GENERON
Food Safety in our Genes

PATHfinder E.coli shiga-toxin producing (STEC) Multiplex Detection Kits

Real-Time PCR kit for ***STEC*** detection

According to

ISO 13136-1: 2012 Detection in PCR of Escherichia coli Shiga toxins producers (STEC) and determination of O157, O111, O26, O103 and O145 serogroups

EU-RL VTEC_Method_10_Rev 0: Detection of Escherichia coli producing the Stx2f subtype by Real-Time PCR

Research Use Only

not for in vitro diagnostics

READ SAFETY INFORMATION AND DISCLAIMERS BEFORE USING THE KIT

This manual refers to the following part numbers, the description of each part number briefly indicates the targets detected by the kits:

PMB10A-50	PATHfinder - 2x2-Plex qPCR kit for the detection of STEC - [stx1]/[stx2] and [eae]/IAC
PMB10A-F-50	PATHfinder - 2x2-Plex qPCR kit for the detection of STEC - [stx1+stx2]/[stx2f] e [eae]/IAC
PMB10M-50	PATHfinder - 3-Plex qPCR kit for the detection of STEC - [stx1+stx2]/[eae]/IAC
PMB10A-EFSA-50	PATHfinder - 4-Plex qPCR kit for the detection of STEC - [stx1]/[stx2 + stx2f]/[eae]/IAC
PMB10M-V1C2E-50	PATHfinder - 4-Plex qPCR kit for the detection of STEC - [stx1]/[stx2]/[eae]/IAC

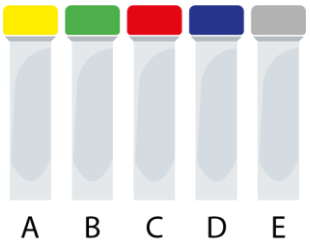
The table below indicates the reaction set-up procedure to follow for each kit:

Cat#	Target	Reaction
PMB10A-50	Stx1, stx2 and eae	18 Mix + 12 DNA
PMB10A-F-50	Stx1, stx2, stx2f and eae	18 Mix + 12 DNA
PMB10M-50	Stx1, stx2 and eae	**15 Mix + 5 DNA**
PMB10A-EFSA-50	Stx1, stx2, stx2f and eae	**15 Mix + 5 DNA**
PMB10M-V1C2E-50	Stx1, stx2 and eae	**15 Mix + 5 DNA**

Quick guide

Assay Box 50 reactions content		N. vials	
		PMB10A-50 PMB10A-F-50	PMB10M-50 PMB10M-V1C2E-50 PMB10A-EFSA-50
A	**PATHfinder** OLIGO Mix*	2	1
B	GENERase ULTRA Mastermix*	2	1
C	Positive Control*	2	1
D	Negative Control	1	1
E	Diluent*	2	1

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



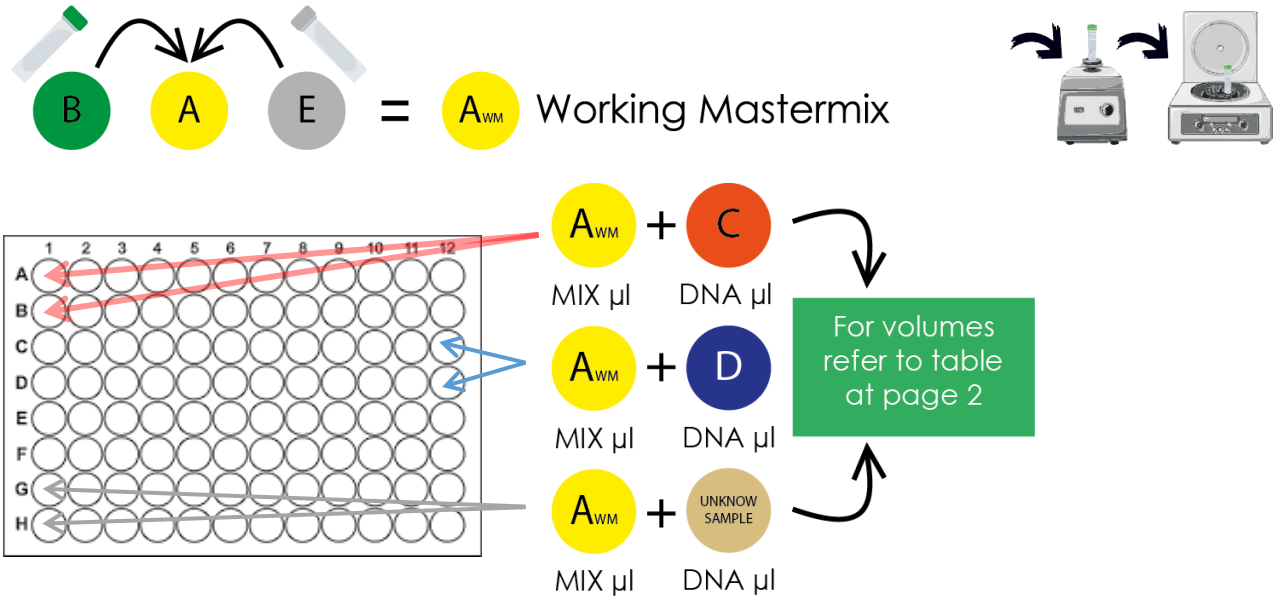
Reaction Set-Up

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.

1 - Introduction

E. coli are facultatively anaerobic gram negative bacteria that are naturally present in humans and animals as part of the intestinal microflora. Some strains are, however, able to cause disease ranging from mild to cholera-like diarrhea and may lead to potentially fatal complications such as hemolytic uremic syndrome (HUS). On the basis of pathogenic features, the most important diarrheagenic E. coli are classified into 6 distinct groups: enteropathogenic (EPEC), enterotoxigenic (ETEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), diffuse-adhering (DAEC), and enteroaggregative (EAEC). These strains are known to produce Shiga-toxin 1 (Stx1) and Shiga-toxin 2 (Stx2) and, in addition, other virulence-associated factors as hemolysin and the enterocyte effacement locus containing the intimin gene (eaeA).

These PATHfinder STEC multiplex Detection kits allow the detection of VTEC virulence factors in both pre-enriched food samples or colony isolates using sequences reported in ISO/TS 13136:2012, titled "Microbiology of food and animal feed - Real-time polymerase chain reaction (PCR)-based method for the detection of food-borne pathogens -- Horizontal method for the detection of Shiga toxin-producing Escherichia coli (STEC) and the determination of O157, O111, O26, O103 and O145 serogroups".

Each kit contains reagents sufficient for 50 tests. All kits include an internal amplification control (IAC) 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.

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)

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.

2 – Sample DNA

The DNA of STEC can be obtained from:

- **Enrichment broth(s):** food (but also swab, sponges and filters) samples should be enriched according to the corresponding International Standards (ISO 13136 indictes BPW and mTSB) 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 FASTfood 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 FASTfood 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.

3 – 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.

4 - 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.

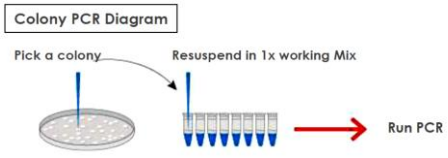
4.3 Reaction set-up

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** or to **15 Mix + 5 DNA**. According to the case, in the following steps use the volumes in brackets.
2. Transfer **18 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 (5 µL)** of positive control into wells acting as positive PCR control.
4. Add **12 µL (5 µL)** of each sample to wells testing the unknown samples.
5. Add **12 µL (5 µL)** of positive and negative extraction controls when present.
6. Add **12 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 (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 (2.5 µL)** of positive control into wells acting as positive control.
3. Add **4 µL (2.5 µL)** of negative control into wells acting as negative control.
4. Add **4 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 different fluorophores quenched with non-fluorescent quenchers**, set the instrument detector according to the table below. If HEX dye is not included in the list of calibrated dyes in the qPCR instrument, select alternatively VIC or JOE.

Kit		FAM	HEX	TexasRed	Cy5
PMB10A-50	Mix 1	stx1	stx2		
	Mix 2	eae	IAC		
PMB10A-F-50	Mix 1	stx1 + stx2	stx2f		
	Mix 2	eae	IAC		
PMB10M-50		stx1 + stx2	IAC		eae
PMB10A-EFSA-50		stx1	IAC	stx2 + stx2f	eae
PMB10M-V1C2E-50		stx1	IAC	stx2	eae

4.5 Thermal Cycling Conditions

These PATHfinder multiplex detection systems share a common thermal protocol and use degradative probes. Find below the protocol associated to these kits.

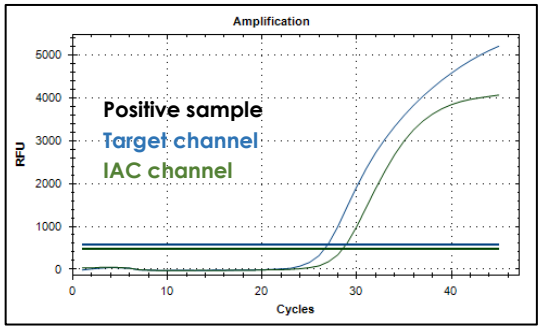
Step	T (°C)	Duration	Loops
Taq Activation	95	3 min	1
Denaturation	95	10 sec	45*
Annealing/Extension + Plate Reading	60	45 sec	

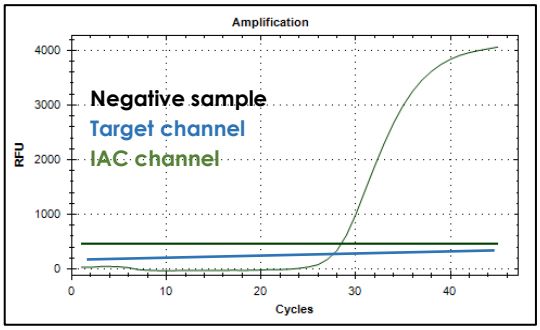
***IMPORTANT!** **When running colony PCR experiments reduce the cycling loops to 30.**

5 – 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.

6 – 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, all 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.

7 – 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.

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.

8 – 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.**

9 – 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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