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Detection of *Brettanomyces/Dekkera bruxellensis* using real time-PCR

VALIDATION REPORT

ASSAY

Brettanomyces/Dekkera bruxellensis detection by 5' nuclease real time-PCR assay.

VALIDATED EQUIPMENT

Applied Biosystems, model 7500; Thermo Scientific, model PikoReal; Condalab, model CDL-96.

SAMPLES (N)

Wine

VALIDATION ASSAYS

Start – 29-12-2014
Finish – 07-04-2015

DEFINITIONS

Analyte

DNA of the microorganism detected by the analysis method

Qualitative method

Analysis method which response is either the presence or absence of the microorganism either directly or indirectly in a certain amount of sample.

True positive (TP)

Sample presenting a positive result for the test and that has equal or higher amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

True negative (TN)

Sample presenting a negative result for the test and that has a lower amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

False Positive (FP)

Sample presenting a positive result for the test in the absence of target analyte.

False Negative (FN)

Sample presenting a negative result for the test in the presence of equal or higher amount of the target analyte than the detection limit.

Detection Limit (LOD)

- Reaction detection limit: lowest concentration of the target DNA that can be consistently detected in the specified experimental conditions.
- Method reaction limit: lowest concentration or content of the target microorganism relative to the defined amount of matrix that can be consistently detected in the specified experimental conditions.

Inclusivity

Detection of the target microorganism.

Exclusivity

Non-detection of non-target microorganisms.

Selectivity

Measure of the inclusivity and exclusivity.

Sensitivity (S)

Measure of the lowest number of analyte cells which can be detected in a single test reaction.

Specificity (Sp)

Ability to exclusivity recognize the target microorganism to be detected and distinguish it from other similar organisms and impurities.

Trueness (T)

Closeness of agreement between the expectation of a test result and the corresponding true value.

Precision (P)

Closeness of agreement between independent tests obtained under defined conditions. It depends only on the distribution of random errors and does not relate to the true value of the test.

Positive predictive value (PPV)

Probability of a positive test be referent to a sample presenting an analyte concentration above.

Negative predictive value (NPV)

Probability of a positive test be referent to a sample presenting an analyte concentration above.

SCOPE OF THE METHOD

The yeast Brettanomyces bruxellensis (teleomorph Dekkera bruxellensis) is a spoilage yeast found in wine and various alcoholic beverages that has been described as the principal agent responsible for the formation of volatile phenols which leads to the characteristic "Brett" taints associated with red wine.
The present method is based on a 5' nuclease real-time PCR reactions and amplify a unique genomic sequence of *Brettanomyces/Dekkera bruxellensis*, aiming the detection of this spoilage organism DNA in wine samples.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a second fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 7 raw wine samples.

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing genomic DNA of the *Brettanomyces/Dekkera bruxellensis*

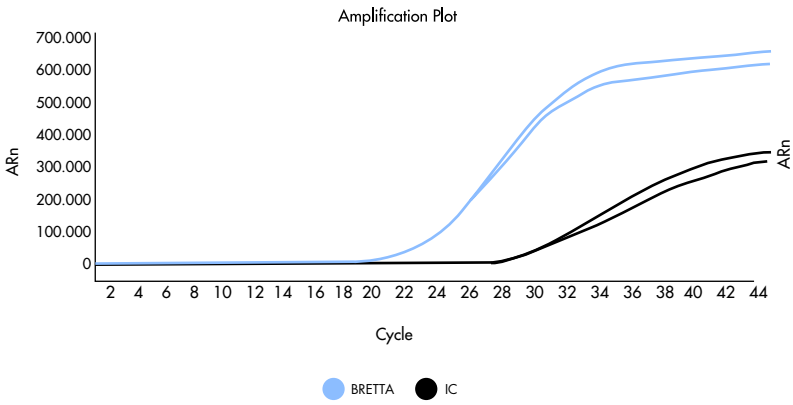
Negative control (NC): reaction without any target DNA
Each individual reaction includes:

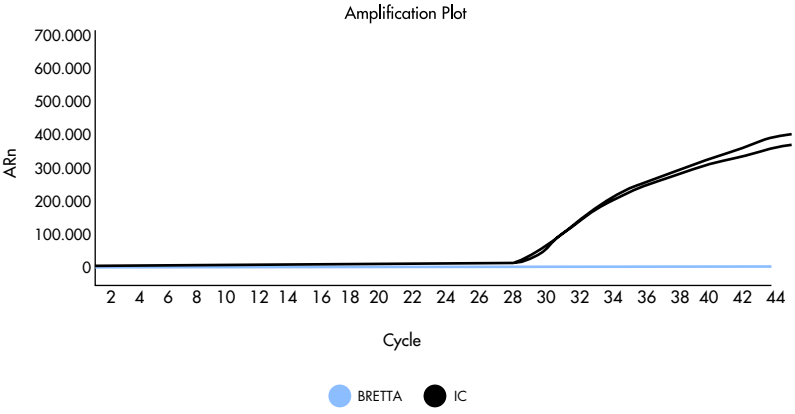
Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

To validate the tests, the controls must show the following results:

	Target detection Chanel 1	Target detection Chanel 2
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

If the controls do not match these results, the experiment must be repeated.





The results obtained by the present method shall be interpreted as follows:

Target detection Chanel 1	Target detection Chanel 2	Interpretation
Positive	Not significant	Positive
Ct = NA	Positive	Negative
Ct = NA	Ct = NA	Inhibition**

When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.

2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).

The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific). Comparable results were obtained with CFX96 (BioRad).

3. SELECTIVITY

3.1. Inclusivity test

A total of 3 Brettanomyces/Dekkera bruxellensis strains were tested (Table 3.1). The strains were tested using 2 ng of genomic DNA.

Code	Strain identification	Other informations
MF 130	*Dekkera bruxellensis*	
MF 131	*Dekkera bruxellensis*	
MF 132	*Dekkera bruxellensis*	

Table 3.1 – List of *Brettanomyces/Dekkera bruxellensis* strains used for inclusivity test

Result:

All strains were detected with the present method, corresponding to 100% inclusivity.

3.2. Exclusivity test

A total of 34 non target microorganisms related with the target or occurring in the same habitat and that could cause interference with test results were de tested (Table 3.2). The strains were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the 16S rRNA bacterial gene, using universal primers. The identification of all strains was confirmed by 16S rRNA gene sequencing. All strains were tested in triplicate.

Code	Strain identification	Other informations
MF 22	*Aspergillus flavus*	
MF 23	*Aspergillus fumigatus*	
MF 24	*Aspergillus fumigatus*	
MF 33	*Fusarium sp*	
MF 39	*Trichophyton tonsurans*	
MF 46	*Sporothrix schenckii*	
MF 49	*Scopulariopsis brevicaulis*	
MF 55	*Candida parapsilosis*	PYCC 2545T
MF 58	*Candida glabrata*	PYCC 2418T
MF 61	*Candida tropicalis*	PYCC 3097T
MF 64	*Candida lusitaneae*	PYCC 2705T
MF 67	*Candida krusei*	
MF 70	*Cryptococcus neoformans*	
MF 71	*Trichosporon sp*	
MF 75	*Trichophyton erinacei*	
MF 86	*Trichophyton verrucosum*	
MF 90	*Arthroderma benhamiae*	
MF 98	*Trichophyton mentagrophytes*	
MF 128	*Saccharomyces cerevisae*	
MF 129	*Aspergillus nigger*	
MF 133	*Dekkera anómala*	
MF 134	*Bretanomyces naardenensis*	
MF 135	*Zygosaccharomyces bailii*	
MF 136	*Zygosaccharomyces bailii*	
MF 137	*Zygosaccharomyces bailii*	
MF 138	*Zygosaccharomyces bailii*	
MF 139	*Zygosaccharomyces sp.*	
MF 140	*Zygosaccharomyces bisporus*	
MF 141	*Zygosaccharomyces lentus*	
MF 142	*Zygosaccharomyces rouxii*	
MF 144	*Lactobacillus paracasei subsp paracasei*	
(not classified)	*Oenococcus oeni*	
(not classified)	*Pichia membranifaciens*	
(not classified)	*Schizosaccharomyces pombe*	

Table 3.2 – List of strains used for exclusivity test

Result:
The method presented 100% specificity as none of the species showed a positive result.

4. SENSITIVITY

4.1. Sensitivity of the Real-Time PCR reaction

The sensitivity of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1 ng, 500 fg, 100 fg, 50 fg, 20 fg and 15 fg.

Result:
Target amplification was observed with DNA amounts of 20 fg or higher. Thus, the limit of detection of the reaction was established in 20 fg of target genomic DNA.

4.2. Sensitivity of the test in DNA mixtures

The sensitivity of the test was determined in raw wine samples artificially contaminated with decreasing number of fungal cells. The sensitivity was tested in 4 raw wine samples. The sample' concentration was performed with 45 mL of each sample, after inoculation with 10^2-10^3 , 10^3-10^4 , 10^4-10^5 and 10^5-10^6 cfu of *Brettanomyces/Dekkera bruxellensis*. All procedures starting from DNA extraction were performed in triplicate.

Result:
Target amplification was observed in samples contaminated with 102-103 cfu/45 mL or more. No amplification was obtained in non-contaminated samples (inoculated with 0 cfu). Thus, the method can detect 102-103 cfu/45 mL of raw wine (LOD).

5. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 5.1, using two positive and two negative samples and performed in triplicate.

Parameter	N° variations	Description
Annealing temperature	2	+ 2°C; - 2°C
MgCl2 concentration	2	+ 0,5mM; - 0,5mM
Independent performer	2	Not applicable
Different Real Time equipment	3	ABI 7500 PikoReal, Thermo Scientific BioRad CFX96
Independent laboratories	2	Biopremier R&D laboratory Portuguese Food Safety Authority laboratory

Table 5.1 – Method's parameters submitted to variations

Result:
Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

6. TRUENESS

Trueness of the method was evaluated using 5 positive and 2 negative raw wine samples for the target. All the samples were tested in triplicate.

Result:
All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%

Performance Characteristics for validation

For the determination of false positive and false negative rate, the method was performed in 7 samples (5 positive and 2 negative samples).

For the determination of method's limit of detection, the sample was inoculated considering 4 contamination levels: 10^2-10^3 , 10^3-104 , 10^4-10^5 and 10^5-10^6 cfu in 45 mL of raw wine sample. All the procedures were performed as defined for the present method. All

experiments starting from DNA extraction were performed in triplicate.

1. FALSE POSITIVE RATE

This corresponds to the probability that a known negative sample has been classified as positive by the method. The false positive rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fpos}=n_{fpos}/n_{rneg}+n_{fpos}$ x 100%

In which:

P_{fpos}: False positive rate

n_{rneg}: Number of real negative test results

n_{fpos}: Number of misclassified known negative samples

Result:

For the present method, false positive rate was calculated based on 21 real samples. The final result was 0%

2. FALSE NEGATIVE RATE

This corresponds to the probability that a known positive sample has been classified as negative by the method. The false negative rate (that can be expressed as a percentage) is calculated by the expression:

P_{fneg}=nfneg/$n_{rneg}+n_{fneg}$ x 100%

In which:

P_{fneg}: False negative rate

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:

For the present method, false positive rate was calculated based on 7 real samples. The final result was 0%

3. LIMIT OF DETECTION

3.1. Limit of detection of the Real-Time PCR reaction

The limit of detection of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1 ng, 500 fg, 100 fg, 50 fg, 20 fg and 15 fg.

Result:

The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 20 fg of Brettanomyces/Dekkera bruxellensis genomic DNA.

3.2. Limit of detection of the method

The limit of detection of the test was determined in food samples artificially contaminated with decreasing number of bacterial cells, following the requirements of ISO 22118, using five different food matrices: minced meat, frozen fish, frozen vegetables, eggs and cheese. The enrichment was performed according to ISO 6579:2012 / ISO 6579:2017, with 25 g of each sample, after inoculation with 0, 1-10, 10-10^2 and 10^2-10^3 cfu of *Brettanomyces/Dekkera bruxellensis*. All procedures starting from DNA extraction were performed in triplicate.

Result:

The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 102-103 cfus *Brettanomyces/Dekkera bruxellensis*.

Positive predictive value (PPV)

The PPV is calculated by the expression:

$PPV=n_{rpos}/n_{rpos}+n_{fpos}$ x 100%

In which:

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:

The positive predictive value determined was 100%.

Negative predictive value (NPV)

The PPV is calculated by the expression:

$NPV=n_{rneg}/n_{rneg}+n_{fneg}$ x 100%
In which:
n_{rneg}: Number of real negative test results
n_{fneg}: Number of misclassified known negative samples

Result:
The negative predictive value determined was 100%.

Criteria for acceptance

Inclusivity: 95%

Exclusivity: 95%

Selectivity: 95%

Trueness: 95%

False positive rate: 5%

False negative rate: 5%

Performance declaration

Accordingly, with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results in agreement with the Condalab requirements and is validated.
The present test allows the "*Detection of Brettanomyces/Dekkera bruxellensis using real time-PCR*" in wine and beverages, cultures and food samples.

Detection of *Campylobacter jejuni* using real-time PCR

VALIDATION REPORT

ASSAY

Campylobacter jejuni detection by 5' nuclease real time-PCR assay.

VALIDATED EQUIPMENT

Applied Biosystems, model 7500; Thermo Scientific, model PikoReal; Condalab, model CDL-96.

SAMPLES (N)

Enrichments prepared from suitable food samples. During re-validation samples from external quality assessment schemes from FAPAS, Instand and INSA-PHE were tested.

VALIDATION ASSAYS

Start – 01-10-2013
Finish – 01-05-2014

DEFINITIONS

Analyte

DNA of the microorganism detected by the analysis method

Qualitative method

Analysis method which response is either the presence or absence of the microorganism either directly or indirectly in a certain amount of sample.

True positive (TP)

Sample presenting a positive result for the test and that has equal or higher amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

True negative (TN)

Sample presenting a negative result for the test and that has a lower amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

False Positive (FP)

Sample presenting a positive result for the test in the absence of target analyte.

False Negative (FN)

Sample presenting a negative result for the test in the presence of equal or higher amount of the target analyte than the detection limit.

Detection Limit (LOD)

- Reaction detection limit: lowest concentration of the target DNA that can be consistently detected in the specified experimental conditions.
- Method reaction limit: lowest concentration or content of the target microorganism relative to the defined amount of matrix that can be consistently detected in the specified experimental conditions.

Inclusivity

Detection of the target microorganism.

Exclusivity

Non-detection of non-target microorganisms.

Selectivity

Measure of the inclusivity and exclusivity.

Sensitivity (S)

Measure of the lowest number of analyte cells which can be detected in a single test reaction.

Specificity (Sp)

Ability to exclusivity recognize the target microorganism to be detected and distinguish it from other similar organisms and impurities.

Trueness (T)

Closeness of agreement between the expectation of a test result and the corresponding true value.

Precision (P)

Closeness of agreement between independent tests obtained under defined conditions. It depends only on the distribution of random errors and does not relate to the true value of the test.

Positive predictive value (PPV)

Probability of a positive test be referent to a sample presenting an analyte concentration above.

Negative predictive value (NPV)

Probability of a positive test be referent to a sample presenting an analyte concentration above.

SCOPE OF THE METHOD

The present method is based on a 5' nuclease real-time PCR reactions and amplify a unique genomic sequence of *Campylobacter jejuni*, aiming the detection of this pathogen DNA in food samples.

The test is designed for the detection of the target microorganism DNA in food products after enrichment in buffered peptone water and DNA extraction. It can also be used with animal feedstuff and environmental samples. Is not suitable for human and clinical diagnostics. The method's performance was tested according ISO 22118:2011 and fulfils the requirements set in this International Standard.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a second fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 22 food samples enriched according the method described in ISO 10272-1:2006. These food samples included 7 different matrices, aleatory taken from commercial shops:

- Cooked meal
- Meat
- Eggs
- Crustaceous
- Herbs mix
- Chicken meat
- Turkey meat

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing genomic DNA of the *Campylobacter jejuni*

Negative control (NC): reaction without any target DNA

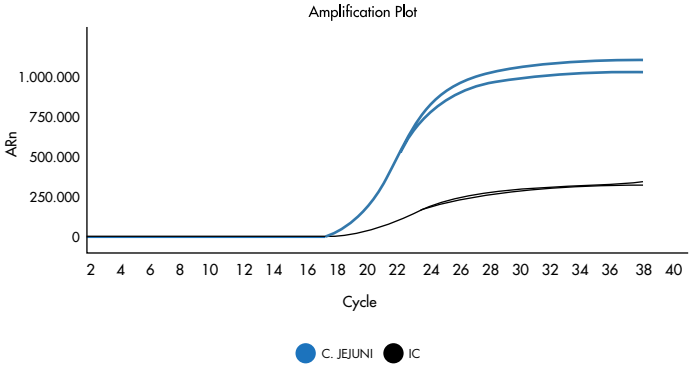
Each individual reaction includes:

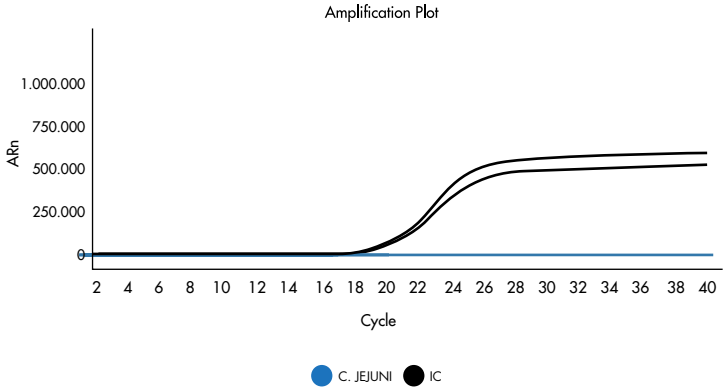
Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

To validate the tests, the controls must show the following results:

	Target detection Chanel 1	Target detection Chanel 2
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

If the controls do not match these results, the experiment must be repeated.





The results obtained by the present method shall be interpreted as follows:

Target detection Chanel 1	Target detection Chanel 2	Interpretation
Positive	Not significant	Positive
Ct = NA	Positive	Negative
Ct = NA	Ct = NA	Inhibition**

When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.

2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).

The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific). Comparable results were obtained with CFX96 (BioRad).

3. SELECTIVITY

3.1. Inclusivity test

A total of 23 *Campylobacter jejuni* strains were tested (Table 3.1). The strains were tested using 2 ng of genomic DNA.

Code	Strain identification	Other informations
MB 454	*Campylobacter jejuni*	
MB 458	*Campylobacter jejuni*	
MB 459	*Campylobacter jejuni*	
MB 460	*Campylobacter jejuni*	
MB 461	*Campylobacter jejuni*	
MB 462	*Campylobacter jejuni*	
MB 463	*Campylobacter jejuni*	
MB 464	*Campylobacter jejuni*	
MB 466	*Campylobacter jejuni*	
MB 467	*Campylobacter jejuni*	
MB 468	*Campylobacter jejuni*	
MB 470	*Campylobacter jejuni*	
MB 471	*Campylobacter jejuni*	
MB 472	*Campylobacter jejuni*	
MB 474	*Campylobacter jejuni*	
MB 476	*Campylobacter jejuni*	
MB 477	*Campylobacter jejuni*	
MB 478	*Campylobacter jejuni*	
MB 479	*Campylobacter jejuni*	
MB 484	*Campylobacter jejuni*	
MB 485	*Campylobacter jejuni*	
MB 359	*Campylobacter jejuni*	
MB 372	*Campylobacter jejuni subsp jejuni*	NCTC 11351

Table 3.1 – List of *Campylobacter jejuni* strains used for inclusivity test

Result:

All strains were detected with the present method, corresponding to 100% inclusivity.

3.2. Exclusivity test

The strains for exclusivity test were chosen following the recommendations of International Standard ISO 22118:2011. A total of 30 non target microorganisms related with the target or occurring in the same habitat and that could cause interference with test results were de tested (Table 3.2). The strains were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the 16S rRNA bacterial gene, using universal primers. The identification of all strains was confirmed by 16S rRNA gene sequencing. All strains were tested in triplicate.

Code	Strain identification	Other informations
MB 385	*Listeria monocytogenes*	CECT 4031
MB 388	*Listeria monocytogenes*	Serotype 1/2a ou 3a
MB 381	*Listeria innocua*	CECT 910
MB 240	*Escherichia coli O157:H7*	CDC 337
MB 357	*Escherichia coli*	NCTC 9001
MB 399	*Salmonella Typhimurium*	CECT 443
MB 373	*Campylobacter coli*	NCTC 11366
MB 22	*Vibrio cholerae*	Local isolate
MB 356	*Vibrio parahaemolyticus*	NCTC 10885
MB 363	*Bacillus cereus*	IFM1600
MB 115	*Citrobacter freundii*	Local isolate
MB 451	*Cronobacter muytjensii*	ATCC 51329
MB 141	*Lactobacillus plantarum*	CECT 748 T
MB 144	*Lactobacillus paracasei subsp paracasei*	CECT 4022T
MB 150	*Staphylococcus aureus*	NCTC 6571
MB 302	*Pseudomonas aeruginosa*	Local isolate
MB 13	*Serratia marcescens*	Local isolate
MB 163	*Proteus vulgaris*	Local isolate
MB 365	*Enterococcus faecalis*	Local isolate
MB 306	*Enterococcus hirae*	Local isolate
MB 147	*Staphylococcus epidermidis*	Local isolate
MB 267	*Shigella flexneri*	Local isolate
MB 249	*Yersinia enterocolitica*	Local isolate
MB 178	*Klebsiella pneumoniae*	Local isolate
MB 449	*Brochothrix thermosphacta*	ATCC 11509
MB 274	*Legionella pneumophila*	Local isolate
MB 278	*Legionella micdadei*	Local isolate
MB 362	*Clostridium perfringens*	Local isolate
MF 128	*Saccharomyces cerevisae*	CECT
MF 129	*Aspergillus niger*	Local isolate

Table 3.2 – List of strains used for exclusivity test

Result:
The method presented 100% exclusivity as none of the strains showed a positive results and positive result.

4. SENSITIVITY

4.1. Sensitivity of the Real-Time PCR reaction

The sensitivity of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 10 ng, 5 ng, 1 ng, 500 pg, 50 pg, 5 pg, 500 fg, 50 fg, 25 fg and 5 fg.

Result:
Target amplification was observed with DNA amounts of 500 fg or higher. Thus, the limit of detection of the reaction was established in 500 fg of target genomic DNA.

4.2. Sensitivity of the test

The sensitivity of the test was determined in food samples artificially contaminated with decreasing number of bacterial cells, following the requirements of ISO 22118. The sensitivity was tested in four different food matrices: chicken meat, turkey meat, eggs and cooked meal. The enrichment was performed according to ISO 10272-1:2006, with 25 g of each sample, after inoculation with 0, 1-10, 10-102 and 102-103 cfu of ampylobacter jejuni (strain NCTC 11351). All procedures starting from DNA extraction were performed in triplicate.

Result:
Target amplification was observed in samples contaminated with 1-10 cfu/25 g or more. No amplification was obtained in non-contaminated samples (inoculated with 0 cfu). Thus, the method can detect 1-10 cfu in 25 g of food sample (LOD).

5. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 5.1, using two positive and two negative samples and performed in triplicate.

Parameter	N° variations	Description
Annealing temperature	2	+ 2°C; - 2°C
MgCl2 concentration	2	+ 0,5mM; - 0,5mM
Independent performer	2	Not applicable
Different Real Time equipment	3	ABI 7500 PikoReal, Thermo Scientific BioRad CFX96
Independent laboratories	2	Biopremier R&D laboratory Portuguese Food Safety Authority laboratory

Table 5.1 – Method's parameters submitted to variations

Result:
Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

6. TRUENESS

Trueness of the method was evaluated using 7 positive and 15 negative food samples for the target, respectively, corresponding to the following food matrices:

- Cooked meal
- Meat
- Eggs
- Crustaceous
- Herbs mix
- Chicken meat
- Turkey meat

All the samples were tested in triplicate.

Result:
All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%

Performance Characteristics for validation

For the determination of false positive and false negative rate, the method was performed in 22 samples (7 positive and 15 negative samples), corresponding to 7 food matrices.
For the determination of method's limit of detection, the sample was inoculated considering 3 contamination levels: 0, 1 to 10 and 10 to 100 cfus in 25 g of sample. All the procedures were performed as defined for the present method. All experiments starting from DNA extraction were performed in triplicate.

1. FALSE POSITIVE RATE

This corresponds to the probability that a known negative sample has been classified as positive by the method. The false positive rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fpos}=n_{fpos}/n_{rneg}+n_{fpos}$ x 100%

In which:

P_{fpos}: False positive rate
n_{rneg}: Number of real negative test results
n_{fpos}: Number of misclassified known negative samples

Result:
For the present method, false positive rate was calculated based on 22 real samples. The final result was 0%

2. FALSE NEGATIVE RATE

This corresponds to the probability that a known positive sample has been classified as negative by the method. The false negative rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fneg}=n_{fneg}/n_{rneg}+n_{fneg}$ x 100%

In which:

P_{fneg}: False negative rate

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:
For the present method, false positive rate was calculated based on 22 real samples. The final result was 0%

3. LIMIT OF DETECTION

3.1. Limit of detection of the Real-Time PCR reaction

The limit of detection of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 10 ng, 5 ng, 1 ng, 500 pg, 50 pg, 5 pg, 500 fg, 50 fg, 25 fg and 5 fg.

Result:
The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 500 fg of Campylobacter jejuni genomic DNA.

3.2. Limit of detection of the method

The limit of detection of the test was determined in food samples artificially contaminated with decreasing number of bacterial cells, following the requirements of ISO 22118, using four different food matrices: chicken meat, turkey meat, eggs and cooked meal. The enrichment was performed according to ISO 10272-1:2006, with 25 g of each sample, after inoculation with 0, 1-10, 10-102 and 102-103 cfu of Campylobacter jejuni. All procedures starting from DNA extraction were performed in triplicate.

Result:
The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 1-10 cfus of *Campylobacter jejuni*.

Positive predictive value (PPV)

The PPV is calculated by the expression:

$PPV=n_{rpos}/n_{rpos}+n_{fpos}$ x 100%

In which:

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:
The positive predictive value determined was 100%.

Negative predictive value (NPV)

The PPV is calculated by the expression:

$NPV=n_{rneg}/n_{rneg}+n_{fneg}$ x 100%

In which:

n_{rneg}: Number of real negative test results

n_{fneg}: Number of misclassified known negative samples

Result:
The negative predictive value determined was 100%.

Criteria for acceptance

Inclusivity: 95%
Exclusivity: 95%
Selectivity: 95%
Trueness: 95%
False positive rate: 5%
False negative rate: 5%

Performance declaration

Accordingly, with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results in agreement with the Condalab requirements and is validated.
The present test allows the "*Detection of Campylobacter jejuni using real time-PCR*" in food samples after enrichment.

Detection of *Celery DNA* using real-time PCR

VALIDATION REPORT

ASSAY

Detection of *Celery DNA*. using real-time PCR

VALIDATED EQUIPMENT

Applied Biosystems, model 7500; Thermo Scientific, model PikoReal; Condalab, model CDL-96.

SAMPLES (N)

Food products and samples from external quality assessment schemes.

VALIDATION ASSAYS

Start – 03-03-2016
Finish – 19-08-2016

DEFINITIONS

Analyte

DNA of the microorganism detected by the analysis method

Qualitative method

Analysis method which response is either the presence or absence of the microorganism either directly or indirectly in a certain amount of sample.

True positive (TP)

Sample presenting a positive result for the test and that has equal or higher amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

True negative (TN)

Sample presenting a negative result for the test and that has a lower amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

False Positive (FP)

Sample presenting a positive result for the test in the absence of target analyte.

False Negative (FN)

Sample presenting a negative result for the test in the presence of equal or higher amount of the target analyte than the detection limit.

Detection Limit (LOD)

- Reaction detection limit: lowest concentration of the target DNA that can be consistently detected in the specified experimental conditions.
- Method reaction limit: lowest concentration or content of the target microorganism relative to the defined amount of matrix that can be consistently detected in the specified experimental conditions.

Inclusivity

Detection of the target species.

Exclusivity

Non-detection of non-target species.

Selectivity

Measure of the inclusivity and exclusivity.

Sensitivity (S)

Measure of the lowest number of analyte cells which can be detected in a single test reaction.

Specificity (Sp)

Ability to exclusivity recognize the target species to be detected and distinguish it from other similar organisms and impurities.

Trueness (T)

Closeness of agreement between the expectation of a test result and the corresponding true value.

Positive predictive value (PPV)

Probability of a positive test to be referent to a sample presenting the DNA of the target species.

Negative predictive value (NPV)

Probability of a positive test to be referent to a sample presenting the DNA of the target species in a concentration above the detection limit.

SCOPE OF THE METHOD

The present method is based on a 5' nuclease real-time PCR reactions and amplify a unique celery genomic sequence aiming the detection of this species DNA in food samples after DNA extraction. It can also be used with other samples.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a second fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 21 food samples obtained from different commercial sources. These food samples included different 19 matrices:

- Cooked pasta with chicken
- Stuffed tomato
- Soy dessert
- Smoked sausage
- Cereal bar
- Juice
- Sesame breadsticks
- Chocolate snacks
- Energy bar
- Chocolate and peanut drops
- Sausage roll
- Soy hamburger
- Tea
- Cooked Pork
- Pizza
- Soup
- Integral biscuits
- Cooked rice
- Dehydrated broth

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing celery DNA

Negative control (NC): reaction without any target DNA

Each individual reaction includes:

Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

To validate the tests, the controls must show the following results:

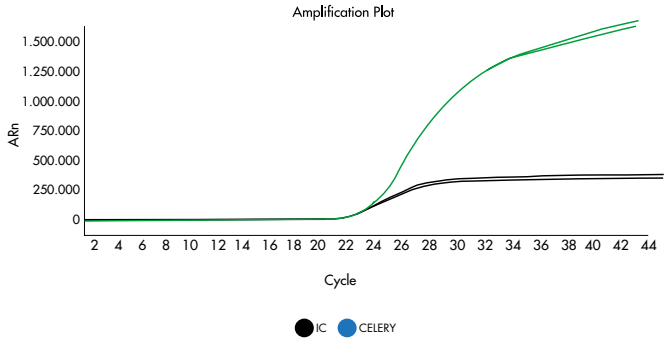
If the controls do not match these results, the experiment must be repeated.

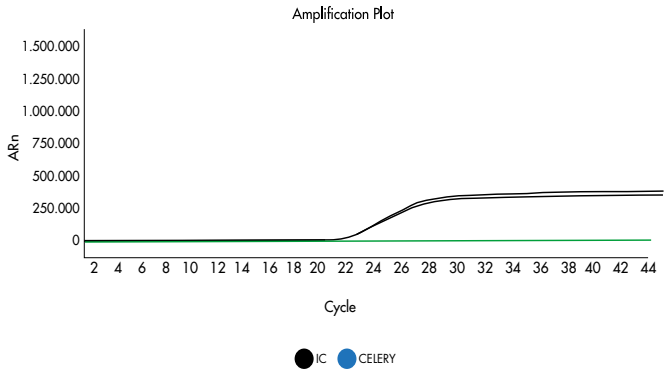
	Target detection Chanel 1	Target detection Chanel 2
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

The results obtained by the present method shall be interpreted as follows:

Target detection Chanel 1	Target detection Chanel 2	Interpretation
Positive	Not significant	Positive
Ct = NA	Positive	Negative
Ct = NA	Ct = NA	Inhibition**

**When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.





2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).

The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific).

3. SPECIFICITY

3.1. Inclusivity test

A total of 51 non target species related with the target or occurring in the food product and that could cause interference with test results were de tested (Table 3.1). The species were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the ITS region, using universal primers. The identification of all species was confirmed by sequencing mitochondrial genes. All DNA extracts were tested in duplicate.

4. SENSITIVITY

4.1. Sensitivity of the Real-Time PCR reaction

The sensitivity of the reaction was determined by testing in duplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg, 1pg,

Code	Species name	Code	Species name
CR1	*Ovis aries*	VR12	Sinapis alba
CR4	*Sus domesticus*	VR2	Prunus dulcis
CR7	*Gallus gallus*	VR3	Arachis hypogaea L.
CR8	*Bos taurus*	VR4	Avena sativa
CR9	*Meleagris gallopavo*	VR5	Corylus avellana, L.
CR13	*Equus equus*	VR6	Lycium barbarum
PR1	*Auxis rochei*	VR7	Anacardium occidentale
PR3	*Sarda sarda/chiliensis*	VR8	Bertholletia excelsa
PR7	*Hymenopenaeus muelleri*	VR9	Secale cereale
PR8	*Litopenaeus vannamei*	VR10	Hordeum vulgare
PR9	*Salmo salar*	VR11	Macadamia ternifolia
PR10	*Gadus morhua*	VR13	Brassica nigra
PR12	*Sepiella japonica*	VR14	Juglans regia L.
PR13	*Merluccius senegalensis*	VR15	Carya illinoensis K.
PR14	*Illex argentinus*	VR16	Curcubita pepo L.
PR15	*Sepia aculeata*	VR17	Pinus pinea
PR16	*Merluccius paradoxus*	VR18	Pistacia vera
PR17	*Meretrix lyrata*	VR19	Sesamum indicum
PR19	*Ensis directus*	VR20	Glycine max
PR21	*Metapenaeus sp. (affinis/ensis)*	VR21	Lupinus albus
PR22	*Fenneropenaeus indicus*	VR22	Triticum aestivum
PR24	*Penaeus japonicus*	VR23	Allium sativum
PR37	*Uroteuthis duvauceli*	VR24	Oryza sativa
PR32	*Cerastoderma edule*	VR25	Solanum tuberosum
PR49	*Argopecten furfuratus*	VR29	Pisum sativum

Table 3.1 – List of species used for exclusivity test

Result:

The method presented 100% specificity as none of the species showed a positive result.

500 fg, and 100fg.

Result:
Target amplification was observed with DNA amounts of 10 pg or higher. Thus, the limit of detection of the reaction was established in 10 pg of target genomic DNA.

4.2. Sensitivity of the test in DNA mixtures

The sensitivity of the test was determined in a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05%, 0,01%, 005% and 0,001%. All determinations were performed duplicate.

Result:
Target amplification was observed in samples containing 0,01% of target DNA. Thus, the method can detect 0,01% of celery DNA in 100 ng of total DNA.

5. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 5.1, using two positive and two negative samples and performed in triplicate.

Parameter	Nº variations	Description
Annealing temperature	2	+ 2ºC; - 2ºC
MgCl2 concentration	2	+ 0,5mM; - 0,5mM
Independent performer		Not applicable
Different Real Time equipment	2	ABI 7500 PikoReal, Thermo Scientific
Independent laboratories	1	Biopremier R&D laboratory

Table 5.1 – Method's parameters submitted to variations

Result:
Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

6. TRUENESS

Trueness of the method was evaluated using 7 positive and 14 negative food samples.

All the samples were tested in triplicate.

Result:
All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%

Performance Characteristics for validation

The determination of false positive and false negative rate, the method was performed in 21 food samples (7 positive and 14 negative samples).
The method's limit of detection was determined in a mixture containing a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05%, 0,01%, 005% and 0,001%. All determinations were performed in duplicate.

1. FALSE POSITIVE RATE

This corresponds to the probability that a known negative sample has been classified as positive by the method. The false positive rate (that can be expressed as a percentage) is calculated by the expression:
$P_{fpos}=n_{fpos}/n_{rneg}+n_{fpos}$ x 100%
In which:
P_{fpos}: False positive rate
n_{rneg}: Number of real negative test results
n_{fpos}: Number of misclassified known negative samples

Result:

For the present method, false positive rate was calculated based on 21 samples. The final result was 0%

2. FALSE NEGATIVE RATE

This corresponds to the probability that a known positive sample has been classified as negative by the method. The false negative rate (that can be expressed as a percentage) is calculated by the expression:

P_{fneg}=nfneg/n_{rneg}+n_{fneg} x 100%

In which:

P_{fneg}: False negative rate

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:

For the present method, false positive rate was calculated based on 21 samples. The final result was 0%

3. LIMIT OF DETECTION

3.1. Limit of detection of the Real-Time PCR reaction

The limit of detection of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg, 1pg, 500 fg, and 100fg.

Result:

The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 10 pg of the target genomic DNA.

3.2. Limit of detection of the method

The method's limit of detection was determined in a mixture containing a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05%, 0,01%, 005% and 0,001%. All determinations were performed in duplicate.

Result:

The limit of detection determined with a probability 0,95% (false positive rate < 0,05) was 0,01% of celery DNA in 100 ng of total DNA.

Positive predictive value (PPV)

The PPV is calculated by the expression:

PPV=n_{rpos}/n_{rpos}+n_{fpos} x 100%

In which:

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:

The positive predictive value determined was 100%.

Negative predictive value (NPV)

The PPV is calculated by the expression:

NPV=n_{rneg}/n_{rneg}+n_{fneg} x 100%

In which:

n_{rneg}: Number of real negative test results

n_{fneg}: Number of misclassified known negative samples

Result:

The negative predictive value determined was 100%.

Criteria for acceptance

Inclusivity: 95%

Exclusivity: 95%

Selectivity: 95%

Trueness: 95%
False positive rate: 5%
False negative rate: 5%

Performance declaration

Accordingly, with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results in agreement with the Condalab requirements and is validated.
The present test allows the "Detection of *Celery* using real-time PCR".

Detection of *Cronobacter* spp. using real-time PCR

VALIDATION REPORT

ASSAY

Cronobacter spp. detection by 5' nuclease real time-PCR assay.

VALIDATED EQUIPMENT

Applied Biosystems, model 7500; Thermo Scientific, model PikoReal; Condalab, model CDL-96.

SAMPLES (N)

Enrichments prepared from suitable food samples. During re-validation samples from external quality assessment schemes from FAPAS, Instand and INSA-PHE were tested.

VALIDATION ASSAYS

Start – 27-05-2015
Finish – 21-08-2015

DEFINITIONS

Analyte

DNA of the microorganism detected by the analysis method

Qualitative method

Analysis method which response is either the presence or absence of the microorganism either directly or indirectly in a certain amount of sample.

True positive (TP)

Sample presenting a positive result for the test and that has equal or higher amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

True negative (TN)

Sample presenting a negative result for the test and that has a lower amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

False Positive (FP)

Sample presenting a positive result for the test in the absence of target analyte.

False Negative (FN)

Sample presenting a negative result for the test in the presence of equal or higher amount of the target analyte than the detection limit.

Detection Limit (LOD)

- Reaction detection limit: lowest concentration of the target DNA that can be consistently detected in the specified experimental conditions.
- Method reaction limit: lowest concentration or content of the target microorganism relative to the defined amount of matrix that can be consistently detected in the specified experimental conditions.

Inclusivity

Detection of the target microorganism.

Exclusivity

Non-detection of non-target microorganisms.

Selectivity

Measure of the inclusivity and exclusivity.

Sensitivity (S)

Measure of the lowest number of analyte cells which can be detected in a single test reaction.

Specificity (Sp)

Ability to exclusivity recognize the target microorganism to be detected and distinguish it from other similar organisms and impurities.

Trueness (T)

Closeness of agreement between the expectation of a test result and the corresponding true value.

Precision (P)

Closeness of agreement between independent tests obtained under defined conditions. It depends only on the distribution of random errors and does not relate to the true value of the test.

Positive predictive value (PPV)

Probability of a positive test be referent to a sample presenting an analyte concentration above.

Negative predictive value (NPV)

Probability of a positive test be referent to a sample presenting an analyte concentration above.

SCOPE OF THE METHOD

The present method is based on a 5' nuclease real-time PCR reactions and amplify a unique genomic sequence of *Cronobacter* spp. , aiming the detection of this pathogen DNA in food samples.
The test is designed for the detection of the target microorganism DNA in food products after enrichment in buffered peptone water and DNA extraction. It can also be used with animal feedstuff and environmental samples. Is not suitable for human and clinical diagnostics. The method's performance was tested according ISO 22118:2011 and ISO 16140:2003 and fulfils the requirements set in this International Standard.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a second fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 10 food samples enriched according the method described in ISO 22964:2006.
These food samples included 4 different matrices, aleatory taken from commercial shops:

· Neonates milk powders
· Powder infant formula
· Cheese
· Milk

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing genomic DNA of the *Cronobacter* spp.

Negative control (NC): reaction without any target DNA

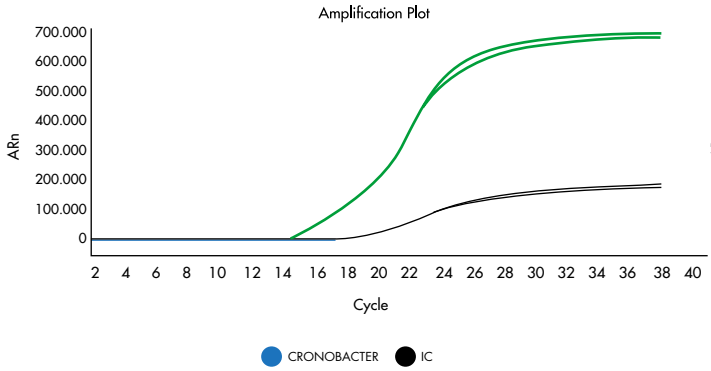
Each individual reaction includes:

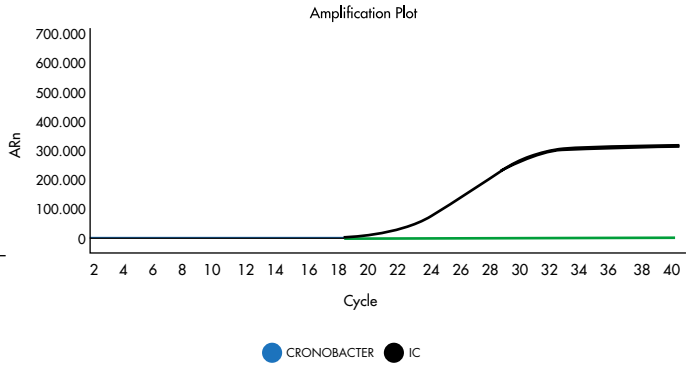
Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

To validate the tests, the controls must show the following results:

	Target detection Chanel 1	**Target detection Chanel 2**
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

If the controls do not match these results, the experiment must be repeated.





The results obtained by the present method shall be interpreted as follows:

Target detection Chanel 1 (*Cronobacter* spp)	**Target detection Chanel 2 (IC)**	**Interpretation**
Positive	Not significant	Positive
Ct = NA	Positive	Negative
Ct = NA	Ct = NA	Inhibition**

**When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.

2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).

The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific). Comparable results were obtained with CFX96 (BioRad).

3. SELECTIVITY

3.1. Inclusivity test

A total of 2 *Cronobacter* spp. strains were tested (Table 3.1). The strains were tested using 2 ng of genomic DNA.

Code	Strain identification	Other informations
MB 450	*Cronobacter sakazakii*	SO670
MB 451	*Cronobacter muytjensii*	ATCC 51329

Table 3.1 – List of *Cronobacter* spp. strains used for inclusivity test

Result:

All strains were detected with the present method, corresponding to 100% inclusivity.

3.2. Exclusivity test

The strains for exclusivity test were chosen following the recommendations of International Standard ISO 22118:2011. A total of 31 non target microorganisms related with the target or occurring in the same habitat and that could cause interference with test results were de tested (Table 3.2). The strains were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the 16S rRNA bacterial gene, using universal primers. The identification of all strains was confirmed by 16S rRNA gene sequencing. All strains were tested in triplicate.

Code	Strain identification	Other informations
MB 372	*Campylobacter jejuni subsp jejuni*	NCTC 11351
MB 164	*Salmonella enteritidis*	Local isolate
MB 381	*Listeria innocua*	CECT 910
MB 240	*Escherichia coli O157:H7*	CDC 337
MB 357	*Escherichia coli*	NCTC 9001
MB 399	*Salmonella Typhimurium*	CECT 443
MB 373	*Campylobacter coli*	NCTC 11366
MB 22	*Vibrio cholerae*	Local isolate
MB 356	*Vibrio parahaemolyticus*	NCTC 10885
MB 223	*Bacillus cereus*	IFM1600
MB 115	*Citrobacter freundii*	Local isolate
MB 141	*Lactobacillus plantarum*	CECT 748 T
MB 144	*Lactobacillus paracasei subsp paracasei*	CECT 4022T
MB 150	*Staphylococcus aureus*	NCTC 6571
MB 302	*Pseudomonas aeruginosa*	Local isolate
MB 13	*Serratia marcescens*	Local isolate
MB 163	*Proteus vulgaris*	Local isolate
MB 365	*Enterococcus faecalis*	Local isolate
MB 306	*Enterococcus hirae*	Local isolate
MB 147	*Staphylococcus epidermidis*	Local isolate
MB 267	*Shigella flexneri*	Local isolate
MB 249	*Yersinia enterocolitica*	Local isolate
MB 178	*Klebsiella pneumoniae*	Local isolate
MB 449	*Brochothrix thermosphacta*	ATCC 11509
MB 274	*Legionella pneumophila*	Local isolate
MB 278	*Legionella micdadei*	Local isolate
MB 607	*E. coli stx2*	Local isolate
MF 128	*Saccharomyces cerevisae*	CECT
MF 129	*Aspergillus niger*	Local isolate
MB 385	*Listeria monocytogenes*	CECT 4031
MB 388	Listeria monocytogenes	Serotype 1/2a ou 3a

Table 3.2 – List of strains used for exclusivity test

Result:
The method presented 100% specificity as none of the species showed a positive result.

4. SENSITIVITY

4.1. Sensitivity of the Real-Time PCR reaction

The sensitivity of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 10 ng, 5 ng, 1 ng, 500 pg, 50 pg, 5 pg, 500 fg, 50 fg, 25 fg and 5 fg.

Result:
Target amplification was observed with DNA amounts of 25 fg or higher. Thus, the limit of detection of the reaction was established in 500 fg of target genomic DNA.

4.2. Sensitivity of the test

The sensitivity of the test was determined in food samples artificially contaminated with decreasing number of bacterial cells, following the requirements of ISO 22118. The sensitivity was tested in five different food matrices: Chicken meat, salad, yogurt, cheese and cottage cheese. The enrichment was performed according to ISO 22964:2006, with 25 g of each sample, after inoculation with 0, 1-10 and $10\text{-}10^2$ cfu of *Cronobacter*. All procedures starting from DNA extraction were performed in triplicate.

Result:
Target amplification was observed in samples contaminated with 1-10 cfu/25 g or more. No amplification was obtained in non-contaminated samples (inoculated with 0 cfu). Thus, the method can detect 1-10 cfu in 25 g of food sample (LOD).

5. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 5.1, using two positive and two negative samples and performed in triplicate.

Parameter	N° variations	Description
Annealing temperature	2	+ 2°C; - 2°C
MgCl2 concentration	2	+ 0,5mM; - 0,5mM
Independent performer	2	Not applicable
Different Real Time equipment	3	ABI 7500 PikoReal, Thermo Scientific BioRad CFX96
Independent laboratories	2	Biopremier R&D laboratory Portuguese Food Safety Authority laboratory

Table 5.1 – Method's parameters submitted to variations

Result:
Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

6. TRUENESS

Trueness of the method was evaluated using 5 positive and 5 negative food samples for the target, respectively, corresponding to the following food matrices:

· Neonates milk powders
· Powder infant formula
· Vegetables
· Cheese
· Milk

All the samples were tested in triplicate.

Result:
All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%

Performance Characteristics for validation

For the determination of false positive and false negative rate, the method was performed in 17 samples (8 positive and 9 negative samples), corresponding to 4 food matrices.

For the determination of method's limit of detection, 5 of the positive samples were inoculated considering 3 contamination levels: 0, 1 to 10 and 10 to 100 cfus in 25 g of sample. All the procedures were performed as defined for the present method. All experiments starting from DNA extraction were performed in triplicate.

1. FALSE POSITIVE RATE

This corresponds to the probability that a known negative sample has been classified as positive by the method. The false positive rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fpos}=n_{fpos}/n_{rneg}+n_{fpos}$ x 100%

In which:

P_{fpos}: False positive rate

n_{rneg}: Number of real negative test results

n_{fpos}: Number of misclassified known negative samples

Result:

For the present method, false positive rate was calculated based on 17 real samples. The final result was 0%

2. FALSE NEGATIVE RATE

This corresponds to the probability that a known positive sample has been classified as negative by the method. The false negative rate (that can be expressed as a percentage) is calculated by the expression:

P_{fneg}=nfneg/$n_{rneg}+n_{fneg}$ x 100%

In which:

P_{fneg}: False negative rate

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:

For the present method, false positive rate was calculated based on 17 real samples. The final result was 0%

3. LIMIT OF DETECTION

3.1. Limit of detection of the Real-Time PCR reaction

The limit of detection of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1 ng, 500 pg, 50 pg, 5 pg, 500 fg, 50 fg, 25 fg and 5 fg.

Result:

The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 25 fg of Cronobacter spp. genomic DNA.

3.2. Limit of detection of the method

The limit of detection of the test was determined in food samples artificially contaminated with decreasing number of bacterial cells, following the requirements of ISO 22118:2011, using five baby food samples. The enrichment was performed according to ISO 22964:2006, with 25 g of each sample, after inoculation with 0, 1-10, 10-10^2 and 102-103 cfu of *Cronobacter* spp. All procedures starting from DNA extraction were performed in triplicate.

Result:

The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 1-10 cfus of *Cronobacter* spp.

Positive predictive value (PPV)

The PPV is calculated by the expression:

$PPV=n_{rpos}/n_{rpos}+n_{fpos}$ x 100%

In which:

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:
The positive predictive value determined was 100%.

Negative predictive value (NPV)
The PPV is calculated by the expression:
$NPV=n_{rneg}/n_{rneg}+n_{fneg} \times 100\%$
In which:
n_{rneg}: Number of real negative test results
n_{fneg}: Number of misclassified known negative samples

Result:
The negative predictive value determined was 100%.

Criteria for acceptance
Inclusivity: 95%
Exclusivity: 95%
Selectivity: 95%
Trueness: 95%
False positive rate: 5%
False negative rate: 5%

Performance declaration
Accordingly, with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results in agreement with the Condalab requirements and is validated.
The present test allows the "Detection of *Cronobacter* spp. using real time-PCR" in food samples after enrichment.

Detection of *Escheria coli* eae, vtx1 and vtx2 using real-time PCR

VALIDATION REPORT

ASSAY

Escherichia coli eae, vtx1 and vtx2 detection by 5' nuclease real time-PCR assay.

VALIDATED EQUIPMENT

Applied Biosystems, model 7500; Thermo Scientific, model PikoReal; Condalab, model CDL-96.

SAMPLES (N)

Enrichments prepared from suitable food samples. During re-validation samples from external quality assessment schemes from FAPAS, Instand and INSA-PHE were tested.

VALIDATION ASSAYS

Start – 01-10-2013
Finish – 01-05-2014

DEFINITIONS

Analyte

DNA of the microorganism detected by the analysis method

Qualitative method

Analysis method which response is either the presence or absence of the microorganism either directly or indirectly in a certain amount of sample.

True positive (TP)

Sample presenting a positive result for the test and that has equal or higher amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

True negative (TN)

Sample presenting a negative result for the test and that has a lower amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

False Positive (FP)

Sample presenting a positive result for the test in the absence of target analyte.

False Negative (FN)

Sample presenting a negative result for the test in the presence of equal or higher amount of the target analyte than the detection limit.

Detection Limit (LOD)

- Reaction detection limit: lowest concentration of the target DNA that can be consistently detected in the specified experimental conditions.
- Method reaction limit: lowest concentration or content of the target microorganism relative to the defined amount of matrix that can be consistently detected in the specified experimental conditions.

Inclusivity

Detection of the target microorganism.

Exclusivity

Non-detection of non-target microorganisms.

Selectivity

Measure of the inclusivity and exclusivity.

Sensitivity (S)

Measure of the lowest number of analyte cells which can be detected in a single test reaction.

Specificity (Sp)

Ability to exclusivity recognize the target microorganism to be detected and distinguish it from other similar organisms and impurities.

Trueness (T)

Closeness of agreement between the expectation of a test result and the corresponding true value.

Precision (P)

Closeness of agreement between independent tests obtained under defined conditions. It depends only on the distribution of random errors and does not relate to the true value of the test.

Positive predictive value (PPV)

Probability of a positive test be referent to a sample presenting an analyte concentration above.

Negative predictive value (NPV)

Probability of a positive test be referent to a sample presenting an analyte concentration above.

SCOPE OF THE METHOD

The present method is based on a 5' nuclease real-time PCR reactions and amplify a unique genomic sequence of the genes eae, vtx1 (verocytoxin) and vtx2 Escherichia coli, aiming the detection of this pathogen DNA in food samples. The test is designed for the detection of the target microorganism DNA in food products after enrichment in buffered peptone water and DNA extraction. It can also be used with animal feedstuff and environmental samples. Is not suitable for human and clinical diagnostics. The method's performance was tested according ISO 22118:2011 and ISO 16140:2003 and fulfils the requirements set in this International Standard.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a second fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 34 food samples enriched according the method described in ISO 16654:2001. These food samples included 5 different matrices, aleatory taken from commercial shops:

- Cheese
- Milk
- Meat
- Processed meat
- Soil

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing genomic DNA of the eae, vtx1 and vtx2 genes of E. coli

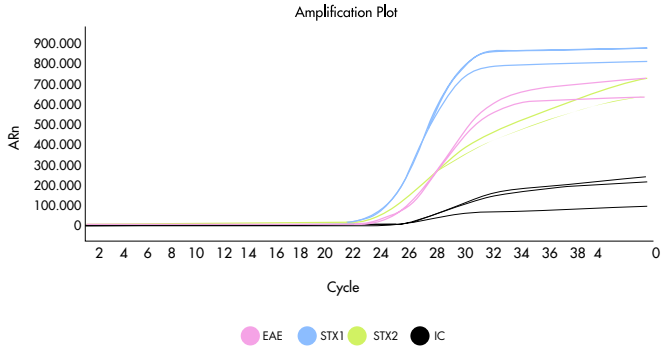
Negative control (NC): reaction without any target DNA
Each individual reaction includes:

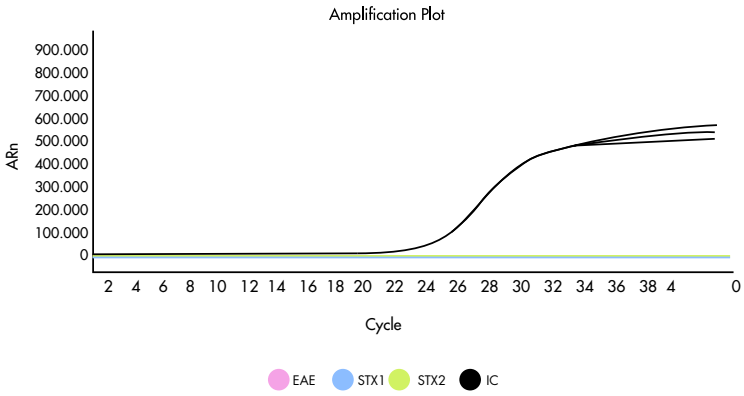
Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

To validate the tests, the controls must show the following results:

	Target detection Chanel 1	**Target detection Chanel 2**
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

If the controls do not match these results, the experiment must be repeated.





The results obtained by the present method shall be interpreted as follows:

***E. coli* detection 026, 0103, 0111, 0145 o 0157 with FAM**	**SIC detection with ROX**	**Interpretation**
Positive	Positive/ negative**	DNA detected, positive sample in *E.coli* serogroup.
Negative	Positive	No DNA detected, sample negative for E.coli serogroup.
Negative	Negative	The result is invalid*.

**When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.

2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).

The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific). Comparable results were obtained with CFX96 (BioRad).

3. SELECTIVITY

3.1. Inclusivity test

For this propose we used pre- enriched positive samples for eae, vtx1 and/or vtx2 genes of Escherichia coli that were identified by an accredited method. A total of 18 pre-enriched samples were tested (Table 3.1).

Code	Sample	Other informations
E 2	*Enriched cheese*	
E 5	*Enriched cheese*	
E 110	*Enriched cheese*	
E 112	*Enriched milk*	
E 214	*Enriched cheese*	
E 217	*Enriched cheese*	
E 218	*Enriched processed meat*	
E 222	*Enriched cheese*	
E 223	*Enriched cheese*	
E 240	*Enriched milk*	
E 430	*Enriched processed meat*	
E 498	*Enriched milk*	
E 500	*Enriched milk*	
E 600	*Enriched soil*	
E 601	*Enriched soil*	
E 644	*Enriched meat*	
E 661	*Enriched meat*	
E 662	*Enriched meat*	

Result:
All strains were detected with the present method, corresponding to 100% inclusivity.

3.2. Exclusivity test

The strains for exclusivity test were chosen following the recommendations of International Standard ISO 22118:2011. A total of 30 non target microorganisms related with the target or occurring in the same habitat and that could cause interference with test results were de tested (Table 3.2). The strains were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the 16S rRNA bacterial gene, using universal primers. The identification of all strains was confirmed by 16S rRNA gene sequencing. All strains were tested in triplicate.

Code	Strain identification	Other informations
MB 385	*Listeria monocytogenes*	CECT 4031
MB 388	*Listeria monocytogenes*	Serotype 1/2a ou 3a
MB 381	*Listeria innocua*	CECT 910
MB 240	*Escherichia coli O157:H7*	CDC 337
MB 357	*Escherichia coli*	NCTC 9001
MB 237	*Escherichia coli*	Local isolate
MB 372	*Campylobacter jejuni subsp jejuni*	NCTC 11351
MB 373	*Campylobacter coli*	NCTC 11366
MB 22	*Vibrio cholerae*	Local isolate
MB 356	*Vibrio parahaemolyticus*	NCTC 10885
MB 363	*Bacillus cereus*	IFM1600
MB 115	*Citrobacter freundii*	Local isolate
MB 451	*Cronobacter muytjensii*	ATCC 51329
MB 141	*Lactobacillus plantarum*	CECT 748 T
MB 144	*Lactobacillus paracasei subsp paracasei*	CECT 4022T
MB 150	*Staphylococcus aureus*	NCTC 6571
MB 302	*Pseudomonas aeruginosa*	Local isolate
MB 13	*Serratia marcescens*	Local isolate
MB 163	*Proteus vulgaris*	Local isolate
MB 365	*Enterococcus faecalis*	Local isolate
MB 306	*Enterococcus hirae*	Local isolate
MB 147	*Staphylococcus epidermidis*	Local isolate
MB 267	*Shigella flexneri*	Local isolate
MB 249	*Yersinia enterocolitica*	Local isolate
MB 178	*Klebsiella pneumoniae*	Local isolate
MB 449	*Brochothrix thermosphacta*	ATCC 11509
MB 164	*Salmonella Enteritidis*	Local isolate
MB 399	*Salmonella Typhimurium*	CECT 443
MF 128	*Saccharomyces cerevisae*	CECT 12928
MF 129	*Aspergillus niger*	Local isolate

Table 3.2 – List of strains used for exclusivity test

Result:
The method presented 100% specificity as none of the species showed a positive result.

4. SENSITIVITY

4.1. Sensitivity of the Real-Time PCR reaction

The sensitivity of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 10 ng, 5 ng, 1 ng, 500 pg, 50 pg, 5 pg, 500 fg, 50 fg, 25 fg and 5 fg.

Result:
Target amplification was observed with DNA amounts of 25 pg or higher for the genes vtx1 and vtx2. For the gene eae was observed with DNA amounts of 2,5 pg or higher. Thus, the limit of detection of the reaction was established in 25 pg of target genomic DNA.

4.2. Sensitivity of the test

The sensitivity of the test was determined in food samples artificially contaminated with decreasing number of bacterial cells, following the requirements of ISO 22118. The sensitivity was tested in four different food matrices: meat, frozen fish, frozen vegetables, eggs and cheese. The enrichment was performed according to ISO 16654:2001, with 25 g of each sample, after inoculation with 0, 1-10,

10-10^2 and 10^2-10^3 cfu of Escherichia coli (strain CDC 337). All procedures starting from DNA extraction were performed in triplicate.

Result:
Target amplification was observed in samples contaminated with 1-10 cfu/25 g or more. No amplification was obtained in non-contaminated samples (inoculated with 0 cfu). Thus, the method can detect 1-10 cfu in 25 g of food sample (LOD).

5. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 5.1, using two positive and two negative samples and performed in triplicate.

Parameter	N° variations	Description
Annealing temperature	2	+ 2°C; - 2°C
MgCl2 concentration	2	+ 0,5mM; - 0,5mM
Independent performer	2	Not applicable
Different Real Time equipment	3	ABI 7500 PikoReal, Thermo Scientific BioRad CFX96
Independent laboratories	2	Biopremier R&D laboratory Portuguese Food Safety Authority laboratory

Table 5.1 – Method's parameters submitted to variations

Result:
Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

6. TRUENESS

Trueness of the method was evaluated using 18 positive and 16 negative food samples for the target, respectively, corresponding to the following food matrices:

- Cheese
- Milk
- Meat
- Processed meat
- Soil

All the samples were tested in triplicate.

Result:
All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%

Performance Characteristics for validation

For the determination of false positive and false negative rate, the method was performed in 34 samples (18 positive and 16 negative samples), corresponding to 5 food matrices.
For the determination of method's limit of detection, the sample was inoculated considering 3 contamination levels: 0, 1 to 10 and 10 to 100 cfus in 25 g of sample. All the procedures were performed as defined for the present method. All experiments starting from DNA extraction were performed in triplicate.

1. FALSE POSITIVE RATE

This corresponds to the probability that a known negative sample has been classified as positive by the method. The false positive rate (that can be expressed as a percentage) is calculated by the expression:
$P_{fpos}=n_{fpos}/n_{rneg}+n_{fpos}$ x 100%
In which:
P_{fpos}: False positive rate
n_{rneg}: Number of real negative test results
n_{fpos}: Number of misclassified known negative samples

Result:
For the present method, false positive rate was calculated based on 10 samples. The final result was 0%

2. FALSE NEGATIVE RATE

This corresponds to the probability that a known positive sample has been classified as negative by the method. The false negative rate (that can be expressed as a percentage) is calculated by the expression:

P_{fneg}=nfneg/n_{rneg}+n_{fneg} x 100%
In which:

P_{fneg}: False negative rate
n_{rpos}: Number of real positive test results
n_{fneg}: Number of misclassified known positive samples

Result:
For the present method, false positive rate was calculated based on 10 samples. The final result was 0%

3. LIMIT OF DETECTION

3.1. Limit of detection of the Real-Time PCR reaction

The limit of detection of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 10 ng, 5 ng, 1 ng, 500 pg, 50 pg, 5 pg, 500 fg, 50 fg, 25 fg and 5 fg.

Result:
The limit of detection determined with a probability 0,95% (false positive rate < 0,05) was 5 fg of the target genomic DNA.

3.2. Limit of detection of the method

The limit of detection of the test was determined in water samples and swabs artificially contaminated with decreasing number of bacterial cells, following the requirements of ISO 22118, using three water samples. For water, the samples' concentration was performed according to ISO 11731:1998, with 1 L of each sample, after inoculation with and 10^2-10^3, 10^3-10^4 10^4-10^5 10^5-10^6 cfu of *Legionella pneumophila* (strain DSM 7513). All procedures starting from DNA extraction were performed in triplicate.

Result:
The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 2.5 pg of the eae gene and 25 pg of the vtx1 and vtx2 genes of *Escherichia coli* genomic DNA.

Positive predictive value (PPV)

The PPV is calculated by the expression:
PPV=n_{rpos}/n_{rpos}+n_{fpos} x 100%
In which:
n_{rpos}: Number of real positive test results
n_{fneg}: Number of misclassified known positive samples

Result:
The positive predictive value determined was 100%.

Negative predictive value (NPV)

The PPV is calculated by the expression:
NPV=n_{rneg}/n_{rneg}+n_{fneg} x 100%
In which:
n_{rneg}: Number of real negative test results
n_{fneg}: Number of misclassified known negative samples

Result:
The negative predictive value determined was 100%.

Criteria for acceptance
Inclusivity: 95%
Exclusivity: 95%
Selectivity: 95%
Trueness: 95%
False positive rate: 5%
False negative rate: 5%

Performance declaration

Accordingly, with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results in agreement with the Condalab requirements and is validated.

The present test allows the "Detection of the eae, vtx1 and vtx2 genes of E. coli spp. using real time-PCR" in food samples after enrichment.

Detection of *Escherichia coli* serogroups DNA using real-time PCR

VALIDATION REPORT

ASSAY

Escherichia coli O157, O26, O111, O103, and O145 detection by 5' nuclease real time-PCR assay.

VALIDATED EQUIPMENT

Applied Biosystems, 7500 model; Thermo Scientific, model PikoReal.

SAMPLES (N)

Enrichments prepared from suitable food samples.

VALIDATION ASSAYS

Start – 01-10-2013
Finish – 01-05-2014

SCOPE OF THE METHOD

The test is designed for the detection the genes associated with the serogroups O157, O111, O26, O103, and O145, based on a 5' nuclease real-time PCR reactions. The method's performance was tested according ISO 22118:2011 and ISO/TS 13136:2012 and fulfils the requirements set in this International Standard.

Conventional serotyping is a method based in antibody-antigen agglutination reactions. These is a very complex and time-consuming method. Recently, rapid and sensitive methods have been found to be attractive alternatives to the *E. coli* conventional serotyping method. These methods are PCR-based methods, in particular real-time-PCR-based, that allows the rapid detection of different antigen genes, which in turn allow the determination of *E. coli* serogroups.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a second fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 5 food samples enriched according the method described in ISO 16654:2001 and 45 *Escherichia coli* strains. After enrichment, STEC colonies suspected were isolated, resuspended in the lysis solution and DNA extracted. These food samples included 5 different matrices, aleatory taken from commercial shops:

- Cheese
- Milk
- Meat
- Processed meat
- Soil

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing mustard DNA

Negative control (NC): reaction without any target DNA

Each individual reaction includes:

Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

To validate the tests, the controls must show the following results:

	Target detection Chanels 1	Target detection Chanel 2
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

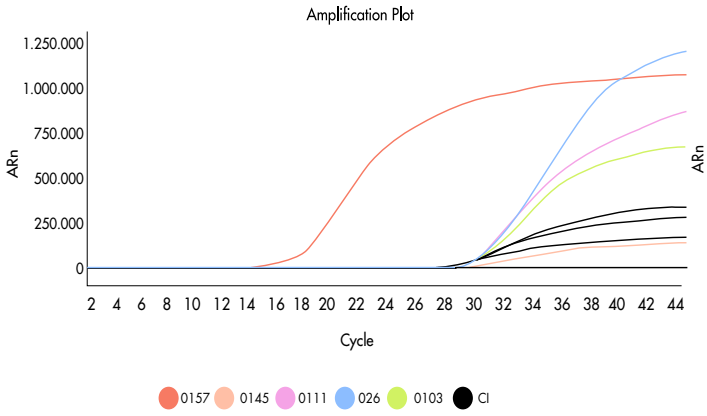
If the controls do not match these results, the experiment must be repeated.

The results obtained by the present method shall be interpreted as follows:

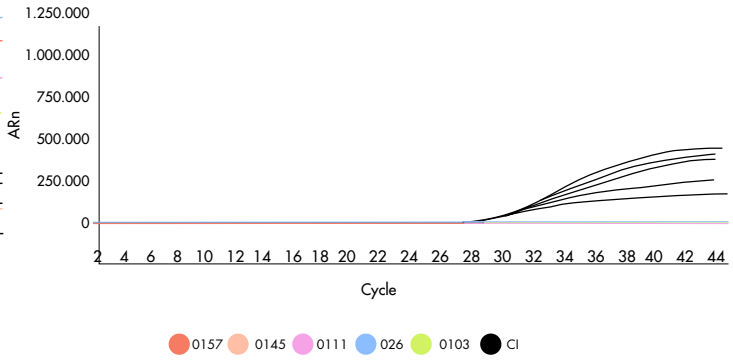
Target detection Chanels 1	Target detection Chanels 2	Interpretation
Positive	Not significant	Positive
Ct = NA	Positive	Negative
Ct = NA	Ct = NA	Inhibition**

**When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.

Positive control



Negative control



2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).

The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific).

3. SPECIFICITY

For this propose we used pre- enriched positive samples for O157, O26, O111, O103, and O145 of Escherichia coli that were identified by an accredited method. A total of 5 pre-enriched samples were tested (Table 3.1).

Code	Strain identification	Other informations
E 2	*Enriched cheese*	
E 112	*Enriched milk*	
E 218	*Enriched processed meat*	
E 600	*Enriched soil*	
E 662	*Enriched meat*	

Table 3.1 – List of Escherichia coli strains used for inclusivity test

Result:

All strains were detected with the present method, corresponding to 100% inclusivity.

3.2. Exclusivity test

The strains for exclusivity test were chosen following the recommendations of International Standard ISO 22118:2011. A total of 30 non target microorganisms related with the target or occurring in the same habitat and that could cause interference with test results were de tested (Table 3.2). The strains were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the 16S rRNA bacterial gene, using universal primers. The identification of all strains was confirmed by 16S rRNA gene sequencing. All strains were tested in triplicate.

Code	Strain identification	Other informations
MB 385	*Listeria monocytogenes*	CECT 4031
MB 388	*Listeria monocytogenes*	Serotype 1/2a ou 3a
MB 381	*Listeria innocua*	CECT 910
MB 357	*Escherichia coli*	NCTC 9001
MB 372	*Campylobacter jejuni subsp jejuni*	NCTC 11351
MB 373	*Campylobacter coli*	NCTC 11366
MB 399	*Salmonella Typhimurium*	CECT 443
MB 22	*Vibrio cholerae*	Local isolate
MB 356	*Vibrio parahaemolyticus*	NCTC 10885
MB 363	*Bacillus cereus*	IFM1600
MB 115	*Citrobacter freundii*	Local isolate
MB 451	*Cronobacter muytjensii*	ATCC 51329
MB 141	*Lactobacillus plantarum*	CECT 748 T
MB 144	*Lactobacillus paracasei subsp paracasei*	CECT 4022T
MB 150	*Staphylococcus aureus*	NCTC 6571
MB 302	*Pseudomonas aeruginosa*	Local isolate
MB 13	*Serratia marcescens*	Local isolate
MB 163	*Proteus vulgaris*	Local isolate
MB 365	*Enterococcus faecalis*	Local isolate
MB 306	*Enterococcus hirae*	Local isolate
MB 147	*Staphylococcus epidermidis*	Local isolate
MB 267	*Shigella flexneri*	Local isolate
MB 249	*Yersinia enterocolitica*	Local isolate
MB 178	*Klebsiella pneumoniae*	Local isolate
MB 449	*Brochothrix thermosphacta*	ATCC 11509
MB 274	*Legionella pneumophila*	Local isolate
MB 278	*Legionella micdadei*	Local isolate
MB 362	*Clostridium perfringens*	Local isolate
MF 128	*Saccharomyces cerevisae*	CECT
MF 129	*Aspergillus niger*	Local isolate

Table 3.2 – List of strains used for exclusivity test

Result:
The method presented 100% exclusivity as none of the strains showed a positive results and positive result.

4. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 4.1, using two positive and two negative samples and performed in triplicate.

Parameter	N° variations	Description
Annealing temperature	2	+ 2°C; - 2°C
MgCl2 concentration	2	+ 0,5mM; - 0,5mM
Independent performer		Not applicable
Different Real Time equipment	2	ABI 7500 PikoReal, Thermo Scientific
Independent laboratories	1	Biopremier R&D laboratory

Table 4.1 – Method's parameters submitted to variations

Result:
Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

5. TRUENESS

Trueness of the method was evaluated using 6 positive and 11 negative food samples. All the samples were tested in triplicate.

Result:
All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%.

Performance declaration

Accordingly with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results in agreement with the Condalab requirements and is validated.

The present test allows the "Detection of *E. coli* serogroups O157, O26, O111, O103, and O145 using real-time PCR".

Detection of Almond DNA using real-time PCR

VALIDATION REPORT

ASSAY

Detection of almond DNA, using real-time PCR

VALIDATED EQUIPMENT

Applied Biosystems, model 7500; Thermo Scientific, model PikoReal; Condalab, model CDL-96.

SAMPLES (N)

Food products and samples from external quality assessment schemes.

VALIDATION ASSAYS

Start – 01-02-2016
Finish – 19-08-2016

DEFINITIONS

Analyte

DNA of the target species detected by the analysis method

Qualitative method

Analysis method which response is either the presence or absence of the target species either directly or indirectly in a certain amount of sample.

True positive (TP)

Sample presenting a positive result for the test and that has equal or higher amount of the target analyte (target species DNA) than the detection limit established in the specified experimental conditions.

True negative (TN)

Sample presenting a negative result for the test and that has a lower amount of the target analyte (target species DNA) than the detection limit established in the specified experimental conditions.

False Positive (FP)

Sample presenting a positive result for the test in the absence of target analyte.

False Negative (FN)

Sample presenting a negative result for the test in the presence of equal or higher amount of the target analyte than the detection limit.

Detection Limit (LOD)

- Reaction detection limit: lowest concentration of the target DNA that can be consistently detected in the specified experimental conditions.
- Method reaction limit: lowest concentration or content of the target microorganism relative to the defined amount of matrix that can be consistently detected in the specified experimental conditions.

Inclusivity

Detection of the target species.

Exclusivity
Non-detection of non-target species.

Selectivity
Measure of the inclusivity and exclusivity.

Sensitivity (S)
Measure of the lowest of analyte DNA concentration which can be detected in a single test reaction.

Specificity (Sp)
Ability to exclusivity recognize the target species to be detected and distinguish it from other similar organisms and impurities.

Trueness (T)
Closeness of agreement between the expectation of a test result and the corresponding true value.

Positive predictive value (PPV)
Probability of a positive test to be referent to a sample presenting the DNA of the target species.

Negative predictive value (NPV)
Probability of a positive test to be referent to a sample presenting the DNA of the target species in a concentration above the detection limit.

SCOPE OF THE METHOD
The present method is based on a 5' nuclease real-time PCR reactions and amplify a unique almond genomic sequence aiming the detection of this species DNA in food samples after DNA extraction. It can also be used with other samples.

SCIENTIFIC BASIS
The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a second fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES
For the validation of the present test were used 21 food samples obtained from different commercial sources. These food samples included 9 different matrices:
- Cereal mix
- Cereal bread
- Energy bar
- Chocolate snacks
- Cookies
- Hazelnut drink
- Sweetened hazelnut chocolate spread
- Breadsticks with sesame
- Passionfruit bar with cashew

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS
Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing almond DNA

Negative control (NC): reaction without any target DNA

Each individual reaction includes:

Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

To validate the tests, the controls must show the following results:

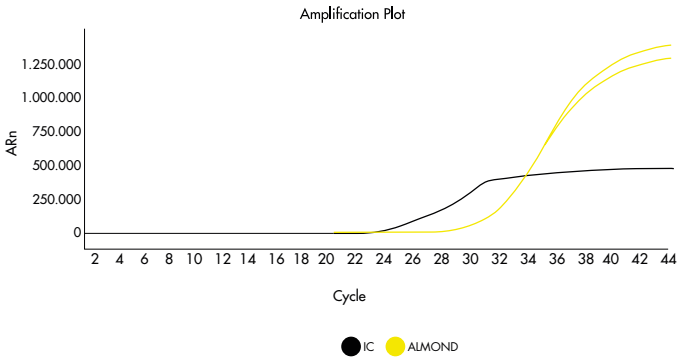
	Target detection Chanel 1	Target detection Chanel 2
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

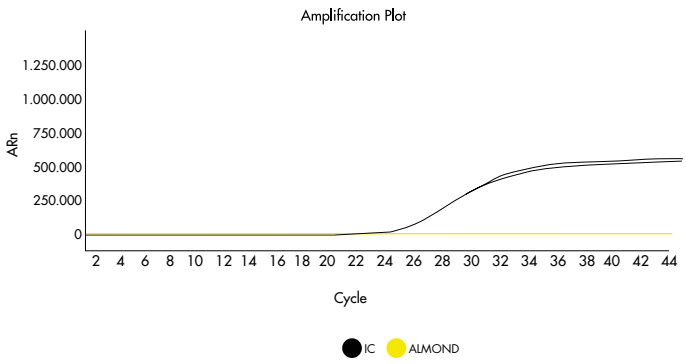
If the controls do not match these results, the experiment must be repeated.

The results obtained by the present method shall be interpreted as follows:

Target detection Chanel 1	Target detection Chanel 2	Interpretation
Positive	Not significant	Positive
Ct = NA	Positive	Negative
Ct = NA	Ct = NA	Inhibition**

When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.





2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).
The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific).

3. SPECIFICITY

A total of 53 non target species related with the target or occurring in the food product and that could cause interference with test results were de tested (Table 3.1). The species were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the ITS region, using universal primers. The identification of all species was confirmed by sequencing mitochondrial genes. All DNA extracts were tested in triplicate.

Code	Species name	Code	Species name
CR1	*Ovis aries*	VR3	*Arachis hypogaea L.*
CR4	*Sus domesticus*	VR4	*Avena sativa*
CR7	*Gallus gallus*	VR5	*Corylus avellana, L.*
CR8	*Bos taurus*	VR6	*Lycium barbarum*
CR9	*Meleagris gallopavo*	VR7	*Anacardium occidentale*
CR13	Equus equus	VR8	*Bertholletia excelsa*
PR1	*Auxis rochei*	VR9	*Secale cereale*
PR3	*Sarda sarda/chiliensis*	VR10	*Hordeum vulgare*

PR7	*Hymenopenaeus muelleri*	VR11	*Macadamia ternifolia*
PR8	*Litopenaeus vannamei*	VR12	*Sinapis alba*
PR9	*Salmo salar*	VR13	*Brassica nigra*
PR10	*Gadus morhua*	VR14	*Juglans regia L.*
PR12	*Sepiella japonica*	VR15	*Carya illinoensis K.*
PR13	*Merluccius senegalensis*	VR16	*Curcubita pepo L.*
PR14	*Illex argentinus*	VR17	*Pinus pinea*
PR15	*Sepia aculeata*	VR18	*Pistacia vera*
PR16	*Merluccius paradoxus*	VR20	*Glycine max*
PR17	*Meretrix lyrata*	VR21	*Lupinus albus*
PR19	*Ensis directus*	VR22	*Triticum aestivum*
PR21	*Metapenaeus sp. (affinis/ensis)*	VR23	*Allium sativum*
PR22	*Fenneropenaeus indicus*	VR24	*Oryza sativa*
PR24	*Penaeus japonicus*	VR25	*Solanum tuberosum*
PR32	*Cerastoderma edule*	VR29	*Pisum sativum*
PR37	*Uroteuthis duvauceli*	VR30	*Vigna unguiculata*
PR49	*Argopecten furfuratus*	VR77	*Prunus pérsica*
VR1	*Apium graveolens*	VR78	*Prunus armeniaca*
		VR83	*Prunus domestica*

Table 3.1 – List of species used for exclusivity test

Result:
The method presented 92% specificity, 5 of the species showed a positive result (VR6, VR7, VR77, VR78 and VR83).

4. SENSITIVITY

4.1. Sensitivity of the Real-Time PCR reaction

The sensitivity of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg, 1pg, 500 fg. 50 fg and 10 fg.

Result:
Target amplification was observed with DNA amounts of 1 pg or higher. Thus, the limit of detection of the reaction was established in 1 pg of target genomic DNA.

4.2. Sensitivity of the test in DNA mixtures

The sensitivity of the test was determined in a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 0,5%, 0,1%, 0,05%, 0,01%, 0,005%, 0,001% and 0,0005%. All determinations were performed in duplicate.

Result:
Target amplification was observed in samples containing 0,001% of target DNA. Thus, the method can detect 0,001% of almond DNA in 100 ng of total DNA.

5. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in

Parameter	N° variations	Description
Annealing temperature	2	+ 2°C; - 2°C
MgCl2 concentration	2	+ 0,2mM; - 0,2mM
Independent performer		Not applicable

Different Real Time equipment	2	ABI 7500 PikoReal, Thermo Scientific
Independent laboratories	1	Biopremier R&D laboratory

Table 5.1 – Method's parameters submitted to variations

Result:
Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

6. TRUENESS

Trueness of the method was evaluated using 6 positive and 15 negative food samples. All the samples were tested in duplicate.

Result:
All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%

PERFORMANCE CHARACTERISTICS FOR VALIDATION

The determination of false positive and false negative rate, the method was performed in 21 food samples (6 positive and 15 negative samples).
The method's limit of detection was determined in a mixture containing a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 0,5%, 0,1%, 0,05%, 0,01%, 0,005%, 0,001% and 0,0005%. All determinations were performed in duplicate.

1. FALSE POSITIVE RATE

This corresponds to the probability that a known negative sample has been classified as positive by the method. The false positive rate (that can be expressed as a percentage) is calculated by the expression:
$P_{fpos}=n_{fpos}/n_{rneg}+n_{fpos} \times 100\%$

In which:
P_{fpos}: False positive rate
n_{rneg}: Number of real negative test results
n_{fpos}: Number of misclassified known negative samples

Result:
For the present method, false positive rate was calculated based on 21 samples. The final result was 0%

2. FALSE NEGATIVE RATE

This corresponds to the probability that a known positive sample has been classified as negative by the method. The false negative rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fneg}=n_{fneg}/n_{rpos}+n_{fneg} \times 100\%$

In which:
P_{fneg}: False negative rate
n_{rpos}: Number of real positive test results
n_{fneg}: Number of misclassified known positive samples

Result:
For the present method, false negative rate was calculated based on 21 samples. The final result was 0%

3. LIMIT OF DETECTION

3.1. Limit of detection of the Real-Time PCR reaction

The limit of detection of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg, 1pg, 500 fg, 100 fg, 50 fg and 10 fg.

Result:
The limit of detection determined with a probability 0,95% (false positive rate < 0,05) was 1 pg of the target genomic DNA.

3.2. Limit of detection of the test in DNA mixtures

The method's limit of detection was determined in a mixture containing a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 0,5%, 0,1%, 0,05%, 0,01%, 0,005%, 0,001% and 0,0005%. All determinations were performed in duplicate.

Result:

The limit of detection determined with a probability 0,95% (false positive rate < 0,05) was 0,001% of almond DNA in 100 ng of total DNA.

Positive predictive value (PPV)

The PPV is calculated by the expression:

$PPV=n_{rpos}/n_{rpos}+n_{fpos} \times 100\%$

In which:

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:

The positive predictive value determined was 100%.

Negative predictive value (NPV)

The PPV is calculated by the expression:

$NPV=n_{rneg}/n_{rneg}+n_{fneg} \times 100\%$

In which:

n_{rneg}: Number of real negative test results

n_{fneg}: Number of misclassified known negative samples

Result:

The negative predictive value determined was 100%.

Criteria for acceptance

Inclusivity: 95%
Exclusivity: 95%
Selectivity: 95%
Trueness: 95%
False positive rate: 5%
False negative rate: 5%

PERFORMANCE DECLARATION

Accordingly with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results, despite the exclusivity value being slightly lower than the Condalab requirements.

The present test allows the "Detection of Almond using real-time PCR".

Detection of Cashew DNA using real-time PCR

VALIDATION REPORT

ASSAY

Detection of cashew DNA, using real-time PCR

VALIDATED EQUIPMENT

Applied Biosystems, 7500 model; Thermo Scientific, model

SAMPLES (N)

Food products and samples from external quality assessment schemes.

VALIDATION ASSAYS

Start – 22-09-2015
Finish – 19-08-2016

DEFINITIONS

Analyte

DNA of the target species detected by the analysis method

Qualitative method

Analysis method which response is either the presence or absence of the target species either directly or indirectly in a certain amount of sample.

True positive (TP)

Sample presenting a positive result for the test and that has equal or higher amount of the target analyte (target species DNA) than the detection limit established in the specified experimental conditions.

True negative (TN)

Sample presenting a negative result for the test and that has a lower amount of the target analyte (target species DNA) than the detection limit established in the specified experimental conditions.

False Positive (FP)

Sample presenting a positive result for the test in the absence of target analyte.

False Negative (FN)

Sample presenting a negative result for the test in the presence of equal or higher amount of the target analyte than the detection limit.

Detection Limit (LOD)

- Reaction detection limit: lowest concentration of the target DNA that can be consistently detected in the specified experimental conditions.
- Method reaction limit: lowest concentration or content of the target microorganism relative to the defined amount of matrix that can be consistently detected in the specified experimental conditions.

Inclusivity

Detection of the target species.

Exclusivity

Non-detection of non-target species.

Selectivity

Measure of the inclusivity and exclusivity.

Sensitivity (S)

Measure of the lowest of analyte DNA concentration which can be detected in a single test reaction.

Specificity (Sp)

Ability to exclusivity recognize the target species to be detected and distinguish it from other similar organisms and impurities.

Trueness (T)

Closeness of agreement between the expectation of a test result and the corresponding true value.

Positive predictive value (PPV)

Probability of a positive test to be referent to a sample presenting the DNA of the target species.

Negative predictive value (NPV)

Probability of a positive test to be referent to a sample presenting the DNA of the target species in a concentration above the detection limit.

SCOPE OF THE METHOD

The present method is based on a 5' nuclease real-time PCR reactions and amplify a unique cashew genomic sequence aiming the detection of this species DNA in food samples after DNA extraction. It can also be used with other samples.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a second fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 19 food samples obtained from different commercial sources. These food samples included 14 different matrices:

- Cereal mix
- Cereal bread
- Soup
- Cooked rice
- Soy schnitzel
- Sausage roll
- Soy hamburger
- Cookies
- Cooked hamburger
- Pizza
- Dehydrated broth
- Cereal bar
- Cashew drink
- Spice dough

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing cashew DNA

Negative control (NC): reaction without any target DNA

Each individual reaction includes:

Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

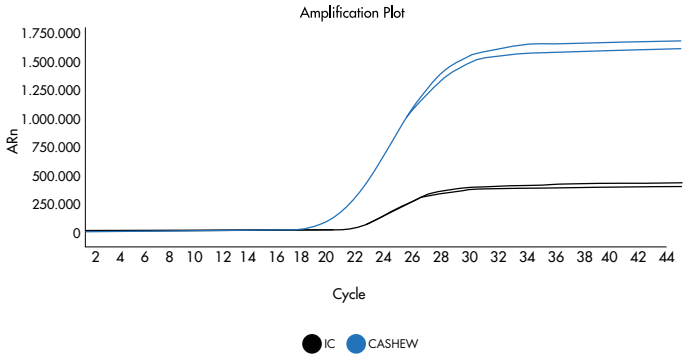
To validate the tests, the controls must show the following results:

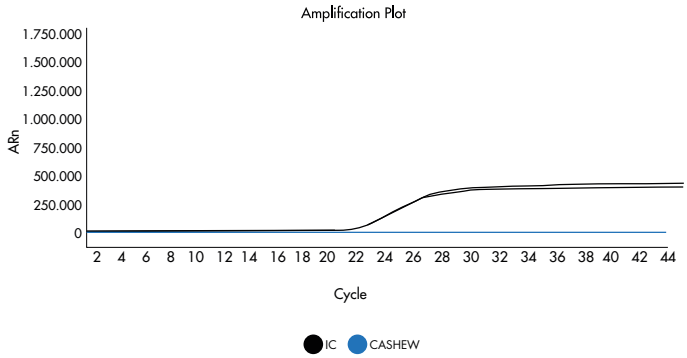
	Target detection Chanel 1	Target detection Chanel 2
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

The results obtained by the present method shall be interpreted as follows:

Target detection Chanel 1	Target detection Chanel 2	Interpretation
Positive	Not significant	Positive
Ct = NA	Positive	Negative
Ct = NA	Ct = NA	Inhibition**

When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.





2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).
The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific).

3. SPECIFICITY

A total of 51 non target species related with the target or occurring in the food product and that could cause interference with test results were de tested (Table 3.1). The species were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the ITS region, using universal primers. The identification of all species was confirmed by sequencing mitochondrial genes. All DNA extracts were tested in triplicate.

Code	Species name	Code	Species name
CR1	*Ovis aries*	VR12	*Sinapis alba*
CR4	*Sus domesticus*	VR2	*Prunus dulcis*
CR7	*Gallus gallus*	VR20	*Glycine max*
CR8	*Bos taurus*	VR4	*Avena sativa*
CR9	*Meleagris gallopavo*	VR3	*Arachis hypogaea L*
CR13	Equus equus	VR6	*Lycium barbarum*
PR1	*Auxis rochei*	VR5	*Corylus avellana, L.*
PR3	*Sarda sarda/chiliensis*	VR8	*Bertholletia excelsa*
PR7	*Hymenopenaeus muelleri*	VR9	*Secale cereale*
PR8	*Litopenaeus vannamei*	VR10	*Hordeum vulgare*
PR9	*Salmo salar*	VR11	*Macadamia ternifolia*
PR10	*Gadus morhua*	VR13	*Brassica nigra*
PR12	*Sepiella japonica*	VR14	*Juglans regia L.*
		VR15	*Carya illinoensis K.*
PR13	*Merluccius senegalensis*	VR16	*Curcubita pepo L.*
PR14	*Illex argentinus*	VR17	*Pinus pinea*
PR15	*Sepia aculeata*	VR18	*Pistacia vera*
PR16	*Merluccius paradoxus*	VR19	*Sesamum indicum*
PR17	*Meretrix lyrata*	VR1	*Apium graveolens*
PR19	*Ensis directus*	VR21	*Lupinus albus*
PR21	*Metapenaeus sp. (affinis/ensis)*	VR22	*Triticum aestivum*
PR22	*Fenneropenaeus indicus*	VR23	*Allium sativum*
PR24	*Penaeus japonicus*	VR24	*Oryza sativa*
PR37	*Uroteuthis duvauceli*	VR25	*Solanum tuberosum*
PR42	*Pandalus montagui*	VR29	*Pisum sativum*
PR49	*Argopecten furfuratus*	VR30	*Vigna unguiculata*

Table 3.1 – List of species used for exclusivity test

Result:
The method presented 100% specificity as none of the species showed a positive result.

4. SENSITIVITY

4.1. Sensitivity of the Real-Time PCR reaction

The sensitivity of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1 ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg, 1 pg, 500 fg, 100 fg, 50fg, 10 fg.

Result:
Target amplification was observed with DNA amounts of 1 pg or higher. Thus, the limit of detection of the reaction was established in 1 pg of target genomic DNA.

4.2. Sensitivity of the test in DNA mixtures

The sensitivity of the test was determined in a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05%, 0,01%, 0,005%, 0,001%, 0,0005%, 0,0001% and 0,00005. All determinations were performed in triplicate

Result:
Target amplification was observed in samples containing 0,001% of target DNA. Thus, the method can detect 0,001% of cashew DNA in 100 ng of total DNA.

5. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 5.1, using two positive and two negative samples and performed in triplicate.

Parameter	N° variations	Description
Annealing temperature	2	+ 2°C; - 2°C
MgCl2 concentration	2	+ 0,5mM; - 0,5mM
Independent performer		Not applicable
Different Real Time equipment	2	ABI 7500 PikoReal, Thermo Scientific
Independent laboratories	1	Biopremier R&D laboratory

Table 5.1 – Method's parameters submitted to variations

Result:

Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

6. TRUENESS

Trueness of the method was evaluated using 5 positive and 14 negative food samples. All the samples were tested in triplicate.

Result:

All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%

PERFORMANCE CHARACTERISTICS FOR VALIDATION

The determination of false positive and false negative rate, the method was performed in 19 food samples (5 positive and 14 negative samples).

The method's limit of detection was determined in a mixture containing a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05%, 0,01%, 0,005%, 0,001%, 0,0005%, 0,0001% and 0,00005. All determinations were performed in triplicate.

1. FALSE POSITIVE RATE

This corresponds to the probability that a known negative sample has been classified as positive by the method. The false positive rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fpos}=n_{fpos}/n_{rneg}+n_{fpos} \times 100\%$

In which:

P_{fpos}: False positive rate

n_{rneg}: Number of real negative test results

n_{fpos}: Number of misclassified known negative samples

Result:

For the present method, false positive rate was calculated based on 19 samples. The final result was 0%

2. FALSE NEGATIVE RATE

This corresponds to the probability that a known positive sample has been classified as negative by the method. The false negative rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fneg}=n_{fneg}/n_{rpos}+n_{fneg} \times 100\%$

In which:

P_{fneg}: False negative rate

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:

For the present method, false negative rate was calculated based on 19 samples. The final result was 0%

3. LIMIT OF DETECTION

3.1. Limit of detection of the Real-Time PCR reaction

The limit of detection of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1 ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg, 1pg, 500 fg, 100 fg, 50fg, 10 fg.

Result:

The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 1 pg of the target genomic DNA.

3.2. Limit of detection of the method

The method's limit of detection was determined in a mixture containing a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05%, 0,01%, 0,005%, 0,001%, 0,0005% and 0,0001%. All determinations were performed in triplicate.

Result:

The limit of detection determined with a probability 0,95% (false positive rate < 0,05) was 0,001% of cashew DNA in 100 ng of total DNA.

Positive predictive value (PPV)

The PPV is calculated by the expression:

$PPV=n_{rpos}/n_{rpos}+n_{fpos} \times 100\%$

In which:

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:

The positive predictive value determined was 100%.

Negative predictive value (NPV)

The PPV is calculated by the expression:

$NPV=n_{rneg}/n_{rneg}+n_{fneg} \times 100\%$

In which:

n_{rneg}: Number of real negative test results

n_{fneg}: Number of misclassified known negative samples

Result:

The negative predictive value determined was 100%.

Criteria for acceptance

Inclusivity: 95%
Exclusivity: 95%
Selectivity: 95%
Trueness: 95%
False positive rate: 5%
False negative rate: 5%

PERFORMANCE DECLARATION

Accordingly with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results in agreement with the Condalab requirements and is validated.

The present test allows the "Detection of Cashew using real-time PCR".

Detection of Hazelnut DNA using real-time PCR

VALIDATION REPORT

ASSAY

Detection of hazelnut DNA, using real-time PCR

VALIDATED EQUIPMENT

Applied Biosystems, model 7500; Thermo Scientific, model PikoReal; Condalab, model CDL-96.

SAMPLES (N)

Food products and samples from external quality assessment schemes.

VALIDATION ASSAYS

Start – 08-09-2015
Finish – 19-08-2016

DEFINITIONS

Analyte

DNA of the target species detected by the analysis method

Qualitative method

Analysis method which response is either the presence or absence of the target species either directly or indirectly in a certain amount of sample.

True positive (TP)

Sample presenting a positive result for the test and that has equal or higher amount of the target analyte (target species DNA) than the detection limit established in the specified experimental conditions.

True negative (TN)

Sample presenting a negative result for the test and that has a lower amount of the target analyte (target species DNA) than the detection limit established in the specified experimental conditions.

False Positive (FP)

Sample presenting a positive result for the test in the absence of target analyte.

False Negative (FN)

Sample presenting a negative result for the test in the presence of equal or higher amount of the target analyte than the detection limit.

Detection Limit (LOD)

- Reaction detection limit: lowest concentration of the target DNA that can be consistently detected in the specified experimental conditions.
- Method reaction limit: lowest concentration or content of the target microorganism relative to the defined amount of matrix that can be consistently detected in the specified experimental conditions.

Inclusivity

Detection of the target species.

Exclusivity

Non-detection of non-target species.

Selectivity

Measure of the inclusivity and exclusivity.

Sensitivity (S)

Measure of the lowest of analyte DNA concentration which can be detected in a single test reaction.

Specificity (Sp)

Ability to exclusivity recognize the target species to be detected and distinguish it from other similar organisms and impurities.

Trueness (T)

Closeness of agreement between the expectation of a test result and the corresponding true value.

Positive predictive value (PPV)

Probability of a positive test to be referent to a sample presenting the DNA of the target species.

Negative predictive value (NPV)

Probability of a positive test to be referent to a sample presenting the DNA of the target species in a concentration above the detection limit.

SCOPE OF THE METHOD

The present method is based on a 5' nuclease real-time PCR reactions and amplify a unique hazelnut genomic sequence aiming the detection of this species DNA in food samples after DNA extraction. It can also be used with other samples.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a second fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 20 food samples obtained from different commercial sources. These food samples included 14 different matrices:

- Cereal mix
- Cereal bread
- Soup
- Cooked rice
- Chocolate snacks
- Soy schnitzel
- Sausage roll
- Soy hamburger
- Cookies
- Cooked hamburger
- Pizza
- Dehydrated broth
- Hazelnut drink
- Sweetened hazelnut chocolate spread

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing peanut DNA

Negative control (NC): reaction containing hazelnut DNA

Each individual reaction includes:

Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.
To validate the tests, the controls must show the following results:

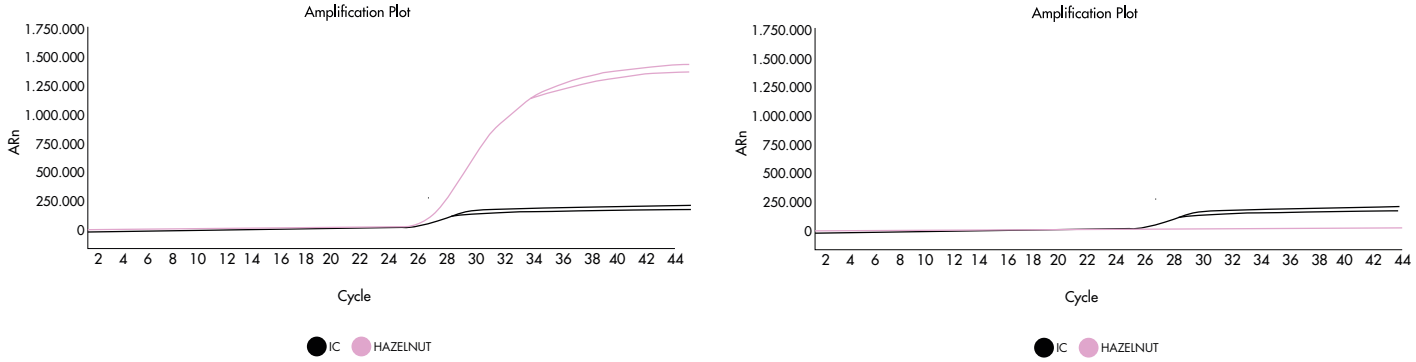
	Target detection Chanel 1	Target detection Chanel 2
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

If the controls do not match these results, the experiment must be repeated.

The results obtained by the present method shall be interpreted as follows:

Target detection Chanel 1	Target detection Chanel 2	Interpretation
Positive	Not significant	Positive
Ct = NA	Positive	Negative
Ct = NA	Ct = NA	Inhibition**

When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.



2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).
The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific).

3. SPECIFICITY

A total of 51 non target species related with the target or occurring in the food product and that could cause interference with test results were de tested (Table 3.1). The species were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the ITS region, using universal primers. The identification of all species was confirmed by sequencing mitochondrial genes. All DNA extracts were tested in triplicate.

Code	Species name	Code	Species name
CR1	*Ovis aries*	VR12	*Sinapis alba*
CR4	*Sus domesticus*	VR2	*Prunus dulcis*
CR7	*Gallus gallus*	VR20	*Glycine max*
CR8	*Bos taurus*	VR4	*Avena sativa*
CR9	*Meleagris gallopavo*	VR3	*Arachis hypogaea L*
CR13	Equus equus	VR6	*Lycium barbarum*
PR1	*Auxis rochei*	VR7	*Anacardium occidentale*
PR3	*Sarda sarda/chiliensis*	VR8	*Bertholletia excelsa*
PR7	*Hymenopenaeus muelleri*	VR9	*Secale cereale*
PR8	*Litopenaeus vannamei*	VR10	*Hordeum vulgare*
PR9	*Salmo salar*	VR11	*Macadamia ternifolia*
PR10	*Gadus morhua*	VR13	*Brassica nigra*
PR12	*Sepiella japonica*	VR14	*Juglans regia L.*
		VR15	*Carya illinoensis K.*
PR13	*Merluccius senegalensis*	VR16	*Curcubita pepo L.*
PR14	*Illex argentinus*	VR17	*Pinus pinea*
PR15	*Sepia aculeata*	VR18	*Pistacia vera*
PR16	*Merluccius paradoxus*	VR19	*Sesamum indicum*
PR17	*Meretrix lyrata*	VR1	*Apium graveolens*
PR19	*Ensis directus*	VR21	*Lupinus albus*
PR21	*Metapenaeus sp. (affinis/ensis)*	VR22	*Triticum aestivum*
PR22	*Fenneropenaeus indicus*	VR23	*Allium sativum*
PR24	*Penaeus japonicus*	VR24	*Oryza sativa*
PR37	*Uroteuthis duvauceli*	VR25	*Solanum tuberosum*
PR42	*Pandalus montagui*	VR29	*Pisum sativum*
PR49	*Argopecten furfuratus*	VR30	*Vigna unguiculata*

Table 3.1 – List of species used for exclusivity test

Result:
The method presented 100% specificity as none of the species showed a positive result.

4. SENSITIVITY

4.1. Sensitivity of the Real-Time PCR reaction

The sensitivity of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1 ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg, 1 pg, 500 fg, 100 fg, 50fg, 10 fg.

Result:
Target amplification was observed with DNA amounts of 100 fg or higher. Thus, the limit of detection of the reaction was established in 100 fg of target genomic DNA.

4.2. Sensitivity of the test in DNA mixtures

The sensitivity of the test was determined in a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05%, 0,01%, 0,005%, 0,001%, 0,0005%, 0,0001% and 0,00005. All determinations were performed in triplicate

Result:
Target amplification was observed in samples containing 0,0001% of target DNA. Thus, the method can detect 0,0001% of hazelnut DNA in 100 ng of total DNA.

5. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 5.1, using two positive and two negative samples and performed in triplicate.

Parameter	Nº variations	Description
Annealing temperature	2	+ 2ºC; - 2ºC
MgCl2 concentration	2	+ 0,5mM; - 0,5mM
Independent performer		Not applicable
Different Real Time equipment	2	ABI 7500 PikoReal, Thermo Scientific
Independent laboratories	1	Biopremier R&D laboratory

Table 5.1 – Method's parameters submitted to variations

Result:

Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

6. TRUENESS

Trueness of the method was evaluated using 5 positive and 15 negative food samples. All the samples were tested in triplicate.

Result:

All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%

PERFORMANCE CHARACTERISTICS FOR VALIDATION

The determination of false positive and false negative rate, the method was performed in 20 food samples (5 positive and 15 negative samples).

The method's limit of detection was determined in a mixture containing a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05%, 0,01%, 0,005%, 0,001%, 0,0005%, 0,0001% and 0,00005. All determinations were performed in triplicate.

1. FALSE POSITIVE RATE

This corresponds to the probability that a known negative sample has been classified as positive by the method. The false positive rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fpos}=n_{fpos}/n_{rneg}+n_{fpos} \times 100\%$

In which:

P_{fpos}: False positive rate

n_{rneg}: Number of real negative test results

n_{fpos}: Number of misclassified known negative samples

Result:

For the present method, false positive rate was calculated based on 20 samples. The final result was 0%

2. FALSE NEGATIVE RATE

This corresponds to the probability that a known positive sample has been classified as negative by the method. The false negative rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fneg}=n_{fneg}/n_{rpos}+n_{fneg} \times 100\%$

In which:

P_{fneg}: False negative rate

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:

For the present method, false negative rate was calculated based on 20 samples. The final result was 0%

3. LIMIT OF DETECTION

3.1. Limit of detection of the Real-Time PCR reaction

The limit of detection of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1 ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg, 1pg, 500 fg, 100 fg, 50fg, 10 fg.

Result:

The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 100 fg of the target genomic DNA.

3.2. Limit of detection of the method

The method's limit of detection was determined in a mixture containing a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05%, 0,01%, 0,005%, 0,001%, 0,0005% and 0,0001%. All determinations were performed in triplicate.

Result:

The limit of detection determined with a probability 0,95% (false positive rate < 0,05) was 0,0001% of hazelnut DNA in 100 ng of total DNA.

Positive predictive value (PPV)

The PPV is calculated by the expression:

$PPV=n_{rpos}/n_{rpos}+n_{fpos} \times 100\%$

In which:

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:

The positive predictive value determined was 100%.

Negative predictive value (NPV)

The PPV is calculated by the expression:

$NPV=n_{rneg}/n_{rneg}+n_{fneg} \times 100\%$

In which:

n_{rneg}: Number of real negative test results

n_{fneg}: Number of misclassified known negative samples

Result:

The negative predictive value determined was 100%.

Criteria for acceptance

Inclusivity: 95%

Exclusivity: 95%

Selectivity: 95%

Trueness: 95%

False positive rate: 5%

False negative rate: 5%

PERFORMANCE DECLARATION

Accordingly with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results in agreement with the Condalab requirements and is validated.

The present test allows the "Detection of Hazelnut using real-time PCR".

Detection of Lupin DNA using real-time PCR

VALIDATION REPORT

ASSAY

Detection of lupin DNA, using real-time PCR

VALIDATED EQUIPMENT

Applied Biosystems, model 7500; Thermo Scientific, model PikoReal; Condalab, model CDL-96.

SAMPLES (N)

Food products and samples from external quality assessment schemes.

VALIDATION ASSAYS

Start – 11-09-2015
Finish – 19-08-2016

DEFINITIONS

Analyte

DNA of the target species detected by the analysis method

Qualitative method

Analysis method which response is either the presence or absence of the target species either directly or indirectly in a certain amount of sample.

True positive (TP)

Sample presenting a positive result for the test and that has equal or higher amount of the target analyte (target species DNA) than the detection limit established in the specified experimental conditions.

True negative (TN)

Sample presenting a negative result for the test and that has a lower amount of the target analyte (target species DNA) than the detection limit established in the specified experimental conditions.

False Positive (FP)

Sample presenting a positive result for the test in the absence of target analyte.

False Negative (FN)

Sample presenting a negative result for the test in the presence of equal or higher amount of the target analyte than the detection limit.

Detection Limit (LOD)

- Reaction detection limit: lowest concentration of the target DNA that can be consistently detected in the specified experimental conditions.
- Method reaction limit: lowest concentration or content of the target microorganism relative to the defined amount of matrix that can be consistently detected in the specified experimental conditions.

Inclusivity

Detection of the target species.

Exclusivity

Non-detection of non-target species.

Selectivity

Measure of the inclusivity and exclusivity.

Sensitivity (S)

Measure of the lowest of analyte DNA concentration which can be detected in a single test reaction.

Specificity (Sp)

Ability to exclusivity recognize the target species to be detected and distinguish it from other similar organisms and impurities.

Trueness (T)

Closeness of agreement between the expectation of a test result and the corresponding true value.

Positive predictive value (PPV)

Probability of a positive test to be referent to a sample presenting the DNA of the target species.

Negative predictive value (NPV)

Probability of a positive test to be referent to a sample presenting the DNA of the target species in a concentration above the detection limit.

SCOPE OF THE METHOD

The present method is based on a 5' nuclease real-time PCR reactions and amplify a unique lupin genomic sequence aiming the detection of this species DNA in food samples after DNA extraction. It can also be used with other samples.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a second fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 20 food samples obtained from different commercial sources. These food samples included 15 different matrices:

- Cereal mix
- Cereal bread
- Soup
- Cooked rice
- Chocolate snacks
- Soy schnitzel
- Sausage roll
- Soy hamburger
- Cookies
- Cooked hamburger
- Pizza
- Dehydrated broth
- Lupin flour
- Cooked meal
- Gluten-free crips rolls

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing lupin DNA

Negative control (NC): reaction without any target DNA

Each individual reaction includes:

Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

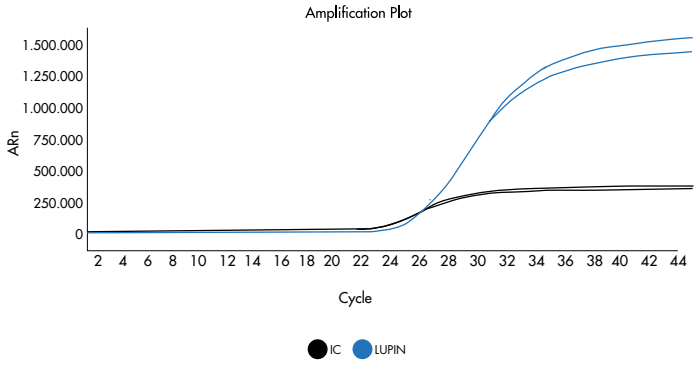
To validate the tests, the controls must show the following results:

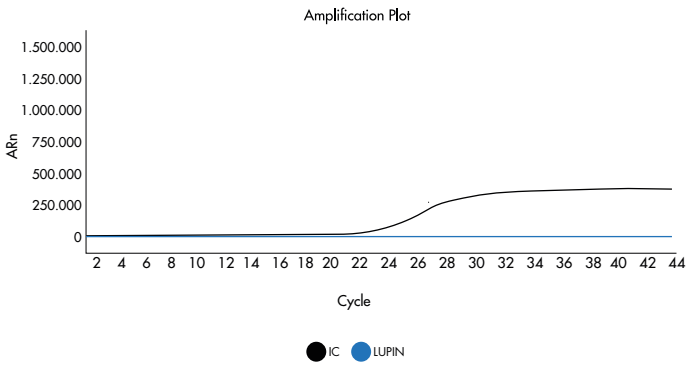
	Target detection Chanel 1	Target detection Chanel 2
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

The results obtained by the present method shall be interpreted as follows:

Target detection Chanel 1	Target detection Chanel 2	Interpretation
Positive	Not significant	Positive
Ct = NA	Positive	Negative
Ct = NA	Ct = NA	Inhibition**

When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.





2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).
The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific).

3. SPECIFICITY

A total of 51 non target species related with the target or occurring in the food product and that could cause interference with test results were de tested (Table 3.1). The species were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the ITS region, using universal primers. The identification of all species was confirmed by sequencing mitochondrial genes. All DNA extracts were tested in triplicate.

Code	Species name	Code	Species name
CR1	*Ovis aries*	VR12	*Sinapis alba*
CR4	*Sus domesticus*	VR2	*Prunus dulcis*
CR7	*Gallus gallus*	VR20	*Glycine max*
CR8	*Bos taurus*	VR4	*Avena sativa*
CR9	*Meleagris gallopavo*	VR3	*Arachis hypogaea L*
CR13	Equus equus	VR6	*Lycium barbarum*
PR1	*Auxis rochei*	VR5	*Corylus avellana, L.*
PR3	*Sarda sarda/chiliensis*	VR8	*Bertholletia excelsa*
PR7	*Hymenopenaeus muelleri*	VR9	*Secale cereale*
PR8	*Litopenaeus vannamei*	VR10	*Hordeum vulgare*
PR9	*Salmo salar*	VR11	*Macadamia ternifolia*
PR10	*Gadus morhua*	VR13	*Brassica nigra*
PR12	*Sepiella japonica*	VR14	*Juglans regia L.*
		VR15	*Carya illinoensis K.*
PR13	*Merluccius senegalensis*	VR16	*Curcubita pepo L.*
PR14	*Illex argentinus*	VR17	*Pinus pinea*
PR15	*Sepia aculeata*	VR18	*Pistacia vera*
PR16	*Merluccius paradoxus*	VR19	*Sesamum indicum*
PR17	*Meretrix lyrata*	VR1	*Apium graveolens*
PR19	*Ensis directus*	VR7	*Anacardium occidentale*
PR21	*Metapenaeus sp. (affinis/ensis)*	VR22	*Triticum aestivum*
PR22	*Fenneropenaeus indicus*	VR23	*Allium sativum*
PR24	*Penaeus japonicus*	VR24	*Oryza sativa*
PR37	*Uroteuthis duvauceli*	VR25	*Solanum tuberosum*
PR42	*Pandalus montagui*	VR29	*Pisum sativum*
PR49	*Argopecten furfuratus*	VR30	*Vigna unguiculata*

Table 3.1 – List of species used for exclusivity test

Result:
The method presented 100% specificity as none of the species showed a positive result.

4. SENSITIVITY

4.1. Sensitivity of the Real-Time PCR reaction

The sensitivity of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1 ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg, 1 pg, 500 fg, 100 fg, 50fg, 10 fg.

Result:
Target amplification was observed with DNA amounts of 500 fg or higher. Thus, the limit of detection of the reaction was established in 500 fg of target genomic DNA.

4.2. Sensitivity of the test in DNA mixtures

The sensitivity of the test was determined in a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05%, 0,01%, 0,005%, 0,001%, 0,0005%, 0,0001% and 0,00005. All determinations were performed in triplicate

Result:
Target amplification was observed in samples containing 0,0005% of target DNA. Thus, the method can detect 0,0005% of lupin DNA in 100 ng of total DNA.

5. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 5.1, using two positive and two negative samples and performed in triplicate.

Parameter	N° variations	Description
Annealing temperature	2	+ 2°C; - 2°C
MgCl2 concentration	2	+ 0,5mM; - 0,5mM
Independent performer		Not applicable
Different Real Time equipment	2	ABI 7500 PikoReal, Thermo Scientific
Independent laboratories	1	Biopremier R&D laboratory

Table 5.1 – Method's parameters submitted to variations

Result:

Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

6. TRUENESS

Trueness of the method was evaluated using 5 positive and 15 negative food samples. All the samples were tested in triplicate.

Result:

All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%

PERFORMANCE CHARACTERISTICS FOR VALIDATION

The determination of false positive and false negative rate, the method was performed in 20 food samples (5 positive and 15 negative samples).

The method's limit of detection was determined in a mixture containing a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05%, 0,01%, 0,005%, 0,001%, 0,0005%, 0,0001% and 0,00005. All determinations were performed in triplicate.

1. FALSE POSITIVE RATE

This corresponds to the probability that a known negative sample has been classified as positive by the method. The false positive rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fpos}=n_{fpos}/n_{rneg}+n_{fpos} \times 100\%$

In which:

P_{fpos}: False positive rate

n_{rneg}: Number of real negative test results

n_{fpos}: Number of misclassified known negative samples

Result:

For the present method, false positive rate was calculated based on 20 samples. The final result was 0%

2. FALSE NEGATIVE RATE

This corresponds to the probability that a known positive sample has been classified as negative by the method. The false negative rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fneg}=n_{fneg}/n_{rpos}+n_{fneg} \times 100\%$

In which:

P_{fneg}: False negative rate

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:

For the present method, false negative rate was calculated based on 20 samples. The final result was 0%

3. LIMIT OF DETECTION

3.1. Limit of detection of the Real-Time PCR reaction

The limit of detection of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1 ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg, 1pg, 500 fg, 100 fg, 50fg, 10 fg.

Result:

The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 500 fg of the target genomic DNA.

3.2. Limit of detection of the method

The method's limit of detection was determined in a mixture containing a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05%, 0,01%, 0,005%, 0,001%, 0,0005% and 0,0001%. All determinations were performed in triplicate.

Result:

The limit of detection determined with a probability 0,95% (false positive rate < 0,05) was 0,0005% of lupin DNA in 100 ng of total DNA.

Positive predictive value (PPV)

The PPV is calculated by the expression:

$PPV=n_{rpos}/n_{rpos}+n_{fpos} \times 100\%$

In which:

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:

The positive predictive value determined was 100%.

Negative predictive value (NPV)

The PPV is calculated by the expression:

$NPV=n_{rneg}/n_{rneg}+n_{fneg} \times 100\%$

In which:

n_{rneg}: Number of real negative test results

n_{fneg}: Number of misclassified known negative samples

Result:

The negative predictive value determined was 100%.

Criteria for acceptance

Inclusivity: 95%
Exclusivity: 95%
Selectivity: 95%
Trueness: 95%
False positive rate: 5%
False negative rate: 5%

PERFORMANCE DECLARATION

Accordingly with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results in agreement with the Condalab requirements and is validated.

The present test allows the "Detection of Lupin using real-time PCR".

Detection of Mustard DNA using real-time PCR

VALIDATION REPORT

ASSAY

Detection of mustard DNA, using real-time PCR

VALIDATED EQUIPMENT

Applied Biosystems, model 7500; Thermo Scientific, model PikoReal; Condalab, model CDL-96.

SAMPLES (N)

Food products and samples from external quality assessment schemes.

VALIDATION ASSAYS

Start – 17-04-2015
Finish – 11-08-2016

DEFINITIONS

Analyte

DNA of the target species detected by the analysis method

Qualitative method

Analysis method which response is either the presence or absence of the target species either directly or indirectly in a certain amount of sample.

True positive (TP)

Sample presenting a positive result for the test and that has equal or higher amount of the target analyte (target species DNA) than the detection limit established in the specified experimental conditions.

True negative (TN)

Sample presenting a negative result for the test and that has a lower amount of the target analyte (target species DNA) than the detection limit established in the specified experimental conditions.

False Positive (FP)

Sample presenting a positive result for the test in the absence of target analyte.

False Negative (FN)

Sample presenting a negative result for the test in the presence of equal or higher amount of the target analyte than the detection limit.

Detection Limit (LOD)

- Reaction detection limit: lowest concentration of the target DNA that can be consistently detected in the specified experimental conditions.
- Method reaction limit: lowest concentration or content of the target microorganism relative to the defined amount of matrix that can be consistently detected in the specified experimental conditions.

Inclusivity

Detection of the target species.

Exclusivity

Non-detection of non-target species.

Selectivity

Measure of the inclusivity and exclusivity.

Sensitivity (S)

Measure of the lowest of analyte DNA concentration which can be detected in a single test reaction.

Specificity (Sp)

Ability to exclusivity recognize the target species to be detected and distinguish it from other similar organisms and impurities.

Trueness (T)

Closeness of agreement between the expectation of a test result and the corresponding true value.

Positive predictive value (PPV)

Probability of a positive test to be referent to a sample presenting the DNA of the target species.

Negative predictive value (NPV)

Probability of a positive test to be referent to a sample presenting the DNA of the target species in a concentration above the detection limit.

SCOPE OF THE METHOD

The present method is based on a 5' nuclease real-time PCR reactions and amplify a unique mustard genomic sequence aiming the detection of this species DNA in food samples after DNA extraction. It can also be used with other samples.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a second fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 17 food samples obtained from different commercial sources. These food samples included 15 different matrices:

- Soy schnitzel
- Soy hamburger
- Dehydrated broth
- Cooked meat hamburger
- Stuffed tomato
- Swab
- Cereal mix
- Soup
- Energy bar
- Cooked rice
- Pizza
- Cereal bar
- Cooked pasta with chicken
- Shrimp patty
- Smoked sausage

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing mustard DNA

Negative control (NC): reaction without any target DNA

Each individual reaction includes:

Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

To validate the tests, the controls must show the following results:

	Target detection Chanel 1	Target detection Chanel 2
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

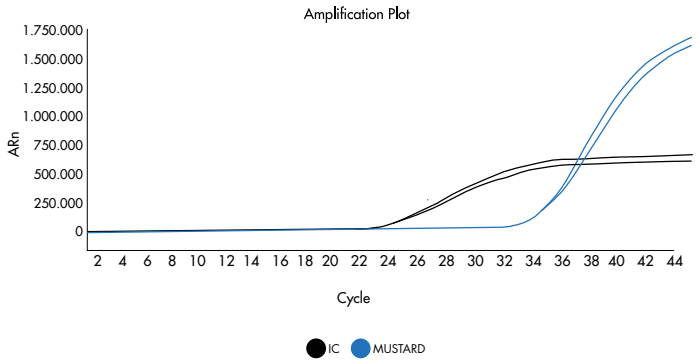
If the controls do not match these results, the experiment must be repeated.

The results obtained by the present method shall be interpreted as follows:

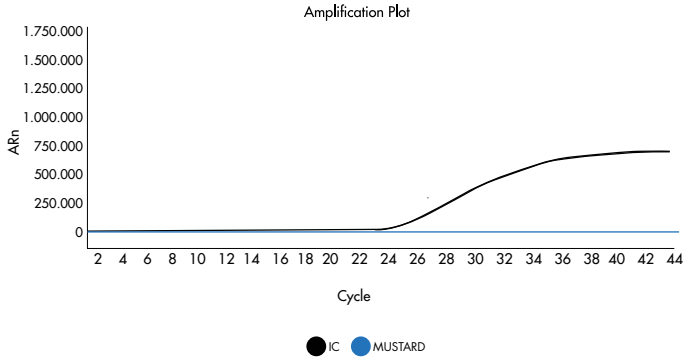
Target detection Chanel 1	Target detection Chanel 2	Interpretation
Positive	Not significant	Positive
Ct = NA	Positive	Negative
Ct = NA	Ct = NA	Inhibition**

When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.

Positive control



Negative control



2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).
The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific).

3. SPECIFICITY

A total of 51 non target species related with the target or occurring in the food product and that could cause interference with test results were de tested (Table 3.1). The species were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the ITS region, using universal primers. The identification of all species was confirmed by sequencing mitochondrial genes. All DNA extracts were tested in triplicate.

Code	Species name	Code	Species name
CR1	*Ovis aries*	VR1	*Apium graveolens*
CR4	*Sus domesticus*	VR2	*Prunus dulcis*
CR7	*Gallus gallus*	VR3	*Arachis hypogaea L.*
CR8	*Bos taurus*	VR4	*Avena sativa*
CR9	*Meleagris gallopavo*	VR5	*Corylus avellana, L.*
CR13	Equus equus	VR6	*Lycium barbarum*
PR1	*Auxis rochei*	VR7	*Anacardium occidentale*
PR3	*Sarda sarda/chiliensis*	VR8	*Bertholletia excelsa*
PR7	*Hymenopenaues muelleri*	VR9	*Secale cereale*
PR8	*Litopenaeus vannamei*	VR10	*Hordeum vulgare*
PR9	*Salmo salar*	VR11	*Macadamia ternifolia*
PR10	*Gadus morhua*	VR13	*Brassica nigra*
PR12	*Sepiella japonica*	VR14	*Juglans regia L.*
PR13	*Merluccius senegalensis*	VR15	*Carya illinoensis K.*
PR14	*Illex argentinus*	VR16	*Cucurbita pepo L.*
PR15	*Sepia aculeata*	VR17	*Pinus pinea*
PR16	*Merluccius paradoxus*	VR18	*Pistacia vera*
PR17	*Meretrix lyrata*	VR19	*Sesamum indicum*
PR19	*Ensis directus*	VR20	*Glycine max*
PR21	*Metapenaeus sp. (affinis/ensis)*	VR21	*Lupinus albus*
PR22	*Fenneropenaeus indicus*	VR22	*Triticum aestivum*
PR24	*Penaeus japonicus*	VR23	*Allium sativum*
PR37	*Uroteuthis duvauceli*	VR24	*Oryza sativa*
PR42	*Pandalus montagui*	VR25	*Solanum tuberosum*
PR49	*Argopecten furfuratus*	VR29	*Pisum sativum*
		VR30	*Vigna unguiculata*

Table 3.1 – List of species used for exclusivity test

Result:
The method presented 100% specificity as none of the species showed a positive result.

4. SENSITIVITY

4.1. Sensitivity of the Real-Time PCR reaction

The sensitivity of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg.

Result:
Target amplification was observed with DNA amounts of 10 pg or higher. Thus, the limit of detection of the reaction was established in 10 pg of target genomic DNA.

4.2. Sensitivity of the test in DNA mixtures

The sensitivity of the test was determined in a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05% and 0,01%. All determinations were performed in triplicate.

Result:
Target amplification was observed in samples containing 0,01% of target DNA. Thus, the method can detect 0,01% of mustard DNA in 100 ng of total DNA.

5. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 5.1, using two positive and two negative samples and performed in triplicate.

Parameter	N° variations	Description
Annealing temperature	2	+ 2°C; - 2°C
MgCl2 concentration	2	+ 0,5mM; - 0,5mM
Independent performer		Not applicable
Different Real Time equipment	2	ABI 7500 PikoReal, Thermo Scientific
Independent laboratories	1	Biopremier R&D laboratory

Table 5.1 – Method's parameters submitted to variations

Result:

Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

6. TRUENESS

Trueness of the method was evaluated using 6 positive and 11 negative food samples. All the samples were tested in triplicate.

Result:

All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%

PERFORMANCE CHARACTERISTICS FOR VALIDATION

The determination of false positive and false negative rate, the method was performed in 17 food samples (6 positive and 11 negative samples).

The method's limit of detection was determined in a mixture containing a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05% and 0,01%. All determinations were performed in triplicate.

1. FALSE POSITIVE RATE

This corresponds to the probability that a known negative sample has been classified as positive by the method. The false positive rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fpos}=n_{fpos}/n_{rneg}+n_{fpos} \times 100\%$

In which:

P_{fpos}: False positive rate

n_{rneg}: Number of real negative test results

n_{fpos}: Number of misclassified known negative samples

Result:

For the present method, false positive rate was calculated based on 17 samples. The final result was 0%

2. FALSE NEGATIVE RATE

This corresponds to the probability that a known positive sample has been classified as negative by the method. The false negative rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fneg}=n_{fneg}/n_{rpos}+n_{fneg} \times 100\%$

In which:

P_{fneg}: False negative rate

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:

For the present method, false negative rate was calculated based on 17 samples. The final result was 0%%

3. LIMIT OF DETECTION

3.1. Limit of detection of the Real-Time PCR reaction

The limit of detection of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg.

Result:

The limit of detection determined with a probability 0,95% (false positive rate < 0,05) was 10 pg of the target genomic DNA.

3.2. Limit of detection of the test in DNA mixtures

The method's limit of detection was determined in a mixture containing a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05% and 0,01%. All determinations were performed in triplicate.

Result:

The limit of detection determined with a probability 0,95% (false positive rate < 0,05) was 0,01% of mustard DNA in 100 ng of total DNA.

Positive predictive value (PPV)

The PPV is calculated by the expression:

$PPV=n_{rpos}/n_{rpos}+n_{fpos} \times 100\%$

In which:

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:

The positive predictive value determined was 100%.

Negative predictive value (NPV)

The PPV is calculated by the expression:

$NPV=n_{rneg}/n_{rneg}+n_{fneg} \times 100\%$

In which:

n_{rneg}: Number of real negative test results

n_{fneg}: Number of misclassified known negative samples

Result:

The negative predictive value determined was 100%.

Criteria for acceptance

Inclusivity: 95%
Exclusivity: 95%
Selectivity: 95%
Trueness: 95%
False positive rate: 5%
False negative rate: 5%

PERFORMANCE DECLARATION

Accordingly with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results in agreement with the Condalab requirements and is validated.

The present test allows the "Detection of Mustard using real-time PCR".

Detection of Peanut DNA using real-time PCR

VALIDATION REPORT

ASSAY

Detection of peanut DNA, using real-time PCR

VALIDATED EQUIPMENT

Applied Biosystems, model 7500; Thermo Scientific, model PikoReal; Condalab, model CDL-96.

SAMPLES (N)

Food products and samples from external quality assessment schemes.

VALIDATION ASSAYS

Start – 07-10-2015
Finish – 10-08-2016

DEFINITIONS

Analyte

DNA of the target species detected by the analysis method

Qualitative method

Analysis method which response is either the presence or absence of the target species either directly or indirectly in a certain amount of sample.

True positive (TP)

Sample presenting a positive result for the test and that has equal or higher amount of the target analyte (target species DNA) than the detection limit established in the specified experimental conditions.

True negative (TN)

Sample presenting a negative result for the test and that has a lower amount of the target analyte (target species DNA) than the detection limit established in the specified experimental conditions. .

False Positive (FP)

Sample presenting a positive result for the test in the absence of target analyte.

False Negative (FN)

Sample presenting a negative result for the test in the presence of equal or higher amount of the target analyte than the detection limit.

Detection Limit (LOD)

- Reaction detection limit: lowest concentration of the target DNA that can be consistently detected in the specified experimental conditions.
- Method reaction limit: lowest concentration or content of the target microorganism relative to the defined amount of matrix that can be consistently detected in the specified experimental conditions.

Inclusivity

Detection of the target species.

Exclusivity

Non-detection of non-target species.

Selectivity

Measure of the inclusivity and exclusivity.

Sensitivity (S)

Measure of the lowest of analyte DNA concentration which can be detected in a single test reaction.

Specificity (Sp)

Ability to exclusivity recognize the target species to be detected and distinguish it from other similar organisms and impurities.

Trueness (T)

Closeness of agreement between the expectation of a test result and the corresponding true value.

Positive predictive value (PPV)

Probability of a positive test to be referent to a sample presenting the DNA of the target species.

Negative predictive value (NPV)

Probability of a positive test to be referent to a sample presenting the DNA of the target species in a concentration above the detection limit.

SCOPE OF THE METHOD

The present method is based on a 5' nuclease real-time PCR reactions and amplify a unique peanut genomic sequence aiming the detection of this species DNA in food samples after DNA extraction. It can also be used with other samples.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a second fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 22 food samples obtained from different commercial sources. These food samples included 17 different matrices:

- Cereal bar
- Energy bar
- Chocolate snacks
- Nougat
- Cereal mix
- Cereal bread
- Soup
- Juice
- Cooked rice
- Soy schnitzel
- Sausage roll
- Soy dessert
- Soy hamburger
- Dehydrated broth
- Cookies
- Sesame breadstick
- Instant coffee

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing peanut DNA

Negative control (NC): eaction without any target DNA
Each individual reaction includes:

Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.
To validate the tests, the controls must show the following results:

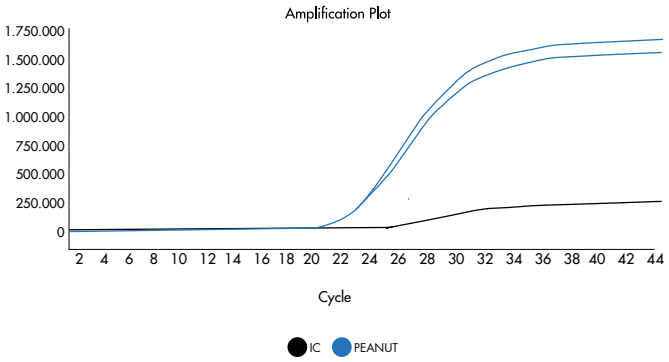
	Target detection Chanel 1	Target detection Chanel 2
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

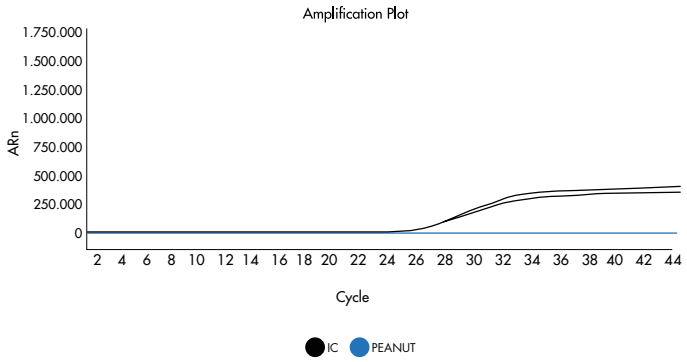
If the controls do not match these results, the experiment must be repeated.

The results obtained by the present method shall be interpreted as follows:

Target detection Chanel 1	Target detection Chanel 2	Interpretation
Positive	Not significant	Positive
Ct = NA	Positive	Negative
Ct = NA	Ct = NA	Inhibition**

When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.





2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).
The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific).

3. SPECIFICITY

A total of 51 non target species related with the target or occurring in the food product and that could cause interference with test results were de tested (Table 3.1). The species were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the ITS region, using universal primers. The identification of all species was confirmed by sequencing mitochondrial genes. All DNA extracts were tested in triplicate.

Code	Species name	Code	Species name
CR1	*Ovis aries*	VR12	*Sinapis alba*
CR4	*Sus domesticus*	VR2	*Prunus dulcis*
CR7	*Gallus gallus*	VR20	*Glycine max*
CR8	*Bos taurus*	VR4	*Avena sativa*
CR9	*Meleagris gallopavo*	VR5	*Corylus avellana, L.*
CR13	Equus equus	VR6	*Lycium barbarum*
PR1	*Auxis rochei*	VR7	*Anacardium occidentale*
PR3	*Sarda sarda/chiliensis*	VR8	*Bertholletia excelsa*
PR7	*Hymenopenaeus muelleri*	VR9	*Secale cereale*
PR8	*Litopenaeus vannamei*	VR10	*Hordeum vulgare*
PR9	*Salmo salar*	VR11	*Macadamia ternifolia*
PR10	*Gadus morhua*	VR13	*Brassica nigra*
PR12	*Sepiella japonica*	VR14	*Juglans regia L.*
PR13	*Merluccius senegalensis*	VR15	*Carya illinoensis K.*
PR14	*Illex argentinus*	VR16	*Curcubita pepo L.*
PR15	*Sepia aculeata*	VR17	*Pinus pinea*
PR16	*Merluccius paradoxus*	VR18	*Pistacia vera*
PR17	*Meretrix lyrata*	VR19	*Sesamum indicum*
PR19	*Ensis directus*	VR1	*Apium graveolens*
PR21	*Metapenaeus* sp. *(affinis/ensis)*	VR21	*Lupinus albus*
PR22	*Fenneropenaeus indicus*	VR22	*Triticum aestivum*
PR24	*Penaeus japonicus*	VR23	*Allium sativum*
PR37	*Uroteuthis duvauceli*	VR24	*Oryza sativa*
PR42	*Pandalus montagui*	VR25	*Solanum tuberosum*
PR49	*Argopecten furfuratus*	VR29	*Pisum sativum*
		VR30	*Vigna unguiculata*

Table 3.1 – List of species used for exclusivity test

Result:

The method presented 100% specificity as none of the species showed a positive result.

4. SENSITIVITY

4.1. Sensitivity of the Real-Time PCR reaction

The sensitivity of the reaction was determined by testing in duplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg and 1 pg.

Result:

Target amplification was observed with DNA amounts of 10 pg or higher. Thus, the limit of detection of the reaction was established in 10 pg of target genomic DNA.

4.2. Sensitivity of the test in DNA mixtures

The sensitivity of the test was determined in a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05%, 0,01%, 005% and 0,001%. All determinations were performed duplicate.

Result:
Target amplification was observed in samples containing 0,01% of target DNA. Thus, the method can detect 0,01% of peanut DNA in 100 ng of total DNA.

5. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 5.1, using two positive and two negative samples and performed in triplicate.

Parameter	N° variations	Description
Annealing temperature	2	+ 2°C; - 2°C
MgCl2 concentration	2	+ 0,5mM; - 0,5mM
Independent performer		Not applicable
Different Real Time equipment	2	ABI 7500 PikoReal, Thermo Scientific
Independent laboratories	1	Biopremier R&D laboratory

Table 5.1 – Method's parameters submitted to variations

Result:
Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

6. TRUENESS

Trueness of the method was evaluated using 5 positive and 17 negative food samples. All the samples were tested in triplicate.

Result:
All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%

PERFORMANCE CHARACTERISTICS FOR VALIDATION

The determination of false positive and false negative rate, the method was performed in 22 food samples (5 positive and 17 negative samples).
The method's limit of detection was determined in a mixture containing a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05%, 0,01%, 005% and 0,001%. All determinations were performed in duplicate.

1. FALSE POSITIVE RATE

This corresponds to the probability that a known negative sample has been classified as positive by the method. The false positive rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fpos}=n_{fpos}/n_{rneg}+n_{fpos} \times 100\%$

In which:
P_{fpos}: False positive rate
n_{rneg}: Number of real negative test results
n_{fpos}: Number of misclassified known negative samples

Result:
For the present method, false positive rate was calculated based on 22 samples. The final result was 0%

2. FALSE NEGATIVE RATE

This corresponds to the probability that a known positive sample has been classified as negative by the method. The false negative rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fneg}=n_{fneg}/n_{rpos}+n_{fneg} \times 100\%$

In which:
P_{fneg}: False negative rate
n_{rpos}: Number of real positive test results
n_{fneg}: Number of misclassified known positive samples

Result:
For the present method, false negative rate was calculated based on 22 samples. The final result was 0%

3. LIMIT OF DETECTION

3.1. Limit of detection of the Real-Time PCR reaction

The limit of detection of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg and 1 pg.

Result:
The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 10 pg of the target genomic DNA.

3.2. Limit of detection of the method

The method's limit of detection was determined in a mixture containing a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05%, 0,01%, 005% and 0,001%. All determinations were performed in duplicate.

Result:
The limit of detection determined with a probability 0,95% (false positive rate < 0,05) was 0,01% of peanut DNA in 100 ng of total DNA.

Positive predictive value (PPV)

The PPV is calculated by the expression:
$PPV=n_{rpos}/n_{rpos}+n_{fpos} \times 100\%$

In which:
n_{rpos}: Number of real positive test results
n_{fneg}: Number of misclassified known positive samples

Result:
The positive predictive value determined was 100%.

Negative predictive value (NPV)

The PPV is calculated by the expression:

$NPV=n_{rneg}/n_{rneg}+n_{fneg} \times 100\%$

In which:
n_{rneg}: Number of real negative test results
n_{fneg}: Number of misclassified known negative samples

Result:
The negative predictive value determined was 100%.

Criteria for acceptance

Inclusivity: 95%
Exclusivity: 95%
Selectivity: 95%
Trueness: 95%
False positive rate: 5%
False negative rate: 5%

PERFORMANCE DECLARATION

Accordingly with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results in agreement with the Cpndalab requirements and is validated.

The present test allows the "Detection of Peanut using real-time PCR".

Detection of *Salmonella* spp. using real-time PCR

VALIDATION REPORT

ASSAY

Salmonella spp. detection by 5' nuclease real time-PCR assay.

VALIDATED EQUIPMENT

Applied Biosystems, model 7500; Thermo Scientific, model PikoReal; Condalab, model CDL-96.

SAMPLES (N)

Enrichments prepared from suitable food samples. During re-validation samples from external quality assessment schemes from FAPAS, Instand and INSA-PHE were tested.

VALIDATION ASSAYS

Start – 01-10-2013
Finish – 01-05-2014

DEFINITIONS

Analyte

DNA of the microorganism detected by the analysis method

Qualitative method

Analysis method which response is either the presence or absence of the microorganism either directly or indirectly in a certain amount of sample.

True positive (TP)

Sample presenting a positive result for the test and that has equal or higher amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

True negative (TN)

Sample presenting a negative result for the test and that has a lower amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

False Positive (FP)

Sample presenting a positive result for the test in the absence of target analyte.

False Negative (FN)

Sample presenting a negative result for the test in the presence of equal or higher amount of the target analyte than the detection limit.

Detection Limit (LOD)

- Reaction detection limit: lowest concentration of the target DNA that can be consistently detected in the specified experimental conditions.
- Method reaction limit: lowest concentration or content of the target microorganism relative to the defined amount of matrix that can be consistently detected in the specified experimental conditions.

Inclusivity

Detection of the target microorganism.

Exclusivity

Non-detection of non-target microorganisms.

Selectivity

Measure of the inclusivity and exclusivity.

Sensitivity (S)

Measure of the lowest number of analyte cells which can be detected in a single test reaction.

Specificity (Sp)

Ability to exclusivity recognize the target microorganism to be detected and distinguish it from other similar organisms and impurities.

Trueness (T)

Closeness of agreement between the expectation of a test result and the corresponding true value.

Precision (P)

Closeness of agreement between independent tests obtained under defined conditions. It depends only on the distribution of random errors and does not relate to the true value of the test.

Positive predictive value (PPV)

Probability of a positive test be referent to a sample presenting an analyte concentration above.

Negative predictive value (NPV)

Probability of a positive test be referent to a sample presenting an analyte concentration above.

SCOPE OF THE METHOD

The present method is based on a 5' nuclease real-time PCR reactions and amplify a unique genomic sequence of *Salmonella* spp., aiming the detection of this pathogen DNA in food samples. It was designed to detect both inv positive and inv negative *Salmonella* spp. variants, increasing the inclusivity potential of the method.
The test is designed for the detection of the target microorganism DNA in food products after enrichment in buffered peptone water and DNA extraction. It can also be used with animal feedstuff and environmental samples. Is not suitable for human and clinical diagnostics. The method's performance was tested according ISO 22118:2011 and ISO 16140:2003 and fulfils the requirements set in this International Standard.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a second fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 21 food samples enriched according the method described in ISO 6579:2012 / ISO 6579:2017. These food samples included 10 different matrices, aleatory taken from commercial shops:

- Minced meat
- Frozen fish
- Frozen vegetables
- Eggs
- Cheese
- Herbs mix
- Mayonnaise
- Pastry
- Processed fish
- Cooked meal

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing genomic DNA of the Salmonella enterica, strain CECT 443

Negative control (NC): reaction without any target DNA

Each individual reaction includes:

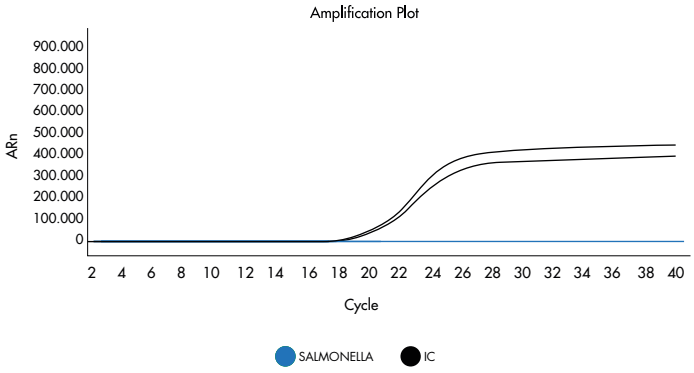
Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

To validate the tests, the controls must show the following results:

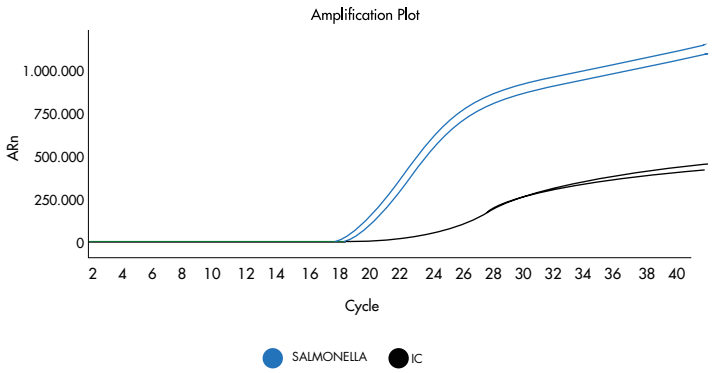
	Target detection Chanel 1	Target detection Chanel 2
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

If the controls do not match these results, the experiment must be repeated.

Positive control



Negative control



The results obtained by the present method shall be interpreted as follows:

Target detection Chanel 1	Target detection Chanel 2	Interpretation
Positive	Not significant	Positive
Ct = NA	Positive	Negative
Ct = NA	Ct = NA	Inhibition**

When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.

2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).

The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific). Comparable results were obtained with CFX96 (BioRad).

3. SELECTIVITY

Code	Strain identification	Other informations
MB 353	*Salmonella Adelaide*	IFM 2312
MB 164	*Salmonella Enteritidis*	
MB 399	*Salmonella Typhimurium*	CECT 443
MB 400	*Salmonella spp.*	
MB 402	*Salmonella* spp.	
MB 403	*Salmonella* spp.	
MB 404	*Salmonella* spp.	
MB 405	*Salmonella* spp.	
MB 406	*Salmonella* spp.	
MB 407	*Salmonella* spp.	
MB 408	*Salmonella* spp.	
MB 409	*Salmonella* spp.	
MB 410	*Salmonella* spp.	
MB 411	*Salmonella* spp.	
MB 412	*Salmonella* spp.	
MB 413	*Salmonella* spp.	
MB 414	*Salmonella* spp.	
MB 415	*Salmonella* spp.	
MB 416	*Salmonella* spp.	
MB 417	*Salmonella* spp.	
MB 418	*Salmonella* spp.	
MB 419	*Salmonella* spp.	
MB 420	*Salmonella* spp.	
MB 421	*Salmonella* spp.	
MB 422	*Salmonella* spp.	
MB 423	*Salmonella* spp.	
MB 426	*Salmonella* spp.	
MB 434	*Salmonella* spp.	
MB 435	*Salmonella* spp.	
MB 436	*Salmonella* spp.	
MB 437	*Salmonella Enteritidis*	
MB 438	*Salmonella* spp.	
MB 439	*Salmonella Typhimurium*	
MB 440	*Salmonella* spp.	
MB 441	*Salmonella* spp.	
MB 442	*Salmonella* spp.	
MB 443	*Salmonella Typhimurium*	
MB 444	*Salmonella* spp.	
MB 445	*Salmonella* spp.	

MB 446	*Salmonella* spp.
MB 497	*Salmonella* spp.
MB 498	*Salmonella* spp.
MB 499	*Salmonella* spp.
MB 500	*Salmonella* spp.
MB 501	*Salmonella* spp.
MB 1002	*Salmonella enterica*
MB 1007	*Salmonella enterica*
MB 1008	*Salmonella enterica*
MB 1009	*Salmonella enterica*
MB 1010	*Salmonella enterica*
MB 1011	*Salmonella enterica*
MB 1012	*Salmonella enterica subsp. enterica serovar Saintpaul strain*
MB 1013	*Salmonella enterica*
MB 1014	*Salmonella enterica subsp. enterica serovar Rissen strain*
MB 1016	*Salmonella enterica*
MB 1017	*Salmonella enterica*
MB 1018	*Salmonella enterica subsp. enterica serovar Saintpaul strain*
MB 1019	*Salmonella enterica subsp. enterica serovar Saintpaul strain*
MB 1020	*Salmonella enterica*
MB 1021	*Salmonella enterica subsp. enterica serovar Heidelberg strain*

Table 3.1 – List of *Salmonella* spp. strains used for inclusivity test

3.1. Inclusivity test

A total of 60 *Salmonella* spp. strains were tested, including the most important serotypes and inv negative strains (Table 3.1). The strains were tested using 2 ng of genomic DNA

Result:

All strains were detected with the present method, corresponding to 100% inclusivity.

3.2. Exclusivity test

The strains for exclusivity test were chosen following the recommendations of International Standard ISO 22118:2011. A total of 30 non target microorganisms related with the target or occurring in the same habitat and that could cause interference with test results were de tested (Table 3.2). The strains were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the 16S rRNA bacterial gene, using universal primers. The identification of all strains was confirmed by 16S rRNA gene sequencing. All strains were tested in triplicate.

Code	Strain identification	Other informations
MB 385	*Listeria monocytogenes*	CECT 4031
MB 388	*Listeria monocytogenes*	Serotype 1/2a ou 3a
MB 381	*Listeria innocua*	CECT 910
MB 240	*Escherichia coli O157:H7*	CDC 337
MB 357	*Escherichia coli*	NCTC 9001
MB 372	*Campylobacter jejuni subsp jejuni*	NCTC 11351
MB 373	*Campylobacter coli*	NCTC 11366
MB 22	*Vibrio cholerae*	Local isolate
MB 356	*Vibrio parahaemolyticus*	NCTC 10885
MB 363	*Bacillus cereus*	IFM1600

MB 115	*Citrobacter freundii*	Local isolate
MB 451	*Cronobacter muytjensii*	ATCC 51329
MB 141	*Lactobacillus plantarum*	CECT 748 T
MB 144	*Lactobacillus paracasei subsp paracasei*	CECT 4022T
MB 150	*Staphylococcus aureus*	NCTC 6571
MB 302	*Pseudomonas aeruginosa*	Local isolate
MB 13	*Serratia marcescens*	Local isolate
MB 163	*Proteus vulgaris*	Local isolate
MB 365	*Enterococcus faecalis*	Local isolate
MB 306	*Enterococcus hirae*	Local isolate
MB 147	*Staphylococcus epidermidis*	Local isolate
MB 267	*Shigella flexneri*	Local isolate
MB 249	*Yersinia enterocolitica*	Local isolate
MB 178	*Klebsiella pneumoniae*	Local isolate
MB 449	*Brochothrix thermosphacta*	ATCC 11509
MB 274	*Legionella pneumophila*	Local isolate
MB 278	*Legionella micdadei*	Local isolate
MB 362	*Clostridium perfringens*	Local isolate
MF 128	*Saccharomyces cerevisae*	CECT
MF 129	*Aspergillus niger*	Local isolate

Table 3.2 – List of strains used for exclusivity test

Result:
The method presented 100% specificity as none of the species showed a positive result.

4. SENSITIVITY

4.1. Sensitivity of the Real-Time PCR reaction

The sensitivity of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 10 ng, 5 ng, 1 ng, 500 pg, 50 pg, 5 pg, 500 fg, 50 fg, 25 fg and 5 fg.

Result:
Target amplification was observed with DNA amounts of 500 fg or higher. Thus, the limit of detection of the reaction was established in 500 fg of target genomic DNA.

4.2. Sensitivity of the test in DNA mixtures

The sensitivity of the test was determined in food samples artificially contaminated with decreasing number of bacterial cells, following the requirements of ISO 22118. The sensitivity was tested in five different food matrices: minced meat, frozen fish, frozen vegetables, eggs and cheese. The enrichment was performed according to ISO 6579:2012 / ISO 6579:2017, with 25 g of each sample, after inoculation with 0, 1-10, 10-10^2 and 10^2-10^3 cfu of *Salmonella enterica* (strain CECT 443). All procedures starting from DNA extraction were performed in triplicate.

Result:
Target amplification was observed in samples contaminated with 1-10 cfu/25 g or more. No amplification was obtained in non-contaminated samples (inoculated with 0 cfu). Thus, the method can detect 1-10 cfu in 25 g of food sample (LOD).

5. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 5.1, using two positive and two negative samples and performed in triplicate.

Parameter	Nº variations	Description
Annealing temperature	2	+ 2ºC; - 2ºC
MgCl2 concentration	2	+ 0,5mM; - 0,5mM
Independent performer	2	Not applicable
Different Real Time equipment	3	ABI 7500 PikoReal, Thermo Scientific BioRad CFX96
Independent laboratories	2	Biopremier R&D laboratory Portuguese Food Safety Authority laboratory

Table 5.1 – Method's parameters submitted to variations

Result:
Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

6. TRUENESS

Trueness of the method was evaluated using 7 positive and 11 negative food samples for the target, respectively, corresponding to the following food matrices:

- Minced meat
- Frozen fish
- Frozen vegetables
- Eggs
- Cheese
- Herbs mix
- Mayonnaise
- Pastry
- Processed fish
- Cooked meal

All the samples were tested in triplicate.

Result:
All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%

PERFORMANCE CHARACTERISTICS FOR VALIDATION

For the determination of false positive and false negative rate, the method was performed in 21 samples (12 positive and 9 negative samples), corresponding to 10 food matrices.
For the determination of method's limit of detection, the sample was inoculated considering 3 contamination levels: 0, 1 to 10 and 10 to 100 cfus in 25 g of sample. All the procedures were performed as defined for the present method. All experiments starting from DNA extraction were performed in triplicate.

1. FALSE POSITIVE RATE

This corresponds to the probability that a known negative sample has been classified as positive by the method. The false positive rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fpos}=n_{fpos}/n_{rneg}+n_{fpos}$ x 100%

In which:

P_{fpos}: False positive rate
n_{rneg}: Number of real negative test results
n_{fpos}: Number of misclassified known negative samples

Result:
For the present method, false positive rate was calculated based on 21 real samples. The final result was 0%

2. FALSE NEGATIVE RATE

This corresponds to the probability that a known positive sample has been classified as negative by the method. The false negative rate (that can be expressed as a percentage) is calculated by the expression:

P_{fneg}=nfneg/n_{rneg}+n_{fneg} x 100%

In which:

P_{fneg}: False negative rate
n_{rpos}: Number of real positive test results
n_{fneg}: Number of misclassified known positive samples

Result:
For the present method, false positive rate was calculated based on 21 real samples. The final result was 0%

3. LIMIT OF DETECTION

3.1. Limit of detection of the Real-Time PCR reaction

The limit of detection of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 100 pg, 50 pg, 10 pg, 5 pg, 1pg, 500 fg. 50 fg, 10 fg, 5 fg.

Result:
The limit of detection determined with a probability 0,95% (false positive rate < 0,05) was 10 fg of the target genomic DNA.

3.2. Limit of detection of the method

The limit of detection of the test was determined in food samples artificially contaminated with decreasing number of bacterial cells, following the requirements of ISO 22118, using five different food matrices: minced meat, frozen fish, frozen vegetables, eggs and cheese. The enrichment was performed according to ISO 6579:2012 / ISO 6579:2017, with 25 g of each sample, after inoculation with 0, 1-10, 10-10^2 and 10^2-10^3 cfu of *Salmonella enterica* (strain CECT 443). All procedures starting from DNA extraction were performed in triplicate.

Result:
The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 1-10 cfus of *Salmonella* spp.

Positive predictive value (PPV)

The PPV is calculated by the expression:
PPV=n_{rpos}/n_{rpos}+n_{fpos} x 100%
In which:
n_{rpos}: Number of real positive test results
n_{fneg}: Number of misclassified known positive samples

Result:
The positive predictive value determined was 100%.

Negative predictive value (NPV)

The PPV is calculated by the expression:
NPV=n_{rneg}/n_{rneg}+n_{fneg} x 100%
In which:
n_{rneg}: Number of real negative test results
n_{fneg}: Number of misclassified known negative samples

Result:
The negative predictive value determined was 100%.

Criteria for acceptance
Inclusivity: 95%
Exclusivity: 95%
Selectivity: 95%
Trueness: 95%
False positive rate: 5%
False negative rate: 5%

PERFORMANCE DECLARATION

Accordingly, with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results in agreement with the Condalab requirements and is validated.
The present test allows the "Detection of *Salmonella* spp. using real time-PCR" in food samples after enrichment.

Detection of Soybean/ Soy DNA using real-time PCR

VALIDATION REPORT

ASSAY

Detection of soybean/ soy DNA, using real-time PCR

VALIDATED EQUIPMENT

Applied Biosystems, model 7500; Thermo Scientific, model PikoReal; Condalab, model CDL-96.

SAMPLES (N)

Food products and samples from external quality assessment schemes.

VALIDATION ASSAYS

Start – 11-12-2015
Finish – 08-02-2016

DEFINITIONS

Analyte

DNA of the target species detected by the analysis method

Qualitative method

Analysis method which response is either the presence or absence of the target species either directly or indirectly in a certain amount of sample.

True positive (TP)

Sample presenting a positive result for the test and that has equal or higher amount of the target analyte (target species DNA) than the detection limit established in the specified experimental conditions.

True negative (TN)

Sample presenting a negative result for the test and that has a lower amount of the target analyte (target species DNA) than the detection limit established in the specified experimental conditions.

False Positive (FP)

Sample presenting a positive result for the test in the absence of target analyte.

False Negative (FN)

Sample presenting a negative result for the test in the presence of equal or higher amount of the target analyte than the detection limit.

Detection Limit (LOD)

- Reaction detection limit: lowest concentration of the target DNA that can be consistently detected in the specified experimental conditions.
- Method reaction limit: lowest concentration or content of the target microorganism relative to the defined amount of matrix that can be consistently detected in the specified experimental conditions.

Inclusivity

Detection of the target species.

Exclusivity

Non-detection of non-target species.

Selectivity

Measure of the inclusivity and exclusivity.

Sensitivity (S)

Measure of the lowest of analyte DNA concentration which can be detected in a single test reaction.

Specificity (Sp)

Ability to exclusivity recognize the target species to be detected and distinguish it from other similar organisms and impurities.

Trueness (T)

Closeness of agreement between the expectation of a test result and the corresponding true value.

Positive predictive value (PPV)

Probability of a positive test to be referent to a sample presenting the DNA of the target species.

Negative predictive value (NPV)

Probability of a positive test to be referent to a sample presenting the DNA of the target species in a concentration above the detection limit.

SCOPE OF THE METHOD

The present method is based on a 5' nuclease real-time PCR reactions and amplify a unique soybean genomic sequence aiming the detection of this species DNA in food samples after DNA extraction. It can also be used with other samples.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a second fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 23 food samples obtained from different commercial sources. These food samples included 15 different matrices:

- Cookies
- Cooked rice
- Soy schnitzel
- Soy hamburger
- Cereal mix
- Cereal bread
- Soup
- Energy bar
- Chocolate snacks
- Nougat
- Sausage roll
- Dehydrated broth
- Gluten-free crips rolls
- Juice
- Meet hamburger

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing soybean DNA

Negative control (NC): reaction without any target DNA
Each individual reaction includes:

Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.
To validate the tests, the controls must show the following results:

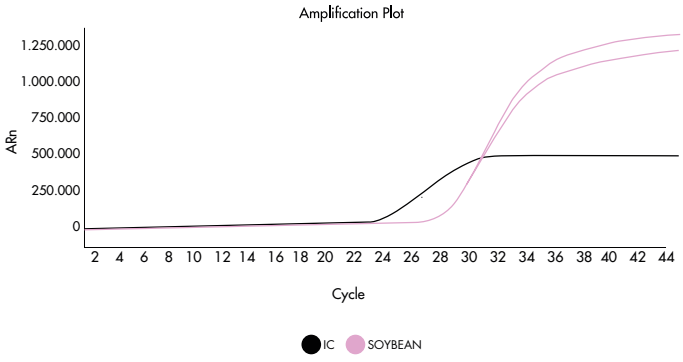
	Target detection Chanel 1	Target detection Chanel 2
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

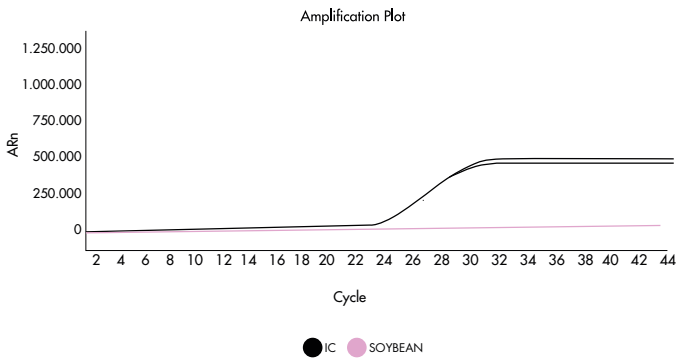
If the controls do not match these results, the experiment must be repeated.

The results obtained by the present method shall be interpreted as follows:

Target detection Chanel 1	Target detection Chanel 2	Interpretation
Positive	Not significant	Positive
Ct = NA	Positive	Negative
Ct = NA	Ct = NA	Inhibition**

When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.





2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).
The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific).

3. SPECIFICITY

A total of 51 non target species related with the target or occurring in the food product and that could cause interference with test results were de tested (Table 3.1). The species were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the ITS region, using universal primers. The identification of all species was confirmed by sequencing mitochondrial genes. All DNA extracts were tested in triplicate.

Code	Species name	Code	Species name
CR1	*Ovis aries*	VR12	*Sinapis alba*
CR4	*Sus domesticus*	VR2	*Prunus dulcis*
CR7	*Gallus gallus*	VR3	*Arachis hypogaea L.*
CR8	*Bos taurus*	VR4	*Avena sativa*
CR9	*Meleagris gallopavo*	VR5	*Corylus avellana, L.*
CR13	Equus equus	VR6	*Lycium barbarum*
PR1	*Auxis rochei*	VR7	*Anacardium occidentale*
PR3	*Sarda sarda/chiliensis*	VR8	*Bertholletia excelsa*
PR7	*Hymenopenaeus muelleri*	VR9	*Secale cereale*
PR8	*Litopenaeus vannamei*	VR10	*Hordeum vulgare*
PR9	*Salmo salar*	VR11	*Macadamia ternifolia*
PR10	*Gadus morhua*	VR13	*Brassica nigra*
PR12	*Sepiella japonica*	VR14	*Juglans regia L.*
PR13	*Merluccius senegalensis*	VR15	*Carya illinoensis K.*
PR14	*Illex argentinus*	VR16	*Curcubita pepo L.*
PR15	*Sepia aculeata*	VR17	*Pinus pinea*
PR16	*Merluccius paradoxus*	VR18	*Pistacia vera*
PR17	*Meretrix lyrata*	VR19	*Sesamum indicum*
PR19	*Ensis directus*	VR1	*Apium graveolens*
PR21	*Metapenaeus* sp. *(affinis/ensis)*	VR21	*Lupinus albus*
PR22	*Fenneropenaeus indicus*	VR22	*Triticum aestivum*
PR24	*Penaeus japonicus*	VR23	*Allium sativum*
PR37	*Uroteuthis duvauceli*	VR24	*Oryza sativa*
PR32	*Cerastoderma edule*	VR25	*Solanum tuberosum*
PR49	*Argopecten furfuratus*	VR29	*Pisum sativum*
		VR30	*Vigna unguiculata*

Table 3.1 – List of species used for exclusivity test

Result:

The method presented 100% specificity as none of the species showed a positive result.

4. SENSITIVITY

4.1. Sensitivity of the Real-Time PCR reaction

The sensitivity of the reaction was determined by testing in duplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg.

Result:

Target amplification was observed with DNA amounts of 50 pg or higher. Thus, the limit of detection of the reaction was established in 50 pg of target genomic DNA.

4.2. Sensitivity of the test in DNA mixtures

The sensitivity of the test was determined in a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05%, 0,01%, 005% and 0,001%. All determinations were performed duplicate.

Result:
Target amplification was observed in samples containing 0,05% of target DNA. Thus, the method can detect 0,05% of soybean DNA in 100 ng of total DNA.

5. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 5.1, using two positive and two negative samples and performed in triplicate.

Parameter	N° variations	Description
Annealing temperature	2	+ 2°C; - 2°C
MgCl2 concentration	2	+ 0,5mM; - 0,5mM
Independent performer		Not applicable
Different Real Time equipment	2	ABI 7500 PikoReal, Thermo Scientific
Independent laboratories	1	Biopremier R&D laboratory

Table 5.1 – Method's parameters submitted to variations

Result:
Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

6. TRUENESS

Trueness of the method was evaluated using 6 positive and 17 negative food samples. All the samples were tested in triplicate.

Result:
All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%

PERFORMANCE CHARACTERISTICS FOR VALIDATION

The determination of false positive and false negative rate, the method was performed in 23 food samples (6 positive and 17 negative samples).
The method's limit of detection was determined in a mixture containing a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05%, 0,01%, 005% and 0,001%. All determinations were performed in duplicate.

1. FALSE POSITIVE RATE

This corresponds to the probability that a known negative sample has been classified as positive by the method. The false positive rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fpos}=n_{fpos}/n_{rneg}+n_{fpos} \times 100\%$

In which:
P_{fpos}: False positive rate
n_{rneg}: Number of real negative test results
n_{fpos}: Number of misclassified known negative samples

Result:
For the present method, false positive rate was calculated based on 23 samples. The final result was 0%

2. FALSE NEGATIVE RATE

This corresponds to the probability that a known positive sample has been classified as negative by the method. The false negative rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fneg}=n_{fneg}/n_{rpos}+n_{fneg} \times 100\%$

In which:
P_{fneg}: False negative rate
n_{rpos}: Number of real positive test results
n_{fneg}: Number of misclassified known positive samples

Result:
For the present method, false negative rate was calculated based on 23 samples. The final result was 0%

3. LIMIT OF DETECTION

3.1. Limit of detection of the Real-Time PCR reaction

The limit of detection of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg.

Result:
The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 50 pg of the target genomic DNA.

3.2. Limit of detection of the method

The method's limit of detection was determined in a mixture containing a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05%, 0,01%, 005% and 0,001%. All determinations were performed in duplicate.

Result:
The limit of detection determined with a probability 0,95% (false positive rate < 0,05) was 0,05% of soybean DNA in 100 ng of total DNA.

Positive predictive value (PPV)

The PPV is calculated by the expression:
$PPV=n_{rpos}/n_{rpos}+n_{fpos} \times 100\%$

In which:
n_{rpos}: Number of real positive test results
n_{fneg}: Number of misclassified known positive samples

Result:
The positive predictive value determined was 100%.

Negative predictive value (NPV)

The PPV is calculated by the expression:

$NPV=n_{rneg}/n_{rneg}+n_{fneg} \times 100\%$

In which:
n_{rneg}: Number of real negative test results
n_{fneg}: Number of misclassified known negative samples

Result:
The negative predictive value determined was 100%.

Criteria for acceptance

Inclusivity: 95%
Exclusivity: 95%
Selectivity: 95%
Trueness: 95%
False positive rate: 5%
False negative rate: 5%

Performance declaration

Accordingly with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results in agreement with the Condalab requirements and is validated.

The present test allows the "Detection of Soybean using real-time PCR".

Detection of *Legionella pneumophila* using real time-PCR

VALIDATION REPORT

ASSAY

Detection of *Legionella pneumophila* using real time-PCR.

VALIDATED EQUIPMENT

Applied Biosystems, 7500 model; Thermo Scientific, model PikoReal.

SAMPLES (N)

Water and swabs. During re-validation samples from external quality assessment schemes from FAPAS.

VALIDATION ASSAYS

Start – 01-06-2013
Finish – 01-09-2014

DEFINITIONS

Analyte

DNA of the microorganism detected by the analysis method

Qualitative method

Analysis method which response is either the presence or absence of the microorganism either directly or indirectly in a certain amount of sample.

True positive (TP)

Sample presenting a positive result for the test and that has equal or higher amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

True negative (TN)

Sample presenting a negative result for the test and that has a lower amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

False Positive (FP)

Sample presenting a positive result for the test in the absence of target analyte.

False Negative (FN)

Sample presenting a negative result for the test in the presence of equal or higher amount of the target analyte than the detection limit.

Detection Limit (LOD)

- Reaction detection limit: lowest concentration of the target DNA that can be consistently detected in the specified experimental conditions.
- Method reaction limit: lowest concentration or content of the target microorganism relative to the defined amount of matrix that can be consistently detected in the specified experimental conditions.

Inclusivity

Detection of the target microorganism.

Exclusivity

Non-detection of non-target microorganisms.

Selectivity

Measure of the inclusivity and exclusivity.

Sensitivity (S)

Measure of the lowest number of analyte cells which can be detected in a single test reaction.

Specificity (Sp)

Ability to exclusivity recognize the target microorganism to be detected and distinguish it from other similar organisms and impurities.

Trueness (T)

Closeness of agreement between the expectation of a test result and the corresponding true value.

Precision (P)

Closeness of agreement between independent tests obtained under defined conditions. It depends only on the distribution of random errors and does not relate to the true value of the test.

Positive predictive value (PPV)

Probability of a positive test be referent to a sample presenting an analyte concentration above.

Negative predictive value (NPV)

Probability of a positive test be referent to a sample presenting an analyte concentration above.

SCOPE OF THE METHOD

Nowadays, 47 species and more than 60 serogroups of Legionella spp. have been recognized. Legionnaires' disease is the most severe form of infection and the vast majority of Legionnaires' disease cases are due to L. pneumophila, being serogroup 1 responsible for up to 80% of reported cases.

The present method is based on a 5' nuclease real-time PCR reactions that allows the amplification of a unique genomic sequence of Legionella pneumophila, aiming the detection of this pathogen DNA in water and other environmental samples. The method was designed to detect Legionella pneumophila. The test is designed for the detection of the target microorganism DNA in water samples after concentration by filtration and other environmental samples (e.g. sampled with swabs). It can also be used with other samples. Is not suitable for human and clinical diagnostics.

The method's performance was tested based on ISO 22118:2011 and fulfils the requirements set in this International Standard.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

Two probes labeled with different fluorophore are used to detect Legionella pneumophila. The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a third fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 5 water samples concentrated according the method described in ISO 11731:1998 and 5 swab samples. The water samples included water taken from different public sources.

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing genomic DNA of the Legionella pneumophila, strain DSM 7513^T

Negative control (NC): reaction without any target DNA

Each individual reaction includes:

Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

To validate the tests, the controls must show the following results:

	Target detection Chanel 1	Target detection Chanel 2
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

If the controls do not match these results, the experiment must be repeated.

The results obtained by the present method shall be interpreted as follows:

Target detection Channel 1 *(L. pneumophila)*	Target detection Chanel 1 (IC)	Interpretation
Positive	Not significant	Positive for *L. pneumophila*
Ct = NA	Positive	Negative
Ct = NA	Ct = NA	Inhibition**

**When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.

2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).

The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific). Comparable results were obtained with CFX96 (BioRad).

3. SELECTIVITY

3.1. Inclusivity test

A total of 28 *Legionella pneumophila* strains were tested. The strains were tested using 2 ng of genomic DNA.

Code	Strain identification	Other informations
MB 274	*Legionella pneumophila*	DSM 7513T
MB 107	*Legionella pneumophila*	
MB 281	*Legionella pneumophila*	SG1 Philadelphia
MB 282	*Legionella pneumophila*	ATCC 33216, SG1 Knoxville
MB 286	*Legionella pneumophila*	SG5 Dallas
MB 288	*Legionella pneumophila*	SG7 Chicago
MB 293	*Legionella pneumophila*	SG11
MB 294	*Legionella pneumophila*	SG13
MB 296	*Legionella pneumophila*	SG15
MB 572	*Legionella pneumophila*	SG10
MB 573	*Legionella pneumophila*	SG7
MB 574	*Legionella pneumophila*	SG3
MB 575	*Legionella pneumophila*	SG3
MB 576	*Legionella pneumophila*	SG9
MB 577	*Legionella pneumophila*	SG1

MB 578	*Legionella pneumophila*	SG5
MB 579	*Legionella pneumophila*	SG5
MB 580	*Legionella pneumophila*	SG10
MB 581	*Legionella pneumophila*	SG14
MB 582	*Legionella pneumophila*	SG4
MB 583	*Legionella pneumophila*	SG8
MB 584	*Legionella pneumophila*	SG1
MB 585	*Legionella pneumophila*	SG1
MB 586	*Legionella pneumophila*	SG4
MB 587	*Legionella pneumophila*	SG9
MB 588	*Legionella pneumophila*	SG3
MB 589	*Legionella pneumophila*	SG10
MB 590	*Legionella pneumophila*	SG8

Table 3.1 – List of strains used for inclusivity test

Result:
All strains of *Legionella* spp. and *Legionella pneumophila*, respectively, were detected with the present method, corresponding to 100% inclusivity.

3.2. Exclusivity test

The strains for exclusivity test were chosen following the recommendations of International Standard ISO 22118:2011. A total of 30 non target microorganisms related with the target or occurring in the same habitat and that could cause interference with test results were de tested (Table 3.1). The strains were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the 16S rRNA bacterial gene, using universal primers. The identification of all strains was confirmed by 16S rRNA gene sequencing. All strains were tested in triplicate.

Code	Strain identification	Other informations
MB 385	*Listeria monocytogenes*	CECT 4031
MB 388	*Listeria monocytogenes*	Serotype 1/2a ou 3a
MB 381	*Listeria innocua*	CECT 910
MB 240	*Escherichia coli O157:H7*	CDC 337
MB 357	*Escherichia coli*	NCTC 9001
MB 372	*Campylobacter jejuni subsp jejuni*	NCTC 11351
MB 373	*Campylobacter coli*	NCTC 11366
MB 22	*Vibrio cholerae*	Local isolate
MB 356	*Vibrio parahaemolyticus*	NCTC 10885
MB 363	*Bacillus cereus*	IFM1600
MB 115	*Citrobacter freundii*	Local isolate
MB 451	*Cronobacter muytjensii*	ATCC 51329
MB 141	*Lactobacillus plantarum*	CECT 748 T
MB 144	*Lactobacillus paracasei subsp paracasei*	CECT 4022T
MB 150	*Staphylococcus aureus*	NCTC 6571
MB 302	*Pseudomonas aeruginosa*	Local isolate
MB 13	*Serratia marcescens*	Local isolate
MB 163	*Proteus vulgaris*	Local isolate
MB 365	*Enterococcus faecalis*	Local isolate
MB 306	*Enterococcus hirae*	Local isolate
MB 147	*Staphylococcus epidermidis*	Local isolate
MB 267	*Shigella flexneri*	Local isolate
MB 249	*Yersinia enterocolitica*	Local isolate
MB 178	*Klebsiella pneumoniae*	Local isolate
MB 449	*Brochothrix thermosphacta*	ATCC 11509

MB 164	*Salmonella Enteritidis*	Local isolate
MB 399	*Salmonella Typhimurium*	CECT 443
MB 278	*Legionella micdadei*	Local isolate
MF 128	*Saccharomyces cerevisae*	CECT 12928
MF 129	*Aspergillus niger*	Local isolate

Table 3.2 – List of strains used for exclusivity test

Result:
The method presented 100% exclusivity as none of the strains showed a positive results and positive result.

4. SENSITIVITY

4.1. Sensitivity of the Real Time-PCR reaction

The sensitivity of the reaction was determined by testing in triplicate decreasing quantities of Legionella pneumophila (strain DSM 7513) DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 10 ng, 5 ng, 1 ng, 500 pg, 50 pg, 5 pg, 500 fg, 50 fg, 25 fg and 5 fg.

Result:
Target amplification was observed with DNA amounts of 5pg or higher. Thus, the limit of detection of the reaction was established in 5pg of target genomic DNA.

4.2. Sensitivity of the test

The sensitivity of the test was determined in water samples artificially contaminated with decreasing number of bacterial cells, following the requirements of ISO 22118. The sensitivity was tested in three water samples. The sample' concentration was performed according to ISO 11731:1998, with 1 L of each sample, after inoculation with and 102-103, 103-104 104-105 105-106 cfu *Legionella pneumophila* (strain DSM 7513). All procedures starting from DNA extraction were performed in triplicate.

Result:
Target amplification was observed in samples contaminated with at least 103-104 cfu/1 L. No amplification was obtained in non-contaminated samples (inoculated with 0 cfu). Thus, the method can detect 103-104 cfu in 1 L of water sample or in a swab (LOD).

5. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 5.1, using two positive and two negative samples and performed in triplicate.

Parameter	N° variations	Description
Annealing temperature	2	+ 2ºC; - 2ºC
MgCl2 concentration	2	+ 0,5mM; - 0,5mM
Independent performer	2	Not applicable
Different Real Time equipment	3	ABI 7500 PikoReal, Thermo Scientific BioRad CFX96
Independent laboratories	2	Biopremier R&D laboratory Portuguese Food Safety Authority laboratory

Table 5.1 – Method's parameters submitted to variations

Result:
Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

6. TRUENESS

Trueness of the method was evaluated using 3 positive and 2 negative water samples and 3 positive and 2 negative swabs for the target.

All the samples were tested in triplicate.

Result:
All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%

Performance Characteristics for validation
The determination of false positive and false negative rate, the method was performed in 5 water samples 5 swabs (6 positive and 4 negative samples).

For the determination of method's limit of detection, the sample was inoculated considering 4 contamination levels: 102-103, 103-104 104-105 105-106 cfu in 1 L of water sample or in a swab. All the procedures were performed as defined for the present method. All experiments starting from DNA extraction were performed in triplicate.

1. FALSE POSITIVE RATE

This corresponds to the probability that a known negative sample has been classified as positive by the method. The false positive rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fpos}=n_{fpos}/n_{rneg}+n_{fpos}$ x 100%

In which:

P_{fpos}: False positive rate
n_{rneg}: Number of real negative test results
n_{fpos}: Number of misclassified known negative samples

Result:
For the present method, false positive rate was calculated based on 10 samples. The final result was 0%

2. FALSE NEGATIVE RATE

This corresponds to the probability that a known positive sample has been classified as negative by the method. The false negative rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fneg}=n_{fneg}/n_{rpos}+n_{fneg}$ x 100%

In which:

P_{fneg}: False negative rate

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:
For the present method, false positive rate was calculated based on 10 samples. The final result was 0%

3. LIMIT OF DETECTION

3.1. Limit of detection of the Real-Time PCR reaction

The limit of detection of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 10 ng, 5ng, 1 ng, 500 pg, 50 pg, 5 pg, 500 fg, 50 fg, 25 fg and 5 fg.

Result:
The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 5 pg of the target genomic DNA for Legionella pneumophila.

3.2. Limit of detection of the method

The limit of detection of the test was determined in water samples and swabs artificially contaminated with decreasing number of bacterial cells, following the requirements of ISO 22118, using three water samples. For water, the samples' concentration was performed according to ISO 11731:1998, with 1 L of each sample, after inoculation with and 10^2-10^3, 10^3-10^4 10^4-10^5 10^5-10^6 cfu of *Legionella pneumophila* (strain DSM 7513). All procedures starting from DNA extraction were performed in triplicate.

Result:
The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 103-104 cfu of the target for *Legionella pneumophila*.

Positive predictive value (PPV)
The PPV is calculated by the expression:

$PPV=n_{rpos}/n_{rpos}+n_{fpos} \times 100\%$

In which:
n_{rpos}: Number of real positive test results
n_{fneg}: Number of misclassified known positive samples

Result:
The positive predictive value determined was 100%.

Negative predictive value (NPV)
The PPV is calculated by the expression:

$NPV=n_{rneg}/n_{rneg}+n_{fneg} \times 100\%$
In which:
n_{rneg}: Number of real negative test results
n_{fneg}: Number of misclassified known negative samples

Result:
The negative predictive value determined was 100%.

Criteria for acceptance

Inclusivity: 95%

Exclusivity: 95%

Selectivity: 95%

Trueness: 95%

False positive rate: 5%

False negative rate: 5%

Performance declaration

Accordingly, with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results in agreement with the Condalab requirements and is validated.

The present test allows the "Detection of Legionella pneumophila using real time-PCR" in water and swab samples.

Condalab

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Simultaneous detection of *Legionella* spp. and *Legionella pneumophila* using real time-PCR

VALIDATION REPORT

ASSAY

Simultaneous detection of *Legionella spp.* and *Legionella pneumophila* using real time-PCR.

VALIDATED EQUIPMENT

Applied Biosystems, 7500 model; Thermo Scientific, model PikoReal.

SAMPLES (N)

Water and swabs. During re-validation samples from external quality assessment schemes from FAPAS.

VALIDATION ASSAYS

Start – 19-12-2014
Finish – 02-03-2015

DEFINITIONS

Analyte

DNA of the microorganism detected by the analysis method

Qualitative method

Analysis method which response is either the presence or absence of the microorganism either directly or indirectly in a certain amount of sample.

True positive (TP)

Sample presenting a positive result for the test and that has equal or higher amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

True negative (TN)

Sample presenting a negative result for the test and that has a lower amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

False Positive (FP)

Sample presenting a positive result for the test in the absence of target analyte.

False Negative (FN)

Sample presenting a negative result for the test in the presence of equal or higher amount of the target analyte than the detection limit.

Detection Limit (LOD)

- Reaction detection limit: lowest concentration of the target DNA that can be consistently detected in the specified experimental conditions.
- Method reaction limit: lowest concentration or content of the target microorganism relative to the defined amount of matrix that can be consistently detected in the specified experimental conditions

Inclusivity

Detection of the target microorganism.

Exclusivity

Non-detection of non-target microorganisms.

Selectivity

Measure of the inclusivity and exclusivity.

Sensitivity (S)

Measure of the lowest number of analyte cells which can be detected in a single test reaction.

Specificity (Sp)

Ability to exclusivity recognize the target microorganism to be detected and distinguish it from other similar organisms and impurities.

Trueness (T)

Closeness of agreement between the expectation of a test result and the corresponding true value.

Precision (P)

Closeness of agreement between independent tests obtained under defined conditions. It depends only on the distribution of random errors and does not relate to the true value of the test.

Positive predictive value (PPV)

Probability of a positive test be referent to a sample presenting an analyte concentration above.

Negative predictive value (NPV)

Probability of a positive test be referent to a sample presenting an analyte concentration above.

SCOPE OF THE METHOD

Nowadays, 47 species and more than 60 serogroups of Legionella spp. have been recognized. Legionnaires' disease is the most severe form of infection and the vast majority of Legionnaires' disease cases are due to L. pneumophila, being serogroup 1 responsible for up to 80% of reported cases.

The present method is based on a 5' nuclease real-time PCR reactions that allows the simultaneous amplification of a unique genomic sequence of Legionella spp. and a unique genomic sequence of Legionella pneumophila, aiming the detection of these pathogens DNA in water and other environmental samples. The method was designed to detect all Legionella species and, among them, to distinguish alll Legionella pneumophila serogroups from the other Legionella species. The test is designed for the detection of the target microorganism DNA in water samples after concentration by filtration and other environmental samples (e.g. sampled with swabs). It can also be used with other samples. Is not suitable for human and clinical diagnostics.

The method's performance was tested based on ISO 22118:2011 and fulfils the requirements set in this International Standard.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

Two probes labeled with different fluorophore are used to allow the simultaneous detection of Legionella spp. and Legionella pneumophila. The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a third fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 5 water samples concentrated according the method described in ISO 11731:1998 and 5 swab samples. The water samples included water taken from different public sources.

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing genomic DNA of the Legionella pneumophila, strain DSM 7513^T

Negative control (NC): reaction without any target DNA

Each individual reaction includes:

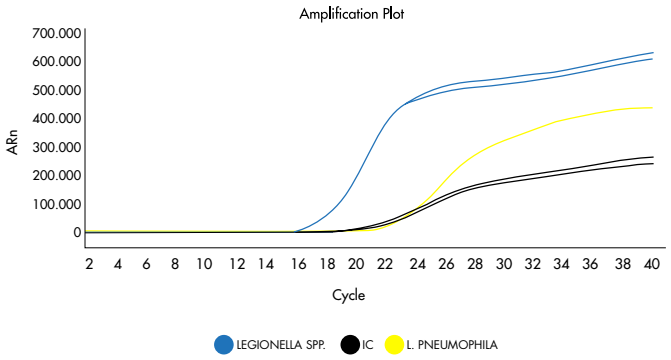
Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

	Target detection Chanels 1 and 2	Target detection Chanel 3
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

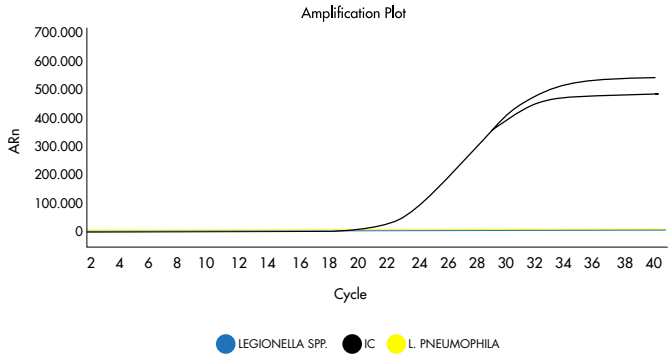
To validate the tests, the controls must show the following results:

If the controls do not match these results, the experiment must be repeated.

Positive control



Negative control



Target detection Channel 1 (*Legionella* spp)	Target detection Chanel 2 (*L. pneumophila*)	Target detection Channel 3 (IC)	Interpretation
Positive	Positive	Not significant	Positive for *L. pneumophila*
Positive	Ct = NA	Not significant	Positive for Legionella spp. But negative for *L. pneumophila*
Ct = NA	Ct = NA	Positive	Negative
Ct = NA	Ct = NA	Ct = NA	Inhibition**

The results obtained by the present method shall be interpreted as follows:

**When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.

2. INSTRUMENTS

Real Time instruments must be equipped with FAM, HEX/JOE and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).

The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific). Comparable results were obtained with CFX96 (BioRad).

3. SELECTIVITY

3.1. Inclusivity test

A total of 30 *Legionella* spp. strains were tested. The strains were tested using 2 ng of genomic DNA.

Code	Strain identification	Other informations
MB 108	*Legionella micdadei*	
MB 278	*Legionella micdadei*	
MB 274	*Legionella pneumophila*	DSM 7513T
MB 107	*Legionella pneumophila*	
MB 281	*Legionella pneumophila*	SG1 Philadelphia
MB 282	*Legionella pneumophila*	ATCC 33216, SG1 Knoxville
MB 286	*Legionella pneumophila*	SG5 Dallas
MB 288	*Legionella pneumophila*	SG7 Chicago
MB 293	*Legionella pneumophila*	SG11
MB 294	*Legionella pneumophila*	SG13
MB 296	*Legionella pneumophila*	SG15
MB 572	*Legionella pneumophila*	SG10
MB 573	*Legionella pneumophila*	SG7
MB 574	*Legionella pneumophila*	SG3
MB 575	*Legionella pneumophila*	SG3
MB 576	*Legionella pneumophila*	SG9
MB 577	*Legionella pneumophila*	SG1
MB 578	*Legionella pneumophila*	SG5
MB 579	*Legionella pneumophila*	SG5
MB 580	*Legionella pneumophila*	SG10
MB 581	*Legionella pneumophila*	SG14
MB 582	*Legionella pneumophila*	SG4
MB 583	*Legionella pneumophila*	SG8
MB 584	*Legionella pneumophila*	SG1
MB 585	*Legionella pneumophila*	SG1
MB 586	*Legionella pneumophila*	SG4
MB 587	*Legionella pneumophila*	SG9
MB 588	*Legionella pneumophila*	SG3
MB 589	*Legionella pneumophila*	SG10
MB 590	*Legionella pneumophila*	SG8

Table 3.1 – List of strains used for inclusivity test

Result:

All strains of *Legionella* spp. and *Legionella pneumophila*, respectively, were detected with the present method, corresponding to 100% inclusivity.

3.2. Exclusivity test

The strains for exclusivity test were chosen following the recommendations of International Standard ISO 22118:2011. A total of 30 non target microorganisms related with the target or occurring in the same habitat and that could cause interference with test results were de tested (Table 3.1). The strains were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the 16S rRNA bacterial gene, using universal primers. The identification of all strains was confirmed by 16S rRNA gene sequencing. All strains were tested in triplicate.

Code	Strain identification	Other informations
MB 385	*Listeria monocytogenes*	CECT 4031
MB 388	*Listeria monocytogenes*	Serotype 1/2a ou 3a
MB 381	*Listeria innocua*	CECT 910

MB 240	*Escherichia coli O157:H7*	CDC 337
MB 357	*Escherichia coli*	NCTC 9001
MB 237	*Escherichia coli*	Local isolate
MB 372	*Campylobacter jejuni subsp jejuni*	NCTC 11351
MB 373	*Campylobacter coli*	NCTC 11366
MB 22	*Vibrio cholerae*	Local isolate
MB 356	*Vibrio parahaemolyticus*	NCTC 10885
MB 363	*Bacillus cereus*	IFM1600
MB 115	*Citrobacter freundii*	Local isolate
MB 451	*Cronobacter muytjensii*	ATCC 51329
MB 141	*Lactobacillus plantarum*	CECT 748 T
MB 144	*Lactobacillus paracasei subsp paracasei*	CECT 4022T
MB 150	*Staphylococcus aureus*	NCTC 6571
MB 302	*Pseudomonas aeruginosa*	Local isolate
MB 13	*Serratia marcescens*	Local isolate
MB 163	*Proteus vulgaris*	Local isolate
MB 365	*Enterococcus faecalis*	Local isolate
MB 306	*Enterococcus hirae*	Local isolate
MB 147	*Staphylococcus epidermidis*	Local isolate
MB 267	*Shigella flexneri*	Local isolate
MB 249	*Yersinia enterocolitica*	Local isolate
MB 178	*Klebsiella pneumoniae*	Local isolate
MB 449	*Brochothrix thermosphacta*	ATCC 11509
MB 164	*Salmonella Enteritidis*	Local isolate
MB 399	*Salmonella Typhimurium*	CECT 443
MF 128	*Saccharomyces cerevisae*	CECT 12928
MF 129	*Aspergillus niger*	Local isolate

Table 3.2 – List of strains used for exclusivity test

Result:
The method presented 100% exclusivity as none of the strains showed a positive results and positive result.

4. SENSITIVITY

4.1. Sensitivity of the Real Time-PCR reaction

The sensitivity of the reaction was determined by testing in triplicate decreasing quantities of Legionella pneumophila (strain DSM 7513) DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1 ng, 500 pg, 50 pg, 5 pg, 500 fg, 50 fg, 25 fg and 5 fg.

Result:
Target amplification was observed with DNA amounts of 500 fg -5pg or higher for both detection of *Legionella* spp. and *Legionella pneumophila*. Thus, the limit of detection of the reaction was established in 500 fg - 5pg of target genomic DNA.

4.2. Sensitivity of the test

The sensitivity of the test was determined in water samples artificially contaminated with decreasing number of bacterial cells, following the requirements of ISO 22118. The sensitivity was tested in three water samples. The sample' concentration was performed according to ISO 11731:1998, with 1 L of each sample, after inoculation with and 10^2-10^3, 10^3-10^4 10^4-10^5 10^5-10^6 cfu *Legionella pneumophila* (strain DSM 7513). All procedures starting from DNA extraction were performed in triplicate.

Result:
Target amplification was observed in samples contaminated with at least 10^3-10^4 cfu/1 L for both Legionella spp. and Legionella pneumophila. No amplification was obtained in non-contaminated samples (inoculated with 0 cfu). Thus, the method can detect 10^3-10^4 cfu in 1 L of water sample or in a swab (LOD).

5. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 5.1, using two positive and two negative samples and performed in triplicate.

Parameter	Nº variations	Description
Annealing temperature	2	+ 2ºC; - 2ºC
MgCl2 concentration	2	+ 0,5mM; - 0,5mM
Independent performer	2	Not applicable
Different Real Time equipment	3	ABI 7500 PikoReal, Thermo Scientific BioRad CFX96
Independent laboratories	2	Biopremier R&D laboratory Portuguese Food Safety Authority laboratory

Table 5.1 – Method's parameters submitted to variations

Result:

Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

6. TRUENESS

Trueness of the method was evaluated using 3 positive and 2 negative water samples and 3 positive and 2 negative swabs for the target.

All the samples were tested in triplicate.

Result:

All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%

Performance Characteristics for validation

The determination of false positive and false negative rate, the method was performed in 5 water samples 5 swabs (6 positive and 4 negative samples).

For the determination of method's limit of detection, the sample was inoculated considering 4 contamination levels: 10^2-10^3, 10^3-10^4 10^4-10^5 10^5-10^6 cfu in 1 L of water sample or in a swab. All the procedures were performed as defined for the present method. All experiments starting from DNA extraction were performed in triplicate.

1. FALSE POSITIVE RATE

This corresponds to the probability that a known negative sample has been classified as positive by the method. The false positive rate (that can be expressed as a percentage) is calculated by the expression:

$$P_{fpos}=n_{fpos}/n_{rneg}+n_{fpos} \times 100\%$$

In which:

P_{fpos}: False positive rate
n_{rneg}: Number of real negative test results
n_{fpos}: Number of misclassified known negative samples

Result:

For the present method, false positive rate was calculated based on 10 samples. The final result was 0%

2. FALSE NEGATIVE RATE

This corresponds to the probability that a known positive sample has been classified as negative by the method. The false negative rate (that can be expressed as a percentage) is calculated by the expression:

$$P_{fneg}=n_{fneg}/n_{rpos}+n_{fneg} \times 100\%$$

In which:

P_{fneg}: False negative rate

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:

For the present method, false positive rate was calculated based on 10 samples. The final result was 0%

3. LIMIT OF DETECTION

3.1. Limit of detection of the Real-Time PCR reaction

The limit of detection of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1 ng, 500 pg, 50 pg, 5 pg, 500 fg, 50 fg, 25 fg and 5 fg.

Result:

The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 500 fg – 5 pg of the target genomic DNA for both *Legionella* spp. and *Legionella pneumophila*.

3.2. Limit of detection of the method

The limit of detection of the test was determined in water samples and swabs artificially contaminated with decreasing number of bacterial cells, following the requirements of ISO 22118, using three water samples. For water, the samples' concentration was performed according to ISO 11731:1998, with 1 L of each sample, after inoculation with and 10^2-10^3, 10^3-10^4 10^4-10^5 10^5-10^6 cfu of Legionella pneumophila (strain DSM 7513). All procedures starting from DNA extraction were performed in triplicate.

Result:

The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 103-104 cfu of the target for both *Legionella* spp. and *Legionella pneumophila*.

Positive predictive value (PPV)

The PPV is calculated by the expression:

$$PPV=n_{rpos}/n_{rpos}+n_{fpos} \times 100\%$$

In which:

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:

The positive predictive value determined was 100%.

Negative predictive value (NPV)

The PPV is calculated by the expression:

$$NPV=n_{rneg}/n_{rneg}+n_{fneg} \times 100\%$$

In which:

n_{rneg}: Number of real negative test results

n_{fneg}: Number of misclassified known negative samples

Result:

The negative predictive value determined was 100%.

Criteria for acceptance

Inclusivity: 95%

Exclusivity: 95%

Selectivity: 95%

Trueness: 95%

False positive rate: 5%

False negative rate: 5%

Performance declaration

Accordingly, with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results in agreement with the Condalab requirements and is validated.

The present test allows the "Simultaneous detection of Legionella spp. and Legionella pneumophila using real time-PCR" in water and swab samples.

Condalab

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Detection of *Legionella* spp. using real-time PCR

VALIDATION REPORT

ASSAY

Detection of *Legionella* spp. using real time-PC

VALIDATED EQUIPMENT

Applied Biosystems, model 7500; Thermo Scientific, model PikoReal; Condalab, model CDL-96.

SAMPLES (N)

Enrichments prepared from suitable food samples. During re-validation samples from external quality assessment schemes from FAPAS, Instand and INSA-PHE were tested.

VALIDATION ASSAYS

Start – 19-12-2014
Finish – 02-03-2015

DEFINITIONS

Analyte

DNA of the microorganism detected by the analysis method

Qualitative method

Analysis method which response is either the presence or absence of the microorganism either directly or indirectly in a certain amount of sample.

True positive (TP)

Sample presenting a positive result for the test and that has equal or higher amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

True negative (TN)

Sample presenting a negative result for the test and that has a lower amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

False Positive (FP)

Sample presenting a positive result for the test in the absence of target analyte.

False Negative (FN)

Sample presenting a negative result for the test in the presence of equal or higher amount of the target analyte than the detection limit.

Detection Limit (LOD)

- Reaction detection limit: lowest concentration of the target DNA that can be consistently detected in the specified experimental conditions.
- Method reaction limit: lowest concentration or content of the target microorganism relative to the defined amount of matrix that can be consistently detected in the specified experimental conditions.

Inclusivity

Detection of the target microorganism.

Exclusivity

Non-detection of non-target microorganisms.

Selectivity

Measure of the inclusivity and exclusivity.

Sensitivity (S)

Measure of the lowest number of analyte cells which can be detected in a single test reaction.

Specificity (Sp)

Ability to exclusivity recognize the target microorganism to be detected and distinguish it from other similar organisms and impurities.

Trueness (T)

Closeness of agreement between the expectation of a test result and the corresponding true value.

Precision (P)

Closeness of agreement between independent tests obtained under defined conditions. It depends only on the distribution of random errors and does not relate to the true value of the test.

Positive predictive value (PPV)

Probability of a positive test be referent to a sample presenting an analyte concentration above.

Negative predictive value (NPV)

Probability of a positive test be referent to a sample presenting an analyte concentration above.

SCOPE OF THE METHOD

Nowadays, 47 species and more than 60 serogroups of Legionella spp. have been recognized. Legionnaires' disease is the most severe form of infection and the vast majority of Legionnaires' disease cases are due to L. pneumophila, being serogroup 1 responsible for up to 80% of reported cases.
The present method is based on a 5' nuclease real-time PCR reactions that allows the amplification of a unique genomic sequence of *Legionella* spp., aiming the detection of this pathogen DNA in water and other environmental samples. The method was designed to detect all *Legionella* species. The test is designed for the detection of the target microorganism DNA in water samples after concentration by filtration and other environmental samples (e.g. sampled with swabs). It can also be used with other samples. Is not suitable for human and clinical diagnostics.
The method's performance was tested based on ISO 22118:2011 and fulfils the requirements set in this International Standard.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

The probe labeled with a fluorophore are used to allow the detection of Legionella spp.. The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a different fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 5 water samples concentrated according the method described in ISO 11731:1998 and 5 swab samples. The water samples included water taken from different public sources

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing genomic DNA of the Legionella pneumophila, strain DSM 7513T

Negative control (NC): reaction without any target DNA

Each individual reaction includes:

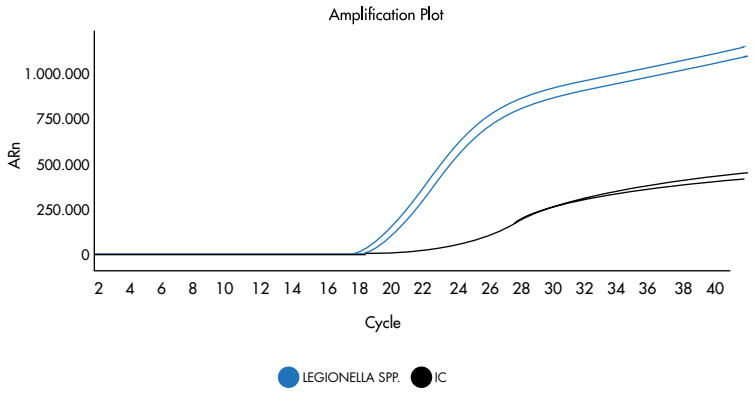
Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

To validate the tests, the controls must show the following results:

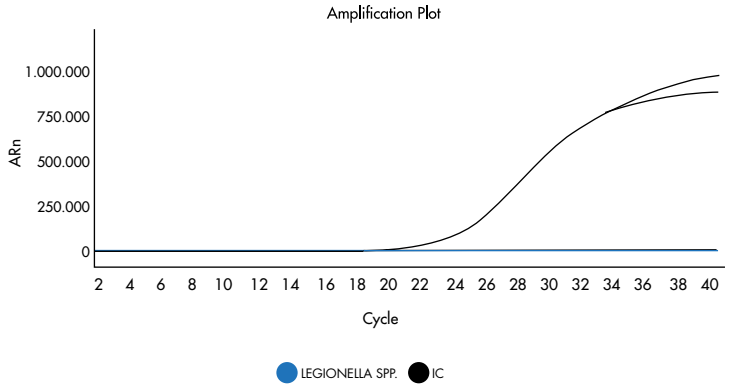
	Target detection Chanel 1	**Target detection Chanel 2**
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

If the controls do not match these results, the experiment must be repeated.

Positive control



Negative control



The results obtained by the present method shall be interpreted as follows:

Target detection Chanel 1 (*Legionella* spp)	**Target detection Chanel 2 (IC)**	**Interpretation**
Positive	Not significant	Positive for *Legionella* spp.
Ct = NA	Positive	Negative
Ct = NA	Ct = NA	Inhibition**

**When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.

2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).

The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific). Comparable results were obtained with CFX96 (BioRad).

3. SELECTIVITY

3.1. Inclusivity test

Code	Strain identification	Other informations
MB 108	*Legionella micdadei*	
MB 278	*Legionella micdadei*	
MB 274	*Legionella pneumophila*	DSM 7513T
MB 107	*Legionella pneumophila*	
MB 281	*Legionella pneumophila*	SG1 Philadelphia
MB 282	*Legionella pneumophila*	ATCC 33216, SG1 Knoxville
MB 286	*Legionella pneumophila*	SG5 Dallas
MB 288	*Legionella pneumophila*	SG7 Chicago
MB 293	*Legionella pneumophila*	SG11
MB 294	*Legionella pneumophila*	SG13
MB 296	*Legionella pneumophila*	SG15
MB 572	*Legionella pneumophila*	SG10
MB 573	*Legionella pneumophila*	SG7
MB 574	*Legionella pneumophila*	SG3
MB 575	*Legionella pneumophila*	SG3
MB 576	*Legionella pneumophila*	SG9
MB 577	*Legionella pneumophila*	SG1
MB 578	*Legionella pneumophila*	SG5
MB 579	*Legionella pneumophila*	SG5
MB 580	*Legionella pneumophila*	SG10
MB 581	*Legionella pneumophila*	SG14
MB 582	*Legionella pneumophila*	SG4
MB 583	*Legionella pneumophila*	SG8
MB 584	*Legionella pneumophila*	SG1
MB 585	*Legionella pneumophila*	SG1
MB 586	*Legionella pneumophila*	SG4
MB 587	*Legionella pneumophila*	SG9
MB 588	*Legionella pneumophila*	SG3
MB 589	*Legionella pneumophila*	SG10
MB 590	*Legionella pneumophila*	SG8

Table 3.1 – List of *Legionella* spp. strains used for inclusivity test

Result:

All strains were detected with the present method, corresponding to 100% inclusivity.

A total of 30 *Legionella* spp. strains were tested. The strains were tested using 2 ng of genomic DNA.

3.2. Exclusivity test

The strains for exclusivity test were chosen following the recommendations of International Standard ISO 22118:2011. A total of 30 non target microorganisms related with the target or occurring in the same habitat and that could cause interference with test results were de tested (Table 3.1). The strains were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the 16S rRNA bacterial gene, using universal primers. The identification of all strains was confirmed by 16S rRNA gene sequencing. All strains were tested in triplicate.

Code	Strain identification	Other informations
MB 385	*Listeria monocytogenes*	CECT 4031
MB 388	*Listeria monocytogenes*	Serotype 1/2a ou 3a
MB 381	*Listeria innocua*	CECT 910
MB 240	*Escherichia coli O157:H7*	CDC 337
MB 357	*Escherichia coli*	NCTC 9001
MB 237	*Escherichia coli*	Local isolate
MB 372	*Campylobacter jejuni subsp jejuni*	NCTC 11351
MB 373	*Campylobacter coli*	NCTC 11366
MB 22	*Vibrio cholerae*	Local isolate
MB 356	*Vibrio parahaemolyticus*	NCTC 10885
MB 363	*Bacillus cereus*	IFM1600
MB 115	*Citrobacter freundii*	Local isolate
MB 451	*Cronobacter muytjensii*	ATCC 51329
MB 141	*Lactobacillus plantarum*	CECT 748 T
MB 144	*Lactobacillus paracasei subsp paracasei*	CECT 4022T
MB 150	*Staphylococcus aureus*	NCTC 6571
MB 302	*Pseudomonas aeruginosa*	Local isolate
MB 13	*Serratia marcescens*	Local isolate
MB 163	*Proteus vulgaris*	Local isolate
MB 365	*Enterococcus faecalis*	Local isolate
MB 306	*Enterococcus hirae*	Local isolate
MB 147	*Staphylococcus epidermidis*	Local isolate
MB 267	*Shigella flexneri*	Local isolate
MB 249	*Yersinia enterocolitica*	Local isolate
MB 178	*Klebsiella pneumoniae*	Local isolate
MB 449	*Brochothrix thermosphacta*	ATCC 11509
MB 164	*Salmonella Enteritidis*	Local isolate
MB 399	*Salmonella Typhimurium*	CECT 443
MF 128	*Saccharomyces cerevisae*	CECT 12928
MF 129	*Aspergillus niger*	Local isolate

Table 3.2 – List of strains used for exclusivity test

Result:

The method presented 100% specificity as none of the species showed a positive result.

4. SENSITIVITY

4.1. Sensitivity of the Real-Time PCR reaction

The sensitivity of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 10 ng, 5 ng, 1 ng, 500 pg, 50 pg, 5 pg, 500

fg, 50 fg, 25 fg and 5 fg.

Result:
Target amplification was observed with DNA amounts of 5pg or higher. Thus, the limit of detection of the reaction was established in 5pg of target genomic DNA.

4.2. Sensitivity of the test

The sensitivity of the test was determined in water samples artificially contaminated with decreasing number of bacterial cells, following the requirements of ISO 22118. The sensitivity was tested in three water samples. The sample' concentration was performed according to ISO 11731:1998, with 1 L of each sample, after inoculation with and 10^2-10^3, 10^3-10^4 10^4-10^5 10^5-10^6 cfu Legionella pneumophila (strain DSM 7513). All procedures starting from DNA extraction were performed in triplicate.

Result:
Target amplification was observed in samples contaminated with at least 103-104 cfu/1 L for both Legionella spp. and Legionella pneumophila. No amplification was obtained in non-contaminated samples (inoculated with 0 cfu). Thus, the method can detect 10^3-10^4 cfu in 1 L of water sample or in a swab (LOD).

5. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 5.1, using two positive and two negative samples and performed in triplicate.

Parameter	N° variations	Description
Annealing temperature	2	+ 2°C; - 2°C
MgCl2 concentration	2	+ 0,5mM; - 0,5mM
Independent performer	2	Not applicable
Different Real Time equipment	3	ABI 7500 PikoReal, Thermo Scientific BioRad CFX96
Independent laboratories	2	Biopremier R&D laboratory Portuguese Food Safety Authority laboratory

Table 5.1 – Method's parameters submitted to variations

Result:
Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

6. TRUENESS

Trueness of the method was evaluated using 3 positive and 2 negative water samples and 3 positive and 2 negative swabs for the target.

All the samples were tested in triplicate.

Result:
All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%

Performance Characteristics for validation

The determination of false positive and false negative rate, the method was performed in 5 water samples 5 swabs (6 positive and 4 negative samples).
For the determination of method's limit of detection, the sample was inoculated considering 4 contamination levels: 10^2-10^3, 10^3-10^4 10^4-10^5 10^5-10^6 cfu in 1 L of water sample or in a swab. All the procedures were performed as defined for the present method. All experiments starting from DNA extraction were performed in triplicate.

1. FALSE POSITIVE RATE

This corresponds to the probability that a known negative sample has been classified as positive by the method. The false positive rate (that can be expressed as a percentage) is calculated by the expression:
$P_{fpos}=n_{fpos}/n_{rneg}+n_{fpos}$ x 100%

In which:
P_{fpos}: False positive rate
n_{rneg}: Number of real negative test results
n_{fpos}: Number of misclassified known negative samples

Result:
For the present method, false positive rate was calculated based on 10 samples. The final result was 0%

2. FALSE NEGATIVE RATE

This corresponds to the probability that a known positive sample has been classified as negative by the method. The false negative rate (that can be expressed as a percentage) is calculated by the expression:

P_{fneg}=nfneg/n_{rneg}+n_{fneg} x 100%
In which:

P_{fneg}: False negative rate
n_{rpos}: Number of real positive test results
n_{fneg}: Number of misclassified known positive samples

Result:
For the present method, false positive rate was calculated based on 10 samples. The final result was 0%

3. LIMIT OF DETECTION

3.1. Limit of detection of the Real-Time PCR reaction

The limit of detection of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 100 pg, 50 pg, 10 pg, 5 pg, 1pg, 500 fg. 50 fg, 10 fg, 5 fg.

Result:
The limit of detection determined with a probability 0,95% (false positive rate < 0,05) was 5 fg of the target genomic DNA.

3.2. Limit of detection of the method

The limit of detection of the test was determined in water samples and swabs artificially contaminated with decreasing number of bacterial cells, following the requirements of ISO 22118, using three water samples. For water, the samples' concentration was performed according to ISO 11731:1998, with 1 L of each sample, after inoculation with and 10^2-10^3, 10^3-10^4 10^4-10^5 10^5-10^6 cfu of *Legionella pneumophila* (strain DSM 7513). All procedures starting from DNA extraction were performed in triplicate.

Result:
The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 103-104 cfu of the target.

Positive predictive value (PPV)

The PPV is calculated by the expression:
PPV=n_{rpos}/n_{rpos}+n_{fpos} x 100%
In which:
n_{rpos}: Number of real positive test results
n_{fneg}: Number of misclassified known positive samples

Result:
The positive predictive value determined was 100%.

Negative predictive value (NPV)

The PPV is calculated by the expression:
NPV=n_{rneg}/n_{rneg}+n_{fneg} x 100%
In which:
n_{rneg}: Number of real negative test results
n_{fneg}: Number of misclassified known negative samples

Result:
The negative predictive value determined was 100%.

Criteria for acceptance
Inclusivity: 95%
Exclusivity: 95%
Selectivity: 95%
Trueness: 95%
False positive rate: 5%
False negative rate: 5%

4. EXTERNAL QUALITY ASSESSMENT SCHEMES

INSTAND 2017:

Positive for Legionella pneumophila 5x104 CFU/mL and Legionella pneumophila 5x103 CFU/mL

FAPAS 2019:

Positive for Legionella pneumophila 20032 CFU/L and Legionella pneumophila 23249 CFU/L

Performance declaration

Accordingly, with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results in agreement with the Condalab requirements and is validated.
The present test allows the "*Detection of Legionella spp. using real time-PCR*" in water and swab samples".

Condalab

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Detection of *Listeria monocytogenes* using real-time PCR

VALIDATION REPORT

ASSAY

Listeria monocytogenes detection by 5' nuclease real time-PCR assay.

VALIDATED EQUIPMENT

Applied Biosystems, model 7500; Thermo Scientific, model PikoReal; Condalab, model CDL-96.

SAMPLES (N)

Enrichments prepared from suitable food samples. During re-validation samples from external quality assessment schemes from FAPAS, Instand and INSA-PHE were tested.

VALIDATION ASSAYS

Start – 01-10-2013
Finish – 01-05-2014

DEFINITIONS

Analyte

DNA of the microorganism detected by the analysis method

Qualitative method

Analysis method which response is either the presence or absence of the microorganism either directly or indirectly in a certain amount of sample.

True positive (TP)

Sample presenting a positive result for the test and that has equal or higher amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

True negative (TN)

Sample presenting a negative result for the test and that has a lower amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

False Positive (FP)

Sample presenting a positive result for the test in the absence of target analyte.

False Negative (FN)

Sample presenting a negative result for the test in the presence of equal or higher amount of the target analyte than the detection limit.

Detection Limit (LOD)

- Reaction detection limit: lowest concentration of the target DNA that can be consistently detected in the specified experimental conditions.
- Method reaction limit: lowest concentration or content of the target microorganism relative to the defined amount of matrix that can be consistently detected in the specified experimental conditions.

Inclusivity

Detection of the target microorganism.

Exclusivity

Non-detection of non-target microorganisms.

Selectivity

Measure of the inclusivity and exclusivity.

Sensitivity (S)

Measure of the lowest number of analyte cells which can be detected in a single test reaction.

Specificity (Sp)

Ability to exclusivity recognize the target microorganism to be detected and distinguish it from other similar organisms and impurities.

Trueness (T)

Closeness of agreement between the expectation of a test result and the corresponding true value.

Precision (P)

Closeness of agreement between independent tests obtained under defined conditions. It depends only on the distribution of random errors and does not relate to the true value of the test.

Positive predictive value (PPV)

Probability of a positive test be referent to a sample presenting an analyte concentration above.

Negative predictive value (NPV)

Probability of a positive test be referent to a sample presenting an analyte concentration above.

SCOPE OF THE METHOD

The present method is based on a 5' nuclease real-time PCR reactions and amplify a unique genomic sequence of *Listeria monocytogenes*, aiming the detection of this pathogen DNA in food samples.
The test is designed for the detection of the target microorganism DNA in food products after enrichment in buffered peptone water and DNA extraction. It can also be used with animal feedstuff and environmental samples. Is not suitable for human and clinical diagnostics.
The method's performance was tested according ISO 22118:2011 and ISO 16140:2003 and fulfils the requirements set in this International Standard.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a second fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 42 food samples enriched according the method described in ISO 11290:2004 / 11290:2017. These food samples included 14 different matrices, aleatory taken from commercial shops:

- Meat
- Salad
- Vegetables
- Icecream
- Cheese
- Pizza
- Pastry
- Butter
- Cooked meal
- Processed meat
- Yogurt
- Codfish
- Herbs mix
- Fish

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing genomic DNA of the Listeria monocytogenes

Negative control (NC): reaction without any target DNA

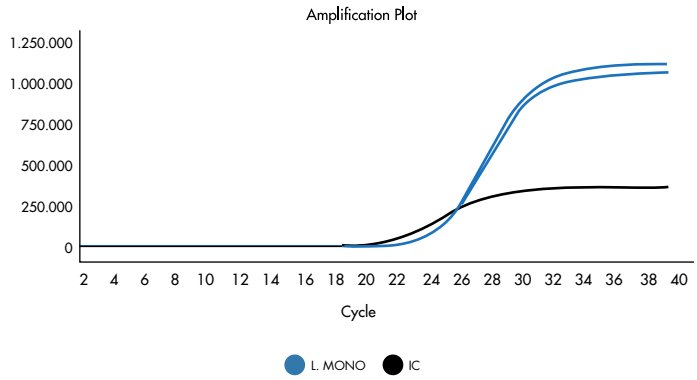
Each individual reaction includes:

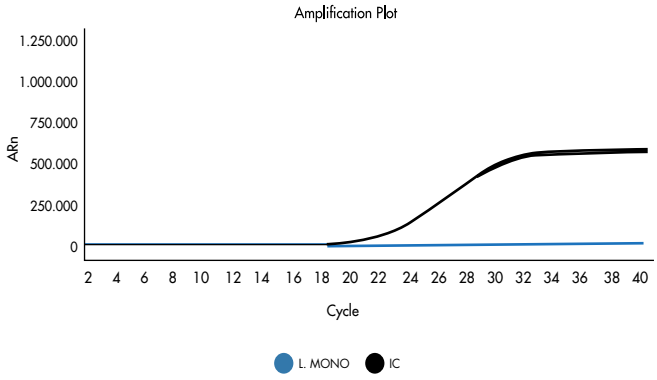
Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

To validate the tests, the controls must show the following results:

	Target detection Chanel 1	Target detection Chanel 2
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

If the controls do not match these results, the experiment must be repeated.





The results obtained by the present method shall be interpreted as follows:

Target detection Chanel 1	Target detection Chanel 2	Interpretation
Positive	Not significant	Positive
Ct = NA	Positive	Negative
Ct = NA	Ct = NA	Inhibition**

When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.

2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).

The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific). Comparable results were obtained with CFX96 (BioRad).

3. SELECTIVITY

3.1. Inclusivity test

A total of 23 Listeria monocytogenes strains were tested (Table 3.1). The strains were tested using 2 ng of genomic DNA.

Code	Strain identification	Other informations
MB 59	*Listeria monocytogenes*	CECT 911T
MB 60	*Listeria monocytogenes*	CECT 937T
MB 62	*Listeria monocytogenes*	SLU 1922
MB 63	*Listeria monocytogenes*	SLU 2157
MB 112	*Listeria monocytogenes*	CECT 4031T
MB 354	*Listeria monocytogenes 4b*	NCTC 11994
MB 385	*Listeria monocytogenes*	CECT 4031
MB 386	*Listeria monocytogenes 1/2a ou 3a*	
MB 387	*Listeria monocytogenes 1/2a ou 3a*	
MB 388	*Listeria monocytogenes 1/2a ou 3a*	
MB 389	*Listeria monocytogenes 1/2b ou 3b*	
MB 391	*Listeria monocytogenes 1/2b ou 3b*	
MB 392	*Listeria monocytogenes 1/2c ou 3c*	
MB 393	*Listeria monocytogenes 1/2c ou 3c*	
MB 394	*Listeria monocytogenes 1/2c ou 3c*	
MB 395	*Listeria monocytogenes 4a ou 4b*	CECT 934
MB 396	*Listeria monocytogenes 4c*	
MB 397	*Listeria monocytogenes 4c*	
MB 398	*Listeria monocytogenes 4c*	
MB 1003	*Listeria monocytogenes*	
MB 1004	*Listeria monocytogenes*	
MB 1005	*Listeria monocytogenes*	
MB 1006	*Listeria monocytogenes*	

Table 3.1 – List of *Listeria monocytogenes* strains used for inclusivity test

Result:

All strains were detected with the present method, corresponding to 100% inclusivity.

3.2. Exclusivity test

The strains for exclusivity test were chosen following the recommendations of International Standard ISO 22118:2011. A total of 30 non target microorganisms related with the target or occurring in the same habitat and that could cause interference with test results were de tested (Table 3.2). The strains were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the 16S rRNA bacterial gene, using universal primers. The identification of all strains was confirmed by 16S rRNA gene sequencing. All strains were tested in triplicate.

Code	Strain identification	Other informations
MB 372	*Campylobacter jejuni subsp jejuni*	NCTC 11351
Mb 164	*Salmonella enteritidis*	
MB 381	*Listeria innocua*	CECT 910
MB 240	*Escherichia coli O157:H7*	CDC 337
MB 357	*Escherichia coli*	NCTC 9001
MB 399	*Salmonella Typhimurium*	CECT 443
MB 373	*Campylobacter coli*	NCTC 11366
MB 22	*Vibrio cholerae*	Local isolate
MB 356	*Vibrio parahaemolyticus*	NCTC 10885
MB 363	*Bacillus cereus*	IFM1600
MB 115	*Citrobacter freundii*	Local isolate
MB 451	*Cronobacter muytjensii*	ATCC 51329
MB 141	*Lactobacillus plantarum*	CECT 748 T
MB 144	*Lactobacillus paracasei subsp paracasei*	CECT 4022T
MB 150	*Staphylococcus aureus*	NCTC 6571
MB 302	*Pseudomonas aeruginosa*	Local isolate
MB 13	*Serratia marcescens*	Local isolate
MB 163	*Proteus vulgaris*	Local isolate
MB 365	*Enterococcus faecalis*	Local isolate
MB 306	*Enterococcus hirae*	Local isolate
MB 147	*Staphylococcus epidermidis*	Local isolate
MB 267	*Shigella flexneri*	Local isolate
MB 249	*Yersinia enterocolitica*	Local isolate
MB 178	*Klebsiella pneumoniae*	Local isolate
MB 449	*Brochothrix thermosphacta*	ATCC 11509
MB 274	*Legionella pneumophila*	Local isolate
MB 278	*Legionella micdadei*	Local isolate
MB 362	*Clostridium perfringens*	Local isolate
MF 128	*Saccharomyces cerevisae*	CECT
MF 129	*Aspergillus niger*	Local isolate

Table 3.2 – List of strains used for exclusivity test

Result:

The method presented 100% specificity as none of the species showed a positive result.

4. SENSITIVITY

4.1. Sensitivity of the Real-Time PCR reaction

The sensitivity of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 10 ng, 5 ng, 1 ng, 500 pg, 50 pg, 5 pg, 500 fg, 50 fg, 25 fg and 5 fg.

Result:
Target amplification was observed with DNA amounts of 500 fg or higher. Thus, the limit of detection of the reaction was established in 500 fg of target genomic DNA.

4.2. Sensitivity of the test in DNA mixtures

The sensitivity of the test was determined in food samples artificially contaminated with decreasing number of bacterial cells, following the requirements of ISO 22118. The sensitivity was tested in five different food matrices: Chicken meat, salad, yogurt, cheese and cottage cheese. The enrichment was performed according to ISO 11290:1996 / ISO 11290:2017, with 25 g of each sample, after inoculation with 0, 1-10, 10-102 and 102-103 cfu of Listeria monocytogenes. All procedures starting from DNA extraction were performed in triplicate.

Result:
Target amplification was observed in samples contaminated with 1-10 cfu/25 g or more. No amplification was obtained in non-contaminated samples (inoculated with 0 cfu). Thus, the method can detect 1-10 cfu in 25 g of food sample (LOD).

5. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 5.1, using two positive and two negative samples and performed in triplicate.

Parameter	N° variations	Description
Annealing temperature	2	+ 2°C; - 2°C
MgCl2 concentration	2	+ 0,5mM; - 0,5mM
Independent performer	2	Not applicable
Different Real Time equipment	3	ABI 7500 PikoReal, Thermo Scientific BioRad CFX96
Independent laboratories	2	Biopremier R&D laboratory Portuguese Food Safety Authority laboratory

Table 5.1 – Method's parameters submitted to variations

Result:
Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

6. TRUENESS

Trueness of the method was evaluated using 7 positive and 11 negative food samples for the target, respectively, corresponding to the following food matrices:

- Meat
- Salad
- Vegetables
- Icecream
- Cheese
- Pizza
- Pastry
- Butter
- Cooked meal
- Processed meat
- Yogurt
- Codfish
- Herbs mix
- Fish

All the samples were tested in triplicate.

Result:
All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%

Performance Characteristics for validation

For the determination of false positive and false negative rate, the method was performed in 42 samples (24 positive and 18 negative samples), corresponding to 14 food matrices.

For the determination of method's limit of detection, the sample was inoculated considering 3 contamination levels: 0, 1 to 10 and 10 to 100 cfus in 25 g of sample. All the procedures were performed as defined for the present method. All experiments starting from DNA extraction were performed in triplicate.

1. FALSE POSITIVE RATE

This corresponds to the probability that a known negative sample has been classified as positive by the method. The false positive rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fpos}=n_{fpos}/n_{rneg}+n_{fpos}$ x 100%

In which:

P_{fpos}: False positive rate

n_{rneg}: Number of real negative test results

n_{fpos}: Number of misclassified known negative samples

Result:

For the present method, false positive rate was calculated based on 21 real samples. The final result was 0%

2. FALSE NEGATIVE RATE

This corresponds to the probability that a known positive sample has been classified as negative by the method. The false negative rate (that can be expressed as a percentage) is calculated by the expression:

P_{fneg}=nfneg/$n_{rneg}+n_{fneg}$ x 100%

In which:

P_{fneg}: False negative rate

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:

For the present method, false positive rate was calculated based on 21 real samples. The final result was 0%

3. LIMIT OF DETECTION

3.1. Limit of detection of the Real-Time PCR reaction

The limit of detection of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 100 pg, 50 pg, 10 pg, 5 pg, 1pg, 500 fg. 50 fg, 10 fg, 5 fg.

Result:

The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 500 fg of *Listeria monocytogenes* genomic DNA.

3.2. Limit of detection of the method

The limit of detection of the test was determined in food samples artificially contaminated with decreasing number of bacterial cells, following the requirements of ISO 22118, using five different food matrices: Chicken meat, salad, yogurt, cheese and cottage cheese. The enrichment was performed according to ISO 11290:2004 / 11290:2017, with 25 g of each sample, after inoculation with 0, 1-10, 10-10^2 and 10^2-10^3 cfu of *Listeria monocytogenes*. All procedures starting from DNA extraction were performed in triplicate.

Result:

The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 1-10 cfus of *Listeria monocytogenes*

Positive predictive value (PPV)

The PPV is calculated by the expression:

$PPV=n_{rpos}/n_{rpos}+n_{fpos}$ x 100%

In which:

n_{rpos}: Number of real positive test results
n_{fneg}: Number of misclassified known positive samples

Result:
The positive predictive value determined was 100%.

Negative predictive value (NPV)
The PPV is calculated by the expression:
$NPV=n_{rneg}/n_{rneg}+n_{fneg}$ x 100%
In which:
n_{rneg}: Number of real negative test results
n_{fneg}: Number of misclassified known negative samples

Result:
The negative predictive value determined was 100%.

Criteria for acceptance

Inclusivity: 95%

Exclusivity: 95%

Selectivity: 95%

Trueness: 95%

False positive rate: 5%

False negative rate: 5%

Performance declaration
Accordingly, with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results in agreement with the Condalab requirements and is validated.
The present test allows the *"Detection of Listeria monocytogenes using real time-PCR"* in food samples after enrichment.

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Detection of Pecan Walnut DNA using real-time PCR

VALIDATION REPORT

ASSAY

Detection of *Pecan walnut DNA* using real time-PC

VALIDATED EQUIPMENT

Applied Biosystems, model 7500; Thermo Scientific, model PikoReal; Condalab, model CDL-96.

SAMPLES (N)

Enrichments prepared from suitable food samples. During re-validation samples from external quality assessment schemes from FAPAS, Instand and INSA-PHE were tested.

VALIDATION ASSAYS

Start – 05-02-2016
Finish – 19-08-2016

DEFINITIONS

Analyte

DNA of the microorganism detected by the analysis method

Qualitative method

Analysis method which response is either the presence or absence of the microorganism either directly or indirectly in a certain amount of sample.

True positive (TP)

Sample presenting a positive result for the test and that has equal or higher amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

True negative (TN)

Sample presenting a negative result for the test and that has a lower amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

False Positive (FP)

Sample presenting a positive result for the test in the absence of target analyte.

False Negative (FN)

Sample presenting a negative result for the test in the presence of equal or higher amount of the target analyte than the detection limit.

Detection Limit (LOD)

- Reaction detection limit: lowest concentration of the target DNA that can be consistently detected in the specified experimental conditions.
- Method reaction limit: lowest concentration or content of the target microorganism relative to the defined amount of matrix that can be consistently detected in the specified experimental conditions.

Inclusivity

Detection of the target species.

Exclusivity

Non-detection of non-target species.

Selectivity

Measure of the inclusivity and exclusivity.

Sensitivity (S)

Measure of the lowest number of analyte cells which can be detected in a single test reaction.

Specificity (Sp)

Ability to exclusivity recognize the target microorganism to be detected and distinguish it from other similar organisms and impurities.

Trueness (T)

Closeness of agreement between the expectation of a test result and the corresponding true value.

Precision (P)

Closeness of agreement between independent tests obtained under defined conditions. It depends only on the distribution of random errors and does not relate to the true value of the test.

Positive predictive value (PPV)

Probability of a positive test to be referent to a sample presenting the DNA of the target species.

Negative predictive value (NPV)

Probability of a positive test to be referent to a sample presenting the DNA of the target species in a concentration above the detection limit.

SCOPE OF THE METHOD

The present method is based on a 5' nuclease real-time PCR reactions and amplify a unique Pecan walnut genomic sequence aiming the detection of this species DNA in food samples after DNA extraction. It can also be used with other samples.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a second fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 15 food samples obtained from different commercial sources. These food samples included 9 different matrices:

- Cereal mix
- Cereal bread
- Energy bar
- Chocolate snacks
- Cookies
- Hazelnut drink
- Sweetened hazelnut chocolate spread
- Sesame breadsticks
- Passionfruit bar with cashew

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing Pecan walnut DNA

Negative control (NC): reaction without any target DNA

Each individual reaction includes:

Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

To validate the tests, the controls must show the following results:

	Target detection Chanel 1	**Target detection Chanel 2**
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

If the controls do not match these results, the experiment must be repeated.

The results obtained by the present method shall be interpreted as follows:

Target detection Chanel 1 (Pecan walnut DNA)	**Target detection Chanel 2 (IC)**	**Interpretation**
Positive	Not significant	Positive
Ct = NA	Positive	Negative
Ct = NA	Ct = NA	Inhibition**

**When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.

2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).

The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific). Comparable results were obtained with CFX96 (BioRad).

3. SPECIFITY

A total of 51 non target species related with the target or occurring in the food product and that could cause interference with test results were de tested (Table 3.1). The species were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the ITS region, using universal primers. The identification of all species was confirmed by sequencing mitochondrial genes. All DNA extracts were tested in triplicate.

3.2. Exclusivity test

The strains for exclusivity test were chosen following the recommendations of International Standard ISO 22118:2011. A total of 30 non target microorganisms related with the target or occurring in the same habitat and that could cause interference with test results were de tested (Table 3.1). The strains were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the 16S rRNA bacterial gene, using universal primers. The identification of all strains was confirmed by 16S rRNA gene sequencing. All strains were tested in triplicate.

Code	Strain identification	Other informations	
CR1	Ovis aries	VR1	Apium graveolens
CR4	Sus domesticus	VR2	Prunus dulcis
CR7	Gallus gallus	VR3	Arachis hypogaea L.
CR8	Bos taurus	VR4	Avena sativa
CR9	Meleagris gallopavo	VR5	Corylus avellana, L.
CR13	Equus equus	VR6	Lycium barbarum
PR1	Auxis rochei	VR7	Anacardium occidentale
PR3	Sarda sarda/chiliensis	VR8	Bertholletia excelsa
PR7	Hymenopenaeus muelleri	VR9	Secale cereale
PR8	Litopenaeus vannamei	VR10	Hordeum vulgare
PR9	Salmo salar	VR11	Macadamia ternifolia
PR10	Gadus morhua	VR12	Sinapis alba
PR12	Sepiella japonica	VR13	Brassica nigra
PR13	Merluccius senegalensis	VR14	Juglans regia L.
PR14	Illex argentinus	VR16	Curcubita pepo L.
PR15	Sepia aculeata	VR17	Pinus pinea
PR16	Merluccius paradoxus	VR18	Pistacia vera
PR17	Meretrix lyrata	VR19	Sesamum indicum
PR19	Ensis directus	VR20	Glycine max
PR21	Metapenaeus sp. (affinis/ensis)	VR21	Lupinus albus
PR22	Fenneropenaeus indicus	VR22	Triticum aestivum
PR24	Penaeus japonicus	VR23	Allium sativum
PR32	Cerastoderma edule	VR24	Oryza sativa
PR37	Uroteuthis duvauceli	VR25	Solanum tuberosum
PR49	Argopecten furfuratus	VR29	Pisum sativum
		VR30	Vigna unguiculata

Table 3.1 – List of species used for exclusivity test

Result:
The method presented 100% specificity as none of the species showed a positive result.

4. SENSITIVITY

4.1. Sensitivity of the Real-Time PCR reaction

The sensitivity of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 100 pg, 50 pg, 10 pg, 5 pg, 1pg, 500 fg. 50 fg , 10 fg and 5 fg.

Result:
Target amplification was observed with DNA amounts of 10 fg or higher. Thus, the limit of detection of the reaction was established in 10 fg of target genomic DNA.

4.2. Sensitivity of the test in DNA mixtures

The sensitivity of the test was determined in a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 0,5%, 0,1%, 0,05%, 0,01%, 0,005% and 0,001%, 0,0005%, 0,0001% and 0,00005%. All determinations were performed in duplicate.

Result:
Target amplification was observed in samples containing 0,00001% of target DNA. Thus, the method can detect 0,00001% of Pecan walnut DNA in 100 ng of total DNA.

5. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 5.1, using two positive and two negative samples and performed in duplicate.

Parameter	N° variations	Description
Annealing temperature	2	+ 2°C; - 2°C
MgCl2 concentration	2	+ 0,2mM; - 0,2mM
Independent performer		Not applicable
Different Real Time equipment	2	ABI 7500 PikoReal, Thermo Scientific
Independent laboratories	1	Biopremier R&D laboratory

Table 5.1 – Method's parameters submitted to variations

Result:

Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

6. TRUENESS

Trueness of the method was evaluated using 1 positive and 14 negative food samples. All the samples were tested in duplicate.

All the samples were tested in triplicate.

Result:

All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%

Performance Characteristics for validation

The determination of false positive and false negative rate, the method was performed in 15 food samples (1 positive and 14 negative samples).

The method's limit of detection was determined in a mixture containing a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 0,5%, 0,1%, 0,05%, 0,01%, 0,005% and 0,001%, 0,0005%, 0,0001% and 0,00005%. All determinations were performed in duplicate.

1. FALSE POSITIVE RATE

This corresponds to the probability that a known negative sample has been classified as positive by the method. The false positive rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fpos}=n_{fpos}/n_{rneg}+n_{fpos}$ x 100%

In which:

P_{fpos}: False positive rate

n_{rneg}: Number of real negative test results

n_{fpos}: Number of misclassified known negative samples

Result:

For the present method, false positive rate was calculated based on 15 samples. The final result was 0%

2. FALSE NEGATIVE RATE

This corresponds to the probability that a known positive sample has been classified as negative by the method. The false negative rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fneg}=nfneg/n_{rneg}+n_{fneg}$ x 100%

In which:

P_{fneg}: False negative rate

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:

For the present method, false positive rate was calculated based on 15 samples. The final result was 0%

3. LIMIT OF DETECTION

3.1. Limit of detection of the Real-Time PCR reaction

The limit of detection of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 100 pg, 50 pg, 10 pg, 5 pg, 1pg, 500 fg. 50 fg, 10 fg, 5 fg.

Result:
The limit of detection determined with a probability 0,95% (false positive rate < 0,05) was 5 fg of the target genomic DNA.

3.2. Limit of detection of the test in DNA mixtures

The method's limit of detection was determined in a mixture containing a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 0,5%, 0,1%, 0,05%, 0,01%, 0,005% and 0,001%, 0,0005%, 0,0001% and 0,00005%. All determinations were performed in duplicate.

Result:
The limit of detection determined with a probability 0,95% (false positive rate < 0,05) was 0,00001% of Pecan walnut DNA in 100 ng of total DNA.

Positive predictive value (PPV)

The PPV is calculated by the expression:
$PPV=n_{rpos}/n_{rpos}+n_{fpos}$ x 100%
In which:
n_{rpos}: Number of real positive test results
n_{fneg}: Number of misclassified known positive samples

Result:
The positive predictive value determined was 100%.

Negative predictive value (NPV)

The PPV is calculated by the expression:
$NPV=n_{rneg}/n_{rneg}+n_{fneg}$ x 100%
In which:
n_{rneg}: Number of real negative test results
n_{fneg}: Number of misclassified known negative samples

Result:
The negative predictive value determined was 100%.

Criteria for acceptance

Inclusivity: 95%
Exclusivity: 95%
Selectivity: 95%
Trueness: 95%
False positive rate: 5%
False negative rate: 5%

Performance declaration

Accordingly, with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results in agreement with the Condalab requirements and is validated.

The present test allows the "*Detection of Pecan walnut using real-time PCR*" in water and swab samples".

Condalab

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Detection of Sesame DNA using real-time PCR

VALIDATION REPORT

ASSAY

Detection of *Sesame DNA*. using real-time PCR

VALIDATED EQUIPMENT

Applied Biosystems, model 7500; Thermo Scientific, model PikoReal; Condalab, model CDL-96.

SAMPLES (N)

Food products and samples from external quality assessment schemes.

VALIDATION ASSAYS

Start – 08-03-2016
Finish – 19-08-2016

DEFINITIONS

Analyte

DNA of the microorganism detected by the analysis method

Qualitative method

Analysis method which response is either the presence or absence of the microorganism either directly or indirectly in a certain amount of sample.

True positive (TP)

Sample presenting a positive result for the test and that has equal or higher amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

True negative (TN)

Sample presenting a negative result for the test and that has a lower amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

False Positive (FP)

Sample presenting a positive result for the test in the absence of target analyte.

False Negative (FN)

Sample presenting a negative result for the test in the presence of equal or higher amount of the target analyte than the detection limit.

Detection Limit (LOD)

- Reaction detection limit: lowest concentration of the target DNA that can be consistently detected in the specified experimental conditions.
- Method reaction limit: lowest concentration or content of the target microorganism relative to the defined amount of matrix that can be consistently detected in the specified experimental conditions.

Inclusivity

Detection of the target species.

Exclusivity

Non-detection of non-target species.

Selectivity

Measure of the inclusivity and exclusivity.

Sensitivity (S)

Measure of the lowest number of analyte cells which can be detected in a single test reaction.

Specificity (Sp)

Ability to exclusivity recognize the target species to be detected and distinguish it from other similar organisms and impurities.

Trueness (T)

Closeness of agreement between the expectation of a test result and the corresponding true value.

Positive predictive value (PPV)

Probability of a positive test to be referent to a sample presenting the DNA of the target species.

Negative predictive value (NPV)

Probability of a positive test to be referent to a sample presenting the DNA of the target species in a concentration above the detection limit.

SCOPE OF THE METHOD

The present method is based on a 5' nuclease real-time PCR reactions and amplify a unique sesame genomic sequence aiming the detection of this species DNA in food samples after DNA extraction. It can also be used with other samples.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a second fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 21 food samples obtained from different commercial sources. These food samples included different 16 matrices:

- Cereal bread
- Energy bar
- Soup
- Juice
- Sesame breadsticks
- Cooked rice
- Chocolate snacks
- Nougat
- Soy schnitzel
- Sausage roll
- Soy hamburger
- Soy and chocolate dessert
- Cooked Pork
- Sesame Integral biscuits
- Cooked meat hamburger
- Instant coffee

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing sesame DNA

Negative control (NC): reaction without any target DNA
Each individual reaction includes:

Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

To validate the tests, the controls must show the following results:

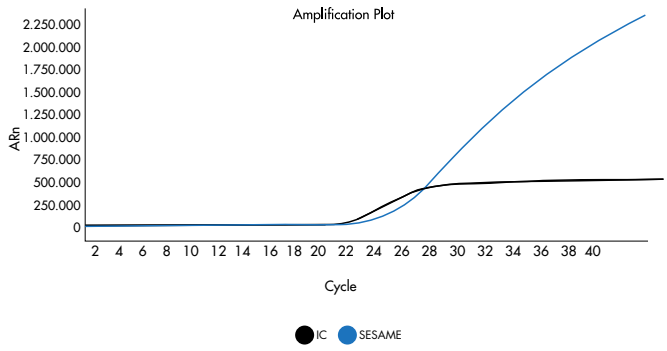
	Target detection Chanel 1	Target detection Chanel 2
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

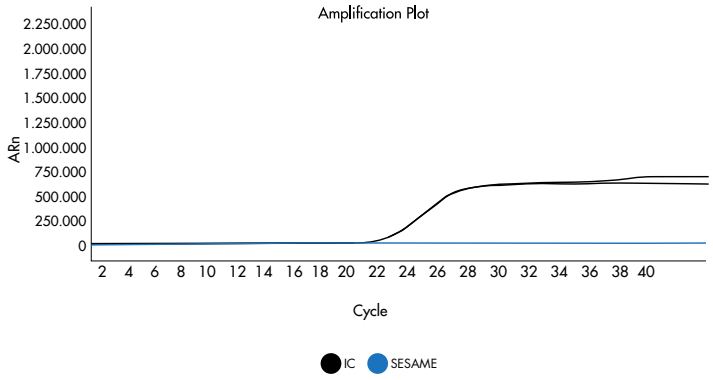
If the controls do not match these results, the experiment must be repeated.

The results obtained by the present method shall be interpreted as follows:

Target detection Chanel 1	Target detection Chanel 2	Interpretation
Positive	Not significant	Positive
Ct = NA	Positive	Negative
Ct = NA	Ct = NA	Inhibition**

**When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.





2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).

The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific).

3. SPECIFICITY

A total of 51 non target species related with the target or occurring in the food product and that could cause interference with test results were de tested (Table 3.1). The species were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the ITS region, using universal primers. The identification of all species was confirmed by sequencing mitochondrial genes. All DNA extracts were tested in duplicate.

Code	Species name	Code	Species name
CR1	*Ovis aries*	VR12	Sinapis alba
CR4	*Sus domesticus*	VR2	Prunus dulcis
CR7	*Gallus gallus*	VR3	Arachis hypogaea L.
CR8	*Bos taurus*	VR4	Avena sativa
CR9	*Meleagris gallopavo*	VR5	Corylus avellana, L.
CR13	*Equus equus*	VR6	Lycium barbarum
PR1	*Auxis rochei*	VR7	Anacardium occidentale
PR3	*Sarda sarda/chiliensis*	VR8	Bertholletia excelsa
PR7	*Hymenopenaeus muelleri*	VR9	Secale cereale
PR8	*Litopenaeus vannamei*	VR10	Hordeum vulgare
PR9	*Salmo salar*	VR11	Macadamia ternifolia
PR10	*Gadus morhua*	VR13	Brassica nigra
PR12	*Sepiella japonica*	VR14	Juglans regia L.
PR13	*Merluccius senegalensis*	VR15	Carya illinoensis K.
PR14	*Illex argentinus*	VR16	Curcubita pepo L.
PR15	*Sepia aculeata*	VR17	Pinus pinea
PR16	*Merluccius paradoxus*	VR18	Pistacia vera
PR17	*Meretrix lyrata*	VR1	Apium graveolens
PR19	*Ensis directus*	VR20	Glycine max
PR21	*Metapenaeus sp. (affinis/ensis)*	VR21	Lupinus albus
PR22	*Fenneropenaeus indicus*	VR22	Triticum aestivum
PR24	*Penaeus japonicus*	VR23	Allium sativum
PR37	*Uroteuthis duvauceli*	VR24	Oryza sativa
PR32	*Cerastoderma edule*	VR25	Solanum tuberosum
PR49	*Argopecten furfuratus*	VR29	Pisum sativum
		VR30	Vigna unguiculata

Result:
The method presented 100% specificity as none of the species showed a positive result.

Table 3.1 – List of species used for exclusivity test

4. SENSITIVITY

4.1. Sensitivity of the Real-Time PCR reaction

The sensitivity of the reaction was determined by testing in duplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg, 1pg, 500 fg, and 100fg.

Result:

Target amplification was observed with DNA amounts of 1 pg or higher. Thus, the limit of detection of the reaction was established in 1 pg of target genomic DNA.

4.2. Sensitivity of the test in DNA mixtures

The sensitivity of the test was determined in a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05%, 0,01%, 005% and 0,001%. All determinations were performed duplicate.

Result:

Target amplification was observed in samples containing 0,001% of target DNA. Thus, the method can detect 0,001% of sesame DNA in 100 ng of total DNA.

5. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 5.1, using two positive and two negative samples and performed in triplicate.

Parameter	N° variations	Description
Annealing temperature	2	+ 2°C; - 2°C
MgCl2 concentration	2	+ 0,5mM; - 0,5mM
Independent performer		Not applicable
Different Real Time equipment	2	ABI 7500 PikoReal, Thermo Scientific
Independent laboratories	1	Biopremier R&D laboratory

Table 5.1 – Method's parameters submitted to variations

Result:

Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

6. TRUENESS

Trueness of the method was evaluated using 4 positive and 14 negative food samples. All the samples were tested in triplicate.

All the samples were tested in triplicate.

Result:

All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%

Performance Characteristics for validation

The determination of false positive and false negative rate, the method was performed in 18 food samples (4 positive and 14 negative samples).

The method's limit of detection was determined in a mixture containing a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05%, 0,01%, 005% and 0,001%. All determinations were performed in duplicate.

1. FALSE POSITIVE RATE

This corresponds to the probability that a known negative sample has been classified as positive by the method. The false positive rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fpos}=n_{fpos}/n_{rneg}+n_{fpos}$ x 100%

In which:

P_{fpos}: False positive rate
n_{rneg}: Number of real negative test results
n_{fpos}: Number of misclassified known negative samples

Result:
For the present method, false positive rate was calculated based on 18 samples. The final result was 0%

2. FALSE NEGATIVE RATE

This corresponds to the probability that a known positive sample has been classified as negative by the method. The false negative rate (that can be expressed as a percentage) is calculated by the expression:

P_{fneg}=nfneg/n_{rneg}+n_{fneg} x 100%
In which:

P_{fneg}: False negative rate
n_{rpos}: Number of real positive test results
n_{fneg}: Number of misclassified known positive samples

Result:
For the present method, false positive rate was calculated based on 18 samples. The final result was 0%

3. LIMIT OF DETECTION

3.1. Limit of detection of the Real-Time PCR reaction

The limit of detection of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1ng, 500 pg, 100 pg, 50 pg, 10 pg, 5 pg, 1pg, 500 fg, and 100fg.

Result:
The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 1 pg of the target genomic DNA.

3.2. Limit of detection of the method

The method's limit of detection was determined in a mixture containing a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 10%, 1%, 0,5%, 0,1%, 0,05%, 0,01%, 005% and 0,001%. All determinations were performed in duplicate.

Result:
The limit of detection determined with a probability 0,95% (false positive rate < 0,05) was 0,01% of sesame DNA in 100 ng of total DNA.

Positive predictive value (PPV)

The PPV is calculated by the expression:
PPV=n_{rpos}/n_{rpos}+n_{fpos} x 100%
In which:
n_{rpos}: Number of real positive test results
n_{fneg}: Number of misclassified known positive samples

Result:
The positive predictive value determined was 100%.

Negative predictive value (NPV)

The PPV is calculated by the expression:
NPV=n_{rneg}/n_{rneg}+n_{fneg} x 100%
In which:
n_{rneg}: Number of real negative test results
n_{fneg}: Number of misclassified known negative samples

Result:
The negative predictive value determined was 100%.

Criteria for acceptance

Inclusivity: 95%
Exclusivity: 95%
Selectivity: 95%
Trueness: 95%
False positive rate: 5%
False negative rate: 5%

Performance declaration

Accordingly, with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results in agreement with the Condalab requirements and is validated.
The present test allows the "Detection of *Sesame* using real-time PCR".

Condalab

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Detection of *Vibrio cholerae, Vibrio parahaemolyticus and Vibrio vulfinicus* using real-time PCR

VALIDATION REPORT

ASSAY

Vibrio cholerae, Vibrio parahaemolyticus and Vibrio vulfinicus detection by 5' nuclease real time-PCR assay.

VALIDATED EQUIPMENT

Applied Biosystems, model 7500; Thermo Scientific, model PikoReal; Condalab, model CDL-96.

SAMPLES (N)

Enrichments prepared from suitable food samples. During re-validation samples from external quality assessment schemes from FAPAS, Instand and INSA-PHE were tested.

VALIDATION ASSAYS

Start – 01-10-2013
Finish – 01-05-2014

DEFINITIONS

Analyte

DNA of the microorganism detected by the analysis method

Qualitative method

Analysis method which response is either the presence or absence of the microorganism either directly or indirectly in a certain amount of sample.

True positive (TP)

Sample presenting a positive result for the test and that has equal or higher amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

True negative (TN)

Sample presenting a negative result for the test and that has a lower amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

False Positive (FP)

Sample presenting a positive result for the test in the absence of target analyte.

False Negative (FN)

Sample presenting a negative result for the test in the presence of equal or higher amount of the target analyte than the detection limit.

Detection Limit (LOD)

- Reaction detection limit: lowest concentration of the target DNA that can be consistently detected in the specified experimental conditions.
- Method reaction limit: lowest concentration or content of the target microorganism relative to the defined amount of matrix that can be consistently detected in the specified experimental conditions.

Inclusivity

Detection of the target microorganism.

Exclusivity

Non-detection of non-target microorganism.

Selectivity

Measure of the inclusivity and exclusivity.

Sensitivity (S)

Measure of the lowest number of analyte cells which can be detected in a single test reaction.

Specificity (Sp)

Ability to exclusivity recognize the target microorganism to be detected and distinguish it from other similar organisms and impurities.

Trueness (T)

Closeness of agreement between the expectation of a test result and the corresponding true value.

Precision (P)

Closeness of agreement between independent tests obtained under defined conditions. It depends only on the distribution of random errors and does not relate to the true value of the test.

Positive predictive value (PPV)

Probability of a positive test be referent to a sample presenting an analyte concentration above.

Negative predictive value (NPV)

Probability of a positive test be referent to a sample presenting an analyte concentration above.

SCOPE OF THE METHOD

The present method is based on a 5' nuclease real-time PCR reactions and amplify a unique genomic sequence of *Vibrio cholerae, Vibrio parahaemolyticus and Vibrio vulfinicus,* aiming the detection of this pathogen DNA in food samples. The test is designed for the detection of the target microorganism DNA in food products after enrichment in buffered peptone water and DNA extraction. It can also be used with animal feedstuff and environmental samples. Is not suitable for human and clinical diagnostics. The method's performance was tested according ISO 22118:2011 and fulfils the requirements set in this International Standard.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a second fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 33 food samples enriched according the method described in ISO 21872:2007. These food samples included 7 different matrices, aleatory taken from commercial shops:

- Shrimp
- Ling
- Mussel
- Fish fillets
- Seafood cocktail
- Herbs mixture
- Crustaceans
- Clams

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing genomic DNA of the *Vibrio cholerae, Vibrio parahaemolyticus and Vibrio vulfinicus.*

Negative control (NC): reaction without any target DNA

Each individual reaction includes:

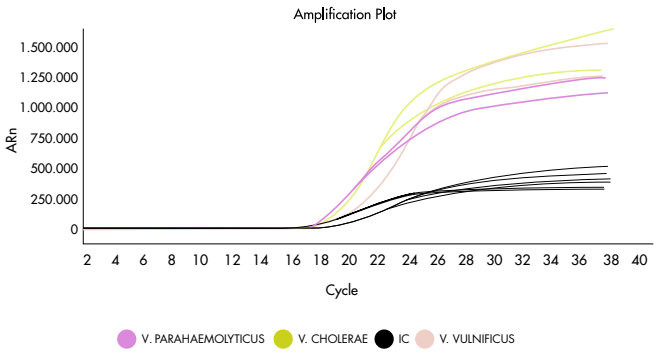
Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

To validate the tests, the controls must show the following results:

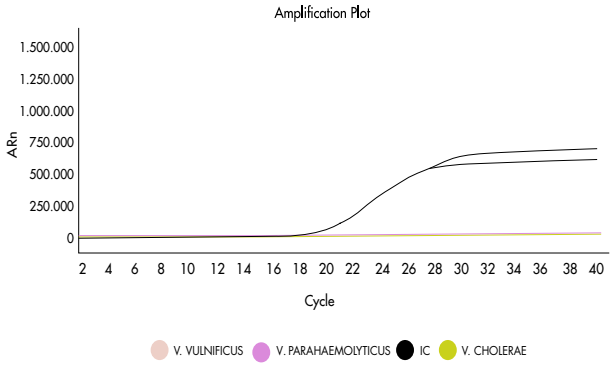
	Target detection Chanel 1	Target detection Chanel 2
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

If the controls do not match these results, the experiment must be repeated.

Positive Control



Negative Control



The results obtained by the present method shall be interpreted as follows:

Target detection Chanel 1	Target detection Chanel 2	Interpretation
Positive	Not significant	Positive
Ct = NA	Positive	Negative
Ct = NA	Ct = NA	Inhibition**

**When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.

2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).

The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific). Comparable results were obtained with CFX96 (BioRad).

3. SELECTIVITY

3.1. Inclusivity test

A total of 10 *Vibrio cholerae* strains, 12 *Vibrio parahaemolyticus* and 1 *Vibrio vulfinicus* were tested (Table 3.1). The strains were tested using 2 ng of genomic DNA.

Code	Sample	Other Information
MB 562	*Vibrio parahaemolyticus*	
MB 563	*Vibrio parahaemolyticus*	
MB 564	*Vibrio parahaemolyticus*	
MB 565	*Vibrio parahaemolyticus*	
MB 566	*Vibrio parahaemolyticus*	
MB 567	*Vibrio parahaemolyticus*	
MB 568	*Vibrio parahaemolyticus*	
MB 569	*Vibrio parahaemolyticus*	
MB 570	*Vibrio parahaemolyticus*	
MB 571	*Vibrio parahaemolyticus*	
MB 356	*Vibrio parahaemolyticus*	NCTC 10885
MB 1029	*Vibrio parahaemolyticus*	
MB 591	*Vibrio cholerae*	
MB 592	*Vibrio cholerae*	
MB 593	*Vibrio cholerae*	
MB 594	*Vibrio cholerae*	
MB 595	*Vibrio cholerae*	
MB 596	*Vibrio cholerae*	
MB 597	*Vibrio cholerae*	
MB 598	*Vibrio cholerae*	
MB22	*Vibrio cholerae*	
MB 1030	*Vibrio cholerae*	
MB 1031	*Vibrio vulfinicus*	

Table 3.1 – List of strains used for inclusivity test

Result:
All strains were detected with the present method, corresponding to 100% inclusivity.

3.2.Exclusivity test

The strains for exclusivity test were chosen following the recommendations of International Standard ISO 22118:2011. A total of 30 non target microorganisms related with the target or occurring in the same habitat and that could cause interference with test results were de tested (Table 3.2). The strains were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the 16S rRNA bacterial gene, using universal primers. The identification of all strains was confirmed by 16S rRNA gene sequencing. All strains were tested in triplicate.

Code	Strain Identification	Other Informations
MB 385	*Listeria monocytogenes*	CECT 4031
MB 388	*Listeria monocytogenes*	Serotype 1/2a ou 3a
MB 381	*Listeria innocua*	CECT 910
MB 357	*Escherichia coli*	NCTC 9001
MB 240	*Escherichia coli O157:H7*	NCTC 9001
MB 372	*Campylobacter jejuni subsp jejuni*	NCTC 11351
MB 373	*Campylobacter coli*	NCTC 11366
MB 399	*Salmonella Typhimurium*	CECT 443
MB 363	*Bacillus cereus*	IFM1600
MB 115	*Citrobacter freundii*	Local isolate
MB 451	*Cronobacter muytjensii*	ATCC 51329
MB 141	*Lactobacillus plantarum*	CECT 748 T
MB 144	*Lactobacillus paracasei subsp paracasei*	CECT 4022T
MB 150	*Staphylococcus aureus*	NCTC 6571
MB 302	*Pseudomonas aeruginosa*	Local isolate
MB 13	*Serratia marcescens*	Local isolate
MB 163	*Proteus vulgaris*	Local isolate
MB 365	*Enterococcus faecalis*	Local isolate
MB 306	*Enterococcus hirae*	Local isolate
MB 147	*Staphylococcus epidermidis*	Local isolate
MB 267	*Shigella flexneri*	Local isolate
MB 249	*Yersinia enterocolitica*	Local isolate
MB 178	*Klebsiella pneumoniae*	Local isolate
MB 449	*Brochothrix thermosphacta*	ATCC 11509
MB 274	*Legionella pneumophila*	Local isolate
MB 278	*Legionella micdadei*	Local isolate
MB 362	*Clostridium perfringens*	Local isolate
MF 128	*Saccharomyces cerevisae*	CECT
MF 129	*Aspergillus niger*	Local isolate

Table 3.2 – List of strains used for exclusivity test

For the assays *Vibrio cholerae* and *Vibrio vulfinicus*:

MB 356	*Vibrio parahaemolyticus*	Local isolate

For the assays *Vibrio parahaemolyticus* and *Vibrio vulfinicus*:

MB 22	*Vibrio cholerae*	Local isolate

For the assays *Vibrio parahaemolyticus* and *Vibrio vulfinicus*:

MB 1031	*Vibrio vulfinicus*	Local isolate

Result:
The method presented 100% exclusivity as none of the strains showed a positive results and positive result.

4. SENSITIVITY

4.1. Sensitivity of the Real-Time PCR reaction

The sensitivity of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 10 ng, 5 ng, 1 ng, 500 pg, 250 pg, 50 pg, 5 pg, 500 fg, 50 fg, 25 fg and 5 fg.

Result:

Target amplification was observed with DNA amounts of 250 pg or higher. Thus, the limit of detection of the reaction was established in 250 pg of target genomic DNA.

4.2. Sensitivity of the test

The sensitivity of the test was determined in food samples artificially contaminated with decreasing number of bacterial cells, following the requirements of ISO 22118. The sensitivity was tested in four different food matrices: shrimp, ling, mussel, fish fillets and seafood cocktail. The enrichment was performed according to ISO 21872:2007, with 25 g of each sample, after inoculation with 0, 1-10, 10-10^2 and 10^2-10^3 cfu of Vibrio spp.All procedures starting from DNA extraction were performed in triplicate.

Result:

Target amplification was observed in samples contaminated with 1-10 cfu/25 g or more. No amplification was obtained in non-contaminated samples (inoculated with 0 cfu). Thus, the method can detect 1-10 cfu in 25 g of food sample (LOD).

5. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 5.1, using two positive and two negative samples and performed in triplicate.

Parameter	N° variations	Description
Annealing temperature	2	+ 2°C; - 2°C
MgCl2 concentration	2	+ 0,5mM; - 0,5mM
Independent performer	2	Not applicable
Different Real Time equipment	3	ABI 7500 PikoReal, Thermo Scientific BioRad CFX96
Independent laboratories	2	Biopremier R&D laboratory Portuguese Food Safety Authority laboratory

Table 5.1 – Method's parameters submitted to variations

Result:

Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

6. TRUENESS

Trueness of the method was evaluated using 16 positive and 17 negative food samples for the target, respectively, corresponding to the following food matrices:

- Shrimp
- Ling
- Mussel
- Fish fillets
- Seafood cocktail
- Herbs mixture
- Crustaceans
- Clams

All the samples were tested in triplicate.

Result:

All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%.

Performance Characteristics for validation

For the determination of false positive and false negative rate, the method was performed in 33 samples (16 positive and 17 negative samples), corresponding to 8 food matrices.
For the determination of method's limit of detection, the sample was inoculated considering 3 contamination levels: 0, 1 to 10 and 10 to 100 cfus in 25 g of sample. All the procedures were performed as defined for the present method. All experiments starting from DNA extraction were performed in triplicate.

1. FALSE POSITIVE RATE

This corresponds to the probability that a known negative sample has been classified as positive by the method. The false positive rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fpos}=n_{fpos}/n_{rneg}+n_{fpos}$ x 100%

In which:

P_{fpos}: False positive rate
n_{rneg}: Number of real negative test results
n_{fpos}: Number of misclassified known negative samples

Result:

For the present method, false positive rate was calculated based on 33 real samples. The final result was 0%

2. FALSE NEGATIVE RATE

This corresponds to the probability that a known positive sample has been classified as negative by the method. The false negative rate (that can be expressed as a percentage) is calculated by the expression:

P_{fneg}=nfneg/$n_{rneg}+n_{fneg}$ x 100%

In which:

P_{fneg}: False negative rate
n_{rpos}: Number of real positive test results
n_{fneg}: Number of misclassified known positive samples

Result:

For the present method, false positive rate was calculated based on 33 real samples. The final result was 0%

3. LIMIT OF DETECTION

3.1. Limit of detection of the Real-Time PCR reaction

The limit of detection of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 10 ng, 5 ng, 1 ng, 500 pg, 50 pg, 5 pg, 500 fg, 50 fg, 25 fg and 5 fg.

Result:

The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 250 pg of Vibrio genomic DNA.

3.2. Limit of detection of the method

The limit of detection of the test was determined in food samples artificially contaminated with decreasing number of bacterial cells, following the requirements of ISO 22118, using four different food matrices: shrimp, ling, mussel, fish fillets and seafood cocktail. The enrichment was performed according to ISO 21872:2007, with 25 g of each sample, after inoculation with 0, 1-10, 10-10^2 and 10^2-10^3 cfu of *Vibrio*. All procedures starting from DNA extraction were performed in triplicate.

Result:

The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 1-10 cfus of Vibrio genomic DNA.

Positive predictive value (PPV)

The PPV is calculated by the expression:

PPV=$n_{rpos}/n_{rpos}+n_{fpos}$ x 100%

In which:

n_{rpos}: Number of real positive test results
n_{fneg}: Number of misclassified known positive samples

Result:
The positive predictive value determined was 100%.

Negative predictive value (NPV)
The PPV is calculated by the expression:
$NPV=n_{rneg}/n_{rneg}+n_{fneg}$ x 100%
In which:
n_{rneg}: Number of real negative test results
n_{fneg}: Number of misclassified known negative samples

Result:
The negative predictive value determined was 100%.

Criteria for acceptance

Inclusivity: 95%
Exclusivity: 95%
Selectivity: 95%
Trueness: 95%
False positive rate: 5%
False negative rate: 5%

Performance declaration

Accordingly, with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results in agreement with the Condalab requirements and is validated.
The present test allows the "*Detection of the Vibrio cholerae, Vibrio parahaemolyticus and Vibrio vulfinicus* using real time-PCR" in food samples after enrichment.

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Detection of Walnut DNA using real-time PCR

VALIDATION REPORT

ASSAY

Detection of walnut DNA, using real-time PCR

VALIDATED EQUIPMENT

Applied Biosystems, model 7500; Thermo Scientific, model PikoReal; Condalab, model CDL-96.

SAMPLES (N)

Food products and samples from external quality assessment schemes.

VALIDATION ASSAYS

Start – 05-02-2016
Finish – 19-08-2016

DEFINITIONS

Analyte

DNA of the target species detected by the analysis method

Qualitative method

Analysis method which response is either the presence or absence of the target species either directly or indirectly in a certain amount of sample.

True positive (TP)

Sample presenting a positive result for the test and that has equal or higher amount of the target analyte (target species DNA) than the detection limit established in the specified experimental conditions.

True negative (TN)

Sample presenting a negative result for the test and that has a lower amount of the target analyte (target species DNA) than the detection limit established in the specified experimental conditions.

False Positive (FP)

Sample presenting a positive result for the test in the absence of target analyte.

False Negative (FN)

Sample presenting a negative result for the test in the presence of equal or higher amount of the target analyte than the detection limit.

Detection Limit (LOD)

- Reaction detection limit: lowest concentration of the target DNA that can be consistently detected in the specified experimental conditions.
- Method reaction limit: lowest concentration or content of the target microorganism relative to the defined amount of matrix that can be consistently detected in the specified experimental conditions.

Inclusivity

Detection of the target species.

Exclusivity

Non-detection of non-target species.

Selectivity

Measure of the inclusivity and exclusivity.

Sensitivity (S)

Measure of the lowest of analyte DNA concentration which can be detected in a single test reaction.

Specificity (Sp)

Ability to exclusivity recognize the target species to be detected and distinguish it from other similar organisms and impurities.

Trueness (T)

Closeness of agreement between the expectation of a test result and the corresponding true value.

Positive predictive value (PPV)

Probability of a positive test to be referent to a sample presenting the DNA of the target species.

Negative predictive value (NPV)

Probability of a positive test to be referent to a sample presenting the DNA of the target species in a concentration above the detection limit.

SCOPE OF THE METHOD

The present method is based on a 5' nuclease real-time PCR reactions and amplify a unique walnut genomic sequence aiming the detection of this species DNA in food samples after DNA extraction. It can also be used with other samples.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a second fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 18 food samples obtained from different commercial sources. These food samples included 11 different matrices:

- Cereal mix
- Cereal bread
- Energy bar
- Chocolate snacks
- Cookies
- Hazelnut drink
- Sweetened hazelnut chocolate spread
- Sesame breadsticks
- Walnut bars
- Passionfruit bar with cashew
- Walnut and honey cakes

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing walnut DNA
Negative control (NC): reaction without any target DNA

Each individual reaction includes:

Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

If the controls do not match these results, the experiment must be repeated.

The results obtained by the present method shall be interpreted as follows:

	Target detection Chanel 1	Target detection Chanel 2
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.

2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).

The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific).

3. SPECIFICITY

A total of 51 non target species related with the target or occurring in the food product and that could cause interference with test results were de tested (Table 3.1). The species were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the ITS region, using universal primers. The identification of all species was confirmed by sequencing mitochondrial genes. All DNA extracts were tested in triplicate.

Code	Species name	Code	Species name
CR1	*Ovis aries*	VR1	Apium graveolens
CR4	*Sus domesticus*	VR2	Prunus dulcis
CR7	*Gallus gallus*	VR3	Arachis hypogaea L.
CR8	*Bos taurus*	VR4	Avena sativa
CR9	*Meleagris gallopavo*	VR5	Corylus avellana, L.
CR13	*Equus equus*	VR6	Lycium barbarum
PR1	*Auxis rochei*	VR7	Anacardium occidentale
PR3	*Sarda sarda/chiliensis*	VR8	Bertholletia excelsa
PR7	*Hymenopenaeus muelleri*	VR9	Secale cereale
PR8	*Litopenaeus vannamei*	VR10	Hordeum vulgare
PR9	*Salmo salar*	VR11	Macadamia ternifolia
PR10	*Gadus morhua*	VR12	Sinapis alba
PR12	*Sepiella japonica*	VR13	Brassica nigra
PR13	*Merluccius senegalensis*	VR16	Curcubita pepo L.
PR14	*Illex argentinus*	VR17	Pinus pinea
PR15	*Sepia aculeata*	VR18	Pistacia vera
PR16	*Merluccius paradoxus*	VR20	Glycine max
PR17	*Meretrix lyrata*	VR21	Lupinus albus
PR19	*Ensis directus*	VR22	Triticum aestivum
PR21	*Metapenaeus sp. (affinis/ensis)*	VR23	Allium sativum
PR22	*Fenneropenaeus indicus*	VR24	Oryza sativa
PR24	*Penaeus japonicus*	VR25	Solanum tuberosum

PR32	*Cerastoderma edule*	VR29	Pisum sativum
PR37	*Uroteuthis duvauceli*	V30	Vigna unguiculata
PR49	*Argopecten furfuratus*	VR19	Sesamum indicum
		VR15	Carya illinoensis K.

Table 3.1 – List of species used for exclusivity test

Result:
The method presented 94% specificity, 3 of the species showed a positive result (VR6, VR16 and VR18).

4. SENSITIVITY

4.1. Sensitivity of the Real-Time PCR reaction

The sensitivity of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 100 pg, 50 pg, 10 pg, 5 pg, 1pg, 500 fg. 50 fg , 10 fg, 5 fg and 1fg.

Result:
Target amplification was observed with DNA amounts of 10 fg or higher. Thus, the limit of detection of the reaction was established in 10 fg of target genomic DNA.

4.2. Sensitivity of the test in DNA mixtures

The sensitivity of the test was determined in a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 0,5%, 0,1%, 0,05%, 0,01%, 0,005% and 0,001%, 0,0005%, 0,0001%, 0,00005%, 0,00001% and 0,000005%. All determinations were performed in duplicate.

Result:
Target amplification was observed in samples containing 0,00001% of target DNA. Thus, the method can detect 0,00001% of walnut DNA in 100 ng of total DNA.

5. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 5.1, using two positive and two negative samples and performed in duplicate.

Parameter	N° variations	Description
Annealing temperature	2	+ 2°C; - 2°C
MgCl2 concentration	2	+ 0,2mM; - 0,2mM
Independent performer		Not applicable
Different Real Time equipment	2	ABI 7500 PikoReal, Thermo Scientific
Independent laboratories	1	Biopremier R&D laboratory

Table 5.1 – Method's parameters submitted to variations

Result:
Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

6. TRUENESS

Trueness of the method was evaluated using 5 positive and 13 negative food samples. All the samples were tested in duplicate.

Result:
All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%

Performance Characteristics for validation

The determination of false positive and false negative rate, the method was performed in 18 food samples (5 positive and 13 negative samples).

The method's limit of detection was determined in a mixture containing a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 0,5%, 0,1%, 0,05%, 0,01%, 0,005% and 0,001%, 0,0005%, 0,0001%, 0,00005%, 0,00001% and 0,000005%. All determinations were performed in duplicate.

1. FALSE POSITIVE RATE

This corresponds to the probability that a known negative sample has been classified as positive by the method. The false positive rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fpos}=n_{fpos}/n_{rneg}+n_{fpos}$ x 100%

In which:

P_{fpos}: False positive rate
n_{rneg}: Number of real negative test results
n_{fpos}: Number of misclassified known negative samples

Result:

For the present method, false positive rate was calculated based on 18 samples. The final result was 0%

2. FALSE NEGATIVE RATE

This corresponds to the probability that a known positive sample has been classified as negative by the method. The false negative rate (that can be expressed as a percentage) is calculated by the expression:

$P_{fneg}=n_{fneg}/n_{rpos}+n_{fneg}$ x 100%

In which:

P_{fneg}: False negative rate

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:

For the present method, false negative rate was calculated based on 18 samples. The final result was 0%

3. LIMIT OF DETECTION

3.1. Limit of detection of the Real-Time PCR reaction

The limit of detection of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 100 pg, 50 pg, 10 pg, 5 pg, 1pg, 500 fg. 50 fg , 10 fg, 5 fg and 1fg.

Result:

The limit of detection determined with a probability 0,95% (false positive rate < 0,05) was 10 fg of the target genomic DNA.

3.2. Limit of detection of the test in DNA mixtures

The method's limit of detection was determined in a mixture containing a proportion of the target DNA in a given amount of total DNA. There were tested the following target DNA proportions in 100 ng of total DNA: 0,5%, 0,1%, 0,05%, 0,01%, 0,005% and 0,001%, 0,0005%, 0,0001%, 0,00005%, 0,00001% and 0,000005%. All determinations were performed in duplicate.

Result:

The limit of detection determined with a probability 0,95% (false positive rate < 0,05) was 0,00001% of walnut DNA in 100 ng of total DNA.

Positive predictive value (PPV)

The PPV is calculated by the expression:

$PPV=n_{rpos}/n_{rpos}+n_{fpos}$ x 100%
In which:
n_{rpos}: Number of real positive test results
n_{fneg}: Number of misclassified known positive samples

Result:
The positive predictive value determined was 100%.

Negative predictive value (NPV)
The PPV is calculated by the expression:

$NPV=n_{rneg}/n_{rneg}+n_{fneg}$ x 100%
In which:
n_{rneg}: Number of real negative test results
n_{fneg}: Number of misclassified known negative samples

Result:
The negative predictive value determined was 100%.

Criteria for acceptance
Inclusivity: 95%

Exclusivity: 95%

Selectivity: 95%

Trueness: 95%

False positive rate: 5%

False negative rate: 5%

Performance declaration
Accordingly with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results, despite the exclusivity value being slightly lower than the Condalab requirements. The present test allows the "Detection of Walnut DNA. using real time-PCR" in food samples after enrichment.

Condalab

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Detection of *Zygosaccharomyces bailii* using real-time PCR

VALIDATION REPORT

ASSAY

Zygosaccharomyces bailii detection by 5' nuclease real time-PCR assay.

VALIDATED EQUIPMENT

Applied Biosystems, model 7500; Thermo Scientific, model PikoReal; Condalab, model CDL-96.

SAMPLES (N)

Enrichments prepared from suitable food samples. During re-validation samples from external quality assessment schemes from FAPAS, Instand and INSA-PHE were tested.

VALIDATION ASSAYS

Start – 29-12-2014
Finish – 06-04-2015

DEFINITIONS

Analyte

DNA of the microorganism detected by the analysis method

Qualitative method

Analysis method which response is either the presence or absence of the microorganism either directly or indirectly in a certain amount of sample.

True positive (TP)

Sample presenting a positive result for the test and that has equal or higher amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

True negative (TN)

Sample presenting a negative result for the test and that has a lower amount of the target analyte (microorganism DNA) than the detection limit established in the specified experimental conditions.

False Positive (FP)

Sample presenting a positive result for the test in the absence of target analyte.

False Negative (FN)

Sample presenting a negative result for the test in the presence of equal or higher amount of the target analyte than the detection limit.

Detection Limit (LOD)

- Reaction detection limit: lowest concentration of the target DNA that can be consistently detected in the specified experimental conditions.
- Method reaction limit: lowest concentration or content of the target microorganism relative to the defined amount of matrix that can be consistently detected in the specified experimental conditions.

Inclusivity

Detection of the target microorganism.

Exclusivity

Non-detection of non-target microorganisms.

Selectivity

Measure of the inclusivity and exclusivity.

Sensitivity (S)

Measure of the lowest number of analyte cells which can be detected in a single test reaction.

Specificity (Sp)

Ability to exclusivity recognize the target microorganism to be detected and distinguish it from other similar organisms and impurities.

Trueness (T)

Closeness of agreement between the expectation of a test result and the corresponding true value.

Precision (P)

Closeness of agreement between independent tests obtained under defined conditions. It depends only on the distribution of random errors and does not relate to the true value of the test.

Positive predictive value (PPV)

Probability of a positive test be referent to a sample presenting an analyte concentration above.

Negative predictive value (NPV)

Probability of a positive test be referent to a sample presenting an analyte concentration above.

SCOPE OF THE METHOD

Zygosaccharomyces bailii is a food spoilage yeast with high importance for wine, beverage and food industry, because of its ability to grow in the presence of weak acid preservatives such as benzoic and sorbic acids. *Z. bailii* is responsible for significant economic loss in the wine and food industry and its efficient detection is of most importance.
The present method is based on a 5' nuclease real-time PCR reactions and amplify a unique genomic sequence of *Zygosaccharomyces bailii*, aiming the detection of this spoilage organism DNA in wine samples.

SCIENTIFIC BASIS

The present method is based on a 5' nuclease real-time PCR assay, in which specific fluorescent probes are used to detect the amplified DNA by hybridizing with amplicons. These probes are linked to a fluorophore on one end and a quencher that suppresses fluorescence on the other. If the target sequence is present during the PCR, amplification occurs and the probe is degraded, resulting in fluorescence increase. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus number of cycles, allowing the determination of the presence or absence of the target organism.

The method also includes a DNA Internal Control (IC). This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a second fluorophore. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

SAMPLES

For the validation of the present test were used 6 wine samples obtained from different commercial sources.

PERFORMANCE CHARACTERISTICS OF THE METHOD

1. ANALYTICAL CONTROLS

Each set tests performed simultaneously with the present method include the following controls:

External positive control (PC): reaction containing genomic DNA of the Zygosaccharomyces bailii

Negative control (NC): reaction without any target DNA

Each individual reaction includes:

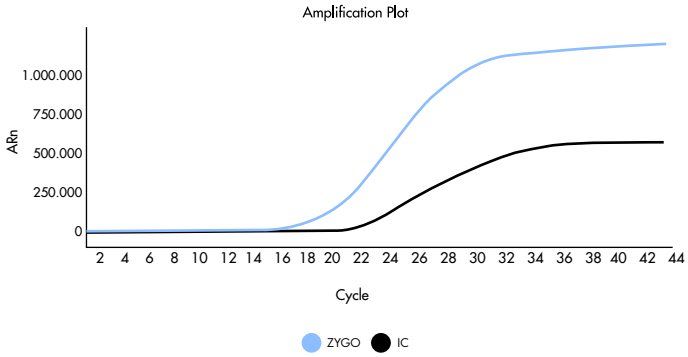
Internal amplification control (IC): all reactions include an IC DNA and the corresponding set of primes and probe, labeled with a second fluorophore. This control is amplified at the same time, but independently from target DNA sequence. The inclusion of the IC in each reaction avoids false negatives due to the presence of substances inhibitory to PCR and validates any negative result.

To validate the tests, the controls must show the following results:

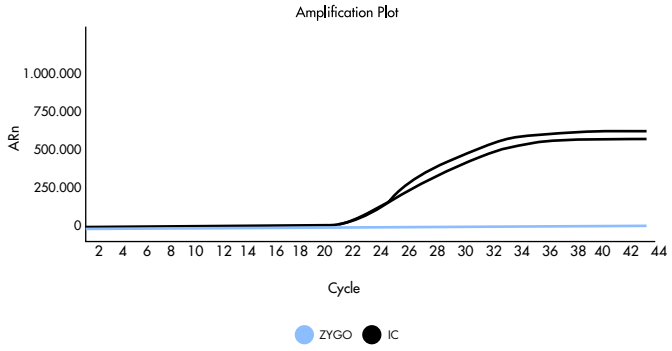
	Target detection Chanel 1	Target detection Chanel 2
Negative Control	Negative	Positive
Positive Control	Positive	Not significant

If the controls do not match these results, the experiment must be repeated.

Positive control



Negative control



The results obtained by the present method shall be interpreted as follows:

Target detection Chanel 1	Target detection Chanel 2	Interpretation
Positive	Not significant	Positive
Ct = NA	Positive	Negative
Ct = NA	Ct = NA	Inhibition**

When both target and IC detection are negative, it indicates the presence of an amplification inhibitor and the sample must be tested again after 1/10 dilution.

2. INSTRUMENTS

Real Time instruments must be equipped with FAM and ROX detection channels. No special requirements are necessary of the other instruments required (centrifuge, heating block and micropipettes).

The method was validated in an ABI 7500 instrument (Applied Biosystems) and PikoReal (Thermo Scientific). Comparable results were obtained with CFX96 (BioRad).

3. SELECTIVITY

3.1. Inclusivity test

A total of 4 *Zygosaccharomyces bailii* strains were tested (Table 3.1). The strains were tested using 2 ng of genomic DNA.

Code	Strain identification	Other informations
MF 135	*Zygosaccharomyces bailii*	
MF 136	*Zygosaccharomyces bailii*	
MF 137	*Zygosaccharomyces bailii*	
MF 138	*Zygosaccharomyces bailii*	

Table 3.1 – List of *Zygosaccharomyces bailii* strains used for inclusivity test

Result:

All strains were detected with the present method, corresponding to 100% inclusivity.
l samples. The final result was 0%

3.2. Exclusivity test

A total of 32 non target microorganisms related with the target or occurring in the same habitat and that could cause interference with test results were de tested (Table 3.2). The strains were tested using 10 ng of genomic DNA. The suitability of the DNA extracts for amplification was confirmed amplification of the 16S rRNA bacterial gene, using universal primers. The identification of all strains was confirmed by 16S rRNA gene sequencing. All strains were tested in triplicate.

Code	Strain identification	Other informations
MF 22	*Aspergillus flavus*	
MF 23	*Aspergillus fumigatus*	
MF 24	*Aspergillus fumigatus*	
MF 33	*Fusarium sp*	
MF 39	*Trichophyton tonsurans*	
MF 46	*Sporothrix schenckii*	
MF 49	*Scopulariopsis brevicaulis*	
MF 55	*Candida parapsilosis*	PYCC 2545T
MF 58	*Candida glabrata*	PYCC 2418T
MF 61	*Candida tropicalis*	PYCC 3097T
MF 64	*Candida lusitaneae*	PYCC 2705T
MF 67	*Candida krusei*	
MF 70	*Cryptococcus neoformans*	
MF 71	*Trichosporon sp*	
MF 75	*Trichophyton erinacei*	
MF 86	*Trichophyton verrucosum*	
MF 90	*Arthroderma benhamiae*	
MF 98	*Trichophyton mentagrophytes*	
MF 128	*Saccharomyces cerevisae*	
MF 129	*Aspergillus nigger*	
MF 133	*Dekkera anomala*	
MF 134	*Bretanomyces naardenensis*	
MF 130	*Dekkera bruxellensis*	
MF 131	*Dekkera bruxellensis*	
MF 132	*Dekkera bruxellensis*	
MF 140	*Zygosaccharomyces bisporus*	
MF 141	*Zygosaccharomyces lentus*	
MF 142	*Zygosaccharomyces rouxii*	
MF 144	*Lactobacillus paracasei subsp paracasei*	
(not classified)	*Oenococcus oeni*	
(not classified)	*Pichia membranifaciens*	
(not classified)	*Schizosaccharomyces pombe*	

Table 3.2 – List of strains used for exclusivity test

Result:

The method presented 100% specificity as none of the species showed a positive result.

4. SENSITIVITY

4.1. Sensitivity of the Real-Time PCR reaction

The sensitivity of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1 ng, 500 pg, 50 pg, 5pg, 500 fg, 50 fg, 25 fg and 10 fg.

Result:
Target amplification was observed with DNA amounts of 50 fg or higher. Thus, the limit of detection of the reaction was established in 50 fg of target genomic DNA.

4.2. Sensitivity of the test in DNA mixtures

The sensitivity of the test was determined in wine samples artificially contaminated with decreasing number of fungal cells. The sensitivity was tested in 4 wine samples. The sample' concentration was performed with 45 mL of each sample, after inoculation with 102-103 , 10^3-10^4 , 10^4-10^5 and 10^5-10 cfu of Zygosaccharomyces bailii. All procedures starting from DNA extraction were performed in triplicate.

Result:
Target amplification was observed in samples contaminated with 102-103 cfu/45 mL or more. No amplification was obtained in non-contaminated samples (inoculated with 0 cfu). Thus, the method can detect 102-103 cfu/45 mL of wine (LOD).

5. ROBUSTNESS

Robustness of the present method was determined introducing experimental and technical variations in the parameters described in Table 5.1, using two positive and two negative samples and performed in triplicate.

Parameter	N° variations	Description
Annealing temperature	2	+ 2°C; - 2°C
MgCl2 concentration	2	+ 0,5mM; - 0,5mM
Independent performer	2	Not applicable
Different Real Time equipment	3	ABI 7500 PikoReal, Thermo Scientific BioRad CFX96
Independent laboratories	2	Biopremier R&D laboratory Portuguese Food Safety Authority laboratory

Table 5.1 – Method's parameters submitted to variations

Result:
Congruent results were obtained for all samples in all parameters variants tested. All samples also presented the expected test results.

6. TRUENESS

Trueness of the method was evaluated using 4 positive and 2 negative wine samples for the target. All the samples were tested in triplicate.

Result:
All samples presented the expected test results for all the replicates, corresponding to a trueness of 100%

Performance Characteristics for validation

For the determination of false positive and false negative rate, the method was performed in 6 samples (4 positive and 2 negative samples).
For the determination of method's limit of detection, the sample was inoculated considering 4 contamination levels: 10^2-10^3 , 10^3-10^4 , 10^4-10^5 and 10^5-10^6 cfu in 45 mL of wine sample. All the procedures were performed as defined for the present method. All experiments starting from DNA extraction were performed in triplicate.

1. FALSE POSITIVE RATE

This corresponds to the probability that a known negative sample has been classified as positive by the method. The false positive rate (that can be expressed as a percentage) is calculated by the expression:
$P_{fpos}=n_{fpos}/n_{rneg}+n_{fpos}$ x 100%
In which:
P_{fpos}: False positive rate
n_{rneg}: Number of real negative test results
n_{fpos}: Number of misclassified known negative samples

3. LIMIT3.1. Limit of detection of the Real-Time PCR reaction

The limit of detection of the reaction was determined by testing in triplicate decreasing quantities of target DNA under the experimental conditions of the present method. There were tested the following genomic DNA amounts: 1 ng, 500 pg, 50 pg, 5pg, 500 fg, 50 fg, 25 fg and 10 fg.

Result:

The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 50 fg of *Zygosaccharomyces bailii* genomic DNA.

3.2. Limit of detection of the method

The limit of detection of the test was determined in wine samples artificially contaminated with decreasing number of fungal cells, using 4 wine samples. The sample' concentration was performed with 45 mL of each sample, after inoculation with 10^2-10^3 , 10^3-10^4 , 10^4-10^5 and 10^5-10^6 cfu of *Zygosaccharomyces bailii*. All procedures starting from DNA extraction were performed in triplicate.

Result:

The limit of detection determined with a probability 0.95% (false positive rate < 0.05) was 102-103 cfus *Zygosaccharomyces bailii*.

Positive predictive value (PPV)

The PPV is calculated by the expression:

$PPV=n_{rpos}/n_{rpos}+n_{fpos}$ x 100%

In which:

n_{rpos}: Number of real positive test results

n_{fneg}: Number of misclassified known positive samples

Result:

The positive predictive value determined was 100%.

Negative predictive value (NPV)

The PPV is calculated by the expression:

$NPV=n_{rneg}/n_{rneg}+n_{fneg}$ x 100%

In which:

n_{rneg}: Number of real negative test results

n_{fneg}: Number of misclassified known negative samples

Result:

The negative predictive value determined was 100%.

Criteria for acceptance

Inclusivity: 95%

Exclusivity: 95%

Selectivity: 95%

Trueness: 95%

False positive rate: 5%

False negative rate: 5%

Performance declaration

Accordingly, with the performed tests and the indicative results obtained, it is considered that the present test allows to achieve suitable results in agreement with the Condalab requirements and is validated.

The present test allows the "*Detection of of Zygosaccharomyces bailii using real time-PCR*" in wine and beverages, cultures and food samples.

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