Rapid Detection and Identification of Bacteria and Yeasts in Food,Beverages and Water

Abstract

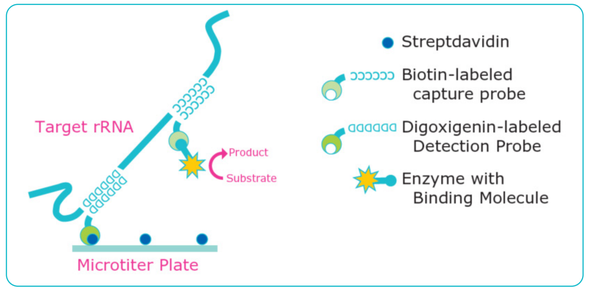
HybriScan® system is a new technology based on the detection of microbe-specific rRNA using sandwich hybridization. It allows comprehensive and reliable routine control of microbial contamination during food production, from raw materials to finished goods. The system is sensitive and specific since the method is based on molecular genetic identification, and it allows detection of a group of microorganisms as well as specific species. The signal read-out is triggered optically by an enzymatically generated color change. No PCR is required because the method is quantitative without cell counting (using standards) and uses standard laboratory equipment.

The HybriScan® method is an economical, high throughput, 96-well microplate format system. The test is performed in less than 3 hours (in addition to the prep time) and offers a time saving of up to 10 days compared to cultivation-based assays. It is ideal for safety and quality control of alcoholic and nonalcoholic beverages, water and food. Specific examples include the detection of food-borne pathogens like *Salmonella, Campylobacter, Listeria* and *Cronobacter* spp., and counting of *Legionella* in water, including the most relevant species, *L. pneumophila*. Organisms can be detected at any level of classification, from species or genus to higher ranks.

Introduction

Methods for a rapid, sensitive and reliable detection and quantification of micro- organisms and pathogens in food, beverages and water are receiving increasing attention. The sandwich hybridization method used in the HybriScan® Test system is a suitable alternative for such analysis. This test method is independent of the influence of sample matrices and is able to distinguish between live and dead cells. Furthermore, the detection of non-culturable microbes is possible.

The HybriScan® method is based on the detection of rRNA via hybridization events and specific capture and detection probes **(Figure 1)**. Specificity is achieved by targeting conserved or unique rRNA sequences. A biotin-labeled capture probe is used to immobilize the target sequence on a solid support plate (streptavidin- coated microtiter plate). A digoxigenin-labeled detection probe provides an enzyme-linked optical signal read out. Detection results from application of anti- DIG-horseradish peroxidase Fab fragments. The bound complex is visualized by horseradish peroxidase substrate TMB (3,3',5,5'-tetramethylbenzidine). Photometric data are measured at 450 nm and compared with standard solutions. The HybriScan® software enables easy measurement and data analysis.



Discussion

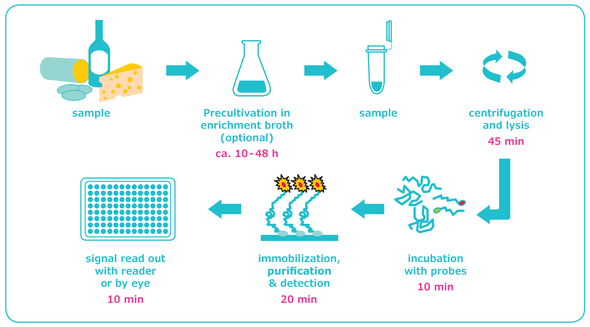
Sensitivity, specificity, flexibility and applicability of HybriScan® technology

Sandwich hybridization is very sensitive, detecting attomoles of the respective target rRNA molecules [1]. The ideal hybridization target for bacteria and yeast is rRNA. These cells contain a large number of rRNA-containing ribosomes; a single cell therefore contains several thousand copies of rRNA but only one DNA. Sandwich hybridization also provides sensitivity in crude biological samples because it is not susceptible to matrix interference.

By using specific probes, the HybriScan® technology allows flexible group- and species-specific detection. It is applicable to many analytical fields, including monitoring the microbial content of beer, wine, non-alcoholic beverages, drinking water, a wide variety of foods and wastewater. The HybriScan® system rapidly and accurately identifies, detects and quantifies many important pathogenic species, including *Salmonella, Campylobacter, Listeria* and *Legionella* including the most relevant species *L. pneumophila*. [2,3,4]. The HybriScan® test system is ideal for the comprehensive and reliable routine control of raw materials and concentrates in all production steps up to the quality check of finished goods.

Figure 2: Workflow of HybriScan® test method

The HybriScan® system is a simple, time-saving assay that can be performed with standard laboratory equipment.



Benefits over conventional detection methods and PCR

The HybriScan® method has significant time- and labor-saving benefits over traditional methods. It also has benefits over PCR and real time PCR, which, although highly sensitive, are susceptible to experimental interferences, like template inhibition from insufficient purification, and lack quantification accuracy due to biases associated with PCR and reverse transcription reactions [5]. In contrast, the HybriScan® method is nearly independent of the influences of sample matrix and detects only living cells. It also permits the detection of non-culturable microbes. **Table 1** compares the benefits and disadvantages of the various methods.

HybriScan® *Listeria monocytogenes*: An example for that rapid and innovative test system

One of the most important foodborne pathogens is *Listeria monocytogenes* **(Figure 3)**, which poses a health threat in foods that have long, refrigerated shelf lives [6]. Listeriosis, caused by ingestion of foods contaminated with *Listeria monocytogenes*, has increased dramatically in recent years, causing a great deal of distress and even death. Milk, cheese, ice cream and meat contaminated with this pathogen have led to recent outbreaks of listeriosis [7].

L. monocytogenes proliferates at refrigeration temperatures and is able to grow over a wide pH range from 4.4 to 9.4.

Table 1: Advantages of HybriScan® system over other detection techniques

Detection technology	Advantage	Disadvantage
HybriScan® system	- detects only living cells - minimal interference by sample matrix - high specificity - ow cross-reactivity - easy handling - cost-efficient read-out devices - quantitative and qualitative - high sample throughput (96-microwell plates) - detects non-culturable microbes	- no differentiation of serotypes or subspecies - limited probe design (rRNA target)
PCR	- high sample throughput - sensitive - quantitative	- no live/dead cell differentiation sensitive to matrix interference (high extraction effort) - susceptible to polymerase inhibition
ELISA	- differentiation of serotypes or subspecies - high sample throughput (96-microwell plates) - quantitative and qualitative	- low sensitivity - low specificity, higher cross- reactivity - slow and expensive assay development
Conventional cultivation-based methods	- relatively inexpensive - simple - specific - widely accepted method	- time-consuming (up to 10 days) - no detection of non-culturable Microbes - low sample throughput - laborious

Figure 3. *Listeria monocytogenes* Colonies Grown on PALCAM Agar

Conventional culture-based methods to detect *L. monocytogenes* generally involve selective enrichment followed by culturing on selective medium, isolation and biochemical identification [8]. This laborious and time-consuming approach often takes several days to show results. Also, compared to molecular biological and immunological methods, culture-based methods often give false negatives.

HybriScan® *Listeria monocytogenes* is an excellent alternative to lengthy culture- based methods. It is as reliable and comprehensive as classical methods, but permits rapid detection and quantification with results available within 48 hours.

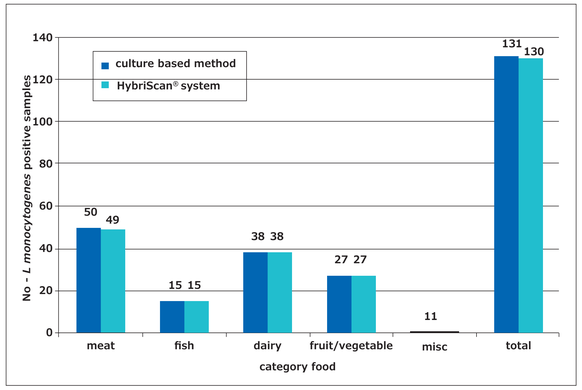
The species-specific probe permits direct detection of *L. monocytogenes*, thereby eliminating false positives caused by other *Listeria* species. Even more compelling, suspected single colonies can be identified within one hour using the HybriScan® identification kit without need for further cultivation.

Figure 4 shows the validation results of HybriScan® *Listeria monocytogenes*. Food samples were analyzed with the HybriScan® method and compared to the culture- based method according to 64-LFGB. Five different food categories were tested. 355 food samples were analyzed and compared to culture-based method according to 64-LFGB. The blue values are the number of analyzed food samples in each category. Validation was according to ISO 16140:2003 (ASU L00.00-22). Results of validation showed a relative accuracy of 99.2 %, relative specificity of 98.5% and relative sensitivity of 99.6%.

Two versions are available. HybriScan® *Listeria monoytogenes* is used for the extremely rapid, sensitive and economical identification of suspect colonies of

L. monocytogenes. HybriScan® *Listeria monocytogenes* is used for the detection, identification and quantification of *L. monocytogenes* in different food matrixes.

Figure 4: Validation of HybriScan® *Listeria monocytogenes*



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Rapid Test System for the Detection of Beer-Spoilage Bacteria

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Abstract

Beer-spoiling microorganisms cause an increase of turbidity and unpleasant sensory changes in beer. Since the improved process technology in modern breweries has resulted in significant reduction of oxygen content in the final product, the role of strictly anaerobic bacteria like *Pectinatus* and *Megasphaera* has increased. Detection of these organisms, which is traditionally done by incubation on culture medium, takes a week or even longer. A rapid molecular test system is desirable for the detection of all known beer-spoiling microorganisms in one test only. Results of this study on a rapid test kit demonstrate that all beer-spoiling microorganisms can be detected and identified in a shorter time and in one test only.

Introduction

Beer continues to be a popular drink. It is important to maintain quality and enhance stability of beer during the production process through early detection of beer-spoiling microbes. The known beer-spoiling species and genera listed in Table 1 can all be detected by HybriScan®***D*** Beer, a rapid molecular test system for the detection of these microorganisms in one test.

Table 1: The Beer Spoiling Species and Genera that can be Detected by the HybriScan®***D*** – Beer Test

Genus *Lactobacillus*
Lactobacillus acidophilus
Lactobacillus brevis
Lactobacillus brevisimilis
Lactobacillus buchneri
Lactobacillus casei
Lactobacillus collinoides
Lactobacillus coryniformis
Lactobacillus curvatus
Lactobacillus fermentum
Lactobacillus fructivorans
Lactobacillus lindneri
Lactobacillus malefermentans
Lactobacillus paracasei
Lactobacillus parabuchneri
Lactobacillus paraplantarum
Lactobacillus plantarum
Lactobacillus rhamnosus

Genus *Pediococcus*
Pediococcus acidilactici
Pediococcus damnosus
Pediococcus inopinatus
Pediococcus parvulus
Pediococcus pentosaceus

Genus *Pectinatus*
Pectinatus cerevisiiphilus
Pectinatus frisingensis
Pectinatus haikare
Pectinatus portalensis
Pectinatus spp.

Genus *Megasphaera*
Megasphaera cerevisiae

HybriScan®***D*** Beer test is based on the detection of target molecules from the microorganisms of interest by means of specific capture and detection probes in a so-called sandwich hybridization. The hybridization reaction of the target molecules with the Biotin-labeled capture and a DIG-labeled detection probe takes place in a streptavidin coated microtiter plate (Figure 1).

After coupling of the target molecule to the microtiter plate, an enzyme is attached in a subsequent incubation step. After several washing steps, reaction with a color substrate gives blue coloration that changes into yellow color after the addition of a stop solution. The yellow color enables highly sensitive photometric measurement at 450 nm (Figure 2). Comparison is made with the standard solutions contained in the test kit.

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Experimental

In this study, beer samples with alcohol content between 2.5% and 6.7% were tested with HybriScan®***D*** beer test kit. The beer samples were spiked with *L. brevis*, *L. coryniformis*, *L. lindneri*, *Ped. damnosus*, *M. cerevisiae,* and *Pec. frisingensis* in a range between 10^3 and 10^6 cfu/sample. The bacteria were cultivated in media according to DSMZ. The cultures were harvested and washed with sterile saline solution. The bacterial counts were then determined by measuring OD_{600} and by counting colony forming units (cfu) on agar.

Afterwards cultures were inoculated into the beer.

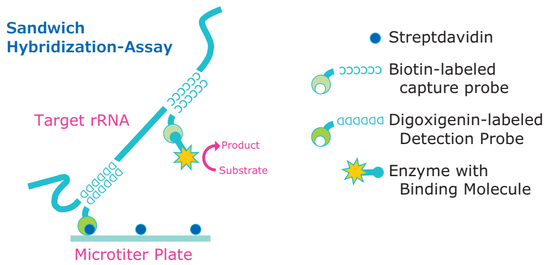
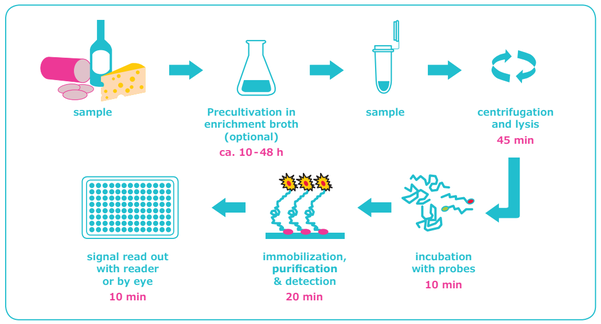


Figure 1: Sandwich Hybridization between Target rRNA Capture and Detection Probe



total analysis time: ca. 2 hours

Figure 2: Test performance – HybriScan®***D*** Beer

Additional 30 brewery samples were examined with the HybriScan®***D*** beer test for beer-spoiling microorganisms. Up to 10 mL of the beer sample was processed according to the HybriScan®***D*** protocol published by Sigma-Aldrich®. The results were controlled by cultivation on NBB agar.

For the development of further genera and species, specific test solution experiments were performed with 11 additional pure bacterial cultures of most common beer-spoiling micro-organisms for detection by HybriScan®***D*** test experiments. These bacterial strains were also cultivated on media according to DSMZ and processed according to HybriScan®***D*** protocol.

Results

In this study, a total of 178 samples, with alcohol content between 2.5% and 6.7%, were tested with HybriScan®***D*** beer test. In order to confirm that it is possible to detect different beer-spoiling species with the test solution beer, the samples were spiked with *L. brevis*, *L. coryniformis*, *L. lindneri*, *Ped. damnosus*, *Pec. frisingensis* and *M. cerevisiae*. Results were compared with inoculated amount of bacteria. Results of 18 samples are presented in Figure 3. The bacterial counts in the beer samples which were determined by the HybriScan®***D*** test were in the same range as the inoculated values.

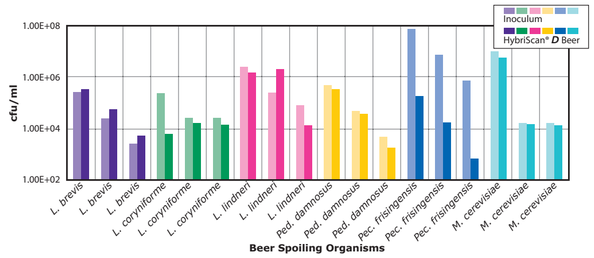


Figure 3: Bacterial Counts of 18 Spiked Beer Samples Determined by the HybriScan®***D*** Beer Compared to the Counts of Inoculated Amounts.

Pec. frisingensis was detected only in beer sample with 2.5% alcohol. Results of the HybriScan®***D*** test were below the counts of the inoculum. The number of microbes in beer is possibly diminished because the strain used in the study was not adapted to beer. *Pec. frisingensis* were not detected in beer with a higher alcohol content (data not shown). It was observed that beer with a low alcohol content is more prone to spoilage with *Pectinatus* and *Megasphaera* species (1). *Pectinatus* species are more resistant and can survive in acidic condition. The pH tolerance of these anaerobic bacteria is influenced by the presence of ethanol (2).

Real beer samples from the brewery were also tested with the HybriScan®***D*** beer test for beer-contaminating bacteria. These samples were also simultaneously checked with NBB agar. The HybriScan®***D*** test gave a positive signal for each sample. These results were confirmed on agar (Figure 4).

For a more sophisticated analysis, seven other test solutions "*Pectinatus spp./Megasphaera spp.*", "*Pediococcus spp.*", "*Lactobacillus brevis*", "*L. lindneri*", "*L. collinoides*", "*L. rossiae*" and "*L. backii*" were developed and optimized. Experiments were performed with 17 beer-spoiling bacteria strains. Eight different test solutions were tested with 10 beer-spoiling bacteria species. The experiment was performed with bacterial cultures which contained bacterial amounts ≥10^6 cfu/sample.

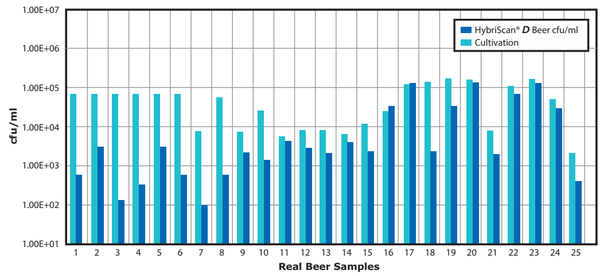


Figure 4: Counts of Real Beer Samples Determinated by HybriScan®***D*** Compared to the Counts of NBB Agar.

The test solution beer gave a positive signal with all used bacteria. The yellow signals at the microtiter plate confirm that the optimized test solutions react specifically and show no cross-reactivity (Figure 5). The experiments for the optimization of the test solutions indicated that the detection limit of those test solutions differs for *Lactobacillus* spp. and the other mentioned beer spoiling genera between 10^4 and 10^5 cfu/sample.

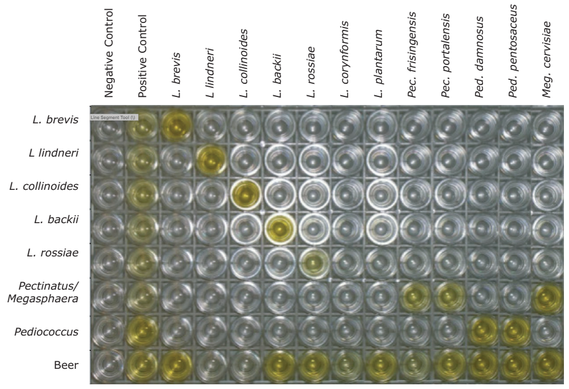


Figure 5: Result of a HybriScan®***D*** Test with 8 Different Test Solutions and 10 Different Beer Contaminating Bacteria after Addition of the Stop Solution following HybriScan®***D*** Test Protocol

Conclusions

- It is a rapid and sensitive molecular biological test system that is comparable to the classical cultivation method.
- It offers qualitative and quantitative detection of the most well-known beer-spoiling organisms in one test only.
- It is highly flexible as it allows combination with different read out technologies, and detection of rRNA and mRNA is possible.
- It is a robust test with easily predictable specificity of the probes, and easy synthesis of the oligonucleotide probes.
- It is a faster and cost-effective test with inexpensive read out device technology.

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Validation of shorter protocol for detection of *Salmonella enterica* subsp. Enterica in peanut butter samples followed by a rRNA detection system

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Abstract

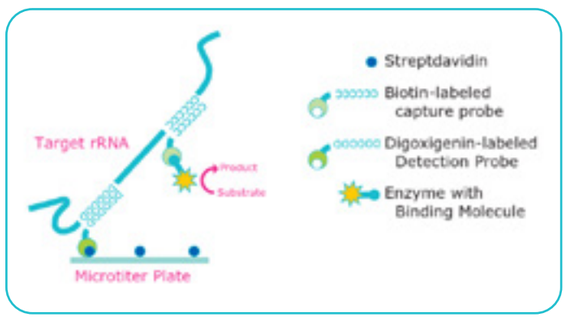
Food-borne pathogens *Salmonella* is commonly evaluated in manufacturing of peanut butter and other food products. For the HybriScan®***D*** *Salmonella* Test (Cat. No. 55662) the ISO based enrichment method is recommended. That means sample pre-enrichment for 18 hours at 37 °C in buffered peptone water (BPW) followed by a selective enrichment step in Rappaport-Vassiliadis (RV) Broth for 24 hours at 41 °C. HybriScan®***D*** *Salmonella* is a rRNA sandwich hybridisation detection system which needs at least 500 cfu/mL for the assay. This rapid molecular test system is desirable for the detection of *Salmonella* species like *S. Enteritidis*, *S. Typhimurium*, *S. Typhi* and *S. Paratyphi*. Results of this study on a rapid test kit demonstrate that *Salmonella* enterica subsp. enterica (ATTC® 13311™) can be detected and identified in a shorter time even in a difficult sample matrix like peanut butter.

Introduction

Peanut butter consist of about 20% carbohydrates, 25% proteins and 50% fat, *Salmonella* cells are just a very small component of the overall sample material and may be attached within the food matrix as single cells or clumps of cells. Normally before rapid detection methods can be used successfully, it is usually necessary to separate the target cells from the food matrix and from the background microflora. But even the HybriScan is as well a rapid molecular biological system it is practically insensitive to the sample matrix.

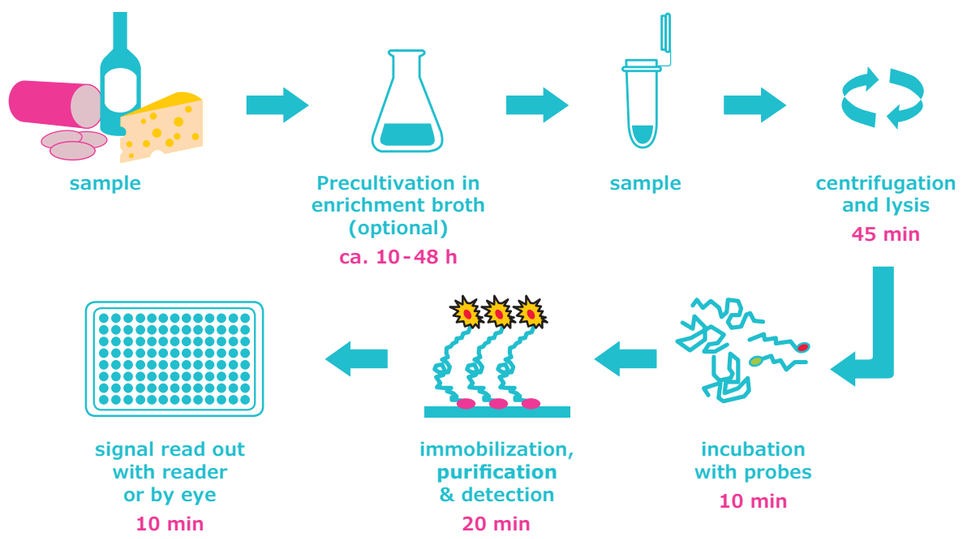
HybriScan®***D*** *Salmonella* test is based on the detection of target molecules from the microorganisms of interest by means of specific capture and detection probes in a so-called sandwich hybridization. The hybridization reaction of the target molecules with the Biotin-labeled capture and a DIG-labeled detection probe takes place in a streptavidin coated microtiter plate (**Figure 1**).

Figure 1: Sandwich Hybridization between Target rRNA Capture and Detection Probe.



After coupling of the target molecule to the microtiter plate, an enzyme is attached in a subsequent incubation step. After several washing steps, reaction with a color substrate gives blue coloration that changes into yellow color after the addition of a stop solution. The yellow color enables highly sensitive photometric measurement at 450 nm (**Figure 2**). Comparison is made with the standard solutions contained in the test kit.

Figure 2: Work flow – HybriScan®*D Salmonella.*



Experimental

The matrix to be examined was Peanut Butter (9 different brand codes with 12 different code dates). The pre-enrichment time described in the protocol of the HybriScan®***D*** *Salmonella* test takes 42 hours. In this experiment one target was to reduce the enrichment time to 24 hours in total. The cultivation time of the pre-enrichment peptone water culture took 18 hours; the incubation time of the selective enrichment culture in Rappaport-Vassiliades Enrichment Broth was shortened to 6 hours. Per peanut butter batch, one sample wasn't inoculated and carried as a negative control (N.C.), 9 to 10 samples were inoculated with *Salmonella enterica* subsp. Enterica (ATTC® 13311™). Each Peanut Butter sample was treated as follows:

- 25 g of Peanut Butter (except the negative controls) were inoculated with 1 to 5 cells of *Salmonella enterica* subsp. Enterica.
- 225 mL buffered peptone water were added to each sample, the mixture was homogenised for 1 minute in a Stomacher and the sample were incubated for 18 hours at 37 °C.
- After 18 hours of incubation 0.1 mL of the pre-enrichment peptone water culture were transferred to 10 mL Rappaport-Vassiliades Enrichment Broth. The selective main enrichment was conducted for 6 hours at 41°C.

Cell lysis and the HybriScan®***D*** *Salmonella* assay were completed as described in the HybriScan®***D*** *Salmonella* test protocol. In addition, each negative control and inoculated sample were tested for *Salmonella* according to EN ISO 6579:2002.

Results

Evaluation of the samples was performed using the following formula as described in the HybriScan®***D*** *Salmonella* assay:

$$\textbf{Sample O.D.\%} = (O.D._{sample} - O.D._{N.C.}) \;/\; (O.D._{P.C.} - O.D._{N.C.}) \times 72.1\%$$

P.C. positive control (S3)

N.C. negative control (S1)

Samples with O.D.% values under 10 are considered negative.
Samples with O.D.% values from 10 to <20 are considered questionable.
Samples with O.D.% values ≥20 are considered positive.

Of the 107 inoculated with *Salmonella enterica* peanut butter samples, 106 were identified as clearly contaminated with *Salmonella* by the use of HybriScan®***D*** *Salmonella* assay. The result of 1 sample was considered questionable. All negative controls gave negative results in the HybriScan®***D*** *Salmonella* assay. See **Table 1– 4**.

Table 1

Code Date	Probe	Average O.D. 450 nm	Sample O.D.%	Cells/ 10 µL
	S1	0.046		0
	S2	0.149		10.0
	S3	0.377		30.0
	S4	1.017		90.0
1624251	1-1 N.C.	0.046	0.0	
	1-2	4.060	874.2	
	1-3	3.955	851.4	
	1-4	3.970	854.6	
	1-5	3.899	839.2	
	1-6	3.891	837.4	
	1-7	3.535	760.0	
	1-8	3.791	815.8	
	1-9	3.736	803.7	
	1-10	3.816	821.1	
1874251	2-1 N.C.	0.048	0.3	
	2-2	3.748	806.4	
	2-3	3.726	801.6	
	2-4	4.072	877.0	
	2-5	3.907	840.9	
	2-6	3.965	853.5	
	2-7	3.824	822.8	
	2-8	4.060	874.2	
	2-9	4.003	861.9	
	2-10	4.032	868.2	

Table 2

Code Date	Probe	Average O.D. 450 nm	Sample O.D.%	Cells/ 10 µL
	S1	0.051		0
	S2	0.169		10.0
	S3	0.409		30.0
	S4	1.171		90.0
01574251	3-1 N.C.	0.057	1.2	
	3-2	0.608	112.1	
	3-3	1.665	324.6	
	3-4	0.289	47.9	
	3-5	0.120	13.9	
	3-6	1.146	220.2	
	3-7	0.701	130.8	
	3-8	0.251	40.2	
	3-9	0.981	187.0	
	3-10	0.359	62.0	
01334252	4-1 N.C.	0.051	0.0	
	4-2	3.737	741.4	
	4-3	3.682	730.3	
	4-4	3.927	779.6	
	4-5	3.700	733.9	
	4-6	3.780	750.1	
	4-7	3.813	756.6	
	4-8	3.915	777.1	
	4-9	3.682	730.4	
	4-10	4.091	812.6	
00864252	5-1 N.C.	0.053	0.4	
	5-2	0.232	36.4	
	5-3	0.267	43.4	
	5-4	0.740	138.7	
	5-5	0.880	166.7	
	5-6	0.736	137.8	
	5-7	0.561	102.7	
	5-8	1.967	385.3	
	5-9	2.093	410.7	
	5-10	3.585	710.7	

S1= Standard1 0 cells/10 µL

S2=Standard2 10.000 cells/µL

S3=Standard3 30.000 cells/10µL

S4= Standard4 90.000 cells/10µL

N.C.= Negative control: sample was not inoculated with *Salmonella spp.*

Table 3

Code Date	Probe	Average O.D. 450 nm	Sample O.D.%	Cells/ 10 µL
	S1	0.058		0
	S2	0.154		10.0
	S3	0.356		30.0
	S4	0.982		90.0
93294252	6-1 N.C.	0.046	-2.8	
	6-2	3.727	886.2	
	6-3	3.994	950.8	
	6-4	3.659	869.8	
	6-5	3.512	834.4	
	6-6	3.430	814.5	
	6-7	3.588	852.8	
	6-8	1.738	405.9	
	6-9	4.067	968.5	
	6-10	3.727	886.2	
01304252	7-1 N.C.	0.046	-2.9	
	7-2	3.787	900.7	
	7-3	3.575	849.5	
	7-4	3.779	898.8	
	7-5	3.875	922.0	
	7-6	4.144	987.1	
	7-7	4.067	968.5	
	7-8	3.988	949.4	
	7-9	4.135	984.9	
	7-10	4.101	976.7	
01304252	8-1 N.C.	0.053	-1.2	
	8-2	3.507	833.2	
	8-3	3.906	929.4	
	8-4	3.708	881.7	
	8-5	3.672	873.1	
	8-6	4.059	966.4	
	8-7	3.914	931.5	
	8-8	4.101	976.7	
	8-9	3.750	891.8	
	8-10	3.852	916.5	

Table 4

Code Date	Probe	Average O.D. 450 nm	Sample O.D.%	Cells/ 10 µL
	S1	0.047		0
	S2	0.131		10.0
	S3	0.339		30.0
00984252	9-1 N.C.	0.060	3.1	
	9-2	2.749	668.2	
	9-3	0.267	54.4	
	9-4	1.231	292.9	
	9-5	0.166	29.3	
	9-6	1.516	363.3	
	9-7	2.969	722.7	
	9-8	1.921	463.5	
	9-9	3.646	890.1	
00894252	10-1 N.C.	0.050	0.7	
	10-2	1.372	327.7	
	10-3	3.943	963.6	
	10-4	4.063	993.3	
	10-5	3.913	956.2	
	10-6	3.543	864.7	
	10-7	3.803	929.0	
	10-8	3.869	945.3	
	10-9	4.112	1005.4	
	10-10	3.615	882.4	
01904252	11-1 N.C.	0.049	0.5	
	11-2	3.537	863.1	
	11-3	3.874	946.6	
	11-4	3.988	974.8	
	11-5	3.979	972.4	
	11-6	4.049	989.7	
	11-7	3.881	948.2	
	11-8	3.421	834.5	
	11-9	3.984	973.7	
	11-10	3.635	887.3	
00704252	12-1 N.C.	0.051	0.9	
	12-2	2.225	538.6	
	12-3	3.100	755.0	
	12-4	2.251	545.0	
	12-5	0.434	95.7	
	12-6	1.037	244.7	
	12-7	0.340	72.5	
	12-8	0.555	125.5	
	12-9	0.293	60.7	