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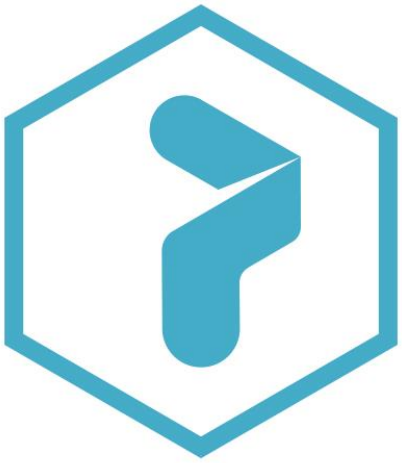

POLYSKOPE

LABS

Polyskope One Multiplex Pathogen Detection Assay

User Guide

Test for the real-time simultaneous PCR detection of *Escherichia coli* O157 STEC, *E. coli* non-O157 STEC, *Salmonella* spp. and *Listeria monocytogenes* in select food and stainless steel environmental samples.

TABLE OF CONTENTS

I. INTRODUCTION

Conventional bacteriological detection methods are often long and labor intensive. In comparison, Polyskope One is a simple and rapid qualitative test, allowing the detection of specific DNA sequences unique to *Escherichia coli* O157 STEC, *E. coli* non-O157 STEC, *Salmonella* spp. and *Listeria monocytogene*s found in stainless steel environmental samples and select food products. Using real-time polymerase chain reaction (PCR), specific DNA sequences are amplified and detected simultaneously by means of fluorescent probes. Up to 94 samples can be processed, with a minimized risk of contamination and an easy to use procedure. The intended users of this kit are trained laboratory personnel who are performing tests to detect *E. coli* O157 STEC, *Salmonella* spp. and *Listeria monocytogenes* (also known as “The Big Three”). The use of this test allows results to be obtained within a few hours following enrichment of a sample.

II. THE Polyskope One. TECHNOLOGY

The Polyskope One kit is a test based on gene amplification and detection by real-time PCR. Ready-to-use PCR reagents contain oligonucleotides (primers and probes) specific to the "Big Three" pathogens as well as DNA polymerase and nucleotides. PCR is a well-established technique used to rapidly generate profuse copies of target DNA. During the PCR reaction, cycles of heating and cooling promote DNA denaturation, followed by primers binding to specific target regions. The DNA polymerase then recognizes these

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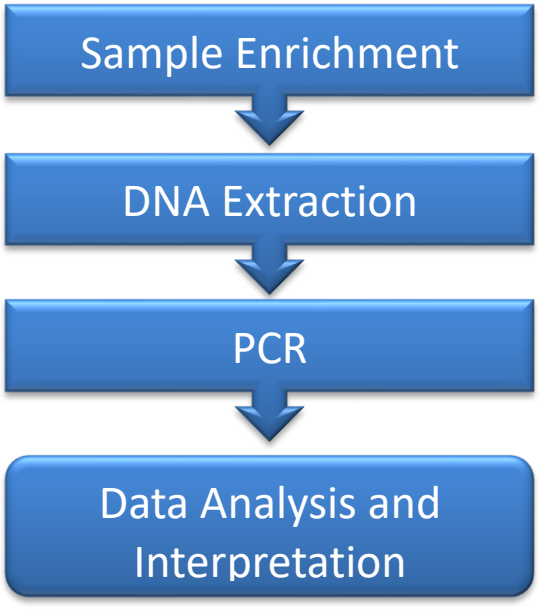
primers and utilizes deoxynucleotide triphosphates (dNTPs) to extend the DNA, creating copies of the target DNA, called amplicons. Next, specific probes are used to detect the DNA during the amplification, by hybridizing to the amplicons. These probes are bound to a fluorophore which fluoresces only when hybridized to the correct target sequence. In the absence of target DNA, no fluorescence will be detected. As the amplicons increase with each round of amplification, fluorescence intensity also increases. At the annealing step of each PCR cycle, the detector measures this fluorescence and the associated software plots the fluorescence intensity versus number of cycles. This method allows a simple determination of the presence, or absence, of up to five targets in a single reaction.
An unrelated DNA "internal control" is included in the reaction mix. This control is amplified with a specific probe at the same time as the other probe target DNA sequences and detected by a specific fluorophore. It allows for the validation of any negative result. .
Polyskope One is specifically designed to detect pathogenic bacteria capable of human infection. The oligonucleotides are targeted to specific pathogen-related genes that are present in these bacteria and distinguish them from closely related non-pathogenic bacteria.

The **PolySkope One method** allows the simultaneous detection of *E. coli* O157 STEC, *E. coli* non-O157 STEC, *Salmonella* spp. and *Listeria monocytogenes* in environmental samples and food products previously enriched by culture in Polyskope Multiplex Enrichment Media (PMEM). It includes the following 4 main steps:



III. KIT COMPONENTS

The Polyskope One kit contains sufficient reagents for 96 tests.

Reference ID	Reagent	Quantity Provided
A	Amplification Mix	1 tube (0.66 ml)
B	Probes	2 tubes (2 X .75 ml)
C	Lysis Component 1	1 bottle (7.5 ml)
D	Lysis Component 2	1 bottle (7.5 ml)
E	Lysis Component 3, Beads	1 bottle (8.8 g)
F	PCR positive control	1 tube (.25 ml)
G	PCR negative control	1 tube (.25 ml)

IV. SHELF LIFE AND STORAGE

Once received, the kit must be stored between +2 ° C and +8° C. Reagents stored between at this temperature can be used until the expiration date indicated on the reagent tube. Shelf life of lysis buffer is 1 month once mixed with lysis beads.

V. MATERIAL REQUIRED BUT NOT SUPPLIED

Equipment

- "Stomacher" masticator or equivalent for homogenizing test samples
- 37°C incubator for microbiological enrichment of sample
- Agitator-thermomixer for deepwell plates, capable of 65 ± 5°C and 95 ± 5°C and shaking at 1,400 RPM – Eppendorf Thermomixer or equivalent
- Vortex apparatus
- Magnetic stir plate
- 1 µl, 20 µl, 200 µl and 1000 µl aerosol resistant micropipette tips
- Combi-tip pipettes or equivalent repeat pipettors
- Real-time PCR system, currently approved on the Quantstudio 5

Supplies

- Polyskope Multiplex Enrichment Media PMEM (available in 500 g to 2.5 kg quantities)
- Stomacher bag with integral mesh filter (or equivalent stomacher bags)
- Environmental swabs and environmental sponges
- 1 ml 96-well deepwell plate
- Pre-pierced sealing film, such as "X-Pierce™ Sealing Films"
- 200 µl wide opening tips for lysis buffer transfer
- 96-well white, sterile PCR plates
- 96-well clear film plate sealer
- Sterile 2.5 ml Eppendorf or similar tubes
- Powder-free gloves.
- Sterile distilled water
- Bleach 5%
- Decontaminating agent such as DNA AWAY® or RNase AWAY®

VI. PRECAUTIONS AND RECOMMENDATIONS FOR BEST RESULTS

- This test must be performed by adequately trained personnel familiar with basic microbiology laboratory techniques as well as handling of potentially infectious material
- Food samples and enrichment cultures must be handled as possibly infectious material and disposed of according to local rules and regulations
- Any and all potentially infectious material should be sterilized before disposal
- Immuno-compromised individuals, the elderly, children and pregnant women should not handle any samples suspected of being positive for the presence of these three pathogens due to the high infection and mortality rate associated with these groups
- The quality of results depends on strict compliance with the Good Laboratory Practice guidelines, especially concerning PCR
    - Laboratory equipment must not circulate between workstations
    - A positive control and a negative control must be used for each series of amplification reactions
    - Do not use reagents past expiration date
    - Vortex all reagents from the kit before use to ensure homogeneity
    - Confirm the accuracy and precision of pipettes, as well as correct functioning of the instruments on a regular basis
    - Change gloves often, especially if contaminated
    - Clean work spaces periodically with at least 5% bleach and a decontaminating agent like DNA AWAY on specialized equipment

- Use powder-free gloves and avoid handling and writing on caps of tubes as this will interfere with data acquisition

Instructions for media preparation

1.) Using aseptic technique, carefully weigh 100 grams of Polyskope PMEM and pour into a sterile one liter flask

2.) Add 1 liter of distilled water and mix thoroughly until powder is dissolved

3.) Autoclave at 121°C for 15 minutes

4.) Store at room temperature (22-25°C), away from light until ready for use, then pre-heat to 37±1°C immediately before use. Prepared PMEM has a shelf life of two weeks.

VII. PROTOCOL

It is strongly recommended to read the entire protocol before starting the test. Please consult the table below for proper applicability before beginning the protocol.

Scope (matrices)	Enrichment	Validations
Fresh raw ground beef (25g), RTE turkey (25g), fresh raw spinach (25g), stainless steel environmental surface	PMEM 23 ± 1h 37±1°C	AOAC-RI

A. Sample Enrichment

Ensure that the enrichment media is at the appropriate incubation temperature (37±1° C) before use

1.) Using an appropriate scale, weigh 25 grams* of sample to be tested and place in a stomacher bag with incorporated mesh filter

2.) Pour 225 ml of Polyskope PMEM into filter bag

3.) Place filter bag in stomacher and homogenize at 130 RPM for 30 seconds. Fold top of filter bag over at least 3 times and close

4.) Incubate without shaking, for 23 ±1 hour at 37±1°C before lysis and PCR analysis

5.) For environmental sponges, the sponge (pre-wetted with neutralizing buffer) is added after sampling the surface area to 100 mL of pre-warmed (37 ± 1ºC) PMEM, homogenized for 30 seconds, and incubated at 37 ± 1ºC for 23 ± 1 hour
* Test portions other than 25 g have not been tested

B. DNA Extraction

- General recommendations:
- Turn on the thermomixer heat block before starting the extraction and preheat it to 65° C
- For food samples with a fatty supernatant, collect the sample just below this layer
- Open tubes and wells carefully to avoid any possible cross contamination
- Cool the deepwell plate before pipetting directly through the pre-pierced sealing film
- Reconstitute the final lysis buffer solution as follows:
  - Carefully pour all the contents from reagent C (Lysis Component 1) into reagent D (Lysis Component 2) and mix thoroughly. Next, carefully pour the contents from reagent E (Lysis Component 3, beads) into the mixed lysis buffer components
  - Use consumables with a wide enough tip to allow pipetting of the homogenized lysis reagent
  - The lysis reagent mixed with lysis beads (reagents C + D + E) has a shelf life of 1 month, when stored at 4° C.
- Before every use, gently agitate the lysis reagent by hand first to resuspend the beads. Then repeat pipette rapidly, in order to keep the lysis buffer in suspension while pipetting from the lysis bottle into the deepwell block.

Lysis Protocol

1.) Using a wide-bore pipette tip, aliquot 150 µl of homogenized lysis reagent (reagents C + D + E) into the wells of a deepwell block

2.) **After removing from the incubator, resuspend the food matrix by repeatedly squeezing/agitating the filter bag by hand (or in the stomacher) for at least 10 seconds.** Add 50 µl of enriched sample. Seal the deepwell block with the X-Pierce sealing film

3.) Incubate deepwell block in the heat block at 65±1°C for 15 minutes, shaking at 1,400 RPM. Secure deepwell block with lab tape if necessary

4.) Remove block from thermomixer and adjust temperature to 95±1°C. After thermomixer has achieved proper temperature, reinsert block and shake at 1,400 RPM for an additional 10 minutes. After 10 minutes, remove the deepwell plate and allow samples to cool to ambient temperature (20-25°C). The deepwell block may be placed in the refrigerator to speed the cooling process.

C. Real-time PCR mix preparation

1.) Prepare PCR mixture containing the amplification solution (reagent A) and the fluorescent probes (reagent B) depending on the number of samples and controls to analyze (at least one positive and one negative control must be included for each PCR run). Use the pipetting table in appendix to find the correct volumes to use for each reagent

- After preparation, the PCR mix (reagent A + B) must be used immediately. It is stable for only 1 hour maximum at 2°C-8°C

3.) Pipette 19 µl of this PCR mix into each well according to your plate setup

4.) Add 1 µl of sample or reagent F (negative control) or reagent E (positive control). Hermetically seal the wells of the plate by lightly applying pressure after the plastic film is in place. It is important to avoid bubbles at the bottom of the wells by pipetting carefully. If necessary, to eliminate any bubbles, centrifuge the sealed PCR plate (quick spin)

5.) Place the plate in the thermal cycler. Be sure to place the plate correctly: A1 well at the upper left corner. Close the reaction module

PCR Start

To start the PCR run, follow instructions in the real-time PCR system user guide for Polyskope One on the Quantstudio 5

D. Data Analysis

Data can be analyzed directly at the end of the PCR run or at a later time by opening the stored data file

Interpreting Results

Once the data analysis parameters have been set, results are interpreted by analyzing the Ct values of each sample (the cycle at which the amplification curve crosses the threshold, also known as the Ct value). The threshold is the solid horizontal line automatically calculated by the software that appears on the data plot when an individual fluorophore (Target 1-5, see **"Fluorophores"** below) is selected.

Fluorophores

Red is the FAM fluorophore (Target 1) and is for *E. coli* STEC STX1/STX2
Blue is the ABY fluorophore (Target 2) and is for *E. coli* STEC EAE
Green is the VIC fluorophore (Target 3) and is for *L. monocytogenes*
Purple is the ALEXA 647 fluorophore (Target 4) and is for *S. enterica*
Yellow is the JUN fluorophore (Target 5) and is the Internal Control, which should be present in every reaction

Controls

Before interpreting sample results, it is necessary to verify the positive and negative controls. For the experiment to be valid, the controls must have the following results, as summarized in the table below, otherwise the PCR reaction needs to be repeated

Test	Target Probe Detection	Internal Control Detection
Positive Control	14 < Ct < 40	14 < Ct < 40
Negative Control	Ct = N/A	14 < Ct < 40

Samples

A positive signal must have a Ct value ≥ 10 for any of the target (non-internal control) fluorophore. Examine the threshold line and verify that it intersects all curves with a typical shape of successful PCR amplification.

If the Ct value is below 10, verify that as raw data the curve is a regular amplification curve (with a flat base line, followed by a rapid increase of fluorescence and then a flattening out). If the curve seems correct, it may be considered a positive sample. If there is no Ct value (Ct=N/A, Not Applicable) for the target fluorophores or the curve is not a typical amplification curve, the internal control for that sample must then be analyzed:

- This sample is considered a negative sample if there is no Ct value in any target fluorophore channels and the internal control has a Ct ≥ 14

- Should the internal control also not have a Ct value (Ct = N/A), this indicates an inhibition of the PCR reaction. Dilute the sample from the lysis block (perform a 1:10 dilution in distilled sterile water using 10 µl of lysate in 90 µl sterile water, then use 1 µl of the dilution) and repeat the PCR

- Should the Ct value for the internal control be < 14 it is not possible to interpret the result. Verify that the threshold was correctly placed, or that the curve as raw data is

a regular amplification curve. If the curve does not have a characteristic shape, it will be necessary to repeat the PCR test

Interpretation of sample results is summarized in the following table:

Target Probe (Any fluorophore)	Internal Control Detection	Interpretation
Ct ≥ 10	Ct ≥14	Positive
Ct = N/A	Ct ≥14	Negative
Ct = N/A	Ct = N/A	Inhibition

The software indicates a Ct value of N/A when the fluorescence of a sample does not rise significantly above the background noise, and hence does not cross the threshold. If results of negative and positive controls differ from those in the table above, it is necessary to repeat the PCR. It should be noted that a 1:10 dilution of the lysate sample must be tested also if both the internal control and target fluorophores give a result of N/A.

Confirmation of positive results

In the context of the food safety regulations, all positive sample results need to be confirmed in one of three ways using Bacteriological Analytical Manual (BAM), USDA FSIS MLG and ISO methods as well as AOAC-approved methods:

1.) Using classic tests described in the BAM, the USDA FSIS MLG and ISO methods

2.) Streaking for isolation on a chromogenic medium. The protocol for these validated chromogenic methods used for confirmation should start from the PMEM enrichment broth. The presence of characteristic pathogen colonies is sufficient to confirm the presence of pathogens

3.) Using any other certified method based on a principle different from that used in the Polyskope One PCR test. The validated protocol of this second method must be followed entirely. In the event of results that are not in agreement between Polyskope One and one of the confirmation options listed above, the laboratory should follow the necessary steps to ensure the validity of their results. It is possible to store the enriched PMEM at 2-8°C, for 72 hours maximum following the incubation at 37° C, before carrying out the confirmation.

VIII. TEST PERFORMANCES AND VALIDATIONS

The Polyskope One Multiplex Pathogen Detection Assay has been validated by the AOAC-Research Institute under the Performance Tested Method Program for simultaneous qualitative detection of *Escherichia coli* O157, non-*E. coli* O157 Shiga-Toxin Producing *E.*

coli (STEC), *Listeria monocytogenes,* and *Salmonella* species from fresh raw ground beef (25 g), deli turkey (25 g), fresh baby spinach (25 g), and stainless steel environmental surface sponges (4 x 4 in test area). A positive result from Polyskope One should be considered presumptive and further confirmation by standard reference method is recommended.

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IX. APPENDIX A

3-in-1 Probe Mixing Guideline

When using the all-in-one version of Polyskope One, refer to the following tables to ensure an accurate ratio of reagents when making the PCR mix

# of Reactions	Reagent A (µls)	Reagent B (µls)
1	5	14
2	10	28
3	15	42
4	20	56
5	28	77
6	33	92
7	39	108
8	44	123
9	50	139
10	55	154
11	61	169
12	66	185
13	72	200
14	77	216
15	83	231
16	88	246
17	94	262
18	99	277
19	105	293
20	110	308
21	116	323
22	121	339
23	127	354
24	132	370
25	138	385

# of Reactions	Reagent A (µls)	Reagent B (µls)
26	143	400
27	149	416
28	154	431
29	160	447
30	165	462
31	171	477
32	176	493
33	182	508
34	187	524
35	193	539
36	198	554
37	204	570
38	209	585
39	215	601
40	220	616
41	226	631
42	231	647
43	237	662
44	242	678
45	248	693
46	253	708
47	259	724
48	264	739
49	270	755
50	275	770

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# of Reactions	Reagent A (µls)	Reagent B (µls)
51	281	785
52	286	801
53	292	816
54	297	832
55	303	847
56	308	862
57	314	878
58	319	893
59	325	909
60	330	924
61	336	939
62	341	955
63	347	970
64	352	986
65	358	1001
66	363	1016
67	369	1032
68	374	1047
69	380	1063
70	385	1078
71	391	1093
72	396	1109
73	402	1124
74	407	1140
75	413	1155

# of Reactions	Reagent A (µls)	Reagent B (µls)
76	418	1170
77	424	1186
78	429	1201
79	435	1217
80	440	1232
81	446	1247
82	451	1263
83	457	1278
84	462	1294
85	468	1309
86	473	1324
87	479	1340
88	484	1355
89	490	1371
90	495	1386
91	501	1401
92	506	1417
93	512	1432
94	517	1448
95	523	1463
96	528	1478

PolySkope One Multiplex Assay Quick Reference Worksheet

PolySkope Media Preparation

100 g PMEM Media into 1 Liter Water

↓

Mix thoroughly until powder is dissolved

↓

Autoclave @ 121°C for 15 minutes

PolySkope Lysis Buffer Preparation

Pour contents (7.5 mL) of Reagent Bottle C (Lysis Component 1) into Reagent Bottle D (7.5 mL)

↓

Pour contents (8.8 g) of Reagent Bottle E (beads) into Reagent Bottle D (15 mL lysis solution)

↓

Mix thoroughly before transfer to keep in suspension

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PolySkope Sample Preparation

225 ml pre-heated (37°C) PMEM media + 25 g sample

Stomach at 130 RPM for 30 seconds

Incubate @ 37°C for 23±1 hours

Add 150 µL lysis buffer to deepwell block

After removing from the incubator, resuspend the food matrix by repeatedly squeezing/agitating the filter bag by hand (or in the stomacher) for at least 10 seconds Transfer 50 µL sample from sample bag to deepwell block

Place block on thermomixer @ 65°C for 15 minutes @ 1400 RPM

Remove deepwell block, adjust thermomixer to 95°C

Shake deepwell block @ 95°C for 10 minutes @ 1400 RPM

Remove deepwell block and allow to cool to room temperature before use. Block may be placed in the refrigerator to speed cooling

Transfer required volume sample lysate for analysis (ensure no lysis beads transferred)

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