strips can be adjusted according to the number of samples to be analyzed by cutting the needed amount with sterilized (flamed) scissors or a knife. Remember to always add 2 tubes and caps for the positive and negative controls.

Preparation and Distribution of the Reconstitution Solution

1. Prepare one PCR reaction tube for each sample. Additionally one positive and one negative control reaction are always necessary per run. Take care **NOT** to touch caps or tubes when not wearing gloves!
2. Prepare the reconstitution solution by pipetting the required amounts of Rehydration buffer and 2 x Master mix into a fresh 1.5 mL reaction tube. Refer to table 1 for the volumes.
 - ✓ When using table 1, consider only the number of samples you want to analyze. Do **NOT** count the control reactions in your calculation
 - ✓ The volume needed for the 2 control reactions per run are already accounted for in the table
 - ✓ A 10% overage to account for pipetting losses is also included in the total calculation
3. After adding both solutions, close the reaction tube, which now contains the reconstitution solution. Mix briefly by vortexing or inverting the tube a couple times. Follow up with a quick spin down to collect the liquid at the bottom of the tube
4. Pipet 25 μ L of the reconstitution solution into each PCR tube needed (number of samples + 2 controls)

5. Prepare the control reactions:
 - ✓ Positive control: Pipet 5.0 μL of the provided DNA into the first PCR tube. Do not add any sample.
 - ✓ Negative control: Pipet 5.0 μL Rehydration buffer into the second PCR tube. Do not add any sample.
6. Prepare the samples: Pipet 5.0 μL of the extracted sample DNA (from part: Sample Preparation) into one PCR tube each, starting with tube 3
7. Close all PCR tubes with the provided cap strips
8. Spin down the PCR tubes shortly (10-15 seconds) to collect the liquid at the bottom (max. 2,000 rpm), and check for trapped air bubbles
9. If trapped bubbles are present, repeat step 8.
10. Transfer all PCR tubes into the thermocycler and follow instructions according to the software

Instrument and Software Setup

For instrument and software setup, follow instructions in the Open qPCR instrument software.

- If you are using a different thermocycler instrument, set the following profile:

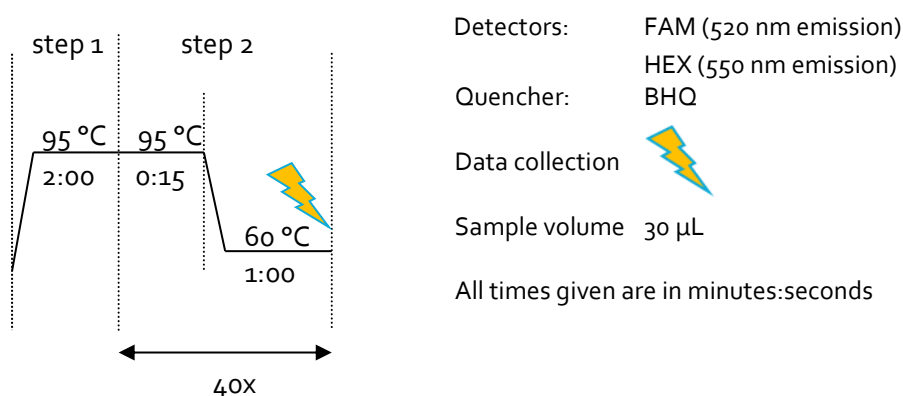


Fig. 2: Temperature scheme of thermocycler

Data Analysis

1. Chai Dual Channel Open qPCR™ real time PCR thermocycler
 - All data can be analyzed directly at the end of the PCR run or at a later time by opening the stored experiment. Follow instructions in the corresponding Open qPCR software manual for opening experiments and setting the data analysis parameters
 - Interpreting Results
 - i. Once the run is complete, the software displays the full data interpretation in a table format which is the standard read-out
 - ii. The full data including amplification curves can be seen under the "Full Results" view
2. Other real time PCR thermocyclers
 - Follow user manual of thermocycler instrument
 - Evaluate the thermocycler results as follows:
 - i. Verify the curves
 - ii. Evaluation of the measured Cq/Ct values:

- FAM channel detects target organisms
- HEX channel detects internal positive control reaction

Detection of target (FAM channel)	Internal control reaction (HEX channel)	Result from analysis
+	+	Positive: DNA of target (ref. Section 1) is present
+	-	Positive: DNA of target (ref. Section 1) is present
-	+	Negative: DNA of target (ref. Section 1) is not detected
-	-	Result is not evaluable: <u>Either</u> : Dilute extracted DNA with rehydration buffer 1:100 and repeat PCR <u>Or</u> : Repeat the DNA extraction with a smaller amount of sample, applying more extensive washing – refer to Section 7, 5.

Table 3: Manually evaluating PCR results

Section 8

Precautions and Recommendations for Best Results

- ✓ This test must be performed by trained persons
- ✓ All potentially infectious material should be autoclaved before disposal
- ✓ The quality of results depends on strict compliance with Good Laboratory Practices (for example, the EN ISO 7218 standard), especially concerning PCR:
 - The laboratory equipment (pipets, tubes, etc.) must not circulate from one workstation to another
 - It is essential to use positive and negative controls for each series of amplification reactions
 - Do not use reagents after their expiration date
 - Periodically verify the accuracy and precision of pipets and the correct functioning of the instruments
- ✓ Change gloves often, especially if you suspect they are contaminated
 - Clean work spaces periodically with at least 5% bleach or other DNA decontaminating agents such as DNA AWAY
 - Use powder-free gloves and avoid fingerprints and writing on tube caps as this can interfere with data acquisition
- ✓ It is strongly advised to follow the general requirements described in the standard EN ISO 22174:2005 (Microbiology of food and animal feeding stuffs Polymerase chain reaction (PCR) for the detection of food pathogens General requirements and definitions)

Section 9 Appendix

PCR Mix Calculation Guide

When using the Chai Dual Channel Open qPCR™ real time PCR thermocycler: To find the correct volumes to pipet when preparing the reconstitution solution, add the total number of samples to be analyzed, and find the corresponding volumes of Rehydration buffer and 2 x Master mix in the table.

Kit 1	volume to pipet in μL		total
number of samples	Rehydration buffer	2x Master mix	Volume in μ L
1	33.0	49.5	82.5
2	44.0	66.0	110.0
3	55.0	82.5	137.5
4	66.0	99.0	165.0
5	77.0	115.5	192.5
6	88.0	132.0	220.0
7	99.0	148.5	247.5
8	110.0	165.0	275.0
9	121.0	181.5	302.5
10	132.0	198.0	330.0
11	143.0	214.5	357.5
12	154.0	231.0	385.0
13	165.0	247.5	412.5
14	176.0	264.0	440.0

Table 4: 1 Kit per Run

Kit 1 + Kit 2	volume to pipet in μL		total
sum of samples from both kits	Rehydration buffer	2x Master mix	Volume in μ L
2	66.0	99.0	165.0
3	77.0	115.5	192.5
4	88.0	132.0	220.0
5	99.0	148.5	247.5
6	110.0	165.0	275.0
7	121.0	181.5	302.5
8	132.0	198.0	330.0
9	143.0	214.5	357.5
10	154.0	231.0	385.0
11	165.0	247.5	412.5
12	176.0	264.0	440.0

Table 5: 2 Kits per Run

Trademarks and Property Rights

Trademarks:

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Chai and Open qPCR are trademarks of Chai Biotechnologies Inc., Santa Clara, CA, USA.

Use of product:

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Property Rights:


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Developed and Manufactured by

PIKA Weihenstephan GmbH
Raiffeisenstrasse 31A
85276 Pfaffenhofen
GERMANY

 +49 (0) 8441 879 48 30

 +49 (0) 8441 879 48 31

www.pika-weihenstephan.com

order@pika-weihenstephan.de