iQ-Check® STEC VirX

Catalog #: 357-8139

User Guide

Test for the real-time PCR detection of virulence genes in Shiga Toxin Producing *E. coli*

BIO-RAD

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I. INTRODUCTION

E. coli bacteria are normal flora in human and animal intestines and are usually harmless. However, some strains can cause diseases to humans. Among them, Shiga toxin-producing *Escherichia coli* (STEC) are known to be highly pathogenic to humans. They can lead to hemorrhagic colitis and hemolytic uremic syndrome (HUS). STECs are defined by the presence of the *stx*1 or *stx*2 (Shiga toxin genes) in their genome. The *eae* (intimin) gene is an additional virulence marker.

The most known of these STEC strains is *E. coli* O157:H7 for which there is a "zero tolerance" policy in North America. Such policy will be implemented by FSIS for non-O157 STEC as well, in the meat sector. Outbreaks are commonly associated with the consumption of raw meat, but also with dairy products and more recently with produce. In the context of the FSIS or the ISO guidelines, a sample positive for both *stx*1/2 and *eae* targets will have to be tested for the presence of the major *E. coli* serogroups.

The iQ-Check STEC VirX kit, based on a multiplex real-time PCR system, allows the detection of the *stx* and *eae* virulence genes in one well, within few hours after the end of the microbiological enrichment. A sample that would be positive for both *stx*1/2 and *eae* would then be tested with the iQ-Check STEC SerO real-time PCR kit.

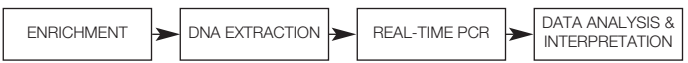
II. THE iQ-Check STEC TECHNOLOGY

PCR is a powerful technique used to generate many copies of target DNA. During the PCR reaction, several cycles of heating and cooling allow DNA denaturation, by heat, followed by primers annealing to the target region. The DNA polymerase then uses these primers and deoxynucleotide triphosphates (dNTPs) to extend the DNA, creating copies of the target DNA. These copies are called amplicons.

In real-time PCR, specific probes are used to detect DNA during the amplification, by hybridizing to the amplicons. These probes are linked to a fluorophore which fluoresces only when hybridized to the target sequence. In the absence of target DNA, no fluorescence will be detected. As the amount of amplicons increases with each round of amplification, fluorescence intensity also increases. During each PCR cycle, at the annealing step, the optical module or detector measures this fluorescence, whereas the associated software plots the fluorescence intensity versus number of cycles.

The iQ-Check STEC VirX kit is a multiplex real-time PCR test. Ready-to-use PCR reagents contain oligonucleotides (primers and fluorescent double strand probes) specific for STEC virulence genes *stx*1, *stx*2 and *eae*, as well as DNA polymerase and nucleotides. Two fluorophores are linked to each probe hybridizing to the target DNA sequences, either to *stx*1 and *stx*2 or to *eae*. A synthetic DNA "internal control", included in the reaction mix, is amplified at the same time as the target DNA sequences, and detected by another probe labeled with a third fluorophore. It allows for the validation of any negative result. Detection and data analysis are optimized for use with a Bio-Rad real-time PCR instrument, such as the CFX96™ system.

This method allows a simple determination of the presence, or absence, of the STEC virulence genes in all food products and environmental samples, previously enriched by culture in specified enrichment broth. It includes the following four main steps:



III. KIT COMPONENTS

The iQ-Check STEC VirX kit contains sufficient reagents for 96 tests.

Reference ID	Reagent	Quantity Provided
A	Lysis reagent	1 bottle (20 mL)
B	Fluorescent probes	1 tube (0.55 mL)
C	Amplification mix	1 tube (1.65 mL)
D	PCR negative control	1 tube (0.5 mL)
E	PCR positive control	1 tube (0.25 mL)
F	Lysis beads	1 bottle (17.6 g)

IV. SHELF LIFE AND STORAGE

Once received, the kit must be stored between +2°C and +8°C. Reagents stored at this temperature can be used until the expiration date indicated on the reagent tube.

V. MATERIAL REQUIRED BUT NOT SUPPLIED

Equipment

- Stomacher®, masticator or equivalent for homogenizing test samples.
- Incubator for sample microbiological enrichment
- Specific for extraction in 1.5 mL tube
  - Bench top centrifuge (max. 10,000-12,000 g).
  - Dry heat block (100°C ± 5°C).
- Specific for extraction in deepwell plate
  - Centrifuge with rotor for 96-wells plates (max. 2,250 g).
  - Dry heat block (100°C ± 5°C).
  - or agitator-incubator for deepwell plates, such as a "Thermomixer" (Eppendorf).
- Magnetic stir plate.
- Vortex apparatus.
- 20 µL, 200 µL and 1000 µL micropipettes.
- Combitip pipettes or equivalent repeat pipettors.
- Bio-Rad real-time PCR system* (Industrial Diagnostic CFX96™ real-time PCR detection system, 96 wells)

* Contact Bio-Rad for detailed information on instruments recommended by our technical department.

Note: We recommend using a universal power source (UPS) with the thermal cycler.

Supplies

- Enrichment medium: SEB STEC Enrichment Broth, Bio-Rad cat.#: 356-4001, 500 g, 356-4002, 5 kg.
- Optional (USDA standard enrichment): mTSB (E.g. Bio-Rad cat. #: 356-4426, 500 g), casamino acids and novobiocin.
- Stomacher bag with incorporated filter.
- Environmental sponges.
- Environmental swabs.
- Specific for extraction in 1.5 mL tube
  - 1.5 mL conical screwcap sterile tubes (E.g. Bio-Rad cat. #: 224-0110).
- Specific for extraction in deepwell plate
  - 1 mL deepwell plate, Bio-Rad cat.#: 359-0132.
  - Plastic sealing film, Bio-Rad cat.#: 359-0139.
  - Pre-pierced sealing film, such as "X-Pierce™ Sealing Films", Bio-Rad cat #: 360-0040, for North America only; cat #: 359-3977, x100.

- Specific for Easy II extraction protocol
  - 200 µL wide opening tips.
- PCR plates, tubes, sealing tape and caps, see real-time PCR system user guide for iQ-Check kits.
- Sterile filter tips, adaptable to 20 µL, 200 µL and 1000 µL micropipettes.
- Tips for Combitip pipettes or equivalent repeat pipettors, sterile, individual package.
- 1 mL and 10 mL pipettes.
- Powder-free gloves.
- Distilled sterile water.
- Bleach 5%.
- Cleaning agent such as DNA AWAY® or RNase AWAY®.

VI. PRECAUTIONS AND RECOMMENDATIONS

- This test must be performed by adequately trained personnel.
- Cultures must be handled as potentially infectious material and eliminated according to local rules and regulations.
- All potentially infectious material should be autoclaved before disposal.
- The quality of results depends on strict compliance with the following Good Laboratory Practice (for example the EN ISO 7218 standard), especially concerning PCR:
  - The laboratory equipment (pipettes, tubes, etc.) must not circulate from one work station to another.
  - It is essential to use a positive control and a negative control for each series of amplification reactions.
  - Do not use reagents after their expiration date.
  - Vortex reagents from the kit before using them to ensure homogeneity.
  - Periodically, verify the accuracy and precision of pipettes, as well as correct functioning of the instruments.
  - Change gloves often, especially if you suspect they are contaminated.
  - Clean work spaces periodically with at least 5% bleach and a decontaminating agent like DNA AWAY.
  - Use powder-free gloves and avoid fingerprints and writing on caps of PCR tubes or plates. Both cases will interfere with data acquisition.

- It is strongly advised to follow the general requirements described in the standard EN ISO 22174:2005 “Microbiology of food and animal feeding stuffs – Polymerase chain reaction (PCR) for the detection of food pathogens – General requirements and definitions”.

VII. PROTOCOL

It is strongly recommended to read the entire protocol before starting the test.

The following table outlines the different protocols that can be used depending on the application:

Scope (matrices)	Enrichment	Protocol	
		Method	Format
Raw beef	Pre-warmed SEB, 41.5°C ± 1°C 10 hrs - 22 hrs	Easy II	Tube Deepwell
Raw milk products, produce, environmental samples	Pre-warmed SEB, 41.5°C ± 1°C 16 hrs - 22 hrs	Easy I	Tube Deepwell

Note: the iQ-Check STEC VirX kit can be used following the enrichment indicated in the USDA MLG 5B.02 standard.

A. Sample Enrichment

Enrichment media must be at the appropriate incubation temperature before use.

For raw beef samples of 375 g, homogenize in 1,125 mL of pre-heated SEB (1/4 dilution), in a stomacher bag with incorporated filter.

For samples of 25 g (and under), homogenize n g of sample in 9 x n mL of pre-heated SEB (1/10 dilution), in a stomacher bag with incorporated filter.

Incubate, without shaking, for times and temperatures indicated in the table above, and then follow the indicated DNA extraction protocol.

B. DNA Extraction

General recommendations:

- Before starting the test, turn on the heat block to preheat it and set it to 95°C - 100°C.
- In general, avoid collecting large fragments of food debris. For food samples with a fatty supernatant, collect the sample just below this layer.
- Open tubes and wells carefully to avoid any possible cross contaminations.
- Cool the deepwell plate before pipetting directly through pre-pierced sealing film.
- Gently shake the lysis reagent by hand first to resuspend the resin. Then pipette while it is stirring at medium speed with the magnetic bar contained in the bottle, in order to keep it in suspension.
- For Easy II protocol, the lysis reagent has to be reconstituted:
  - Carefully pour all the contents from reagent F (lysis beads) into reagent A (lysis reagent).
  - Use consumables with a wide enough tip to allow pipetting of the homogenized lysis reagent.
  - The lysis reagent mixed with lysis beads (reagents A + F) has a shelf life of 6 months, when stored at 4°C.

Easy I protocol

1 - Aliquote 100 µL of homogenized lysis reagent A to tubes or wells of a deepwell plate.
2 - Add 100 µL of the decanted enriched sample. Mix by pipetting up and down and close the tube with caps or seal the deepwell plate with pre-pierced sealing film.
3 - Incubate in the appropriate heat block at 95°C - 100°C for 10 to 15 minutes or in the plate incubator-agitator for 15 to 20 minutes at 1,300 rpm.
4 - Vortex tubes at high speed.
5 - If using a deepwell plate, allow to cool down to room temperature.
6 - Centrifuge at 10,000-12,000 g at least 2 minutes for tubes. Centrifugation is not needed for deepwell plate.

If you choose to temporarily stop the procedure, this is the recommended stopping point. The supernatant can be stored for up to 1 year at -20°C. Before reusing it, always allow it to thaw, homogenize, and then centrifuge tubes at 10,000-12,000 g for 5 minutes.

Easy II protocol (includes a grinding step)

1 - Aliquote 100 µL of homogenized lysis reagent (reagents A + F) to tubes or wells of a deepwell plate.

2 - Add 100 µL of the decanted enriched sample.
Note: shake the suspension to homogenize the culture and then allow any debris to decant before collecting the sample.
Mix the solution by pipetting up and down until homogenized. Close the tubes or seal the deepwell plate with the pre-pierced sealing film.

3 - Place tubes in the Cell Disruptor for 3 min ± 1min (tubes only).

4 - Incubate tubes in the heat block at 95°C - 100°C for 10 to 15 minutes or deepwell plate in the agitator-incubator under agitation at 1,300 rpm at 95°C - 100°C for 15 to 20 minutes

5 - For tubes only, vortex at high speed, centrifuge at 10,000-12,000 g for at least 2 minutes. Centrifugation is not needed for deepwell plate.

If you choose to temporarily stop the procedure, this is the recommended stopping point. The supernatant can be stored for up to 1 year at -20°C. Before reusing it, always allow it to thaw, homogenize, and then centrifuge tubes at 10,000-12,000 g for 5 minutes.

C. Real-time PCR

1. Instrument and software setup

For instrument and software setup, follow instructions in the real-time PCR system user guide for iQ-Check kits.

2. PCR mix preparation

1 . Prepare the PCR mix containing the amplification solution (**reagent C**) and the fluorescent probes (**reagent B**) according to the PCR mix calculation guide found in Appendix. To find the correct volumes to use, add the total number of samples and controls to be analyzed, and find the corresponding volumes in the table. At least one positive and one negative control must be included in each PCR run.

2 . After preparation, the PCR mix (reagent B + C) should be used immediately, or is stable for **1 hour maximum at 2°C - 8°C**.

3 . Pipette **20 µL** of this PCR mix in each well according to your plate setup.

4 . Add **5 µL** of **sample** or **reagent D** (negative control) or **reagent E** (positive controls), and seal the wells.
It is important to avoid bubbles at the bottom of the wells by pipetting carefully. As an optional step to eliminate any bubbles, centrifuge the sealed PCR plate or the PCR strips (quick spin).

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

3. PCR Start

To start the PCR run, follow instructions in the real-time PCR system user guide for iQ-Check STEC kits.

D. Data Analysis and Results Interpretation

Data can be analyzed directly at the end of the PCR run or at a later time by opening the stored data file. Follow instructions in the corresponding real-time PCR system user guide for iQ-Check kits for opening data files and setting the data analysis parameters.

Interpreting Results

Once the data analysis parameters have been set, results are interpreted by analyzing the Cq values of each sample (the cycle at which the amplification curve crosses the threshold).

1. 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.

	***stx*1/*stx*2 detection (FAM)**	***eae* detection (Cy5 channel)**	**Internal control detection (HEX channel)**
Negative control	Cq = N/A*	Cq = N/A*	26 ≤ Cq ≤ 36
Positive control	26 ≤ Cq ≤ 36	26 ≤ Cq ≤ 36	Not significant

* The software indicates a Cq value of N/A (not applicable) 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.

2. Samples:

A sample **positive** for STEC virulence genes must have a Cq value ≥ 10 in both FAM and Cy5 channels.

- If the Cq value for both channel is below 10, verify that as raw data the curve is a regular amplification curve (with a flat base line, followed by a rapid exponential increase of fluorescence and then a flattening out). If the curve seems correct, it may be considered a sample positive for STEC virulence genes.
- If the Cq value of FAM is ≥ 10 and Cq value for Cy5 is N/A, the sample is positive for *stx*1/*stx*2 virulence genes.

- If the Cq value of FAM is N/A and Cq value for Cy5 is ≥ 10, the sample is positive for *eae* virulence gene.

If there is no Cq value (Ct=N/A) for FAM or Cy5, or if the curve is not a typical amplification curve, the internal control for that sample must then be analyzed:

- This sample is considered as a **negative** STEC virulence genes sample if there is no Cq value in FAM, no Cq value in Cy5, and the internal control has a Cq ≥ 26.

- Should the internal control also not have a Cq value (Cq = N/A), this probably indicates an inhibition of the PCR reaction. The sample needs to be diluted (perform a 1/10 dilution in distilled sterile water, using 10 µL of DNA extract, then use 5 µL of the dilution for amplification), and the PCR repeated.

- Should the Cq value for the internal control be < 26 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:

***stx*1/*stx*2 detection (FAM)**	***eae* detection (Cy5 channel)**	**Internal control detection (HEX channel)**	**Interpretation**
Cq ≥ 10	Cq≥ 10	Not significant	Positive. Test with iQ-Check STEC SerO kit
Cq ≥ 10	Cq = N/A	Not significant	Positive for *stx*1/*stx*2, negative for *eae*. Stop sample analysis
Cq = N/A	Cq ≥ 10	Not significant	Positive for *eae*, negative for *stx*1/2. Stop sample analysis
Cq = N/A	Cq = N/A	Cq > 26	Negative
Cq = N/A	Cq = N/A	Cq = N/A	Inhibition**

** When all *stx*1/*stx*2, *eae* virulence genes and internal control detection give a Ct value = N/A, the sample must be tested again but diluted (1/10).

VIII. CONFIRMATION OF SINGLE COLONY USING iQ-Check

1 . Dispense 200 µL of lysis reagent in tubes.
2 . Pick an isolated colony, from the agar plate with a tooth-pick or sterile loop, or other adapted consumable (e.g. pipette tip).

3 . Resuspend the colony in the lysis reagent.
4 . Homogenize using a vortex.
5 . Incubate tubes in the heat block at 95°C - 100°C for 15 minutes.
6 . Centrifuge at 10,000-12,000 g for at least 2 minutes.
Store DNA supernatants at 2°C - 8°C for two weeks maximum. For longer storage, keep samples at -20°C or -70°C.

IX. TEST PERFORMANCES AND VALIDATIONS

iQ-Check STEC VirX is validated by AOAC Research Institute under the Performance Tested Method Program for detection of *stx*1/*stx*2 and *eae* in raw beef trim. A positive result with iQ-Check should be considered presumptive and it is recommended it be confirmed by standard reference methods. (See references 1 and 2, section X). Certificate number: 121203.

iQ-Check STEC VirX has been granted a No Objection Letter from the United States Department of Agriculture Food Safety and Inspection Service (USDA FSIS) for raw beef trim.

X. REFERENCES

1. Centers for Disease Control and Prevention. *Bacterial Foodborne and Diarrheal Disease National Case Surveillance. Annual Report, 2005*. Atlanta: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention; 2007.
2. EFSA Journal 2009; 7(11):1366 [43 pp.]. Technical specifications for the monitoring and reporting of verotoxigenic *Escherichia coli* (VTEC) on animals and food (VTEC surveys on animals and food).
3. Food Safety and Inspection Service, Federal Register, Vol. 76, No. 182, 9 CFR Parts 416, 417, and 430, [Docket No. FSIS–2010–0023], Shiga Toxin-Producing *Escherichia coli* in Certain Raw Beef Products.
4. ISO/TS 13136:2011 (E), Microbiology of food and animal feeding stuffs – Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) belonging to O157, O111, O26, O103 and O145 serogroups - Qualitative Method.
5. United States Department of Agriculture Food Safety And Inspection Service, Office of Public Health Science, MLG 5B.02, Detection and Isolation of non-O157 Shiga-toxin Producing *Escherichia coli* (STEC) from Meat Products.

APPENDIX - PCR Mix Calculation Guide

To find the correct volumes to use when preparing the PCR mix, add the total number of samples and controls to be analyzed, and find the corresponding volumes of reagent B and reagent C in the table.

Total number of samples & controls	Probes Reagent B (µL)	Amplification mix Reagent C (µL)
1	5	15
2	11	33
3	16	48
4	22	66
5	27	81
6	32	96
7	38	115
8	43	130
9	49	147
10	54	160
11	59	177
12	65	195
13	70	210
14	76	230
15	81	245
16	86	260
17	92	275
18	97	290
19	103	310
20	108	325
21	113	340
22	119	357
23	124	370
24	130	390
25	135	405
26	140	420
27	146	440
28	151	450
29	157	470
30	162	485
31	167	500
32	173	520
33	178	535
34	184	550
35	189	565
36	194	580
37	200	600
38	205	615
39	211	635
40	216	650
41	221	665
42	227	680
43	232	696
44	238	715
45	243	730
46	248	745
47	254	760
48	259	777

Total number of samples & controls	Probes Reagent B (µL)	Amplification mix Reagent C (µL)
49	265	795
50	270	810
51	275	825
52	281	845
53	286	860
54	292	880
55	297	890
56	302	905
57	308	925
58	313	940
59	319	960
60	324	970
61	329	990
62	335	1000
63	340	1020
64	346	1040
65	351	1050
66	356	1070
67	362	1090
68	367	1100
69	373	1120
70	378	1130
71	383	1150
72	389	1170
73	394	1180
74	400	1200
75	405	1210
76	410	1230
77	416	1250
78	421	1260
79	427	1280
80	432	1300
81	437	1310
82	443	1330
83	448	1340
84	454	1360
85	459	1380
86	464	1390
87	470	1410
88	475	1420
89	481	1445
90	486	1460
91	491	1470
92	497	1490
93	502	1510
94	508	1520
95	513	1540
96	518	1550

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