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User Guide
nPLEX Food Total

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EC REP

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1. PRODUCT DESCRIPTION AND INTENDED USE

The nPLEX Food Total kit is a multiplex real time PCR kit intended to avoid the delivery of contaminated food products into the market. This kit detects any of the following bacteria: *Listeria monocytogenes, Salmonella spp., Escherichia coli* and *Staphylococcus aureus*; in a single reaction from enriched food samples.

The nPLEX kit is designed to detect food contaminated with any of the above microorganisms, favoring the detection of the most pathogens bacteria: *Salmonella* and *Listeria monocytogenes*, over the less pathogenic *Escherichia coli* and *Staphylococcus aureus*. In all negative Salmonella samples, the nPLEX kit will detect the other 3 bacteria.

2. KIT CONTENT, STORAGE AND STABILITY

The kit contains 3 bags (32 reactions each). The kit was designed for 96 reactions with a final reaction volume of 20 µl each. Up to 93 samples (single sample preparation) plus both positive and negative control reactions can be analyzed per run.

- Store the kit at -20 °C through the expiration date printed on the label
- Avoid repeated freeze-thaw cycles
- Avoid the direct exposure of the solutions to the light
- Once the kit is opened, store the kit components as described in the table 1:

Table 1. nPLEX Food Total content

Vial	Volumen	Descripción
Master mix 5X	140 µl	• Ready-to-use "hot start" reaction mix for PCR (without primers). It contains Taq DNA Polymerase, reaction buffer, dNTP mix and fluorescent dyes • Store at -20°C • Refreeze max. 2 more times. • Protect from light • Avoid repeated freezing and thawing
Primers and probe	420 µl	Ready-to-use primers and fluorescent dyes for specific DNA amplification of L. monocytogenes, Salmonella spp., S. aureus, E. coli and an artificial plasmid DNA used as PCR internal control • Store at -20°C • Refreeze max. 2 more times. • Protect from light • Avoid repeated freezing and thawing
Negative control	110 µl	• Nuclease-free, PCR-grade water for dilution of reaction mixtures for use as a PCR run negative control. • Store at -20°C.
Internal control	352 µl	• Stabilized solution of plasmid DNA used as a PCR run positive control • Store at -20°C • Refreeze max. 2 more times. • Avoid repeated freezing and thawing
Positive control	110 µl	• Stabilized solution of plasmid DNA used as a PCR run positive control • Store at -20°C • Refreeze max. 2 more times. • Avoid repeated freezing and thawing

3. ADDITIONAL EQUIPMENT AND REAGENTS REQUIRED

- Real-time PCR thermal cycler with a FAM detection channel (OpenQPCR real-time PCR instrument, Chaibio).
- Universal enrichment broth (MultiEBroth Food or MultiEbroth Surface, provided by TAAG Genetics)
- DNA extraction kit from enriched food samples (ZyBa, provided by TAAG Genetics)
- Sterile reaction (Eppendorf) tubes for preparing master mixes and dilutions
- Sterile PCR tubes with optically transparent plastic caps
- Pipettes
- Nuclease-free, aerosol-resistant pipette tips
- Standard benchtop microcentrifuge containing a rotor for 2.0 ml reaction tubes.
- Thermoregulated bath or dry bath.

4. BEFORE YOU BEGIN

In order to achieve reliable results, the entire assay procedure must be performed under nuclease free conditions. Follow the instructions below to avoid nuclease-, carry-over-, or cross-contamination:

- Please don't use if the package is damaged
- Keep the kit components separate from other reagents in the laboratory.
- Use nuclease-free labware (e.g., pipettes, pipette tips, reaction vials).
- Wear gloves when performing the assay.
- To avoid cross-contamination of samples and reagents, use fresh aerosol-preventive pipette tips.
- To avoid carry-over contamination, transfer the required solutions for one experiment into a fresh tube, rather than directly pipetting from stock solutions.
- Physically separate the workplaces for DNA preparation, PCR setup, and PCR to minimize the risk of carry-over contamination. Use a PCR hood for all pipetting steps.
- In order to avoid cross-contamination, pipetting of positive controls should be the last step.
- Keep the nPLEX Food Total kit away from light and moisture.

4.1 Enrichment

The food enrichment must be performed as follows. 25 gr. of food sample is incubated for 24 hrs at 35°C with 225 mL of autoclaved MultiEbroth Food or Surface.

Afterwards, 1 mL of the enriched sample is transferred to a 1,5 mL tube (avoid transferring food debris or oils from the enrichment broth into the tube. Samples that have a fatty supernant, be sure to take the necessary volume below that layer). This sample will be used for DNA extraction and PCR using the nPLEX Food total kit (TAAG Genetics). It is recommended to save another tube with 1 mL of the same enriched sample to be used as counter sample.

4.2 DNA extraction

DNA extraction from enriched food samples must be performed using the ZyBa kit (TAAG Genetics). For more product information please contact TAAG Genetics (www.taag-genetics.com).

5. PROTOCOL

5.1. PCR Program setup

The following procedure was optimized for a real-time PCR instrument with FAM detection channel. Program the PCR instrument before preparing the samples. Use the following real-time PCR protocol for the nPLEX Food Total kit (for details about how to program the PCR protocol, see the Instrument Operator's Manual of your real-time PCR thermo cycler):

Table 2. PCR Program setup

PCR Setup

Step	Temperature	Time	Cycles	Acquisition
Denaturation	95°C	10 min	1	
Denaturation	95°C	30 seg		
Anealing	63°C	30 seg	40	FAM
Extension	68°C	30 seg		

Melting curve step

Step	Temperature	Time	Cycles	Acquisition
Denaturation	95°c	30 seg	1	
Dissociation	72°C 98°C	30 seg 30 seg	1	FAM

5.2. PCR Mix preparation

Before the PCR Mix preparation, the sample DNA must be mixed with the Internal control solution. Transfer 10 µl of sample DNA and add 10 µl of the Internal control solution. Use 4 µl of that solution as template DNA for the PCR reactions.

IMPORTANT: Before using any reagent of the nPLEX Food Total kit, it is necessary to homogenize each tube in the vortex for at least 10 seg, or in their replace, homogenize with micropipette taken approximately 50% of the tube volume, and mixing carefully 10 times for pipetting. This step is a milestone to ensure confident results. The homogenization of each reagent must be done each time the kit will be use.

Away from light, prepare a final PCR mix containing Master Mix 5x and Primers and probe, according to the table 3.
Master mix = 4*n*1.1
Primers and probe= 12*n*1.1
n: reactions number
Table 3. Volume for PCR mix

Reactions	Primers and probe (µL)	Master mix (µL)
1	13.2	4.4
2	26.4	8.8
3	39.6	13.2
4	52.8	17.6
5	66	22
6	79.2	26.4
7	92.4	30.8
8	105.6	35.2
9	118.8	39.6
10	132	44
11	145.2	48.4
12	158.4	52.8
13	171.6	57.2
14	184.4	61.6
15	198	66
16	211.2	70.4

It is important to homogenize the final mix in vortex for 10 seg or in their replace, homogenize with micropipette taking approximately 50% of the tube volume, and mix carefully 10 times by pipetting. After complete the preparation of PCR Mix, use this solution immediately.

Add 16 µL of the PCR Mix solution at each PCR tube and then add 4 µL of each template DNA (DNA sample with Internal control solution).

For control samples, transfer 10 µL of Positive control and 10 µL of the negative control, to two different PCR tubes. Mix each of them with 10 µL of Internal control. Use 4 µL of the solutions as PCR controls.

Finally seal the tube, strips or PCR plates with optically transparent plastic caps (do not write on top of the tube caps, since it may interfere with the fluorescence reading). Bubble formation inside the PCR tube must be prevented. To eliminate possible bubbles, it is recommendable perform a spin at the tubes, strips or PCR plate before placing them in the real time PCR machine.

Transfer the PCR tubes to the real time PCR machine and cycle the samples as described in Table 2.

6. DATA INTERPRETATION

When the PCR run is over, export the PCR data to a CSV file.

Users of real-time PCR instrument, ChaiBio, must proceed as described below:

To export the PCR data, click the menu button at the top left of the page. Select "Export" to download the PCR data. A .zip file will be generated and saved to a default directory. After extracting the ZIP file, you will find the following .csv files:

- amplification
- cq
- temperature_log
- melt_curve_analysis
- melt_curve_data
- temperature_log

Enter the website "https://food-nplex.taag-genetics.com/algoritmos/nplex". Select device used "CHAI-BIO", then select file "melt_curve_data" and nPLEX kit used "Multiplex qPCR L. monocytogenes, Salmonella spp, S. aureus and E. coli". Select "Analize!". The software will indicate the result of the qPCR.

"Grey" result can be product of PCR interference or DNA level below the LOD. To confirm the result, transfer 10mL of the first enrichment to 90 mL of the selective broth for the "grey zone" microorganism. Incubate at 35°C for 24 hours and then repeat the DNA extraction and PCR.

IMPORTANT: To validate the result obtained, the negative control must be indicated as negative by the software and the positive control must indicate the presence of *S. aureus, L. monocytogenes, Salmonella spp* and *E. coli.*

7. OPTIONAL.

Procedures for Salmonella positive samples suspected to be additionally contaminated with other pathogens.

7.1. Salmonella positive samples suspected to be additionally contaminated with *L. monocytogenes*.

In the case of a sample contaminated with Salmonella and additionally with a very low concentration of *L. monocytogenes*, the nPLEX Multiplex qPCR *Listeria monocytogenes*, *Salmonella spp*., *Escherichia coli* and

Staphylococcus aureus kit will deliver a positive result for Salmonella and it could deliver a negative result for *L. monocytogenes*. Although this situation is extremely rare, during the enrichment, the fast-growing Salmonella could limit the growing of *L. monocytogenes*. If you want to confirm the absence of *L. monocytogenes* in a Salmonella positive sample, a second selective enrichment for Listeria should be performed. Transfer 10 mL of the first enrichment and add 90 mL of Listeria Express Enrichment Broth (LEE Broth, Neogen corporation). Incubate at 35°C for 24 hours and then repeat the DNA extraction and PCR using the nPLEX Multiplex qPCR *Listeria monocytogenes*, Salmonella spp., *Escherichia coli* and *Staphylococcus aureus* kit.

7.2. Salmonella positive samples suspected to be additionally contaminated with *E. coli*.

In the case of a sample contaminated with Salmonella and additionally with a very low concentration of *E. coli*, the nPLEX Multiplex qPCR *Listeria monocytogenes*, Salmonella spp., *Escherichia coli* and *Staphylococcus aureus* kit will deliver a positive result for Salmonella and it could deliver a negative result for *E. coli*. If you want to confirm the absence of *E. coli* in a Salmonella positive sample, a second qPCR should be performed. Starting from the same DNA already obtained, run a new qPCR using nPLEX qPCR E. coli (TAAG Genetics).

8. TROUBLESHOOTING

Observation	Possible reason	Recommendation
Negative results in positive controls or Internal control	Pipetting errors or omitted reagents	Check for correct pipetting scheme and reaction setup. Repeat the PCR run
	Insufficient vortex or centrifugation of the reagents	Always vortex all reagent before using them
	Air bubble is trapped in the PCR tube	Always centrifuge PCR tubes before loading the PCR machine
	Outer surface of the PCR tube cap is dirty (e.g., by direct skin contact)	• Always wear gloves when handling the PCR tubes • Do not write on the PCR tube cap
	Inappropriate storage of kit components	Keep the kit components at -20 °C, away from light. Avoid repeated freezing and thawing. Replace positive control tubes for new tubes
No signal in Internal control (PCR inhibition)	Internal control solution was not mixed with sample DNA	Check for correct protocol scheme and be sure of mixing Internal control solution with sample DNA. Repeat the PCR run
	Inhibitory effects of the sample material	• Perform a DNA extraction starting from the counter sample, using a bacterial DNA purification kit with silica columns, resins or magnetic particles • Perform a 1:10 dilution from the extracted DNA to decrease the inhibitor concentration in the reaction. The drawback of diluting the sample is that the actual target may be removed if it is present in low copy number
	Inappropriate storage of kit components	Keep the kit components at -20 °C, away from light. Avoid repeated freezing and thawing. Replace positive control tubes for new tubes
Negative control samples are positive	Carry-over contamination	• Exchange all critical solutions • Repeat the complete experiment with fresh aliquots of all reagents • Always handle samples, kit components and consumables in accordance with commonly accepted practices to prevent carry-over contamination • Add positive controls after sample DNA and negative control tubes have been sealed • Carry out decontamination procedures in the work zone, either through cleaning with sodium hypochlorite and/or with some commercial reagent to eliminate DNA fragments