FOR *IN VITRO* USE ONLY

foodproof® *Aspergillus* Detection LyoKit – 5´Nuclease –

Version 1, December 2021

PCR kit for the qualitative detection of *Aspergillus flavus*, *A. terreus*, *A. niger* and *A. fumigatus* DNA using real-time PCR instruments.

Order No. R 602 72-1 / R 602 72-2 / R 602 72-3

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

Store the kit at 2 to 8 °C

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1. What this Product Does

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 control template and negative control reactions can be analyzed per run.

Storage and Stability

- Store the kit at 2 °C 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:

Kit Contents

Component	Label	Contents / Function / Storage
foodproof® *Aspergillus* Detection LyoKit Microplate, prefilled with 96 reactions (lyophilized)	Aluminum bag containing a 8-tube strip mat • R 602 72-1 with white low profile tubes* • R 602 72-2 with clear regular profile tubes* • R 602 72-3 with clear low profile tubes*	• 96 prefilled reactions (lyophilized). • Ready-to-use PCR mix containing primer *and probes specific for DNA of Aspergillus flavus*, *A. terreus*, *A. niger* and *A. fumigatus* and the Internal Control (IC) as well as Taq DNA Polymerase and Uracil-DNA N-Glycosylase (UNG, heat labile) for prevention of carry-over contamination. • Store at 2 °C to 8 °C in the aluminium bag (sealed). • **Protect from light and moisture!**
Positive Control	Vial 2 (purple cap)	• 1 x 300 µl • Contains a stabilized solution of DNA. • For use as a PCR run positive control. • Store at 2 to 8 °C.
Negative Control	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 addition of samples.

*****Tube profile and instrument compatibility chart is available online: www.bc-diagnostics.com/compatibility-chart

Additional Equipment and Reagents Required

- Real-time PCR cycler suitable for detection of FAM, VIC/HEX, ROX and Cy5 labeled probes as well as for using low or regular profile strip tubes. In cases the strip tubes don´t fit for the instrument the samples have to be transferred after resuspension of the lyophilized PCR mix to appropriate PCR vessels.
- Sample Preparation Kit
  foodproof® StarPrep Two Kit (Order No. S 400 08.1)[1]
  foodproof® StarPrep Two 8-Strip Kit (Order No. S 400 17 L)[1]
- Nuclease-free, aerosol-resistant pipette tips
- Pipettes

and optionally

- Vortex centrifuge Multispin MSC-6000 for PCR-strips (Order No. D 110 66)[1] with
- SR-32, Rotor for MSC-3000/6000 (Order No. D 110 65)[1] or
- Vortex centrifuge CVP-2 for PCR-plates (Order No. D 110 67)[1]

[1] Available from BIOTECON Diagnostics; see ordering Information for details

Applicability Statement

The **food**proof® *Aspergillus* Detection LyoKit – 5'Nuclease – is intended for the rapid detection of *Aspergillus flavus*, *A. terreus*, *A. niger* and *A. fumigatus* DNA isolated from enrichment cultures by using the above-mentioned sample preparation methods of all relevant kinds of foods, feeds and environmental that are potentially contaminated with *Aspergillus flavus, A. terreus, A. niger or A. fumigatus.* The **food**proof® *Aspergillus* Detection LyoKit is destined for the food and feed industry and for food testing laboratories.

The kit must not be used in diagnostic procedures.

The kit described in this Instruction Manual has been developed for real-time PCR instruments with a FAM, a VIC/Yakima Yellow or HEX, a ROX or Texas Red and a Cy5 detection channel. The performance of the kit was tested with the following real-time PCR instruments: LightCycler®480, LightCycler®96 (Roche Diagnostics), Mx3005P®, AriaMx (Agilent Technologies), ABI 7500 fast, QuantStudio5 (Thermo Fisher Scientific), CFX96 (Bio-Rad), Dualo 32® R^2 (BIOTECON Diagnostics).

Note: A color compensation (Color Compensation Set 5; Order No. A 500 15) is necessary and will be supplied by BIOTECON Diagnostics for users of the LC 480 Systems II. Please contact BIOTECON Diagnostics for further information.

2. How to Use this Product

2.1 Before You Begin

Precautions

Detection of *Aspergillus flavus*, *A. terreus*, *A. niger* or *A. fumigatus* DNA using the **food**proof® *Aspergillus* 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.,* 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.

Keep the foodproof® **A*spergillus* lyophilized PCR Mix away from light and moisture.**

Sample Material

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

DNA Extraction

BIOTECON Diagnostics provides sample preparation kits suitable for all kind of food samples and PPS (see *"Additional Equipment and Reagents Required"*).
For more product information please refer to www.bc-diagnostics.com.

Positive Control
Always run a positive control with the samples. To prepare a positive control, replace the template DNA with the provided control DNA [**food**proof® *Aspergillus* Control Template (vial 2, purple cap)] or with a positive sample preparation control.

Negative Control
Always run a negative control with the samples. To prepare a negative control, replace the template DNA with **food**proof® *Aspergillus* Negative Control (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

Program Setup

The following procedure is optimized for a real-time PCR instrument with a FAM (*Aspergillus flavus*), VIC/HEX (*Aspergillus terreus*) and ROX (*Aspergillus niger* and *Aspergillus fumigatus*) and Cy5 (Internal Control) detection channel. Program the PCR instrument before preparing the PCR samples. Use the following real-time PCR-protocol for the **food**proof® *Aspergillus* Detection LyoKit. For details on how to program the experimental protocol, see the Instrument Operator's 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
Step 3:	72 °C for 30 seconds

‡ Fluorescence detection in step 2

Melting Curve	**1** cycle
Step 1:	95 °C for 50 seconds
Step 2:	37 °C for 50 seconds
Step 3*:	ramp up to 85 °C

* Fluorescence detection during 50 – 85 °C ramp with 1 measurement/ °C

Notes:
• For some real-time PCR instruments the type of the probe quencher as well as the usage of a passive reference dye has to be specified. The **food**proof® *Aspergillus* Detection LyoKit contains probes with a non-fluorescent ("dark") quencher and no passive reference dye.

• For users of the Agilent Mx3005P instrument: Click "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, HEX and ROX the Filter Set Gain Setting has to be modified to "x1".

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. Seal the zipper of the aluminum bag tightly.

1. Take the needed number of PCR tube strips out of the aluminum bag. Use scissors or scalpel to cut the strips apart. Tightly seal the bag afterwards and store away at 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 the 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 **food**proof® *Aspergillus* Detection LyoKit Control Template (vial 2, purple cap)

   Note: To reduce the risk of cross-contamination, it is recommended to prepare only one PCR tube strip at a time.
5. Seal the vessels accurately and tightly with the colorless cap strips.
6. Mix thoroughly using a vortex centrifuge.

   Note: BIOTECON Diagnostics recommends vortex centrifuges Multispin MSC-6000 (D 110 66) for PCR-strips or vortex centrifuge CVP-2 for PCR-plates (D 110 67). Dedicated protocols are available for these centrifuges.

   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 g in a suitable centrifuge.

   Note: If your centrifuge exceeds 200 g, do not centrifuge for more than 5 seconds. Avoid centrifugation forces exceeding 1000 g!
8. Place the samples in your PCR cycler and run the program as described above.

   Note: For using any LightCycler 480 instrument, a special adapter (Order No. Z 100 24) is necessary. For some PCR instruments, the PCR strips should be placed in a balanced order into the cycler block. For example, two strips can be placed in column 1 and 12.

2.3 Data Interpretation

Procedure – Qualitative Detection
The kit is intended for the qualitative detection of *Aspergillus flavus, Aspergillus terreus, Aspergillus niger* and *Aspergillus fumigatus.*

Detection
The amplification of *Aspergillus flavus* is analyzed in the fluorescence channel suitable for FAM labeled probes detection. The amplification of *Aspergillus terreus* is analyzed in the fluorescence channel suitable for HEX or VIC. The amplification of *Aspergillus niger* and *Aspergillus fumigatus* is analyzed in the fluorescence channel suitable for ROX. The amplification of the Internal Control is analyzed in the fluorescence channel suitable for Cy5.

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

Channel FAM	Channel HEX	Channel ROX	Channel Cy5	Result Interpretation
Positive	Positive or Negative	Positive or Negative	Positive or Negative	Positive for *Aspergillus flavus*
Positive or Negative	**Positive**	Positive or Negative	Positive or Negative	Positive for *Aspergillus terreus*
Positive or Negative	Positive or Negative	**Positive**	Positive or Negative	Positive for *Aspergillus niger* and/or *Aspergillus fumigatus*
Negative	**Negative**	**Negative**	**Positive**	Negative for *Aspergillus flavus, Aspergillus terreus, Aspergillus niger* and *Aspergillus fumigatus*
Negative	**Negative**	**Negative**	**Negative**	Invalid

Note: The Control Template contains a mixture of all target sequences and therefore usually generates significantly higher fluorescent values than samples that are positive for only one or two of the targets. This can affect positive/negative calls in automatic analysis of amplification curves by the respective instrument software. Always check results visually for plausibility.

Differentiation
The amplification product in the ROX channel can be attributed to either *Aspergillus niger* or *Aspergillus fumigatus* by melt curve analysis. The PCR product of *Aspergillus niger* generates a melt peak at 62 ± 3°C, whereas the PCR product of *Aspergillus fumigatus* generates a melt peak at 73 ± 3°C.

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, ROX, CY5
	Pipetting errors.	• Check for correct 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 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., 5 µl instead of 25 µl).
Fluorescence intensity is too low.	Inappropriate storage of kit components.	• Store the **food**proof® *Aspergillus* Detection lyophilized PCR Mix at 2 °C 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 of fluorescence baseline	Resuspension of lyophilized PCR mix not complete	• Always resuspend 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 sample and negative control reaction vessels have been sealed.
Fluorescence intensity varies.	Insufficient centrifugation of the PCR strips. Resuspend 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.	• Store the lyophilized PCR mix always in the aluminum bag with the silica gel pad • Open Strip shortly before filling.

4. Additional Information on this Product

How this Product Works

The **food**proof® *Aspergillus* 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 Cy5 channel, whereas the *Aspergillus flavus* DNA is detected in channel FAM, the *Aspergillus terreus* DNA in the HEX and *Aspergillus niger* and *Aspergillus fumigatus* DNA in ROX channel. 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 *Aspergillus flavus, Aspergillus terreus, Aspergillus niger* or *Aspergillus fumigatus* in the sample. The **food**proof® *Aspergillus* Detection LyoKit minimizes contamination risk and contains all reagents (except for template DNA) needed for the detection of *Aspergillus flavus, Aspergillus terreus, Aspergillus niger* or *Aspergillus fumigatus* -DNA. Primers and probes provide specific detection of *Aspergillus flavus, Aspergillus terreus, Aspergillus niger and Aspergillus fumigatus* DNA in food samples. The described performance of the kit is guaranteed for use on the real-time PCR instruments listed above only.

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 specific sequences for the target *Aspergillus flavus, Aspergillus terreus, Aspergillus niger* and *Aspergillus fumigatus* species.
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.

Prevention of Carry-Over Contamination

The heat-labile Uracil-DNA N-Glycosylase (UNG) is suitable for preventing carry-over contamination between PCRs. 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 *Aspergillus* 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 **food**proof® *Aspergillus* Detection LyoKit, decontamination can be achieved with the provided reagents.

Product characteristics

Specificity:

In- and exclusivity of the **food**proof® *Aspergillus* Detection LyoKit has been tested with 41 strains comprising of the four species *Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus niger* and *Aspergillus fumigatus* and as well as more than 80 non-target strains (47 of closely related *Aspergillus strains*). All *Aspergillus flavus* strains were detected in FAM channel, all *Aspergillus terreus* in HEX/VIC channel and all *Aspergillus niger* and *Aspergillus fumigatus* in ROX channel. None of the non-target strains were detected in any channel.

Sensitivity:

A relative detection limit of 1 to 10 cells per 25/100 g sample can be achieved with all relevant kinds of foods. The **food**proof® *Aspergillus* Detection LyoKit detects down to 10^2 - 10^3 cfu/ml of *Aspergillus flavus, Aspergillus terreus, Aspergillus niger* or *Aspergillus fumigatus* enrichment culture (depending on the sample preparation kit used).

Temperature robustness

The temperature limits of the **food**proof® *Aspergillus* Detection LyoKit are the following:
Denaturation temperature: 95 °C +1.75 / -2.75 °C
Annealing temperature: 60 °C ±1.75 °C
The limits were determined according to Annex C of ISO/DIS 20836:2020.
Real-Time Thermal Cyclers are compatible with the real-time PCR assay when operating within the stated temperature specification limits.

Quality Control

The **food**proof® *Aspergillus* Detection LyoKit is function tested using the LightCycler® 480 System.

5. Supplementary Information

5.1 Ordering Information

BIOTECON Diagnostics is offering a broad range of reagents and services. For a complete overview and for more information, please visit our website at www.bc-diagnostics.com.

5.2 License

License Notice

NOTICE TO PURCHASER: LIMITED LICENSE

Use of this product is covered by one or more of the following US patents and corresponding patent claims outside the US:5,804,375, 5,538,848, 5,723,591, 5,876,930, 6,030,787, 6,258,569. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product solely in Food Testing Applications and Genetically Modified Organism (GMO) Testing Applications, including reporting results of purchaser's activities for a fee or other commercial consideration, and also for the purchaser's own internal research. No right under any other patent claim is conveyed expressly, by implication, or by estoppel. Further information on purchasing licenses may be obtained from the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

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5.3 Trademarks

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5.4 Contact and Support

If you have questions about this or any other product of BIOTECON Diagnostics, please contact our Technical Support staff (for details see www.bc-diagnostics.com). 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.

6. Change Index

First version of the package insert.

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