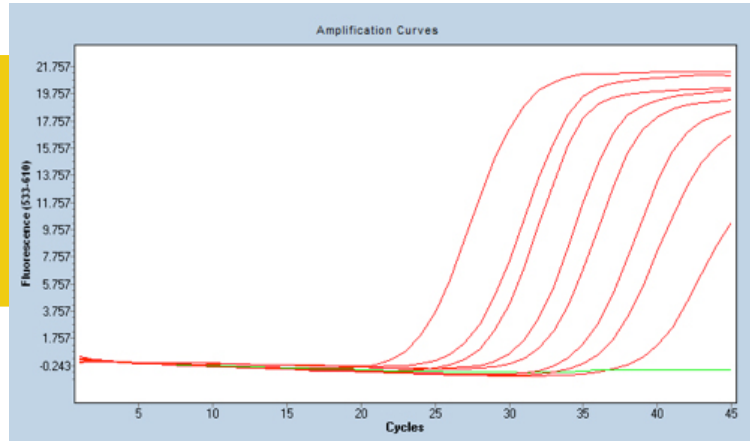


Appendix 2: ASSAY APPLICABILITY

enviroPRO™ has been validated for the detection of *Listeria* and *Salmonella* species in environmental (stainless steel surfaces) samples.

APPENDIX 3: RESULTS INTERPRETATION



Amplification curves have a characteristic shape consisting of an initial lag phase, exponential amplification phase and a final plateau phase. The final plateau phase, which represents a decrease in reaction efficiency as reagents are consumed, may not be reached in reactions containing low levels of *Listeria* or *Salmonella*. Amplification curves that deviate from the characteristic shape should be interpreted with caution. For each enviroPRO™ reaction, the cycle at which fluorescence signal rises above background fluorescence is determined and is called the “threshold cycle” (Ct) “crossing point” (Cp) or “quantification cycle” (Cq), depending on the instrument. The Ct/Cp/Cq will occur at an earlier cycle for samples containing high levels of *Listeria* or *Salmonella* and will be delayed for reactions containing low levels of *Listeria* or *Salmonella*. The ROX channel is designed to detect total *Listeria* organisms in the sample, and the Cy5 channel detects total *Salmonella* organisms. The HEX channel serves as an internal amplification control (IAC) to indicate a successful PCR reaction and should be detected at a Cp value ~<33 cycles.

APPENDIX 4: CONFIRMATION OF RESULTS

Presumptive positive samples can be confirmed by either USDA/FSIS or FDA reference methods for the detection of *Listeria* and *Salmonella* species from environmental samples. Enriched samples from the Veriflow® procedure can be transferred to secondary enrichment and subsequent steps as described in USDA/FSIS Microbiology Laboratory Guidebook Chapters 4 and 8, or alternatively, FDA Bacterial Analysis Manual Chapters 5 and 10.

APPENDIX 5: DISPOSAL

Invisible Sentinel PCR tubes are for single use only. Decontaminate all surfaces, media and reagents and discard in accordance with local, state, and federal regulations.

CUSTOMER SERVICE

Invisible Sentinel customer service and technical assistance can be reached Monday-Friday between 9 AM and 5 PM Eastern Standard Time by calling 215-966-6118 and asking for an Invisible Sentinel sales or technical representative. Training on this product and all Invisible Sentinel test kits is available.

SDS INFORMATION AVAILABLE

Safety Data Sheets (SDS) are available for this test kit and all of Invisible Sentinel's test kits by calling Invisible Sentinel at 215-966-6118.

V. IS2366.1



INVISIBLE SENTINEL

Phone 215.966.6118

Email info@invisiblesentinel.com | www.invisiblesentinel.com



THE POWER OF
MOLECULAR DIAGNOSTICS
IN THE PALM OF YOUR HAND®



ASSAY PRINCIPLES

enviroPRO™ is a real-time PCR assay for the simultaneous presumptive detection of *Listeria* and *Salmonella* species from environmental samples. The assay utilizes a multiplex detection method that detects pan *Listeria* and *Salmonella* species on the ROX and Cy5 channels respectively, and an internal amplification control (IAC) on the HEX channel. The assay can be used for both multiplex analysis and singleplex analysis, as desired. The assay couples the advantages of the real-time format with a streamlined sampling protocol that requires no DNA purification, complicated end user manipulations, or data analysis, in a cost-efficient format.

INTENDED USER

enviroPRO™ is intended for use by personnel familiar with basic sample collection and preparation techniques associated with foodborne pathogen detection during production and packaging. enviroPRO™ is specifically designed to be easy-to-use and eliminate the need for advanced training in molecular biology.

Invisible Sentinel®, veriPRO® and enviroPRO™ are trademarks owned by Invisible Sentinel, Inc., of Philadelphia, PA. U.S. Patent No. 8,183,059, 8,476,082 and patents pending. Purchase and use of this product is subject to Invisible Sentinel's Terms and Conditions of Sale located at <http://www.invisiblesentinel.com>.

INVISIBLE SENTINEL

Phone 215.966.6118

Email info@invisiblesentinel.com | www.invisiblesentinel.com

MATERIALS PROVIDED

1. IS *enviroPRO*TM PCR Reagent – Cat No. IS0563
Also available:
 - a. *enviroPRO*TM *Salmonella* singleplex PCR reagent – Cat. No. IS0564
 - b. *enviroPRO*TM *Listeria* singleplex PCR reagent – Cat. No. IS0565
2. 1.2 mL Sample Tubes – Cat. No. IS2376
3. Buffer ACB – Cat. No. IS0714

MATERIALS NEEDED

1. Real-Time PCR Instrument capable of detecting ROX, Cy5 and HEX fluorophores:
 - a. E.g. Roche LightCycler 480 II or Bio-Rad CFX96 platforms
2. Incubator capable of maintaining 35°C ± 2°C
3. Pipettes and tips capable of 5 µL, 250 µL, 500 µL volume transfers
4. Enrichment bags with sampling sponges
5. Invisible Sentinel Listeria broth (ISLB) – Cat. No. IS0302 (Listeria enrichments)
6. Buffered peptone water (BPW) – Cat. No. IS0305 (Salmonella enrichments, equivalent to FDA BAM M192 formulation)

STORAGE OF MATERIALS

The *enviroPRO*TM kit sampling tubes and Buffer ACB should be stored at room temperature (20°-25°C). The *enviroPRO*TM qPCR tubes should be stored at -20°C ± 2°C.

PRECAUTIONS

1. Enriched samples could contain *Listeria monocytogenes* or *Salmonella* species, human pathogens. All samples collected for use with the *enviroPRO*TM system should be handled with care.
2. Assay users should observe standard BSL-2 microbiological practices and safety precautions when performing this assay. Because high levels of enriched bacteria can result from use, immunocompromised individuals should avoid using the *enviroPRO*TM system.
3. Do not use *enviroPRO*TM system past indicated expiration date.
4. Do not use IS Listeria Broth or Buffered Peptone Water past indicated expiration date.
5. Use rehydrated enrichment medias within 30 days of preparation.
6. Deviations from the assay protocol may impact overall test performance.

MEDIA PREP

ISLB

1. Add 39 grams IS Listeria broth media per 1 Liter dH₂O.
2. Autoclave for 15 minutes at 121°C.
 - a. Media is photosensitive and can be stored at room temperature (20-25°C) in the dark for a maximum of 30 days.
3. Allow media to equilibrate to room temperature (20-25°C) before use.

BPW

1. Add 20 grams BPW media per 1 Liter dH₂O.
2. Autoclave for 15 minutes at 121°C
3. Store media at room temperature for up to 30 days.

SPONGE SURFACE SAMPLING AND ENRICHMENT

1. Squeeze excess moisture from hydrated sponge (e.g. hydrated with Dey-Engley or Hi-cap broths) and remove from incubation bag.
2. Sample surface area of interest using sponge for 15-30 seconds.
3. Transfer sponge back to incubation bag.
4. Media addition:
 - a. For *Salmonella* samples: Transfer 100 mL of BPW into bag containing sponge.
 - b. For *Listeria* samples: Transfer 100 mL of ISLB into bag containing sponge.
5. Seal bag, and briefly (5-10 seconds) massage sponge.
6. Place bag into 35°C ± 2°C incubator.
 - a. Incubate *Listeria* samples: 24-28 hours.
 - b. Incubate *Salmonella* samples: 18-24 hours.

SAMPLE AMPLIFICATION AND ANALYSIS

1. Pipette 500 µL Buffer ACB to a 1.2 mL sample tube (per sample).
2. Remove enrichment bags from incubator and agitate to homogenize sample.
3. Pipette sample to 1.2 mL sample tube.
 - a. FOR MULTIPLEX ANALYSIS: Add 250 µL each from one *Salmonella* enrichment and one *Listeria* enrichment sample.
 - b. FOR SINGLEPLEX ANALYSIS: Add 250 µL to sample tube from desired enrichment bag (either *Listeria* or *Salmonella* enrichment).
 - c. *Note: Singleplex reactions can be run in the Multiplex Master mix, or available Singleplex Master Mix, if desired.*
4. Pipette sample up and down three times to homogenize.
5. Transfer 5 µL of sample to *enviroPRO*TM PCR tube.
 - a. *Note: Open enviroPRO*TM PCR tube only when adding sample and promptly close after to avoid cross contamination between tubes.
6. Place *enviroPRO*TM PCR tube into real-time thermocycler.
7. Initiate program as outlined in the *enviroPRO*TM thermocycler guide, Appendix 1.

APPENDIX 1: REAL-TIME THERMOCYCLER GUIDES

Roche Light Cycler 480 Thermocycler Quick Guide

1. Place *enviroPRO*TM PCR tube into the LC480 II using the LightCycler 8-Tube Strip Adapter Plate.
2. Select “New Experiment from Template” in the LC480 II Overview window and choose the *enviroPRO*TM program.
3. Enter sample ID's into the Sample Editor module, utilizing the Sample Subset module to indicate wells and to discriminate *Salmonella* and *Listeria* samples, if desired.
4. Save the experiment and hit “Start Run” in the Experiment module.
5. Once the run is complete, proceed to the Analysis module. Create a New Analysis by selecting “Abs Quant/2nd Derivative Max” from the list of analysis options provided. If samples are organized as a subset, select the appropriate sample subset from the dropdown menu.
6. Apply color compensation to eliminate signal crosstalk by selecting “In Database” from the Color Comp dropdown menu. Select the *enviroPRO*TM Color Compensation object from the database. Once prompted, ensure that the HEX, ROX and Cy5 channels are selected.
7. Press “Calculate” to obtain the Cp values. Inspect the traces for the characteristic exponential amplification curve shape (see Appendix 3).
8. The sample Cp results table from each channel can be exported as a text file by right clicking on the sample results table. These files can be opened in Microsoft Excel to record, organize and evaluate the data.
9. Perform Step 7 and Step 8 for each channel by toggling to each individual filter using the “Filter Comb” Button.

Bio-Rad CFX96 Thermocycler Quick Guide – Maestro Software

1. Load samples in thermocycler as desired.
2. Using Protocol Editor open saved *enviroPRO*TM program.
3. Using Plate Editor apply the following settings to each sample well:
 - a. Plate Type: BR White
 - b. Scan Mode: All Channels
 - c. Select Fluorophores: Cy5, ROX, HEX (for all samples)
 - d. Highlight desired samples and apply these settings (all samples will need these settings applied):
 - i. Sample type: set as desired
 - ii. Load: Check Cy5, ROX and HEX boxes for each well
 - iii. Optional: Enter sample type and sample name
 - iv. Press OK button to proceed.
4. Using Run Setup Window, initiate run – ensure protocol and plate tabs are programmed correctly, as described above.
5. Analysis Settings:
 - a. Cq Determination Mode: Regression
 - b. Base Line Setting: Choose baseline subtracted curve fit and fluorescence drift correction.
 - c. All other menu items leave under default setting.
6. Any sample with a calculated Cq value should be assigned as positive.