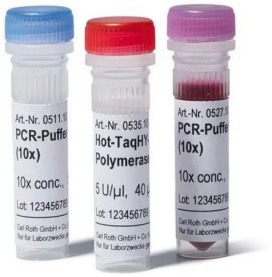


GENERON PATHfinder Real-Time PCR Kits for DNA Detection User Guide

GENERON
Food Safety in our Genes

PATHfinder
Real-Time PCR kits for DNA Detection of Pathogens and spoilage micro-organisms

RUO
Research Use Only

MADE IN ITALY

READ SAFETY INFORMATION AND DISCLAIMERS BEFORE USING THE KIT
Quick guide

Contents

PATHfinder Real-Time PCR Kits for DNA Detection

This manual refers to the following part numbers, the description of each part number briefly indicates the bacteria

detected by the kits, the suggested enrichment and the PCR thermal profile to refer to

Cat#	Target	Gram	Enrichment broth*		PCR profile	Reaction
PMB01A-50	Salmonella Spp	–	BPW	16-20 hrs @ 37°C	Group A (See 4.5.1)	18 Mix + 12 DNA
PMB02A-50	Listeria monocytogenes	+	½ Fraser	23-25 hrs @ 30°C	Group A (See 4.5.1)	18 Mix + 12 DNA
PMB03A-50	Listeria Spp	+	½ Fraser	23-25 hrs @ 30°C	Group A (See 4.5.1)	18 Mix + 12 DNA
PMB04A-50	Thermotolerant Campylobacters (jejunii/coli/lari) (acc. to ISO 10272)	–	Preston	23-25 hrs @ 41.5°C	Group L (See 4.5.10)	**22,5 Mix + 2,5 DNA**
PMB05A-50	Pseudomonas aeruginosa	–	Malachite green	24 hrs @ 36°C	Group A (See 4.5.1)	18 Mix + 12 DNA
PMB06A-50	Clostridium perfringens	+	DRCM	44 hours @ 37°C	Group M (See 4.5.1)	18 Mix + 12 DNA
PMB07A-50	Yersinia Enterocolitica (acc. to ISO18867)	–	PSB	24 hrs @ 25°C	Group K (See 4.5.9)	18 Mix + 12 DNA
PMB08A-50	Cronobacter Spp (acc. to FDA BAM Chapter 29)	–	BPW	23-25 hrs @ 36°C	Group C (See 4.5.3)	**20 Mix + 5 DNA**
PMB09A-50	Bacillus Cereus	+	TSPB	48 hrs @ 30°C	Group A (See 4.5.1)	18 Mix + 12 DNA
PMB10A-H4	Escherichia coli H:4 [fliC]	–	BPW	18-24 hrs @ 37°C	Group A (See 4.5.1)	18 Mix + 12 DNA
PMB10A-H7	Escherichia coli H:7 [fliC]	–	BPW	18-24 hrs @ 37°C	Group A (See 4.5.1)	18 Mix + 12 DNA
PMB10A-P103	Escherichia coli O:103 [wzx] (acc. to ISO13136)	–	BPW	18-24 hrs @ 37°C	Group A (See 4.5.1)	18 Mix + 12 DNA
PMB10A-P104	Escherichia coli O:104 [wzx]	–	BPW	18-24 hrs @ 37°C	Group A (See 4.5.1)	18 Mix + 12 DNA
PMB10A-P111	Escherichia coli O:111 [wbdL] (acc. to ISO13136)	–	BPW	18-24 hrs @ 37°C	Group A (See 4.5.1)	18 Mix + 12 DNA
PMB10A-P121	Escherichia coli O:121 [wzx]	–	BPW	18-24 hrs @ 37°C	Group A (See 4.5.1)	18 Mix + 12 DNA
PMB10A-P145	Escherichia coli O:145 [ihp1] (acc. to ISO13136)	–	BPW	18-24 hrs @ 37°C	Group A (See 4.5.1)	18 Mix + 12 DNA
PMB10A-P157	Escherichia coli O:157 [rfbE] (acc. to ISO13136)	–	BPW	18-24 hrs @ 37°C	Group A (See 4.5.1)	18 Mix + 12 DNA
PMB10A-P26	Escherichia coli O:26 [wzx] (acc. to ISO13136)	–	BPW	18-24 hrs @ 37°C	Group A (See 4.5.1)	18 Mix + 12 DNA

PMB10A-P45	Escherichia coli O:45 [wzx]	–	BPW	18-24 hrs @ 37°C	Group A (See 4.5.1)	18 Mix + 12 DNA
PMB10A-V1-50	E. coli STEC (stx1) (acc. to ISO13136)	–	BPW	18-24 hrs @ 37°C	Group B (See 4.5.2)	18 Mix + 12 DNA
PMB10A-V2-50	E. coli STEC (stx2) (acc. to ISO13136)	–	BPW	18-24 hrs @ 37°C	Group B (See 4.5.2)	18 Mix + 12 DNA
PMB10A-V2F-50	E. coli STEC (stx2f) (according to EURL)	–	BPW	18-24 hrs @ 37°C	Group B (See 4.5.2)	18 Mix + 12 DNA
PMB10A-VE-50	E. coli STEC (eae) (acc. to ISO13136)	–	BPW	18-24 hrs @ 37°C	Group B (See 4.5.2)	18 Mix + 12 DNA
PMB11A-50	Staphylococcus aureus	+	Giolitti&Cantoni	24-48 hrs @ 37°C	Group A (See 4.5.1)	18 Mix + 12 DNA
PMB13A-C-50	Vibrio cholerae	–	ASPW	Variable 5-25 hrs @ 37-41.5°C	Group B (See 4.5.2)	**15 Mix + 5 DNA**
PMB13A-P-50	Vibrio parahemolyticus	–			Group B (See 4.5.2)	**15 Mix + 5 DNA**
PMB13A-V-50	Vibrio vulnificus	–			Group B (See 4.5.2)	**15 Mix + 5 DNA**
PMB15D-SG1-50	Legionella pneumophila Sero group 1	–	No enrichment		Group B (See 4.5.2)	**15 Mix + 5 DNA**
PMB44A-50	Clostridia producing botulinum toxins (A-G)	+	TPGY	24 hrs @ 30°C	Group D (See 4.5.4)	**15 Mix + 5 DNA**
PMB48A-50	Alicyclobacillus spp.	+	BAT	120 hrs @ 45°C	Group H (See 4.5.8)	**15 Mix + 5 DNA**
PMB48A-AC-50	Alicyclobacillus acidocaldarius	+	BAT	120 hrs @ 45°C	Group E (See 4.5.5)	**15 Mix + 5 DNA**
PMB48A-AT-50	Alicyclobacillus acidoterrestris	+	BAT	120 hrs @ 45°C	Group E (See 4.5.5)	**15 Mix + 5 DNA**
PMB60A-50	Yersinia pseudotuberculosis (acc. to ISO18867)	–	TSBY	24 hrs @ 25°C	Group K (See 4.5.9)	18 Mix + 12 DNA
PMB61A-50	Shigella spp	–	Shigella Broth	18 hrs @41.5°C	Group B (See 4.5.2)	**15 Mix + 5 DNA**
PMB62A-50	Xanthomonas campestris	–	No enrichment		Group G (See 4.5.7)	**15 Mix + 5 DNA**
PMB65A-50	Brettanomyces spp.	NA	No enrichment		Group B (See 4.5.2)	**15 Mix + 5 DNA**
PMB67A-50	Clostridium botulinum A, B, E, F (Based on ISO-17919)	+	TPGY	24 hrs @29-31°C	Group F (See 4.5.6)	**20 Mix + 5 DNA**
PMB67A-ID	Clostridium botulinum A, B, E, F (Based on ISO-17919)	+	TPGY	24 hrs @29-31°C	Group F (See 4.5.6)	**20 Mix + 5 DNA**

The manual refers also to all the kits optimized for Hyris Bcube having the standard part number with the prefix

HB3_
* The enrichment method reported represents only a suggestion, other methods are applicable.

Assay Box 50 reactions content		**Number of vials in the kits**	**Number of vials in PMB48A-50**	**Number of vials in PMB67A-ID**
A	**PATHfinder** OLIGO Mix*	1	2	4
B	GENERase ULTRA Mastermix*	1	2	4
C	Positive Control*	1	1	4
D	Negative Control	1	1	4
E	Diluent*	1	2	4

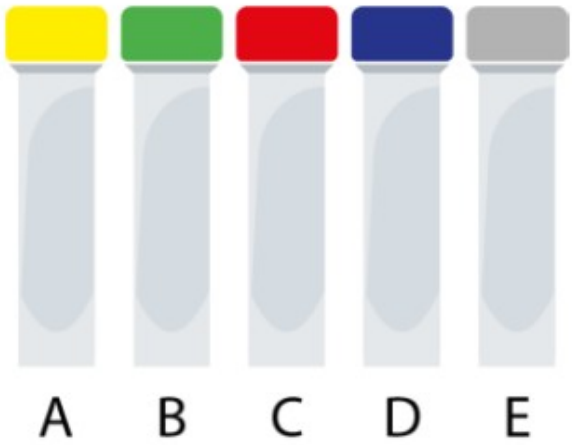
* reagents are supplied with an 5% of extra volume. Generase Mastermix contains ROX as passive reference dye

Reaction Set-Up

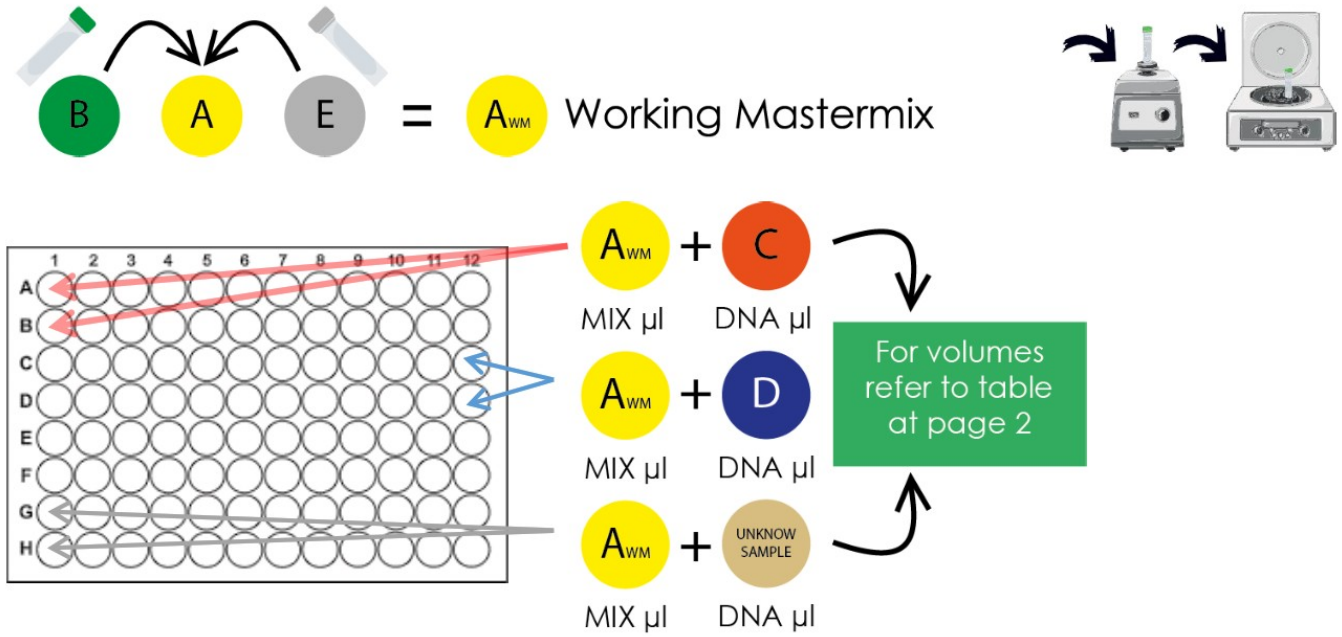
Protect reagents from light exposure as far as OLIGO Mix reagents are photosensitive.

Before Using

1. Leave the reagents to warm up at room temperature
2. Vortex briefly all the reagents
3. Spin (to avoid drops on the cap vials)



Prepare the PATHfinder WORKING Mastermix by adding GENERase Mastermix tube and Diluent tube into the OLIGO Mix (See the workflow below). Then gently vortex the mix and spin briefly to obtain a single volume of PATHfinder WORKING Mastermix for each tube.



This is just an example
You can use any well and place your controls wherever you prefer in the plate
Remember: When setting the analysis, vortex briefly and spin the PATHfinder WORKING Mastermix vial (to avoid drops on the cap vials) then transfer the aliquot of Working Mastermix and samples in the plate wells.

Introduction

Food, feed, water and surfaces contaminated with microorganisms such as bacteria, yeasts, molds, parasites or viruses may pose a risk to the consumer or spoil the products causing economical and reputation losses to industry. In addition to the detection of pathogenic microorganisms, it is important to monitor typical spoilage organisms in order to reduce losses during production.
Traditional culture-based detection of the bacteria is often laborious and time consuming and does not account neither for a rapid risk prevention (e.g. Legionella in communities) nor for the rapid production turn-around time of modern food industry (ready-to-eat products are often consumed in <24 hrs from production).
A major advantage in the application of PCR based methodologies lies in the fact that such assays are generally more specific, informative (e.g. immediate strain identification), sensitive, and faster than conventional microbiological assays.
The following ISO norms under the general title “Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens” provided the guidelines for the development of PATHfinder, the portfolio of Generon kits for the detection of a wide range of pathogenic or spoilage micro-organisms using Real-Time PCR.

- General requirements and definitions (ISO 22174)
- Requirements for sample preparation for qualitative detection (ISO 20837)
- Performance testing for thermal cyclers (ISO/TS 20836)
- Requirements for amplification and detection for qualitative methods (ISO 20838);

This kit detects a highly conserved sequence of the relevant bacteria in any DNA extract. Each kit contains reagents sufficient for 50 duplex reactions: the target microorganism is detected using the fluorophore FAM; the internal amplification control (IAC) is detected using the fluorophore HEX. The IAC allows monitoring the amplification efficiency (in turn avoiding false negative results) by using a control DNA fragment, pre-added to the reagents mastermix, amplified in parallel using a second PCR system. Please refer to the specific technical sheet for further details.
We recommend operating according to the above-mentioned ISO norms. Moreover, a negative control reaction and a positive control (and possibly a positive extraction control) shall be included in each PCR run. Each step of sample preparation must be done according to GLP to minimize risks of cross-contamination between samples. It is recommended to use disposable tools whenever possible.

Generon PATHfinder catalogue includes also: sureXtra, inactivated bacteria pellets to support method validation or as a calibrant in quantitative analysis; DIGIcount, bacterial DNA extracts quantified using ddPCR for best state-of-art accuracy in genomic unit quantification.

Sample DNA

The DNA of micro-organisms can be obtained from:

- Enrichment broth(s): food (but also swab, sponges and filters) samples should be enriched according to the corresponding International Standards or other appropriate standards. Some enrichment media might contain less PCR-inhibitory substances than others, which should be carefully considered in connection with the choice of sample preparation method. For some products, special care should be taken to suppress the growth of competing background micro-organisms (e.g. by addition of selective chemicals or antibiotics). We suggest Generon FAST food Extraction kit (EXD009) for DNA extraction from enrichment broth samples (see specific user manual).
- Concentrates obtained through filtration means: the bacterial content of liquid samples can be concentrated through filtration on filters made of polycarbonate (PC) or Polyethylsulphone (PES) and eventually recovered by rinsing the filter with a buffered solution (PBS or TE). The DNA of bacteria present in the rinsing solution can be extracted using Generon FAST food Extraction kit (EXD009). It is important to note that the absence of an enrichment step gives the possibility to enumerate the bacteria present in the original sample but reduces the possibility to detect pathogens when present in low amount.
- Colony picking using a plastic needle: according to ISO 7218:2007 and following amendments, methods based on nucleic probes can be used for the identification of colony isolates (see ISO 7218 clause 12.5). PATHfinder kits are based on evaluation studies published in international scientific literature (see references in technical datasheet). Hence PATHfinder can be used (using colony PCR approach see paragraph 4.1.2) as an alternative to the reference confirmation tests of colony isolates described in the specific standards unless otherwise stated in specific standards.

Materials and equipment not included

The following materials are necessary to perform sample preparation:
Enrichment broth and bags with filter; 0.22 µm PES Filtering system; Plastic needles; 1.5 or 2 ml tubes; Extraction kit; Heat block for 1.5 ml tubes; precision micropipettes and tips with filter
and Real-Time PCR experiments: Vortex and micro-centrifuge; Real-Time PCR System and PCR hood; DNase/RNase free water; precision micropipettes and tips with filter; optical tubes and seals.

DNA Detection

4.1 General

A complete understanding of this insert is necessary for successful use of the product. Reliable results will only be obtained when following GLP and operating instructions. Protect reagents from light exposure as far as reagents contained in the OLIGO mix are photosensitive.
Do not mix kit components of different lots within one run. Do not use any component beyond the expiration date shown on its label.
After removing reagents from the refrigerator, allow them to thaw slowly and mix them by vortexing or pipetting. Finally, briefly centrifuge before use. Prepare PATHfinder WORKING Mastermix by adding the whole content of one GENERase Mastermix and the whole content of one DILUENT into one PATHfinder OLIGO Mix tube. Gently vortex the mix and spin briefly to obtain a single volume of PATHfinder WORKING Mastermix (AWM).
Before starting the practical work, edit the plate document. For general and more detailed instructions please refer

to the user guide of the instrument and respective software version.

4.2 Controls

According to ISO 22174 appropriate controls should be included in the PCR experiment to monitor the different experimental phases.

	Sampling	**DNA extraction**	**Amplification**
Negative process control	Verified	Verified	Verified
Positive process control*	Verified	Verified	Verified
Negative extraction control		Verified	Verified
Internal amplification control			Verified
Positive PCR control			Verified
Negative PCR control			Verified

* PATHfinder sureXtra can be used for this purpose. For more details contact marketing@generon.it .

4.3 Reaction set-up

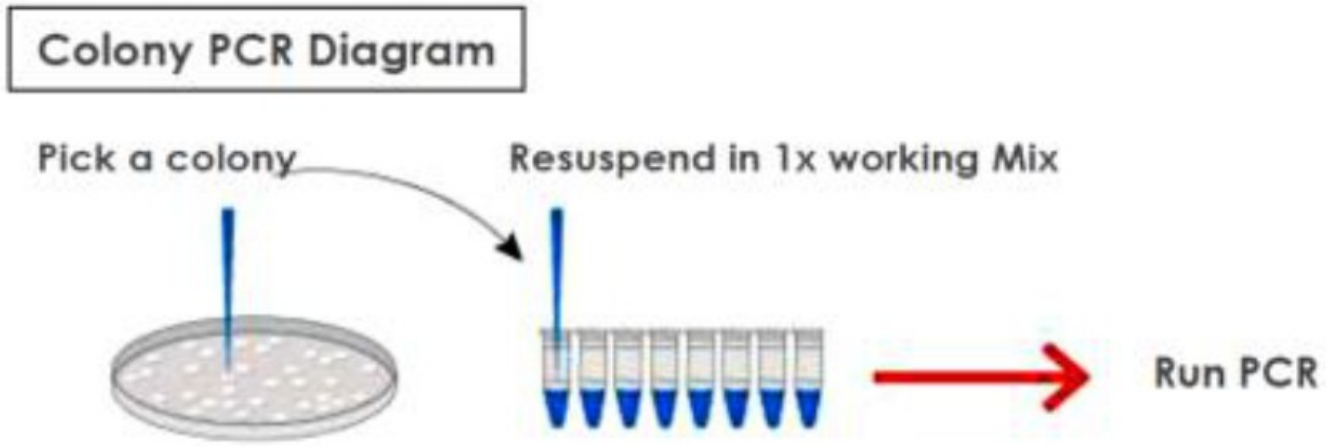
4.3.1 Reaction set-up for DNA extracts

1. Check on table at page 2 if the kit belongs to the group reaction:, 18 Mix +12 DNA, [22.5 Mix + 2.5 DNA], (20 Mix + 5 DNA), or to [15 Mix + 5 DNA]. According to the case, in the following steps use the volumes in brackets.
2. Transfer 18 µL [22.5 µL] (20 µL) [15 µL] of AWMX into PCR plate wells according to the number of unknown samples, plus the number of wells acting as controls.
3. Add 12 µL [2.5 µL] (5 µL) [5 µL] of positive control into wells acting as positive PCR control.
4. Add 12 µL [2.5 µL] (5 µL) [5 µL] of each sample to wells testing the unknown samples.
5. Add 12 µL [2.5 µL] (5 µL) [5 µL] of positive and negative extraction controls when present.
6. Add 12 µL [2.5 µL] (5 µL) [5 µL] of negative control into wells acting as negative PCR control.

4.3.2 Reaction set-up for colony PCR

1. Transfer 6 µL [9 µL] (8 µL) [7.5 µL] of AWMX into PCR plate wells according to the number of colonies to screen, plus the number of wells acting as negative and positive control.
2. Add 4 µL [1 µL] (2 µL) [2.5 µL] of positive control into wells acting as positive control.
3. Add 4 µL [1 µL] (2 µL) [2.5 µL] of negative control into wells acting as negative control.
4. Add 4 µL [1 µL] (2 µL) [2.5 µL] of water into each well for colony screening.
5. Pick one colony with a sterile needle and resuspend it into the mix.

Close tubes and ensure no bubbles are present at the bottom of the wells.



4.4 Instrument set-up

This PATHfinder PCR system uses degradative probes labelled with FAM (Target) and HEX (IAC) quenched with BHQ1 (non-fluorescent quenchers), set the instrument detector accordingly. If HEX dye is not included in the list of calibrated dyes in the qPCR instrument, select alternatively VIC or JOE.

4.5 Thermal Cycling Conditions

The majority of PATHfinder PCR systems share a common thermal protocol and use degradative probes, however some kits are working with peculiar thermal protocols. Find in the paragraph below the protocol associated to the kit in use.

IMPORTANT! When running colony PCR experiments reduce the cycling loops to 30.

4.5.1 Profile Group A

Step	T (°C)	Duration	Loops
Taq Activation	95	10 min	1
Denaturation	95	15 sec	45*
Annealing/Extension + Plate Reading	60	60 sec	

4.5.2 Profile Group B

Step	T (°C)	Duration	Loops
Taq Activation	95	3 min	1
Denaturation	95	10 sec	45*
Annealing/Extension + Plate Reading	60	45 sec	

4.5.3 Profile Group C

Step	T (°C)	Duration	Loops
Taq Activation	95	3 min	1
Denaturation	95	15 sec	45*
Annealing + Plate Reading	52	40 sec	
Extension	72	15 sec	

4.5.4 Profile Group D

Step	T (°C)	Duration	Loops
Taq Activation	95	3 min	1
DNA Denaturation	95	15 sec	45*
Annealing	42	15 sec	
Extension + Plate Reading	55	60 sec	

4.5.5 Profile Group E

Step	T (°C)	Duration	Loops
Taq Activation	95	3 min	1
Denaturation	95	15 sec	40*
Annealing/Extension + Plate Reading	57	20 sec	

4.5.6 Profile Group F

Step	T (°C)	Duration	Loops
Taq Activation	95	3 min	1
Denaturation	95	15 sec	35*
Annealing/Extension + Plate Reading	60	60 sec	

4.5.7 Profile Group G

Step	T (°C)	Duration	Loops
Taq Activation	95	10 min	1
Denaturation	95	15 sec	45*
Annealing/Extension + Plate Reading	60	40 sec	

4.5.8 Profile Group H

Step	T (°C)	Duration	Loops
Taq Activation	95	3 min	1
Denaturation	95	30 sec	40*
Annealing/Extension + Plate Reading	55	30 sec	

4.5.9 Profile Group K

Step	T (°C)	Duration	Loops
Taq Activation	95	10 min	1
Denaturation	95	15 sec	45*
Annealing/Extension + Plate Reading	60	60 sec	

4.5.10 Profile Group L

Step	T (°C)	Duration	Loops
Taq Activation	95	10 min	1
DNA Denaturation	95	15 sec	45*
Annealing	60	60 sec	
Extension + Plate Reading	72	30 sec	

4.5.11 Profile Group M

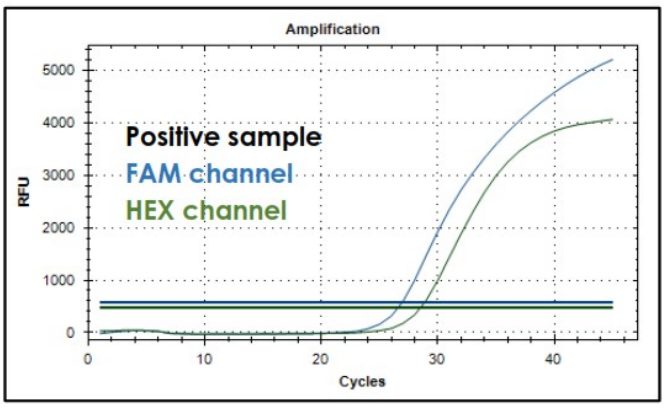
Step	T (°C)	Duration	Loops
Taq Activation	95	3 min	1
Denaturation	95	15 sec	40*
Annealing/Extension + Plate Reading	60	60 sec	

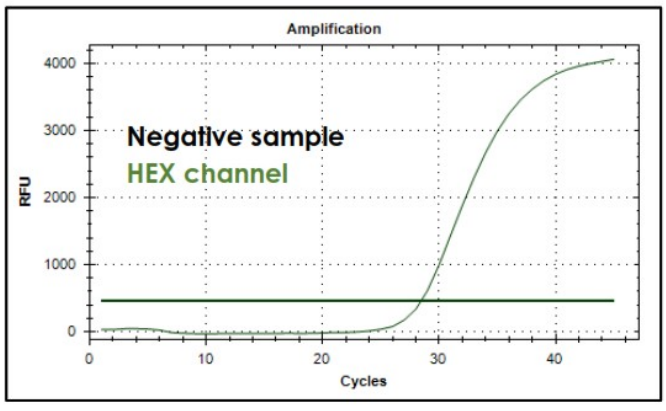
(*) When running colony PCR experiments reduce loops to 30.

Results and Data interpretation

5.1 Curves Interpretation

After performing PCR, each individual sample is analyzed through the instrument software to produce a Cq value (quantification cycle) for each reporter dye. These values are used to determine the presence (Qualitative Test) of bacteria into the sample DNA. See below an example of the graphics obtained for a positive Target and for a negative sample.





5.2 Evaluation

According to ISO 22174 the possible PCR results and their interpretation are given in the following table:

	Positive Result	Negative Result	Contamination	Inhibition
Test Sample	+	–	+	–
Positive process control	+	+	+	–
Positive PCR control	+	+	+	+
All negative controls	–	–	+	–
Internal amplification control	+/-	+	+/-	–

A positive PCR result may also be confirmed by cultural methods.

5.3 Test reporting

The test report should contain the following information:

- all information necessary to identify the laboratory sample including date of receipt and storage conditions;
- any particular point relating to the laboratory sample (e.g. insufficient size, degraded state);
- a reference to the standard used for the test and the methods followed;
- size of the test portion
- analysis start/end date and person responsible for the analysis;
- test results;
- any particular points observed during testing;
- any deviations, additions to or exclusions from the test specification, and any other information relevant to a specific test.

Validation of the product

The kit was validated on Bio-Rad CFX, Bio-Rad MiniOpticon, MyGO mini, MyGO Pro, bCUBE and Applied BioSystems 7500 fast (Quantstudio 5, 7, 8); the assay is not compatible with Roche Light Cycler I and II, but is compatible with other Real-Time PCR instruments as: all Applied BioSystems, Light Cycler 480 Roche, Aria MX Agilent, Rotor-Gene Q Qiagen) inquire **technical.support@generon.it** for details.
A detailed technical datasheet containing relevant validation data including the LOD, is available for each of the PATHfinder kits, inquire **marketing@generon.it** for receiving a copy of it.

Troubleshooting

I. No target nor IAC amplification, or amplification plots grossly abnormal. Possible causes and corrective actions:

- An excess of DNA in the target might inhibit the reaction and endo may be affected due to an excess of DNA and/or PCR inhibitors. Test samples diluted 1:10 and 1:100. Please, use DNase/RNase Free Water to prepare dilutions.
- When running colony PCR pick less bacteria, do not scratch the colony just touch it with the needle
- Inadequate sealing of optical caps/film caused sample evaporation. Redo the analysis using proper tools and proper optical caps/film to secure perfect sealing.
- Did not use the proper consumables. Redo the analysis and use only optical grade 96-well plates and optical adhesive seal or optical 8-well strips and caps.
- Samples were not properly prepared. Re-extract the DNA ensuring method is properly performed.

II. Negative Control reactions are positive. Possible causes and corrective actions:

- Contamination of the negative control vial or the PCR working master mix. mix with PATHfinder-positive DNA. Use more care to prevent contamination while handling assay reagents and setting up PCR plate.

III. Positive Control reactions failed to amplify, but other reactions appear correct (the IAC is amplified). Possible causes and corrective actions:

- Positive Control DNA was not added to the reaction wells or is degraded by multiple thawing or mishandling. If other reactions look normal, there may be no need to repeat the run.

Please, be aware that the intensity of the fluorescence signal is influenced by the type of disposable PCR labware: well plates, tubes, strip tubes, adhesive films, caps, cap strips. On Bio-Rad CFX it is recommended the use of white tubes/plates.
If you have any questions or experience any difficulties regarding this kit, please do not hesitate to contact us **technical.support@generon.it**. Our customers are also a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at Generon. We therefore encourage you to contact us if you have any suggestions regarding product performance or new applications and techniques.

Storage & Expiry information

Expiry date: see date on the packaging, product validity refers to the product kept intact in its original packaging. Protect reagents from light exposure as far as OLIGO Mix reagents are photosensitive. Store frozen. Despite the day of actual use of the kit components and their mixing, all the reagents are considered expired on the date indicated on the Kit box. Avoid repeated thawing and freezing (>5x) and in case subaliquot the working mastermix and positive control.

Disclaimers

Generon warrants the products will be free of defects in materials and workmanship when used in accordance with the instructions and before the expiration date marked on the product packaging under the storage conditions recommended in the instructions and/or on the package. Application protocols published by Generon are intended to be only guidelines for the buyers of the products. Each buyer is expected to validate the applicability to his individual application. Generon makes no other warranty, expressed or implied. There is no warranty of merchantability or fitness for a particular purpose. Generon sole obligation with the respect to the foregoing warranties shall be, at its option, to either replace or to refund the purchase price of the product(s) or part thereof that proves defective in materials or workmanship within the warranty period, provided the customer notifies Generon promptly of any such defect. Generon shall not be liable for any direct, indirect or consequential damages resulting from economic loss or property damages sustained by buyer or any customer from the use of the product(s).

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Product Insert
PATHfinder Detection Assay
update of 27/06/2023

Documents / Resources

	GENERON PATHfinder Real-Time PCR Kits for DNA Detection [pdf] User Guide PMB01A-50, PMB02A-50, PMB03A-50, PMB04A-50, PMB05A-50, PMB06A-50, PMB07A-50, PMB08A-50, PMB09A-50, PMB10A-H4, PMB10A-H7, PMB10A-P103, PMB10A-P104, PMB10A-P111, PMB10A-P121, PMB10A-P145, PMB10A-P157, PMB10A-P26, PMB10A-P45, PMB10A-V1-50, PMB10A-V2-50, PMB10A-V2F-50, PMB10A-VE-50, PMB11A-50, PMB13A-C-50, PATHfinder Real-Time PCR Kits for DNA Detection, Real-Time PCR Kits for DNA Detection, PCR Kits for DNA Detection, DNA Detection