foodproof® Animal Detection 1 LyoKit

Revision A, November 2023

PCR kit for the qualitative detection of porcine (*Sus scrofa*), bovine (*Bos taurus*, *Bos indicus*) and equine (*Equidae*: horse, zebra, donkey) animals by using real-time PCR instruments.

Product No. KIT230127

Product No. KIT230128

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

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1. Product Overview

1.1 Product Information

The **food**proof® Animal Detection 1 LyoKit was developed to reliably detect DNA of porcine (*Sus scrofa*), bovine (*Bos taurus, Bos indicus*), equine (horse, donkey, zebra) species in raw material, food-, feed-, and pharmaceutical products with real-time PCR instruments. Out of religious or ethical convictions or in case of false labeling of food products the foodproof Animal Detection 1 LyoKit enables a very sensitive analysis by using amplification of multi copy targets with porcine, bovine and equine specific primers and probes. The limit of detection amounts to 0.001%/10 ppm pork, beef, horse or donkey in meat products. Because of the high sensitivity of the test, the kit is suitable to analysis products like gelatin products, e.g., pharmaceutical capsule, milk powder or other highly processed products. Control Template and Internal Control as well as Negative Control monitor the PCR run validity. PCR results, including the DNA extraction process, obtain in less than two hours.
If quantification of samples is desired, please use our reference material Animal RM 1 (Product No. KIT230150), tested by IUPAC regimentations: DNA of raw meat mixtures from 1%, 0.1%, 0.01% pork, beef and horse in chicken meat.
Note: For quantification details, please contact us: www.hygiena.com

1.2 Product Characteristics

Specificity	The primers and hydrolysis probes (5' nuclease probes) provided in the lyophilized mix, are sequence-specific for porcine, bovine and equine DNA and the Internal Control, respectively. Specificity of the assay was proven by 45 different animals, microorganism and plant species.
Sensitivity	The limit of detection: 1 genome equivalent of *Sus scrofa, Bos taurus, Bos indicus, Equus caballus and Equus asinus* as well as 10 ppm/0.001% pork, beef or horse spiked in chicken meat.
Matrix	Kit was validated with meat products (e.g. lasagna, tomato sauce, sausages, minced meat), instant food, pastries, crisps, feed, gelatin products, pharmaceutical tablets, milk powder
Robustness	Reproducibility of Cp-values was successfully tested with different real-time PCR instruments, including LightCycler® 480 II, LightCycler 96 (Roche); AriaMx, Agilent Mx3005P (Agilent Technologies); Applied Biosystems® 7500 FAST, PikoReal 24™ (Thermo Fisher Scientific); CFX96™ real-time PCR Cycler (Bio-Rad)

Note: More detailed information is listed in the Validation Data Report of the foodproof Animal Detection 1 LyoKit. Please contact our Technical Support (hygiena.com/support)

1.3 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 one positive (purple cap) and one negative control reactions (colorless cap) can be analyzed per run.

1.4 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:

1.5 Kit contents

Component	Label	Contents / Function / Storage
foodproof Animal Detection 1 LyoKit Microplate, prefilled with 96 reactions (lyophilized)	Aluminum bag containing a 8-tube strip mat • KIT230127 with white low profile tubes* • KIT230128 with clear regular profile tubes*	• 96 prefilled reactions (lyophilized). • Ready-to-use PCR mix containing • primer and hydrolysis probes specific for porcine (*Sus Scrofa)*, bovine (*Bos taurus, Bos indicus)* and equine DNA (Equidae*)* and the Internal Control (IC) • Internal Control Plasmid • Taq DNA Polymerase • Uracil-DNA N-Glycosylase (UNG, heat labile) for prevention of carry-over contamination • For amplification and detection of (*Sus Scrofa)*, bovine (*Bos taurus, Bos indicus)* and equine (Equidae: horse, donkey, zebra*)* DNA, for internal amplification control in case of inhibition • Store at 2 °C to 8 °C in the aluminum bag (sealed) • **Protect from light and moisture!**
Control Template	Vial 2 (purple cap)	• 1 x 250 µL • Contains a stabilized solution of *Sus Scrofa*, *Bos taurus,* • *Equus caballus* and *Equus asinus* 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 addition of samples.

*Tube profile and instrument **compatibility** chart is available on hygiena.com/documents

1.6 Additional Equipment and Reagents Required

- Real-time PCR instrument with a FAM, HEX, ROX, and Cy5 detection channel, capable of performing a melting curve analysis. Without a melting curve analysis porcine, bovine and equine DNA can still be detected, but equine DNA cannot be differentiated in horse, donkey and zebra DNA. In cases the low or regular profile strip tubes do not 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 Five Kit (Product No.: KIT230191)
  - foodproof Magnetic Preparation Kit III (Product No.: KIT230182)
  - foodproof Sample Preparation Kit III (Product No.: KIT230174)
    - Nuclease-free, aerosol-resistant pipette tips
    - Pipettes

1.7 Applicability Statement

The foodproof Animal Detection LyoKit is intended for the rapid detection of porcine, bovine and equine animal DNA, including domestic pig and wild boar, *Bos indicus* and *Bos taurus* in raw meat, processed food or feed samples and pharmaceutical products.

The foodproof Animal Detection 1 LyoKit has been developed for real-time PCR PCR instrument with a FAM, HEX, ROX, and Cy5 detection channel, capable of performing a melting curve analysis. The performance of the kit was tested with the following real-time PCR instruments: LightCycler 480 II, LightCycler 96 (Roche); AriaMx, Agilent Mx3005P (Agilent Technologies); Applied Biosystems 7500 FAST, PikoReal 24™ (Thermo Fisher Scientific); CFX96™ real-time PCR Cycler (Bio-Rad).

2. Procedure

2.1 Before You Begin

2.1.1 Precautions

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 Animal Detection 1 LyoKit 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 preparation of DNA from raw material of animal origin food, feed, pharmaceutical, gelatin and milk products, refer to the corresponding product package inserts of a suitable sample preparation kit (see *"Additional Equipment and Reagents Required"*).

2.1.3 DNA Extraction

Hygiena Diagnostics provides sample preparation kits suitable for all kind of raw materials from animal origin food, feed or pharmaceutical, gelatine and milk products

- foodproof StarPrep Five Kit (Product No.: KIT230191)
- foodproof Magnetic Preparation Kit III (Product No.: KIT230182)
- foodproof Sample Preparation Kit III (Product No.: KIT230174)

(see *"Additional Equipment and Reagents Required"*).
For more product information please refer to www.hygiena.com

2.1.4 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 Animal Detection 1 LyoKit Control Template (vial 2, purple cap)] or with a positive sample preparation control.

2.1.5 Negative Control

Always run a negative control with the samples. To prepare a negative control, replace the template DNA with H_2O PCR-grade water (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 Program

2.2.1 Program Setup

The following procedure is optimized for a real-time PCR instrument with a FAM, HEX, ROX, and Cy5 detection channel, capable of performing a melting curve analysis. Without a melting curve analysis porcine, bovine and equine DNA can still be detected, but equine DNA can´t differentiated in horse, donkey and zebra.
Program the PCR instrument before preparing the samples. Use the following real-time PCR-protocol for the foodproof Animal Detection 1 LyoKit (for details on how to program the experimental protocol, see the operator's manual of your real-time PCR cycler):

Real-time PCR Time-Temperature Protocol	
Pre-incubation	**1** cycle
Step 1:	37 °C for 4 minutes
Step 2:	95 °C for 5 minutes
Amplification	**35** cycles[1]
Step 1:	95 °C for 5 seconds
Step 2*:	60 °C for 60 seconds
* Fluorescence detection in step 2	
Melting curve	**1** cycles
Step 1:	95 °C for 50 seconds
Step 2:	60 °C for 50 seconds
Step 3*:	ramp up to 80 °C
*Fluorescence detection during 60– 80°C ramp with approx. 1 measurement / °C	

[1] Amplification 50 cycles if foodproof Magnetic Preparation Kit III for animal identification (Product No.: KIT230182) was used for DNA extraction out of gelatin, cosmetic or pharmaceutical products

Notes:

- For CFX96™ real-time PCR cycler (Bio-Rad) Step 2 of Melting Curve has to be: 50 °C for 50 seconds.
- For Applied Biosystems 7500 Fast (Thermo Fisher Scientific) Step 2 of Melting Curve has to be: 50 °C for 60 seconds, Step 3: ramp up to 85 °C.

- For some real-time PCR instruments, the type of probe quencher as well as the usage of a passive reference dye must be specified. The foodproof Animal Identification Kit contains probes with a nonfluorescent ("dark") quencher as the quencher and no passive reference dye.
- For users of the Agilent Mx3005P instrument: Choose Experiment Type "SYBR® Green (with Dissociation Curve)" and add HEX, ROX, and Cy5 channels for data collection in the setup section.
- A Color Compensation (Color Compensation Set 3; Product No.: KIT230005) is necessary and will be supplied by Hygiena Diagnostics for users of the LightCycler 480 Systems I and II. Please contact Hygiena Diagnostics for further information.

2.2.2 Preparation of the PCR Mix

Proceed as described below to prepare a 25 µL standard reaction. Always wear gloves when handling PCR strips or caps. The lyophilizate is only stable in the provided aluminum bag with the silica gel pad.

1. Take the needed number of PCR tube strips out of the aluminum bag. Close the bag afterwards.
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 clear 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 and resuspend the pellet by cautiously pipetting up and down:
5. For the samples of interest, add 25 µL sample DNA (if less, then add to 25 µL with H_2O PCR-grade).
   Note: For samples prepared with foodproof StarPrep Five Kit: add 3 µL sample DNA, then add to 25 µL with H_2O PCR-grade. If inhibition of PCR occurs, take less sample volume and add to 25 µL with H_2O.
6. For the negative control, add 25 µL H_2O PCR-grade (vial 3, colorless cap).
7. For the positive control, add 25 µL Control Template (vial 2, purple cap).
8. Seal the vessels accurately with the colorless cap strips.
   Note: Alternatively resuspend the pellet after sealing by mixing thoroughly. To reduce the risk of cross-contamination, it is recommended to prepare only one PCR tube strip at once.
9. Briefly spin the PCR tube strips in a suitable centrifuge.
10. Cycle the samples as described above.
    Note: For using the LightCycler 480 instrument, a special adapter 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 columns 1 and 12.

2.3 Data Interpretation

2.3.1 Result Interpretation of the Amplification Curves

- A positive amplification signal in the FAM detection channel indicates the presence of porcine DNA in the tested sample.
- A positive amplification signal in the HEX detection channel indicates the presence of bovine DNA in the tested sample.
- A positive amplification signal in ROX detection channel indicates the presence equine DNA in the tested sample.
- Positive amplification signals in FAM, HEX, and ROX detection channels indicate the presence of bovine, equine and porcine DNA in the tested sample.

To exclude PCR inhibition, the amplification in the Cy5 detection channel must be checked, if there is a negative result in all channels. If all results are negative, the total result is invalid.

Channel FAM	Channel HEX	Channel ROX	Channel Cy5	Result Interpretation
Positive	Negative	Negative	Positive or Negative	Positive for *Sus scrofa*
Negative	Positive	Negative	Positive or Negative	Positive for *Bos indicus* or/and *Bos taurus*
Negative	Negative	Positive	Positive or Negative	Positive for Equidae
Positive	Positive	Positive	Positive or Negative	Positive for *Sus scrofa, Bos indicus, Bos taurus,* Equidae
Negative	Negative	Negative	Positive	Negative for *Sus scrofa, Bos indicus, Bos taurus,* Equidae
Negative	Negative	Negative	Negative	Invalid

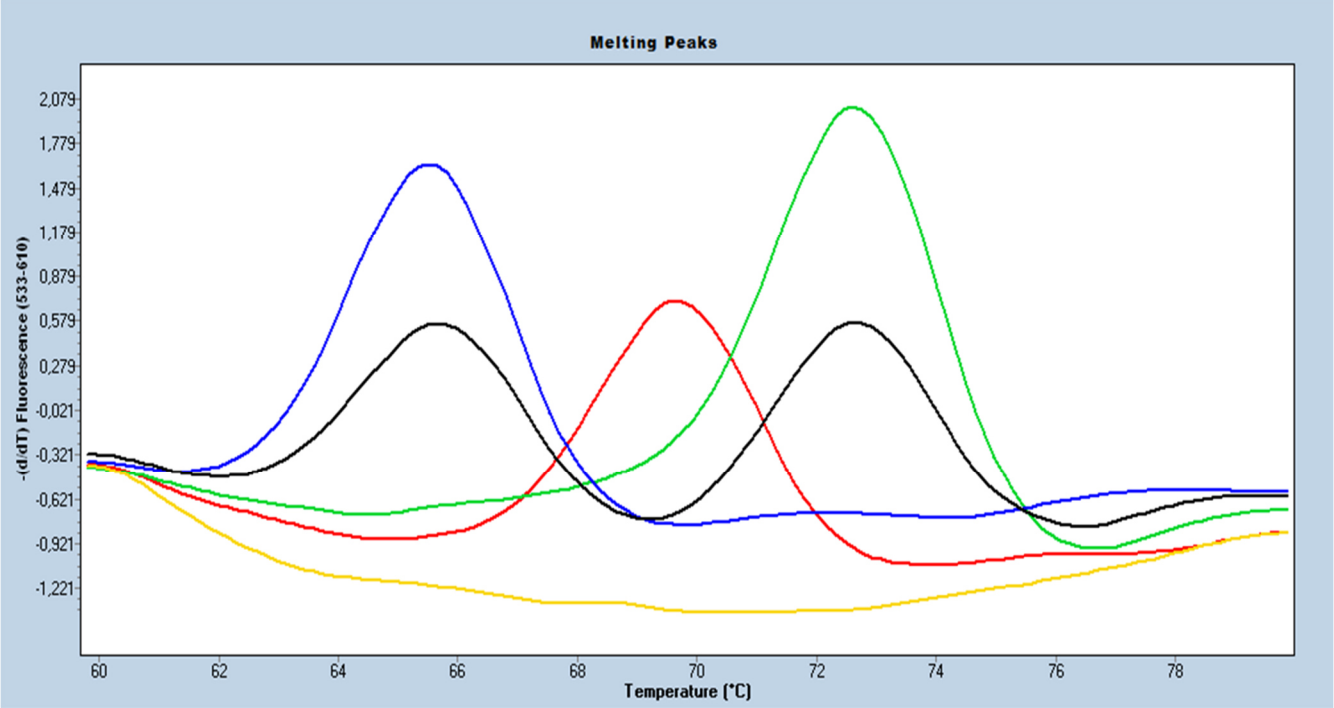
2.4 Melting Curves

Samples that are positive in the ROX detection channel can be further differentiated by using a melting curve analysis in the ROX channel.

2.4.1 Result Interpretation of the Melting Curves

The following table lists the detectable Equidae in the ROX channel and the melting peak range temperature (Tm).

ROX	64 – 67 °C	68 – 70.5 °C	71 – 74 °C
Equidae	donkey	zebra	horse



Melting curves in channel ROX: blue line: donkey, red line: zebra, green line: horse, black line: Control Template, yellow line: Negative Control

The figure shows typical melting curves on a LightCycler 480 Instrument: melting curves in channel ROX: blue line: donkey, red line: zebra, green line: horse, black line: Control Template, yellow line: Negative Control.

For some real-time PCR cyclers, the peak range can be different from the melting peak range temperature of the table. For this case the Control Template contains a mixture of donkey and horse target sequences. This Control Template mixture can be used as an approximate reference for the melting peak ranges in the particular PCR assay. Furthermore, some sample preparation matrices can slightly shift melting peak temperatures (not more than ± 1.5 °C variance from the peak of the Control Template).

The peak height of positive samples may vary according to matrices or number of copies.
Note that the presence or absence of specific melting peaks should be checked manually for all positive samples as the peak finding algorithms of the respective PCR instrument software may not detect all relevant maxima of the melting curve. A guarantee for the identification via melting curves cannot be given.

A prerequisite for a clear assignment of the horse, donkey and zebra is an adequate calibration of the PCR cycler for the channels FAM, HEX, ROX and Cy5. Please refer to the operating manual of your real-time PCR Cycler for further information.

3. Troubleshooting

Observation	Possible Reason	Recommendation
No signal increase, even with positive controls.	Incorrect detection channel has been chosen.	• Set channel settings of FAM, VIC/HEX, ROX and 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 program.
No signal increase in channel Cy5.	Inhibitory effects of the sample material (e.g., caused by insufficient purification).	• Dilute samples or pipet a lower amount of sample DNA (e.g., 20 µL PCR-grade H2O and 5 µL sample DNA instead of 25 µL sample DNA).
	Large amounts of porcine, bovine or equine DNA	• If a positive signal in FAM, HEX or ROX is detected, the PCR run is valid.
Fluorescence intensity is too low.	Inappropriate storage of kit components.	• Store the lyophilized PCR-mix **food**proof® Animal Detection 1 LyoKit 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 control after all sample and negative control reaction tubes have been sealed.
Fluorescence intensity varies.	Insufficient centrifugation of the PCR-strips. Resuspension of the PCR-mix only in the upper part of the reaction tube.	• 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 hard to dissolve.	The lyophilized PCR-mix started to rehydrate.	• Store the lyophilized PCR-mix tightly sealed in the aluminum bag with silica gel pad. • Open strips just before filling.
Amplification curve is positive and melting curve is negative or vice-versa.	Low amounts of target DNA.	• Increase amount of target DNA by increasing the sample volume (max. volume 25 µL), by extending enrichment time (see chapter B) or by subcultivation. • Repeat DNA extraction (depending on the chosen DNA isolation method, inhibitory effects can occur).

4. Additional Information on this Product

4.1 How this Product Works

The foodproof Animal Detection 1 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 porcine DNA is detected in FAM, bovine DNA is detected in HEX, equine DNA is detected 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 porcine, bovine and equine DNA in the sample. The foodproof Animal Detection 1 LyoKit minimizes contamination risk and contains all reagents (except for template DNA) needed for the detection of porcine, bovine and equine DNA. Primers and probes provide specific detection of porcine, bovine and equine DNA in food-, cosmetic- and pharmaceutical samples. The described performance of the kit is guaranteed for use on the real-time PCR instruments listed above.

4.2 Test Principle

1. Using sequence-specific primers of the foodproof Animal Detection 1 LyoKit in a polymerase chain reaction (PCR), the PCR instrument and the supplied reagents amplify fragments of porcine, bovine and equine 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 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's. 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 not longer serve as PCR templates. The heat-labile UNG is inactivated during the initial denaturation step. Native DNA (e.g., the isolated animal 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 Animal Detection 1 LyoKit, decontamination can be achieved with the provided reagents.

4.4 Quality Control

The foodproof Animal Detection 1 LyoKit is function tested using the LightCycler 480 System. More detailed information is listed in the Validation Data Report of the foodproof Animal Detection 1 LyoKit. Please contact our Technical Support (hygiena.com/support).

5. Supplementary Information

5.1 Ordering Information

Hygiena Diagnostics GmbH is offering 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

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

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 Reference Number

The reference number and original Hygiena Diagnostics GmbH article number: R 602 49-1 (KIT230127) and R 602 49-2 (KIT230128).

6. Change Index

Version 1, August 2019:
First version of the package insert.

Revision A, November 2023:
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