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MASTERARBEIT

Optimierung der PCR für Microarray-basierte Pathogendetektion

ausgeführt am

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unter der Anleitung von

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Abstract

The microbial diagnostic microarray (MDM) that was developed by Kostić *et al.* (2007, 2010) allows the highly specific detection of food- and water-borne pathogens using the phylogenetically robust *gyrB* gene as diagnostic marker. However, the system lacks sensitivity; the amplification of the *gyrB* gene being the limiting factor. Thus, the *gyrB* PCR should be optimized in the course of this project. Different approaches were tried in order to do so: i) a nested PCR targeting the sequencing tags of the original, highly degenerate *gyrB* primers that were published by Yamamoto and Harayama (1995) and ii) new primers were designed.

The nested approach failed to yield reproducible results in the initial experiments and had to be discontinued. Therefore, new species-specific primers were designed for the set of selected species (*Salmonella* spp., *E. coli*, *Y. enterocolitica*, *Y. pseudotuberculosis*, *S. aureus*, *C. jejuni*, *C. lari*, *C. coli*, *C. upsaliensis*, *C. perfringens* and *C. difficile)* and tested individually and as primer mix using both single strain gDNA and gDNA mixes. It was found that a primer mix with a concentration of 150nM each performed best, improving the limit of detection (LOD) of the MDM by at least two log steps for *S.* Typhimurium. Tests with a mix containing gDNA from four different organisms showed that all of them could be detected at concentration of 50ng/µl when amplified with the new primers, while only two could be detected after amplification with the old primers. Furthermore, experiments with spiked food samples were conducted, showing that the new primers are suitable for the application in food analysis.

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1 Introduction

1.1 Foodborne Pathogens

The connection between food and diseases such as typhoid fever and tuberculosis was first recognized in the early 1900s (Rosenau, 1926; cited in Tauxe, 2002). Although due to advances in hygiene in food processing these conditions are hardly found in the industrial world anymore, they have been replaced by other diseases (Tauxe, 2002).

There are no exact data on the number of illnesses caused by foodborne pathogens; however, an initiative to close this data gap has been launched by the World Health Organization (WHO) (Newell *et al.*, 2010). There are several reasons for this lack of information: First, underreporting, since mild cases are often not reported. Second, many pathogens present in food can also be transmitted via other routes; and, third, there are still pathogens that remain undetected to date (Mead *et al.*, 1999).

According to the European Food Safety Authority (EFSA), in 2006 a total of 5,705 outbreaks resulting in 5,523 hospitalizations and 50 deaths was reported in the European Union (EU) (Beloeil, 2008). The causative agents responsible for foodborne outbreaks are listed in Table 1:

Table 1: Causative agents responsible for foodborne outbreaks in the EU in 2006 (Beloeil, 2008)

	Outbreaks				Human Cases		
Agent	total cases	% of total	General	House-hold	total cases	Hospital-izations	Deaths
Salmonella	3,131	53.9	1,520	1,611	22,705	3,185	23
Unknown	952	16.4	610	342	9,437	947	2
Foodborne viruses	587	10.2	373	214	13,345	553	3
Campylobacter	400	6.9	116	284	1,304	65	0
Staphylococcus	236	4.1	157	79	2,057	277	2
Toxins	86	1.5	20	66	834	261	3
Clostridium	81	1.4	55	26	1,651	44	2
Bacillus	78	1.3	66	12	964	34	0
Histamine	71	1.2	62	9	370	41	0
pathogenic *E. coli*	48	0.8	25	23	750	103	1
Shigella	33	0.6	19	14	138	22	0
Yersinia	26	0.4	11	15	604	15	2
Giardia	18	0.3	13	5	44	-	0
Trichinella	18	0.3	5	13	202	113	0
Listeria	9	0.2	5	4	120	89	17
Other	9	0.2	5	4	31	2	0
Cryptosporidium	7	0.1	4	3	59	0	0
Brucella	6	0.1	3	3	43	3	0
Flavivirus	6	0.1	2	4	26	25	0

	Outbreaks				Human Cases		
Agent	total cases	% of total	General	House-hold	total cases	Hospital-izations	Deaths
Klebsiella	3	0.1	2	1	109	1	0
Streptococcus	2	<0.1	2	0	236	-	-
EU Total	5,705	98,2	3,000	2,706	53,546	5,523	50
Total	5,907	100.0	3,075	2,732	55,029	5,790	55

Salmonella spp. accounted for 54% of the reported foodborne outbreaks with eggs and meat being the most common food vehicles. *Listeria* was the most severe causative agent resulting in a hospitalization rate of 74% (Beloeil, 2008).

1.2 DNA Arrays

1.2.1 Principle

"Microarrays are orderly miniaturized arrays containing large sets of DNA sequences that have been attached to a solid substrate using automated equipment such that each spot (element) corresponds to unique DNA" (Zhou and Thompson, 2004: 143). Upon hybridization of a labeled target prepared from unknown sample an ensuing hybridization pattern will give detailed information on the nature of this sample.

While the first microarrays were developed for monitoring gene expression, they are now also used in many other areas: cell differentiation, drug discovery, vaccine development, comparative genomics, microbial detection, single nucleotide polymorphism analysis and sequencing (Hashsham *et al.*, 2004; Schrenzel *et al.*, 2009).

Microarrays can be considered an advancement from dot-blotting, the difference being that a solid substrate, usually glass, is used for immobilization of the probes (Southern *et al.*, 1999). Based on the probe density, microarrays can be differentiated into macroarrays, microarrays, high-density oligonucleotide arrays (Gene Chips) and microelectronic arrays. The term macroarray refers to robotically spotted probes on a membrane. Microarrays, on the other hand, have a solid matrix, such as glass slides, and a greater probe density. For high-density oligonucleotide arrays, an *in situ* synthesis approach using photolithography is applied. A more recent development are microelectronic arrays, which are made up of a set of electrodes covered by agarose with an affinity moiety (Freeman *et al.*, 2000). An overview is given in Figure 1:

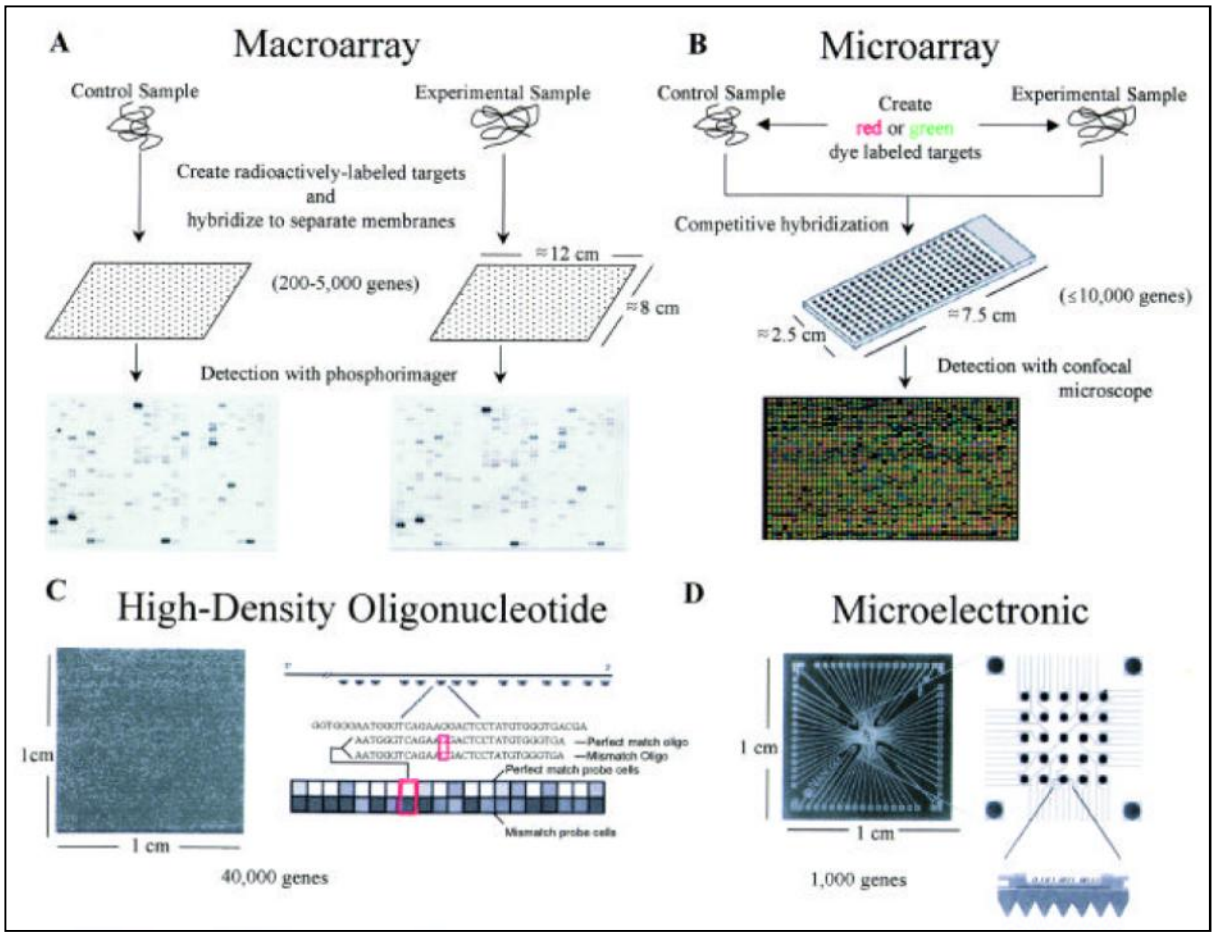

Figure 1: Array types (Freeman *et al.*, 2000)

Microarrays offer a wide range of applications in environmental microbiology and microbial ecology, human and veterinary diagnostics, industrial microbiology and detection of pathogens in food or water (Bodrossy and Sessitsch, 2004, Kim *et al.*, 2008; Kostić *et al.*, 2007; Kostić *et al.*, 2010; Maynard *et al.*, 2005).

1.2.2 Target Labeling and Detection

Only the method used for this work, i.e. Sequence Specific Labeling of Oligonucleotides (SSELO) will be addressed.

1.2.2.1 Sequence Specific Labeling of Oligonucleotides

Sequence-specific labeling of oligonucleotides (SSELO) for array analyses of microbial communities was introduced by Rudi and co-workers (2003) and allows high specificity and sensitivity. In this approach, reverse complement oligos (RC oligos) of the probes are end-labeled with Tamra-ddCTP in a linear amplification reaction, depending on the presence of the target, i.e. PCR products. The labeled RC oligonucleotides are hybridized with the microarray, allowing them to bind to the probes. Since they match the probes perfectly, practically no non-specific binding occurs; thus leading to detection sensitivity in the relative abundance range of 0.1% compared to 1 to 5% using conventional approaches (Kostić and Bodrossy, 2009).

1.2.3 Microarrays in Microbial Diagnostics

Microbial diagnostic Microarrays (MDMs) can be grouped in environmental MDMs used for microbial community analysis and clinical MDMs. The latter are used for detection and/or identification of one or few microorganisms at the level of species, subspecies or strain, while environmental MDMs are required to detect at the level of species, genus or higher taxon (Bodrossy and Sessitsch, 2004).

There are several platforms available for MDMs: planar glass microarray, macroarray (dot blots on nitrocellulose or nylon membranes), Affymetrix gene-chip and three-dimensional platforms. Each of these platforms has specific advantages and disadvantages regarding accessibility, price, probe density, flexibility, throughput and detection (Bodrossy and Sessitsch, 2004).

A schematic representation of the experimental approach is given in Figure 2:

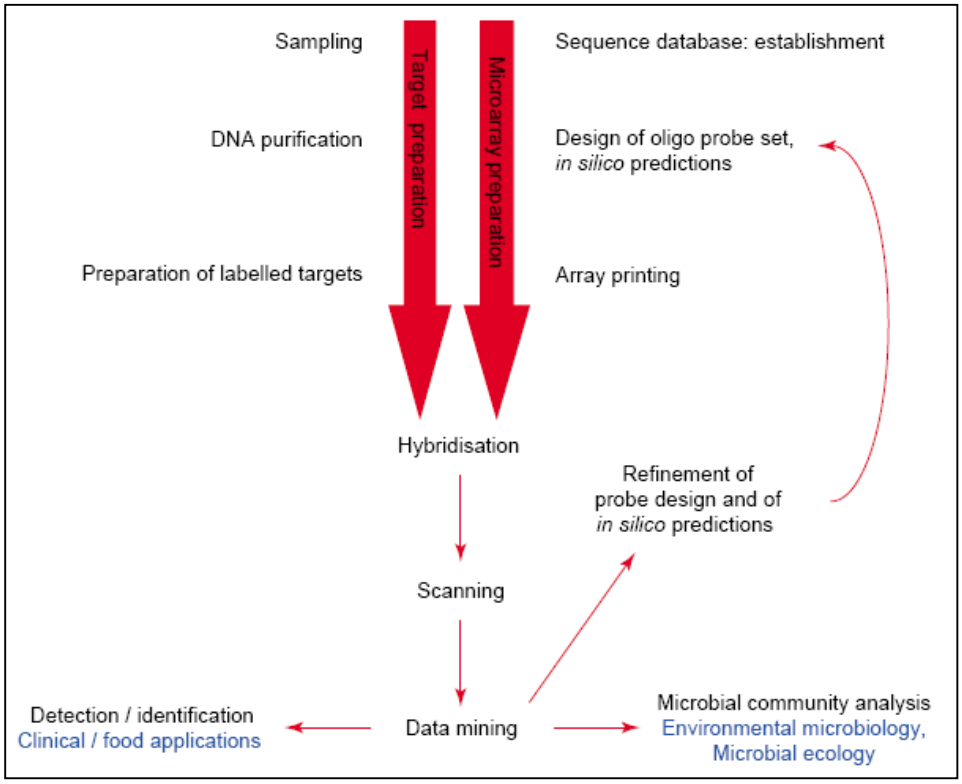

Figure 2: Schematic of the experimental approach (Bodrossy and Sessitsch, 2004)

The resolution of an MDM is determined by the conservation of the marker gene used. The *16S* rRNA marker is widely used, but due to the high overall sequence conservation it allows identification at species level at best (Bodrossy and Sessitsch, 2004; Loy and Bodrossy, 2006). For applications that require higher resolution, other universal markers have been suggested: i) the large-subunit ribosomal RNA (LSU rRNA), ii) the small-subunit-large-subunit rRNA (SSU-LSU rRNA) intergenic spacer region and iii) house-keeping genes (e.g. *rpoB*, *recA*, gyrA, *gyrB*, *groEL*, *atpD*, *tuf*, *ompA*, *gapA*, *pgi* or the tmRNA gene). Furthermore, a range of alternative marker genes such as: i) virulence genes, ii) antibiotic resistance genes and iii) functional genes encoding enzymes responsible for specific metabolic traits was suggested (Bodrossy and Sessitsch, 2004; Loy and Bodrossy, 2006).

The main disadvantage of these alternate markers, however, is that there is substantially less sequence information available as compared to the SSU rRNA databases thus impeding the development of probe sets (Loy and Bodrossy, 2006).

Sensitivity of an MDM is defined as "*lowest relative abundance of the target group detectable"* (Bodrossy and Sessitsch, 2004: 247). Specificity characterizes the ability of the probe to hybridize with a unique target sequence (Draghici *et al.*, 2006).

1.3 Polymerase Chain Reaction

1.3.1 Principle

Polymerase Chain Reaction (PCR) is a method to amplify specific DNA fragments from a mixture of nucleic acids. The reaction principle is based on primer extension by DNA polymerase, synthesizing a DNA strand from a single-stranded template starting from a double-stranded region which is formed by the binding of a complementary oligonucleotide (primer). For PCR, two primers flanking the DNA sequence to be amplified are used. The reagents needed are a template, primers, deoxynucleotides (dNTPs), a thermostable DNA polymerase and a buffer containing magnesium (Taylor, 1991).

By repeating the following three steps (one cycle), exponential amplification of the DNA fragment can be achieved: Each cycle starts with denaturing of the template DNA at 93-97°C, followed by annealing of two primers to complementary regions of the single-stranded DNA (ssDNA) at 50-70°C. In the third step, primer sequences are elongated at around 72°C using a thermostable DNA polymerase resulting in double-stranded DNA (dsDNA) (Willems *et al.*, 2007). The first three cycles of a PCR are shown in Figure 3:

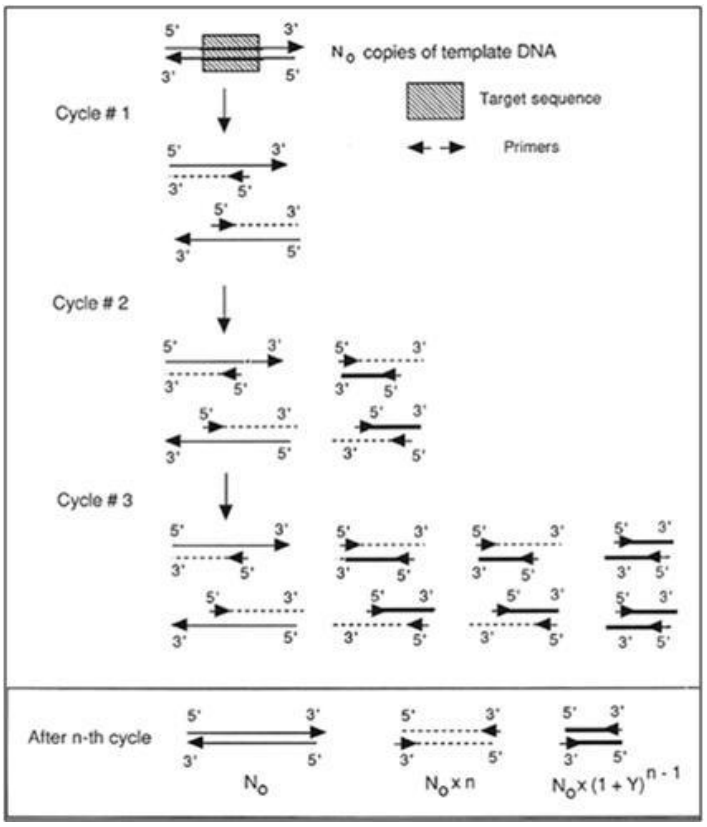

Figure 3: Schematic representation of PCR.
N_0: copies of duplex template. n: number of cycles. (Cha and Tilly, 1993)

The reaction produces fragments of undefined length in the first two cycles; however, from the third cycle on amplicons with defined lengths are produced and amplified exponentially during the following cycles (Cha and W. Thilly, 1993; Ling *et al.*, 1991; Willems *et al.*, 2007).

Figure 4 depicts a typical temperature profile for one cycle:

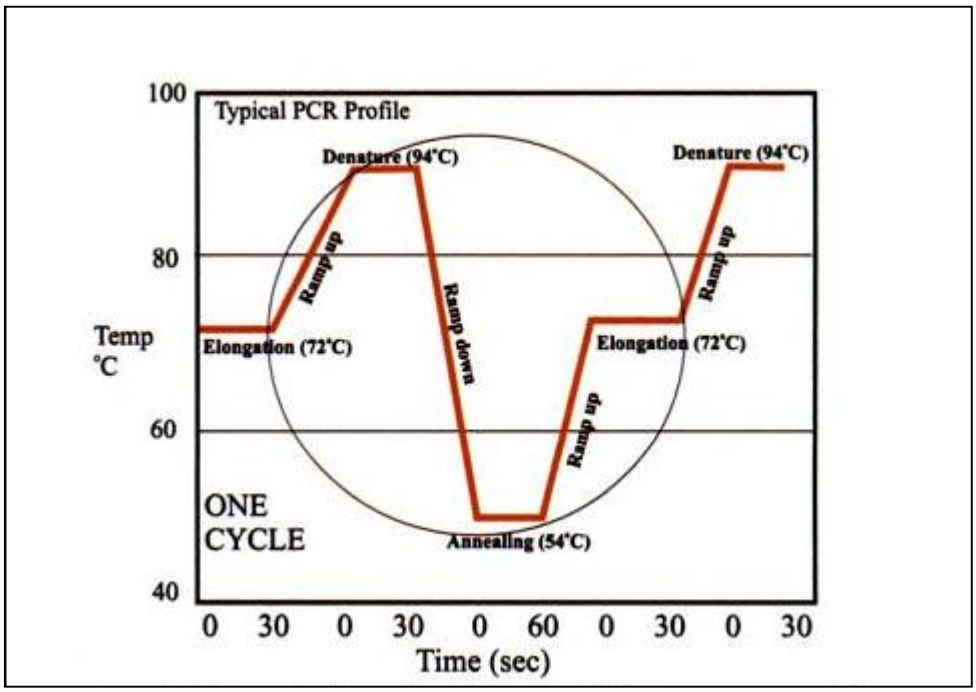

Figure 4: Typical temperature profile (Viljoen *et al.*, 2005)

1.3.2 Evaluation of a PCR

A PCR can be characterized by specificity, i.e. the frequency with which mispriming events occur, fidelity, i.e. accuracy, and efficiency, which gives information on how close the amplification is to the theoretical optimum of twofold increase of the PCR product each cycle (Cha and Thilly, 1993).

Practically, there is an increase by the factor (1+η) each cycle, where η is the efficiency (Booth *et al.*, 2010). Saiki (1985; cited in Booth *et al.* 2010) formulated the relation between overall efficiency (η) and yield (X): $X = (1 + \eta)^n$, where n is the cycle number. Keohavong and Thilly (1989) found efficiencies varying from 56% with T4 DNA polymerase for 30 cycles and 90-93% with modified T7 DNA polymerase for 20 cycles.

The parameters specificity, fidelity and efficiency are influenced by the reaction conditions and DNA polymerases (Cha and Thilly, 1993).

As discussed by Polz and Cavanaugh (1998), template-to-product ratios can be skewed by two mechanisms: First, PCR selection, where the amplification of certain templates is favored due to properties of the genes, flanking sequences or overall genome. Second, PCR drift which is supposedly caused by stochastic variations at the beginning of the reaction. In order to avoid PCR bias, they suggest avoiding degeneracies when universal primers are used. Furthermore, reproducibility between replicates can be improved by using high template concentrations. The combination of several PCR replicates can reduce PCR drift. PCR selection can be circumvented by reducing the number of cycles (Polz and Cavanaugh, 1998).

1.3.3 Primers

1.3.3.1 Primer Design

Primer design is a crucial step in the set-up of a new PCR, since the primers determine both efficiency and specificity (Dieffenbach, 1993).

Willems and co-workers (2007: 9-10) suggest the following parameters for primer design:

1. *A balanced base distribution*
2. *No stretches (more than 4) of bases and/or no repetitive sequence motifs*
3. *No stable secondary structures (self complementarities) especially at the 3' end*
4. *No complementary sequences between primers (primer dimer formation), especially at the 3' end*
5. *15-30 bases in size*
6. *Final concentration 0.05-1µM*
7. *Unique sequence*
8. *A maximum of 1-2Gs and/or Cs at the 3' end*
9. *T_m of both primers should be similar (±2°C)*
10. *The size of the PCR product should be in the range of 100-600bp*

The melting temperature (T_m) is defined as the temperature where one half of the DNA molecules is double-stranded and the other half is single-stranded. It varies with the GC content, the salt concentration and the primer concentration. An approximate calculation of the melting temperature can be performed using the following formula, provided the GC content is roughly 50% (Willems *et al.*, 2007):

$$T_m = 2 \cdot (no.\,A\ and\ T) + 4 \cdot (no.\,G\ and\ C)$$

The formula by Howley *et al.* (1979) takes into account the salt concentration in the reaction mixture:

$$T_m = 81.5 + 16.6 \cdot log(M) + 0.41 \cdot (\%\ G + C) - 0.72 \cdot (\%\ formamide)$$

where M is the monovalent salt molarity.

Another, more sophisticated formula, is based on the nearest-neighbour model and thermodynamic data, a discussion of which can be found in the paper by Santa Lucia (1998). There are significant differences in T_m values depending on the calculation method used (Panjkovich and Melo, 2005).

1.3.3.2 Formation of Primer Dimers

The formation of primer dimers, as well as fragments produced due to non-specific priming and subsequent amplification of non-target DNA result in undesired background fragments (Li *et al.*, 1990). Chou and co-workers (1992) showed that these events occur during mixing of the reactants at room temperature before the start of the amplification. They proposed Hot Start PCR, where one or more reagents are withheld from the mixture until it reaches a temperature of 60-80°C, as a means to increase amplification efficiency and specificity.

Brownie and colleagues (1997) described a method to suppress primer dimer formation by using primers that are genome-specific at the 3' end, but bear additional nucleotides (Tail) at the 5' ends. Furthermore, tail-specific primers (Tags) are added to the reaction mix, which can prime from the

newly synthesized fragments. T_m of the Tag is higher than T_m of the Tail so that switching from genomic priming can be achieved by raising the annealing temperature.

1.3.4 Annealing Temperature

The annealing temperature (T_a) greatly influences the purity and yield of the reaction products. Sub-optimal T_a leads to the amplification of non-specific DNA fragments; a T_a value too high has a negative impact on the yield of the product and may also lead to a reduction in purity (Rychlik, Spencer and Rhoads, 1990).

An annealing temperature 5°C below the true melting temperatures (T_m) of the primers has been found to be applicable for PCR (Innis and Gelfand, 1990). For a more exact calculation of the optimal annealing temperature (T_a^{opt}), an empirical formulation was published by Rychlik and co-workers in 1990:

$$T_a^{OPT} = 0.3T_m^{Primer} + 0.7T_m^{Product} - 14.9$$

where T_m^{Primer} is the calculated T_m of the less stable primer pair and $T_m^{Product}$ is the T_m of the PCR product (Rychlik *et al.*, 1990).

1.3.5 DNA Polymerase

A number of thermostable DNA polymerases are suitable for DNA amplification by PCR; however, they have different characteristics influencing the PCR reaction, e.g. regarding exonuclease activity, error rate and optimal temperature and magnesium concentration (Cha and Thilly, 1993; McPherson and Moller, 2006; Willems *et al.*, 2007).

During polymerization, mutations in the newly synthesized strand occur. The average mutation frequency can be calculated using the following formula:

$$f = \frac{(s_0 \cdot p)(n \cdot 2^{n-1})}{s_0 \cdot 2^n} = \frac{n \cdot p}{2}$$

where p is the error rate per cycle, n is the number of cycles and s_0 the number of target molecules before amplification. A lower number of cycles and the use of a polymerase with 3'-5' exonuclease activity may reduce the error frequency (Willems *et al.*, 2007).

1.3.6 Magnesium Concentration

It has been reported by various authors (Cline *et al.*, 1996; Hillebrand and Beattie, 1984; Innis and Gelfand, 1990; Willems *et al.*, 2007) that the concentration of Magnesium as a co-factor for DNA polymerase is crucial for PCR amplifications. It influences the following parameters: primer annealing, melting temperatures of both template and PCR product, product specificity, formation of primer-dimer artifacts, enzyme activity and fidelity (Innis and Gelfand, 1990).

According to Innis and Gelfand (1990), optimal Mg^{2+} concentration lies between 0.5 to 2.5mM, while Willems and colleagues (2007) suggest a concentration range from 1.0 to 5.0mM.

1.3.7 Deoxynucleotide Concentration

Deoxynucleotides provide the nucleotides and energy needed for the DNA synthesis (Taylor, 1991). The dNTP concentration that should be used depends on the amplicon size and Mg^{2+} concentration. Common concentrations go from 20 to 200µM each. In order to avoid misincorporation errors, dNTPs should be used in equivalent concentrations. Concentrations above 200µM have been found to lead to deterioration in yield and specificity (Viljoen *et al.*, 2005). A concentration too low, on the other hand, compromises PCR efficiency (Cha and Thilly, 1993).

1.3.8 Buffer

A standard reaction buffer for PCR generally consists of 10-50mM *N*-Tris(hydroxymethyl)aminomethane (Tris), pH 8.3-8.8, 25-50mM KCl, 0.01% gelatin and nonionic detergents such as Laureth 12, NP-40 and Tween. The latter have a positive effect on the processivity of the DNA polymerase. The standard protocol should be optimized for each reaction (Innis and Gelfand, 1990; Willems *et al.*, 2007).

1.3.9 Additives

An improved method for directly sequencing PCR amplified material using 10% dimethyl sulphoxide (DMSO) was first published by Winship (1989) and it was shown by Hung and colleagues (1990) that it can also improve DNA amplification by PCR. However, they also found that DMSO inhibits DNA synthesis by *Taq* polymerase by 50%. Furthermore, they reported an increase in specificity by use of $1x10^{-5}$M to $1x10^{-4}$M tetramethylammonium chloride (TMAC) without inhibition of *Taq* polymerase. This result was also supported by another study by Chevet *et al.* (1995). Kovarova and Dráber suggest the use of TMAC and oxalate to improve specificity and yield.

According to Sarkar *et al.* (1990) specificity as well as efficiency can be enhanced by addition of formamide to the reaction mixture. This method was mainly designed for amplification of GC-rich segments. However, as reported by Chakrabarti and Schutt (2001) other light-weight amides, especially 2-pyrrolidone, show much better characteristics with regard to potency, specificity and effective range. Betaine has also been shown to improve the amplification of GC-rich segments (Henke *et al.*, 1997).

1.3.10 Number of Cycles

The number of cycles to be performed mostly depends on the initial concentration of target DNA (Innis and Gelfand, 1990). Some recommendations are given in Table 2:

Table 2: Recommended number of cycles (Innis and Gelfand, 1990)

Number of Target Molecules	Number of cycles
$3x10^5$	25-30
$1.5x10^4$	30-35
$1x10^3$	35-40
50	40-45

Too many cycles lead to formation of non-specific background products; too few cycles have a negative impact on the yield of the amplification reaction (Innis and Gelfand, 1990).

1.3.11 Inhibitors

There are several substances often present in sample material that inhibit amplification of DNA by PCR. These include reagents and components of body fluids in clinical samples, food components and environmental compounds. They either interfere with cell lysis or cause nucleic acid degradation or inhibit polymerase activity. Although inhibition mechanisms are not fully understood yet, it has been found that inhibition may also be a result of poorly controlled reaction mechanisms or contamination (Wilson, 1997).

Some PCR inhibitors as well as facilitators in food samples are listed in Table 3:

Table 3: PCR inhibitors and facilitators in food samples (Wilson, 1997)

Substrate(s)	Target organism(s)	Inhibitor(s)	Facilitators
Milk	*Listeria monocytogenes*	Proteins	BSA, proteinase inhibitors
		unknown	enzymatic digestion, membrane solubilization
		Ca^{2+}	Chelation, [Mg^{2+}]
Skim milk	*Staphylococcus aureus*	Thermonuclease, proteins, bacterial debris	NaOH, NaI, physicochemical extraction, nested PCR
Raw milk	*Clostridium tyrobutyricum*	Unknown	Chemical extraction, centrifugation
	Brucella spp.	Milk proteins	Physicochemical extraction, nested PCR
Soft cheeses	*Listeria monocytogenes*	Brand-specific inhibitors, denatured protein	Phenol extraction, Quiagen column
		Unknown	PEG-dextran extraction
Various foods	*Escherichia coli*	Bean sprouts, oyster meat	Magic Minipreps
Foods and cultures	*Staphylococcus aureus, Escherichia coli, Salmonella* spp.		Lectin-affinity chromatography

Substrate(s)	Target organism(s)	Inhibitor(s)	Facilitators
Meat	*Brochothrix thermosphacta*	Fetuin, meat components	Lectin binding
Drinking water	*Enteroviruses, hepatits A virus*	Humic acid organic compounds	Pro-Cipitate, PEG, antibody capture

1.4 Nested PCR

Nested PCR is used for the amplification of low levels of target. After conventional PCR with an outer primer set, a small amount of PCR product is used as template for another round of amplification with an inner primer pair. The position of the latter influences the specificity and sensitivity of the experiment (Sachse, 2004). The principle is shown in Figure 5:

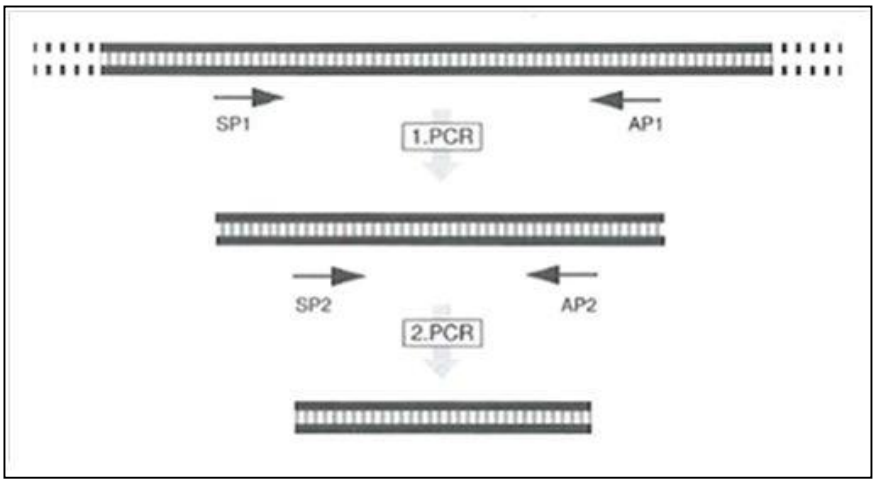

Figure 5: Nested PCR (Mülhardt, 2009)

Nested PCR is not only a means to increase the sensitivity of an assay; it can also be used for differentiation in diagnostic applications. In that case, the outer primer pair binds in a genomic segment common to a group of organisms while the inner primer pair is specific for a species, serovar or biovar (Sachse, 2004).

Special care must be taken when performing nested PCR, because this approach is prone to carry-over contamination from the first to the second amplification round (Mothershed and Whitney, 2006).

2 Objective

A microbial diagnostic microarray for the detection of the most relevant bacterial food- and waterborne pathogens and indicator organisms was developed by Kostić *et al.* (2007, 2010), using the phylogenetically robust *gyrB* gene as diagnostic marker. The selection of this gene allows high specificity and sensitivity in the detection of the target organisms, e.g. *Salmonella* spp., *E. coli*, *Y. enterocolitica*, *Y. pseudotuberculosis*, *S. aureus*, *C. jejuni*, *C. lari*, *C. coli*, *C. upsaliensis*, *C. perfringens* and *C. difficile*. However, the system is less sensitive compared to other systems that utilize the *16S* gene as a diagnostic marker, the lowest number of detectable cells being 10^4 cells and 10^3 cells respectively (Kostić *et al.*, 2010; Lee *et al.*, 2008). The limiting factor with regard to sensitivity is the efficiency of the *gyrB* PCR amplification showing a decrease in sensitivity of two log steps in comparison to the *16S* PCR (preliminary data by T. Kostić). In order to achieve higher sensitivity in this diagnostic system, the *gyrB* PCR should be optimized in the course of this project.

The primers used for the *gyrB* PCR were universal primers UP1 (5' GA AGT CAT CAT GAC CGT TCT GCA (YGC NGG NGG NAA RTT YGA) 3'), UP2r (5' AG CAG GGT ACG GAT GTG CGA GCC (RTC NAC RTC NGC RTC NGT CAT) 3'), UP1G (5' GA AGT CAT CAT GAC CGT TCT GCA (YGS NGG NGG NAA RTT YGG) 3') and UP2Ar (5' AG CAG GGT ACG GAT GTG CGA GCC (RTC NAC RTC NGC RTC NGY CAT) 3') as published by Yamamoto and Harayama (1995). The first 23 residues at the 5' end were intended to be used as tag sequences for sequencing of the PCR product; only the nucleotide sequence in parentheses is complementary to the target *gyrB* sequences. All primers are degenerate in order to amplify DNA fragments from various gram-negative and gram-positive bacteria.

In order to increase sensitivity, a nested PCR should be performed using primers complementary to the sequencing tags of the primers UP1, UP2r, UP1G and UP2Ar. To find the optimum T_a, a gradient PCR should be set up and first be tested on the arbitrarily chosen organisms *E. coli* and *S.* Typhimurium. A dilution series of gDNA would be used for amplification of the *gyrB* gene and the *16S* gene, in order to compare sensitivity. If the desired sensitivity can be achieved, array analysis can be performed with the *gyrB* PCR products. Subsequently, the nested *gyrB* PCR should also be tested on gDNA mixes.

If the nested PCR approach should not work, new primers would have to be designed. Since the PCR products will be analyzed in the microarray, primers have to be in the same region of the *gyrB* gene as the old ones because the probe set on the microarray is specific for the 1200bp fragment of the *gyrB* gene amplified in the *gyrB* PCR. New primers would first be tested with gDNA of single organisms and eventually also as primer mix using gDNA mix template.

3 Materials and Methods

3.1 Test Organisms

The experiments were performed with the following organisms:

Table 4: Strains and gDNA used

Strain		available as:
C. difficile	DSM 1296	*gDNA*
C. jejuni	DSM 4688	*gDNA*
C. perfringens	DSM 628	*gDNA*
E. coli (feacal isolate)	DSM 10757	*strain*
L. monocytogenes	CIP 61.4	*strain*
S. aureus	NCTC 6571	*strain*
S. Typhimurium	DSM 554	*strain*
Y. enterocolitica	NCTC 10460	*strain*
Y. pseudotuberculosis	DSM 8992	*gDNA*

3.1.1 Overnight Cultures

Overnight cultures (ONCs) of the strains were grown in 5ml liquid LB and incubated on a shaker (120rpm) or in an incubator at 37°C. The ONCs were pooled the next day and used for gDNA extraction (see 3.3).

3.1.2 Glycerol Stocks

Two glycerol stocks of each strain were prepared using 200µl 60% glycerol and 800µl ONC. The stocks were stored at -80°C.

3.2 Media

For preparation of the culture media, MilliQ water was used. Media were autoclaved at 121°C for at least 20 minutes. Media composition can be found in Table 5 and Table 6:

Table 5: LB medium (liquid)

LB medium (liquid)	g/L
Bacto trypton (Merck)	10
Yeast extract (Merck)	5
NaCl (Merck)	5

Table 6: LB medium (solid)

LB medium (solid)	g/L
Bacto trypton (Merck)	10
Yeast extract (Merck)	5
NaCl (Merck)	5
Agar agar (Gerbu)	14

3.3 DNA Extraction

DNA extraction was performed using the GenElute™Bacterial Genomic DNA Kit by Sigma-Aldrich according to manufacturer's instructions for gram-positive bacteria. Deviating from the protocol, the second elution was performed with the eluate from the first elution.

3.4 Agarose Gel Electrophoresis

1% agarose gels containing 0.5% ethidium bromide (EtBr) were prepared by dissolving the agarose in 1xTris-Borat-EDTA (TBE) buffer and adding EtBr after the mixture had cooled off. The solution was cast into a gel tray and transferred into an electrophoresis tank after solidifying.

The electrophoresis was performed in 1xTBE applying 120V for 50 to 60 minutes.

3.5 PCR Protocols

PCRs were performed with Aqua ad iniectabilia Braun which was sterile-filtered through a 0.22µm filter, autoclaved and stored at -20°C. Primers were synthesized by Microsynth AG. Other reagents and corresponding manufacturers are listed in Table 7 through Table 11.

3.5.1 *16S* PCR

Forward primer 8f: 5′ AG AGT TTG ATC CTG GCT CAG 3′
Reverse primer 1520r: 5′ AA GGA GGT GAT CCA GCC GCA 3′

Table 7: *16S* PCR

Reaction Mixture		Program		
dH_2O	11.3 µl	95°C	5 min	
10x PCR Rxn buffer (-$MgCl_2$) (Invitrogen)	2.5 µl			
$MgCl_2$ [50mM] (Invitrogen)	2.5 µl	95°C	30 s	25x
dNTP-Mix [2mM] (Fermentas)	2.5 µl	54°C	1 min	
8f [1.5µM]	2.5 µl	72°C	1 min	
1520r [1.5µM]	2.5 µl			
gDNA [50ng/µl]	1.0 µl	72°C	10 min	
Taq DNA Polymerase Recombinant [5U/µl] (Invitrogen)	0.2 µl			
	25.0 µl			

3.5.2 *gyrB* PCR

Forward primer UP1: 5' GA AGT CAT CAT GAC CGT TCT GCA (YGC NGG NGG NAA RTT YGA) 3'
Forward primer UP1G: 5' GA AGT CAT CAT GAC CGT TCT GCA (YGS NGG NGG NAA RTT YGG) 3'
Reverse primer UP2r: 5' AG CAG GGT ACG GAT GTG CGA GCC (RTC NAC RTC NGC RTC NGT CAT) 3'
Reverse primer UP2Ar: 5' AG CAG GGT ACG GAT GTG CGA GCC (RTC NAC RTC NGC RTC NGY CAT) 3'

N=G/A/T/C, R=G/A, Y=C/T, S=C/G

Table 8: *gyrB* PCR

Reaction Mixture		Program		
dH_2O	9.2 µl	95°C	5 min	
2x FailSafe Premix E (Epicentre)	50.0 µl			
UP1 [1.5µM]	10.0 µl	95°C	1 min	35x
UP2r [1.5µM]	10.0 µl	58°C	1 min	
UP1G [1.5µM]	10.0 µl	72°C	2 min	
UP2Ar [1.5µM]	10.0 µl			
gDNA [50ng/µl]	1.0 µl	72°C	10 min	
Taq DNA Polymerase Recombinant [5U/µl] (Invitrogen)	0.8 µl			
	100.0 µl			

3.5.3 Nested *gyrB* PCR

Forward primer UP1_S: 5' GA AGT CAT CAT GAC CGT TCT GCA 3'
Reverse primer UP2r_S: 5' AG CAG GGT ACG GAT GTG CGA GCC 3'

Table 9: Nested *gyrB* PCR

Reaction Mixture		Program		
dH_2O	28.2 µl	95°C	5 min	
2x FailSafe Premix E (Epicentre)	50.0 µl			
UP1_S [1.5µM]	10.0 µl	95°C	1 min	35x
UP2r _S [1.5µM]	10.0 µl	50-70°C	1 min	
gyrB PCR product (from 3.5.2)	1.0 µl	72°C	2 min	
Taq DNA Polymerase Recombinant [5U/µl] (Invitrogen)	0.8 µl			
		72°C	10 min	
	100.0 µl			

3.5.4 *gyrB* PCR with New Primers

Primers are listed in Table 21.

Table 10: *gyrB* PCR with new primers

Reaction Mixture		Program		
dH_2O	28.2 µl	95°C	5 min	
2x FailSafe Premix E (Epicentre)	50.0 µl			
forward primer[1.5µM]	10.0 µl	95°C	1 min	35x
reverse primer [1.5µM]	10.0 µl	52°C	1 min	
gDNA [50ng/µl]	1.0 µl	72°C	2 min	
Taq DNA Polymerase Recombinant [5U/µl] (Invitrogen)	0.8 µl			
		72°C	10 min	
	100.0 µl			

3.5.5 *gyrB* PCR with New Primer Mix

Table 11: *gyrB* PCR with new primer mix

Reaction Mixture		Program		
dH_2O	28.2 µl	95°C	5 min	
2x FailSafe Premix E (Epicentre)	50.0 µl			
primer mix [150nM each primer]	20.0 µl	95°C	1 min	35x
gDNA [50ng/µl]	1.0 µl	52°C	1 min	
Taq DNA Polymerase Recombinant [5U/µl] (Invitrogen)	0.8 µl	72°C	2 min	
	100µl	72°C	10 min	

3.6 Microarrays

3.6.1 Spotting

Microarrays were spotted with an Omnigrid™ microarrayer (GeneMachines) on aldehyde coated slides (CEL Associates). 50% DMSO was used as spotting buffer. Humidity was adjusted to 46 to 48%.

3.6.2 Slide Processing

After spotting, slides were stored in a humidity chamber for 12 to 24 hours. For processing, 0.2% SDS and a blocking solution were prepared. The composition of the blocking solution is given in Table 12:

Table 12: Blocking solution

$NaBH_4$ (Merck)	2.6g/l
PBS (Ambion)	773ml
EtOH *p.a.* (Merck)	227ml

Slides were rinsed twice in 0.2% SDS for two minutes, followed by two rinsings in water. Both steps were performed on the Belly Dancer to ensure vigorous agitation. A DNA denaturation was performed in boiling water for 2 minutes. After the slides had cooled off, they were incubated with blocking solution for 5 minutes. Afterwards, they were rinsed in 0.2% SDS on the Belly Dancer three times for 1 minute. A final rinse with water was performed for 1 minute. The processed slides were either dried with an air gun or in the slide centrifuge for 5 minutes at 900rpm. Slides were stored at room temperature in the dark.

3.6.3 Alkaline Phosphatase Treatment

After PCR, an alkaline phosphatase treatment was performed in order to dephosphorylate remaining nucleotides. The following reaction mixture was prepared and incubated at 37°C for 30 minutes, followed by a 10 minute heat inactivation at 95°C:

Table 13: Alkaline phosphatase treatment

PCR product	10.0 µl
10x Thermo Sequenase buffer (Amersham Biosciences)	1.0 µl
rAPid Alkaline Phosphatase [1U/µl] (Roche Applied Science)	2.0 µl

3.6.4 Sequence-Specific End-Labeling of Oligonucleotides (SSELO)

The labeling was performed in 25 cycles (30 seconds at 95°C, 75 seconds at 60°C) in a thermocycler using the reaction mixture described in Table 14. The RC mix consisted of 1 pmol of each RC oligonucleotide. The *pmoA* PCR product from *Methylosinus trichosporium* OB3b was included as a positive control. The ddNTP-C mix consisted of 10pmol of each ddATP, ddTTP and ddGTP (Roche Diagnostics).

Table 14: SSELO

dH_2O	2.3 µl
10x Thermo Sequenase buffer (Amersham Biosciences)	1.0 µl
RC oligomix	1.0 µl
OB3b PCR product [5ng/µl]	2.0 µl
Tamra ddCTP [0.1 mM] (Perkin Elmer)	0.1 µl
ddNTP-C	1.0 µl
Taq DNA Polymerase Recombinant [5U/µl] (Invitrogen)	0.6 µl
SAP treated PCR template (from 3.6.3)	2.0 µl

3.6.5 Hybridization

The hybridization mix was prepared according to the following table:

Table 15: Hybridization buffer

dH_2O	57.0 µl
10% SDS	1.0 µl
50x Denhardt's solution (Sigma Aldrich)	2.0 µl
20x SSC	30.0 µl

10 µl of SSELO labeled PCR product (from 3.6.4) were mixed with 90 µl hybridization buffer and loaded onto slides using HybriWell chambers (Grace BioLabs).

Hybridization took place for two hours at 55°C in a Belly Dancer Hybridization Water Bath. During hybridization and washing (see 3.6.6), the slides were protected from light.

3.6.6 Washing

After hybridization, the slides were washed in order to remove unbound targets. Washing was performed in four steps:

- 5 minutes in 2xSSC, 0.1% SDS
- 5 minutes in 0.2xSSC (twice)
- 5 minutes in 0.1xSSC

Slides were dried and scanned immediately.

3.6.7 Scan and Data Analysis

Microarrays were scanned using a GenePix 4000B laser scanner (Axon Instruments) with one line to average and a pixelsize of 10µm. Results were analyzed with the GenePix Pro 6.0 software (Axon

Instruments) and displayed in Microsoft Excel 2007. The signals were normalized to the internal control signal (probe Msi_294). Signals equal to the control signal were set to 100%. Normalized signals greater than 25% were considered positive.

3.7 Equipment

Balance BL150	Sartorius
Balance CPA223S	Sartorius
Belly Dancer	Stovall
Belly Dancer Hybridization Water Bath	Stovall
Biospectrum AC Imaging System	UVP
Centrifuge 5415D	Eppendorf
Electrophoresis Tank Sub-Cell GT	Bio-Rad
Freezer (-20°C)	Liebherr MedLine
Freezer UltimaII(-80°C)	Revco
Incubator	Binder
Laboklav autoclave	Steriltechnik AG
Magnetic Stirrer	Variomag
Microcentrifuge Galaxy MiniStar	VWR
Microwave	LG
Milli-Q Synthesis System	Millipore
Power Supply PowerPac Basic	Bio-Rad
Refrigerator / Freezer (-20°C)	Liebherr MedLine
Safety Cabinet Safe 2020	Thermo Scientific
Shaker	GFL
T1 Thermocycler	Biometra
Table autoclave	CertoClav
TGradient Cycler	Biometra
Thermomixer comfort	Eppendorf
Vortexer lab dancer	VWR
NanoDrop spectrophotometer	NanoDrop Technologies
GenePix 4000B laser scanner	Axon Instruments

4 Results

4.1 Comparison of *16S* PCR to *gyrB* PCR

A ten-fold serial dilution of the isolated *E. coli* and *S.* Typhimurium DNA ranging from 50ng/µl to 50fg/µl was prepared for the PCR amplification of the *16S* rRNA gene as well as the *gyrB* gene, as described in 3.5.1 and 3.5.2 respectively. 2µl template DNA of the 50ng/µl dilution was used for the first sample (100ng template DNA) and 1µl of each serial dilution for the other samples (50ng to 50fg template DNA). After amplification, the samples were loaded on a 1% agarose gel. Electrophoresis was performed in 1xTBE buffer applying 120V for one hour.

Figure 6 shows the *16S* PCR products on a 1% agarose gel after gel electrophoresis. PCR amplification from 100ng to 50pg template DNA yielded visible PCR products.

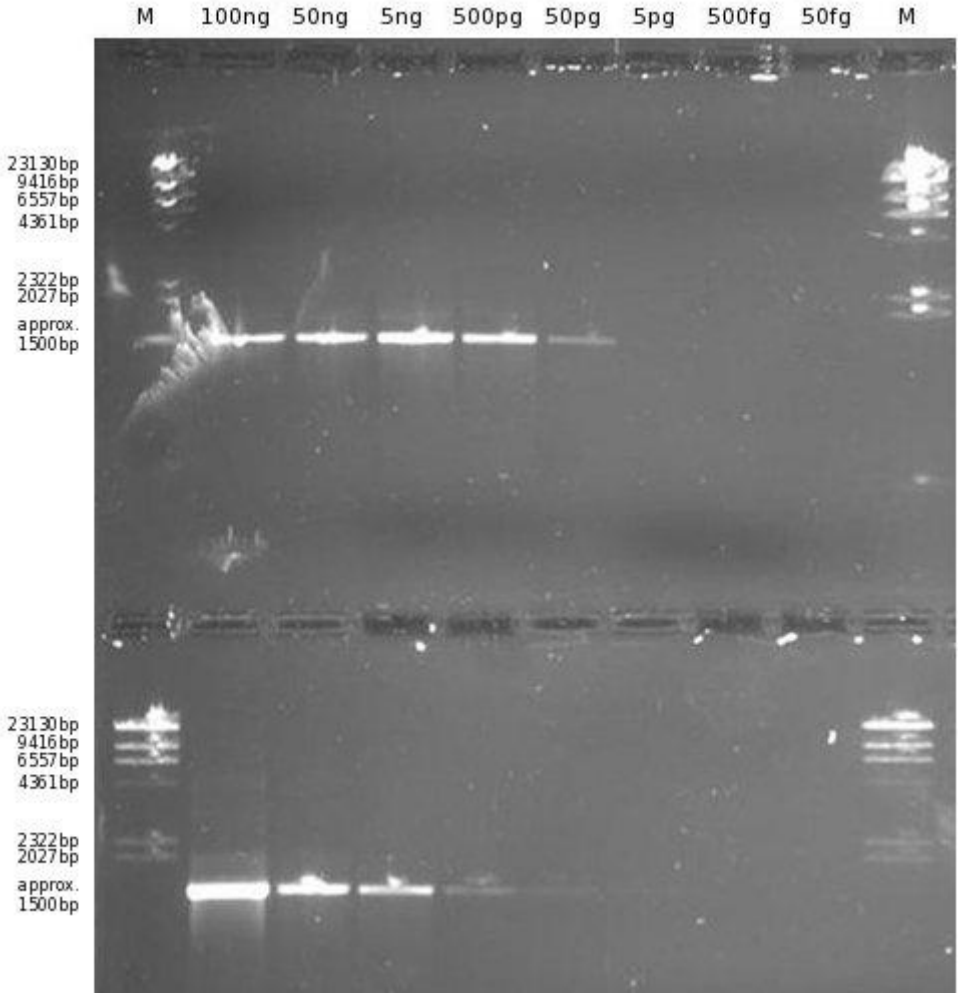

Figure 6: *16S* PCR products (5µl + 5µl loading dye) of *E. coli* (upper part) and *S.* Typhimurium (lower part) on 1% agarose gel. M: Lambda/HindIII Marker. -: negative control.

The PCR products of the *gyrB* PCR are shown in Figure 7. The lowest amount of template DNA from which PCR products can be detected is 5ng.

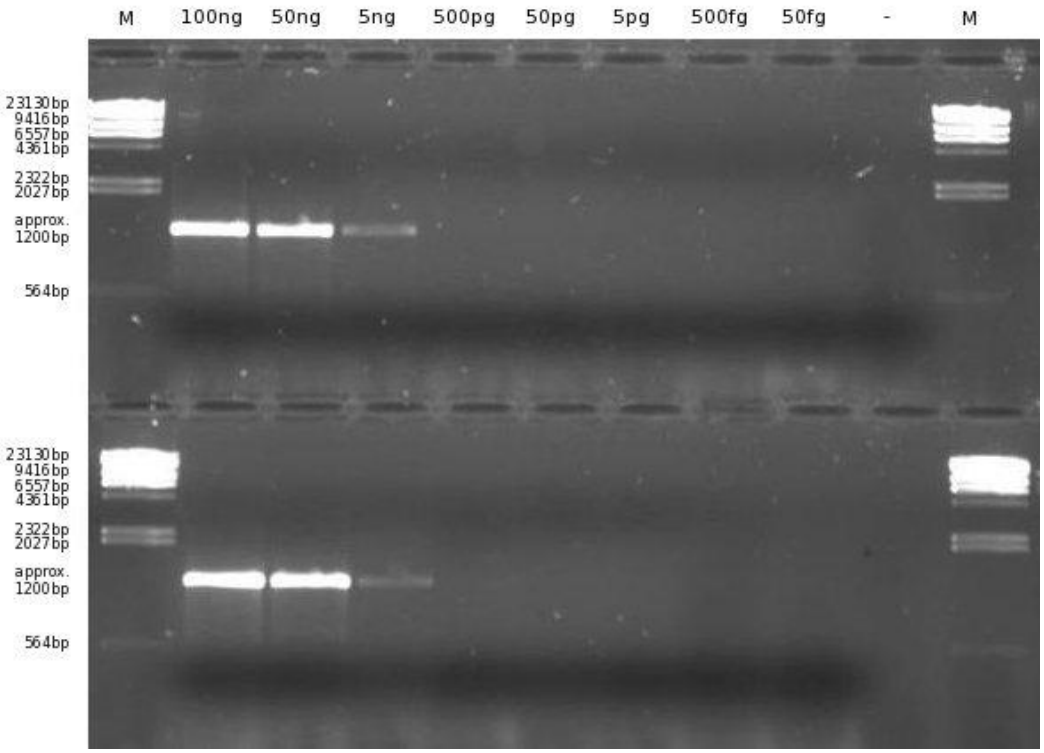

Figure 7: *gyrB* PCR products (5µl + 5µl loading dye) of *E. coli* (upper part) and *S.* Typhimurium (lower part) on 1% agarose gel. M: Lambda/HindIII Marker. -: negative control.

The comparison shows a difference of 10^2 in sensitivity between *16S* and *gyrB* PCR.

4.2 Nested *gyrB* PCR

In order to achieve further amplification of the *gyrB* PCR products, a nested PCR was set up. The primers UP1_S and UP2r_S had previously been designed such that they target the tail region of the *gyrB* primers:

Forward primer UP1:	5' GA AGT CAT CAT GAC CGT TCT GCA (YGC NGG NGG NAA RTT YGA) 3'
Forward primer UP1G:	5' GA AGT CAT CAT GAC CGT TCT GCA (YGS NGG NGG NAA RTT YGG) 3'
Reverse primer UP2r:	5' AG CAG GGT ACG GAT GTG CGA GCC (RTC NAC RTC NGC RTC NGT CAT) 3'
Reverse primer UP2Ar:	5' AG CAG GGT ACG GAT GTG CGA GCC (RTC NAC RTC NGC RTC NGY CAT) 3'
Nested primer UP1_S:	5' GA AGT CAT CAT GAC CGT TCT GCA 3'
Nested primer UP2r_S:	5' AG CAG GGT ACG GAT GTG CGA GCC 3'

In order to find the optimal T_a for the nested *gyrB* PCR, a gradient PCR ranging from 50°C to 70°C was set up. The data for the T_m values were taken from the datasheet and calculated with OligoAnalyzer (Integrated DNA Technology,http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx). The values from the datasheet were calculated with a monovalent salt concentration of 50mM. The manufacturer of the FailSafe PCR 2x PreMix E (Epicentre) states a concentration of 100mM monovalent salt, so this values was used for the calculation with Oligo Analyzer. T_a values were calculated using the formula by Innis and Gelfand (1990), subtracting 5°C from the T_m. The T_m and corresponding T_a values are given in Table 16:

Table 16: T_m and T_a values of the nested primers UP1_S and UP2r_S. ([M]: monovalent salt molarity, NNM: Nearest Neighbour Model)

Primers	Datasheet [M]=50mM	Datasheet (NNM-Method) [M]=50mM	OligoAnalyzer [M]=100mM
UP1_S		Melting Temperature	
	57.8	59.8	66.1
		Annealing Temperature	
	52.8	54.8	61.1
UP2r_S		Melting Temperature	
	64.8	70.0	73.0
		Annealing Temperature	
	59.8	65.0	68.0

As can be seen, the T_m values not only vary depending on the calculation method, there is also a difference of about 7°C between UP1_S and UP2r_S. Therefore, a 20°C gradient was used.

The nested *gyrB* PCR was performed according to the protocol described in Table 9 (see 3.5.3), with a temperature gradient from 50°C to 70°C in the annealing step. *E. coli* and *S.* Typhimurium *gyrB* PCR products from 100ng and 500pg gDNA were used as templates. Afterwards, an agarose gel electrophoresis (1% agarose; 120V, 1h) was performed. The results are shown in Figure 8:

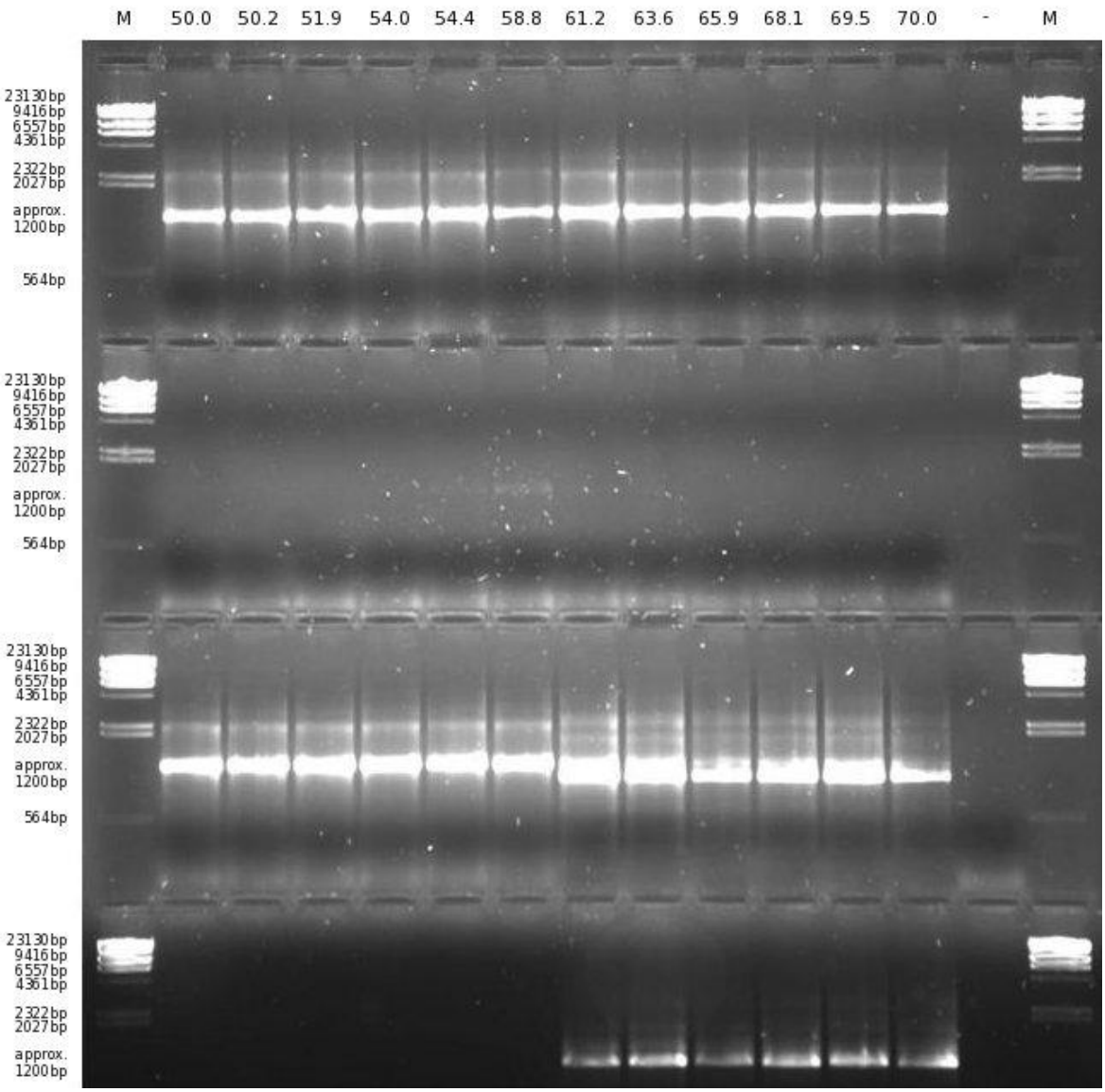

Figure 8: Nested *gyrB* PCR products (5µl + 5µl loading dye) on 1% agarose gel. Top to bottom: *E. coli* (100ng template DNA), *E. coli* (500pg template DNA), *S.* Typhimurium (100ng template DNA), *S.* Typhimurium (500pg template DNA). M: Lambda/HindIII Marker. -: negative control.

As expected, intense bands were visible for both *E. coli* and *S.* Typhimurium samples that used the PCR product from 100ng template. The amplification of the PCR products from 500pg template DNA worked well for *S.* Typhimurium at all temperatures equal to or greater than 61.2°C. With *E. coli*, on the other hand, further amplification was only achieved at 56.4 and 58.8°C; both hardly visible.

In order to narrow down the T_a further, another nested *gyrB* PCR was carried out where the temperature gradient ranged from 55°C to 65°C. Contradictory to the previous results, there was no amplification of the *gyrB* PCR product from 500pg template DNA. The same experimental setup was also used for amplification of the *gyrB* PCR product from 100ng template DNA. Specific bands were observed for *S.* Typhimurium at all temperatures, whereas in the sample with *E. coli* DNA only smears were visible, except for a light band at 55.2°C. Thus, 55°C was chosen as T_a for the subsequent experiments. However, the results could not be reproduced.

In an attempt to improve the results, an additional experiment was performed using shorter primers UP1_S_k (5' CAT CAT GAC CGT TCT GCA 3') and UP2r_S_k (5' GGT ACG GAT GTG CGA GCC 3'), where 5

bases were removed from the 5' end of primers UP1_S and UP2r_S respectively. This was an attempt to move the binding site of the primers away from the end of the template PCR products in order to enable better binding. The melting temperature of the primers and likely annealing temperatures can be found in Table 17:

Table 17: T_m and T_a values of the nested primers UP1_k and UP2r_k. ([M]: monovalent salt molarity, NNM: Nearest Neighbour Model)

Primers	Datasheet [M]=50mM	Datasheet (NNM-Method) [M]=50mM	OligoAnalyzer [M]=100mM
UP1_S_k	52.6	Melting Temperature 50.5	61.4
	48.6	Annealing Temperature 55.5	56.4
UP2r_S_k	59.6	Melting Temperature 57.2	65.1
	54.6	Annealing Temperature 52.2	63.0

Nested *gyrB* PCR was performed with both primer pairs in parallel using a 15°C gradient from 45 to 60°C. As can be seen in Figure 9, the nested PCR did not work at all with *S.* Typhimurium regardless of the primers used, while with *E. coli* only smears were produced at all annealing temperatures.

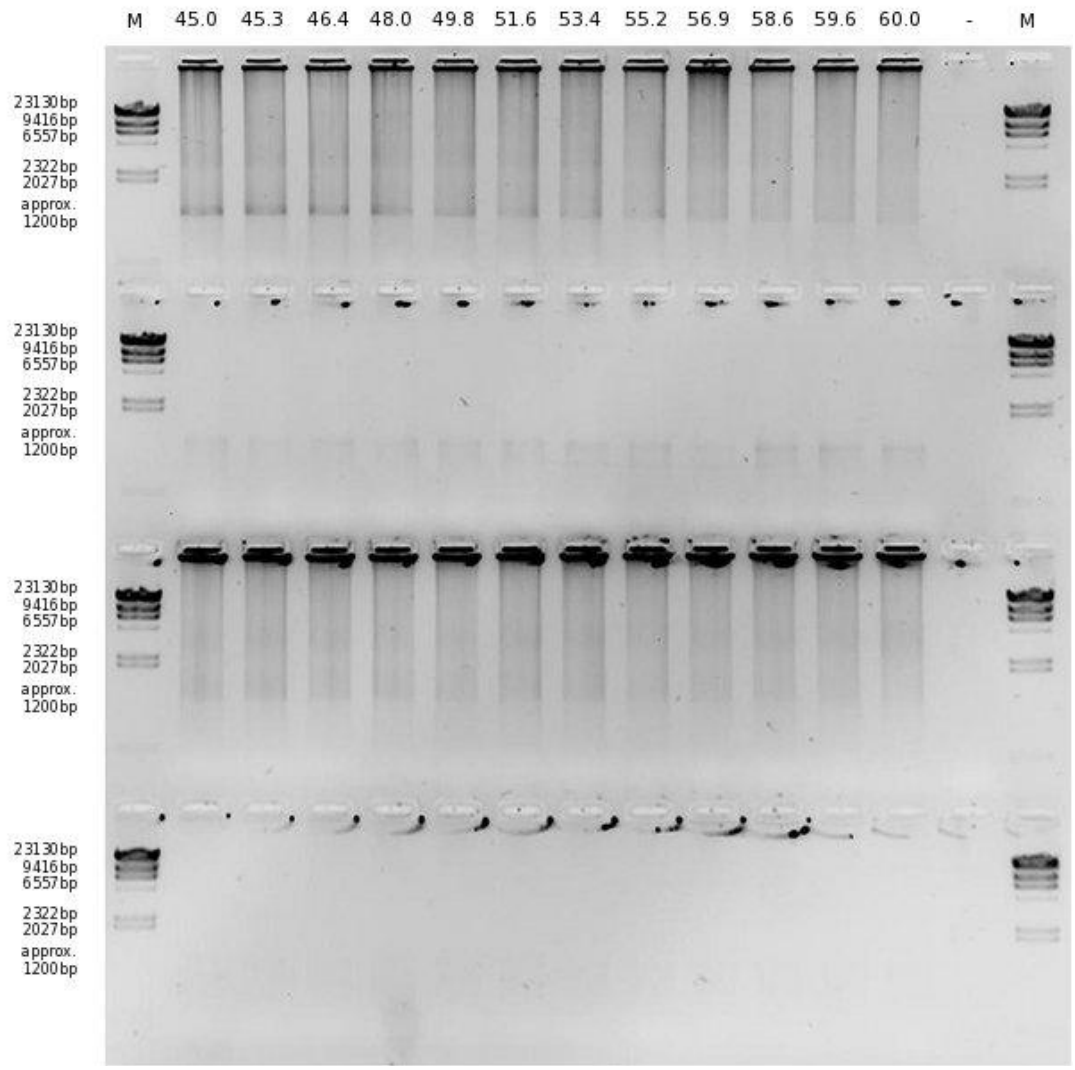

Figure 9: Nested *gyrB* PCR products (5µl + 5µl loading dye) on 1% agarose gel. Top to bottom: *E. coli* (100ng template DNA) with UP1_S and UP2r_S, *S.* Typhimurium (100ng template DNA) with UP1_S and UP2r_S, *E. coli* (100ng template DNA) with UP1_S_k and UP2r_S_k, *S.* Typhimurium (100ng template DNA) with UP1_S_k and UP2r_S_k. M: Lambda/HindIII Marker. -: negative control.

4.3 Design of New Primers for *gyrB* PCR

Due to the highly degenerate primers, *gyrB* PCR did not show the required sensitivity. Primers UP1, UP2r, UP2Ar are 512-fold degenerate, primer UP1G is 1024-fold degenerate. Further amplification with a nested PCR could not be achieved either; thus new primers were designed.

Sequences of the *gyrB* gene were downloaded from the NCBI nucleotide database (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) for the following organisms: *Salmonella* spp., *Escherichia coli*, *Yersinia enterocolitica*, *Y. pseudotuberculosis*, *Staphylococcus aureus*, *Campylobacter jejuni*, *C. lari*, *C. coli*, *C. upsaliensis*, *Listeria monocytogenes*, *Clostridium perfringens* and *C. difficile*. When possible, ten sequences each were downloaded from the assembled complete genome sequences. However, this was not possible in all cases (e.g. only three sequences could be found for *C. upsaliensis)*.

A sequence database was created using the program ARB (W. Ludwig *et al.*, 2004) and the sequences were aligned. Since the PCR products were used for further microarray analysis, the new primers had to be in the same region as the old ones.

4.3.1 Primer Adaptation

In a first approach, the currently used primers should be adapted. For Enterobacteriaceae, UP1 was used as a basis and redesigned such that some wobbles were excluded and some were narrowed down and two bases were added to the 3' end to enhance stability:

Table 18: Suggestion for improvement of primer UP1 (Enterobacteriaceae). (N=A,C,G,T; Y=C,T; R=A,G; D=A,G,T)

UP1	5'	YGCNGGNGGNAARTTYGA	3'
New suggestion	5'	GCDGGCGGTAARTTYGACG	3'

Primer UP1 is 512x degenerate while the new suggestion is 12x degenerate.

When considering Enterobacteriaceae and *Campylobacter* spp. together as Proteobacteria, it turned out that the suggestions made for Enterobacteriaceae do not fit for *Campylobacter* spp. Therefore, this genus was considered separately. UP1 was again used as basis. From 27 sequences, six did not have sequence information in the primer region. Two wobbles could be eliminated and the degree of degeneracy was reduced from 512x to 18x; however, there was no possibility for primer extension in any direction.

Table 19: Suggestion for improvement of Primer UP1 (Campylobacter). (N=A,C,G,T; Y=C,T; R=A,G; D=A,G,T)

UP1	5'	YGCNGGNGGNAARTTYGA	3'
New suggestion	5'	GCAGGDGGDAAATTYGA	3'

Using this approach, wobbles could be reduced to three, but it is questionable whether this would lead to any improvement.

For Firmicutes (*Listeria* spp., *Clostridium* spp., *S. aureus*), primer UP1G was used as a basis. As there were only two *C. difficile* sequences with information in the said region available, adaptation proved difficult. 3' extension of the primer would be possible for *Listeria* spp. and *S. aureus*, for *Clostridium* spp., however, another wobble would be needed.

4.3.2 Redesign at Species Level

Since the adaptation of the primers UP1 and UP1G did not lead to any significant improvement, new primers were designed at species level. Primers were designed at the same position as the previously used primers or with minor shifts to the 5' end if necessary. The possible primers that were found are listed in Table 20:

Table 20: Possible new *gyrB* primers (N=A,C,G,T; Y=C,T; R=A,G; S=C,G; K=G,T; W=A,T)

E. coli		
UP1	5' **Y**GCNGG**N**GG**N**AA**R**TT**Y**GA 3'	pos 2128 – 2148
UP1_Eco	5' CGCAGGCGGTAAATT**Y**GACG 3'	pos 2128 – 2148
UP1_Eco_2	5' CCGTTCTGCACGCAGGCGG 3'	pos 2139 – 2158
UP2r	5' **R**TC**N**AC**R**TC**N**GC**R**TC**N**GTCAT 3'	pos 914 – 934
UP2r_Eco	5' GTCGACGTCCGCATCGGTCATG 3'	pos 914 – 934
Salmonella spp.		
UP1	5' **Y**GC**N**GG**N**GG**N**AA**R**TT**Y**GA 3'	pos 2128 – 2148

UP1_Sal	5′ GCGGGCGGTAAATTTGACG 3′	pos 2128 – 2148	
UP2r	5′ **R**TC**N**AC**R**TC**N**GC**R**TC**N**GTCAT 3′	pos 914 – 934	
UP2r_Sal	5′ GTCGACGTCCGCATCGGTCATG 3′	pos 914 – 934	*same as UP2r_Eco*
Yersinia spp.			
UP1	5′ **Y**GC**N**GG**N**GG**N**AA**R**TT**Y**GA 3′	pos 2128 – 2148	
UP1_Yer	5′ TGC**K**GGCGGTAA**R**TTTGACG 3′	pos 2128 – 2148	
UP1_Yent	5′ TGC**K**GGCGGTAAGTTTGACG 3′	pos 2128 – 2148	
UP1_Ypse	5′ TGCTGGCGGTAAATTTGACG 3′		
UP2r	5′ **R**TC**N**AC**R**TC**N**GC**R**TC**N**GTCAT 3′	pos 914 – 934	
UP2r_Yer	5′ ATC**Y**ACGTCGGCATCGGTCATG 3′	pos 914 – 934	
Campylobacter spp.			
UP1	5′ **Y**GC**N**GG**N**GG**N**AA**R**TT**Y**GA 3′	pos 2128 – 2148	
UP1_Cam	5′ GCAGG**D**GG**D**AAATT**Y**GA 3′	pos 2128 – 2148	
UP1_Ccj	5′ CT**W**CATGCAGGGGGAAAATTCG ‘3	pos 2128 – 2148	
UP1_Clar	5′ CGCAGGTGGTAAATTTG 3′	pos 2128 – 2148	
UP1_Cups	5′ CGCAGGAGGGAAATT**Y**GACC 3′	pos 2128 – 2148	
UP2r	5′ **R**TC**N**AC**R**TC**N**GC**R**TC**N**GTCAT 3′	pos 914 – 934	
UP2_Ccj	5′ ATCAACATCCGCATCTGTCATG 3′	pos 914 – 934	
UP2_Clar	5′ ATCTACATCAGCATCGGTCATG 3′	pos 914 – 934	
UP2_Cups	5′ ATC**R**ACATCAGCATCGGTCAT 3′	pos 914 – 934	
L. monocytogenes			
UP1G	5′ **Y**G**SN**GG**N**GG**N**AA**R**TT**Y**GG 3′	pos 2128 – 2148	
UP1G_Lmo	5′ TGCTGGTGGTAAATTTGG 3′	pos 2128 – 2148	
UP1G_Lmo_2	5′ **R**TC**N**AC**R**TC**N**GC**R**TC**N**G**Y**CAT 3′	pos 2128 – 2148	
UP2Ar	5′ ATC**R**ACATCGGCATC**M**GTCAT 3′	pos 914 – 934	
UP2Ar_Lmo	5′ ATC**R**ACATCGGCATC**M**GTCAT 3′	pos 914 – 934	
UP2Ar_Lmo_2	5′ GTACGAATATGTGCACCATC 3′	pos 896 – 916	
Clostridium spp.			
UP1G	5′ **Y**G**SN**GG**N**GG**N**AA**R**TT**Y**GG 3′	pos 2128 – 2148	
UP1G_Cper	5′ TGCTGGAGGTAAATTCGG 3′	pos 2128 – 2148	
UP1G_Cdiff	5′ TGCAGGAGGAAAGTTTGG 3′	pos 2128 – 2148	
UP2Ar	5′ **R**TC**N**AC**R**TC**N**GC**R**TC**N**G**Y**CAT 3′	pos 914 – 934	
UP2Ar_Cper	5′ TCAACGTCAGCATCAGTCATG 3′	pos 914 – 934	
UP2Ar_Cdiff	5′ TCTACATCAGCATCGGTCAT 3′	pos 914 – 934	
S. aureus			
UP1G	5′ **Y**G**SN**GG**N**GG**N**AA**R**TT**Y**GG 3′	pos 2128 – 2148	
UP1G_Saur	5′ TGCTGGTGGTAAATT**Y**GG 3′	pos 2128 – 2148	
UP1G_Saur_2	5′ TAAATT**Y**GGCGGTGGCGG 3′	pos 2117 – 2139	
UP1G_Saur_3	5′ CTGTTTTACATGCTGGTGG 3′	pos 2139 – 2158	
UP2Ar	5′ **R**TC**N**AC**R**TC**N**GC**R**TC**N**G**Y**CAT 3′	pos 914 – 934	
UP2Ar_Saur	5′ ATCCACATCGGCATCAGTCAT 3′	pos 914 – 934	

The melting temperatures of the primers were checked with OligoAnalyzer (Integrated DNA Technologies, http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx) using default settings and adapted to have the best matching T_m. The final selection is given in Table 21:

Table 21: Selected new *gyrB* primers (Y=C,T; R=A,G; K=G,T; W=A,T)

E. coli		GC Content [%]	T_m [°C]
UP1_Eco	5' GCAGGCGGTAAATT**Y**GACG '3	55,3	55,8
UP2r_Eco	5' CGTCCGCATCGGTCATG '3	64,7	56,5
Salmonella spp.			
UP1_Sal	5' GCGGGCGGTAAATTTGACG '3	57,9	57,4
UP2r_Sal	5' CGTCCGCATCGGTCATG '3	*same as UP2r_Eco*	
Yersinia spp.			
UP1_Yer	5' GC**K**GGCGGTAA**R**TTTGACG '3	57,9	57,1
UP2r_Yer	5' CGTCGGCATCGGTCATG '3	64,7	56,5
Campylobacter spp.			
UP1_Ccj	5' CT**W**CATGCAGGGGGAAAATTCG '3	50,0	56,4
UP1_Clar	5' CAAAACACGCAGGTGGTAAATTTG '3	41,7	55,6
UP1_Cups	5' CGCAGGAGGGAAATT**Y**GACC '3	57,5	57,4
UP2_Ccj	5' CATCAACATCCGCATCTGTCATG '3	47,8	56,4
UP2_Clar	5' CCATCTACATCAGCATCGGTCATG '3	50,0	57,4
UP2_Cups	5' CCATCRACATCAGCATCTGTCAT '3	45,7	56,2
L. monocytogenes			
UP1G_Lmo	5' TTCT**R**CATGCTGGTGGTAAATTTGG '3	42,0	56,9
UP2Ar_Lmo	5' AGTAGTGTACGAATATGTGCACCATC '3	42,3	56,5
Clostridium spp.			
UP1G_Cper	5' TGCTGGAGGTAAATTCGGAGG '3	52,4	56,8
UP1G_Cdiff	5' TGCAGGAGGAAAGTTTGGAGG '3	52,4	57,3
UP2Ar_Cper	5' CATCAACGTCAGCATCAGTCATG '3	47,8	56,2
UP2Ar_Cdiff	5' CCATCTACATCAGCATCGGTCAT '3	47,8	56,6
S. aureus			
UP1G_Saur	5' TAAATT**Y**GGCGGTGGCGG '3	58,3	57,6
UP2Ar_Saur	5' ATCCACATCGGCATCAGTCAT '3	47,6	56,5

4.4 *gyrB* PCR with New Primers

4.4.1 Individual Primers

Primers were tested individually on the following species: *L. monocytogenes*, *S. aureus*, *S.* Typhimurium, *E. coli, C. perfringens, Y. enterocolitica, Y. pseudotuberculosis* and *C. jejuni*. 50ng gDNA were used as template, except for *Y. peudotuberculosis* (34ng). Reaction conditions were the same as for *gyrB* PCR with the old primers (compare 3.5.2), but the annaling temperature was lowered to 52°C. T_a was calculated as described above (see 4.2).

Figure 10 shows the PCR products after electrophoresis:

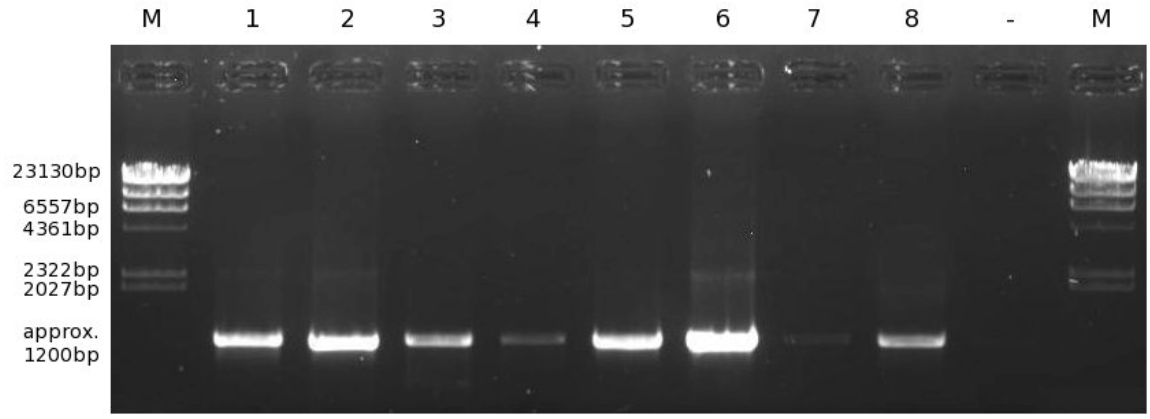

Figure 10: Individual primer test. PCR products (5µl + 5µl loading dye) on 1% agarose gel. Electrophoresis was performed at 120V for 45 minutes. 1: *L. monocytogenes*, 2: *S. aureus*, 3: *S.* Typhimurium, 4: *E. coli*, 5: *C. perfringens*, 6: *Y. enterocolitica*, 7: *Y. pseudotuberculosis*, 8: *C. jejuni*.. M: Lambda/HindIII Marker. -: negative control.

All samples were amplified. The PCR product of *Y. pseudotuberculosis* (7) is only slightly visible.

4.4.2 Primer Mix

A 1.5µM primer mix of all new primers was prepared and tested on *L. monocytogenes*, *S. aureus*, *S.* Typhimurium, *E. coli*, *C. perfringens*, *Y. enterocolitica*, *Y. pseudotuberculosis* and *C. jejuni*.

Figure 11 shows the PCR products after electrophoresis:

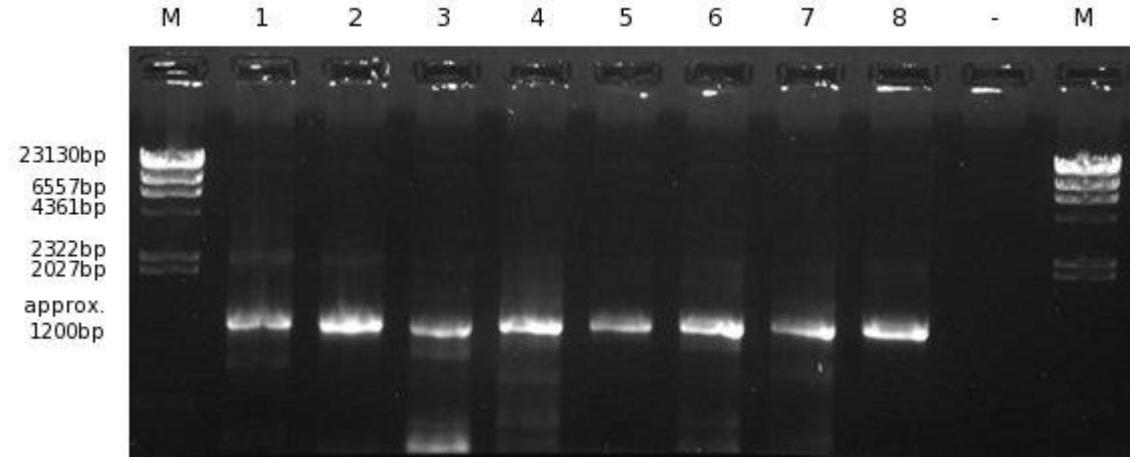

Figure 11: Primer mix (1.5µM) tested on different organisms. PCR products (5µl + 5µl loading dye) on 1% agarose gel. Electrophoresis was performed at 120V for 50 minutes. 1: *L. monocytogenes*, 2: *S. aureus*, 3: *S.* Typhimurium, 4: *E. coli*, 5: *C. perfringens*, 6: *Y. enterocolitica*, 7: *Y. pseudotuberculosis*, 8: *C. jejuni*. M: Lambda/HindIII Marker. -: negative control.

All samples were amplified.

Sensitivity tests were performed with *C. perfringens* as well as *E. coli* by preparing a dilution series of the respective gDNA (50ng to 50fg) and comparing the performances of *16S* PCR, *gyrB* PCR with old primers, *gyrB* PCR with new single primers and *gyrB* PCR with the new primer mix according to the protocols described in 3.5.1 through 3.5.5. PCR products of the *gyrB* PCR with old and new primers of *C. perfringens* are shown in Figure 12

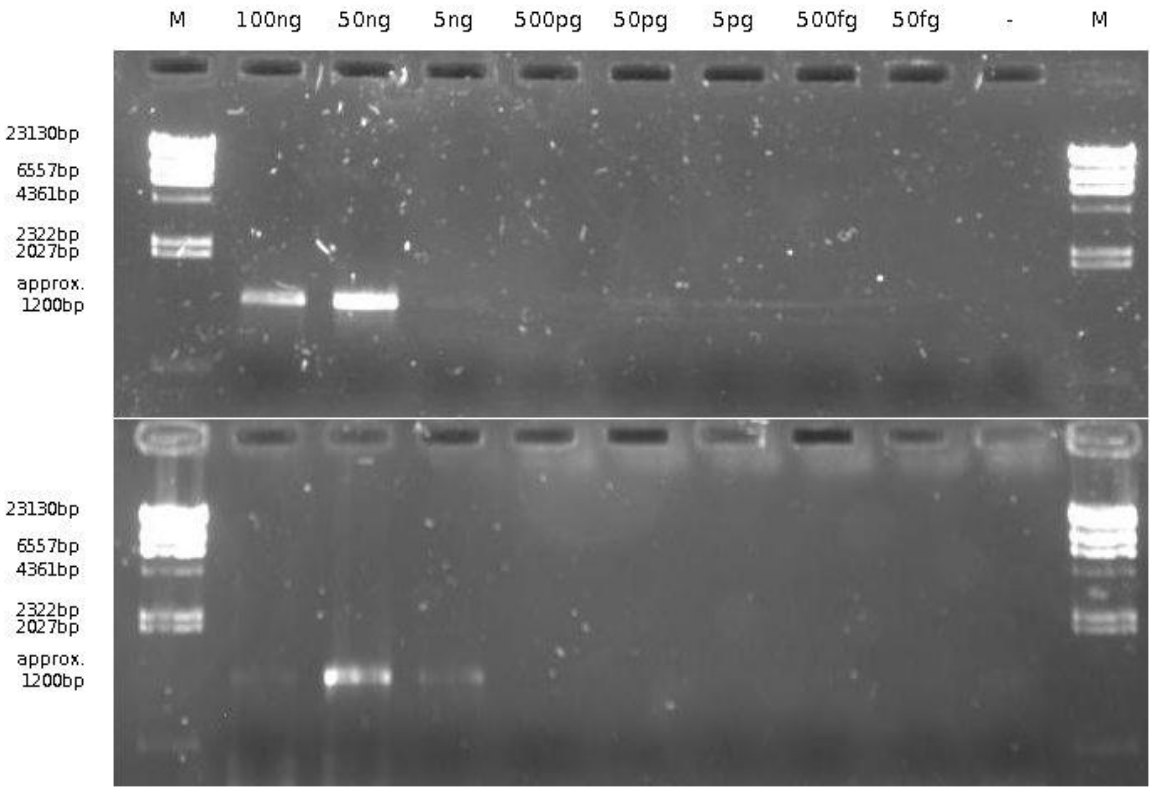

Figure 12: Sensitivity test with *C. perfringens*. PCR products (5µl + 5µl loading dye) on 1% agarose gel. Electrophoresis was performed at 120V for 50 minutes. Top: *gyrB* PCR old. Bottom: *gyrB* PCR new with primer mix (1.5µM). M: Lambda/HindIII marker. -: negative control.

The limit of detection for the *gyrB* PCR with the new primer mix was found to be 5ng template gDNA for both *C. perfringens* and *E. coli*, which is the same LOD as with the old primers.

In order to optimize the reaction, an experiment using different concentrations of the primer mix was set up. 100ng, 5ng and 5pg *E. coli* gDNA were each amplified with single primers, the primer mix [1.5µM] and 1:10 [150nM] and 1:50 [30nM] dilutions of the primer mix. PCR products after electrophoresis are depicted in Figure 13:

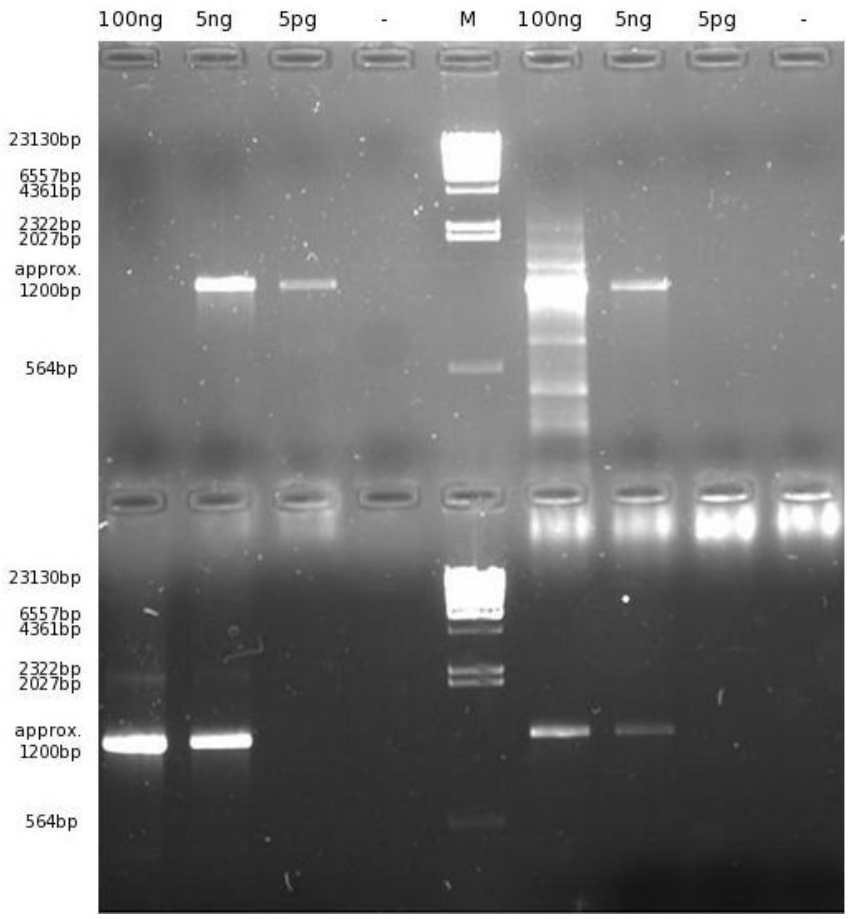

Figure 13: Test of different primer mix concentrations with *E. coli*. PCR products (5µl + 5µl loading dye) on 1% agarose gel. Electrophoresis was performed at 120V for 50 minutes. Top left: new specific *E. coli* primers. Top right: new primer mix 1.5µM. Bottom left: new primer mix 150nM. Bottom right: new primer mix 30nM. M: Lambda/HindIII marker. -: negative control.

The 1:10 dilution [150nM] produced clear and intense bands, whereas the 1:50 dilution [30nM] resulted in weaker bands. Thus, the 1:10 dilution [150nM] was used for further experiments.

4.4.3 Sensitivity Tests

Sensitivity tests were performed with tenfold gDNA dilution series (50ng to 50fg) of *E. coli*, *S.* Typhimurium, *L. monocytogenes* and *C. jejuni*; comparing *16S* PCR, *gyrB* PCR with the old primers, *gyrB* PCR with the new single primers and *gyrB* PCR with the new primer mix (1:10). Figure 14 shows the PCR products of *E. coli* after electrophoresis:

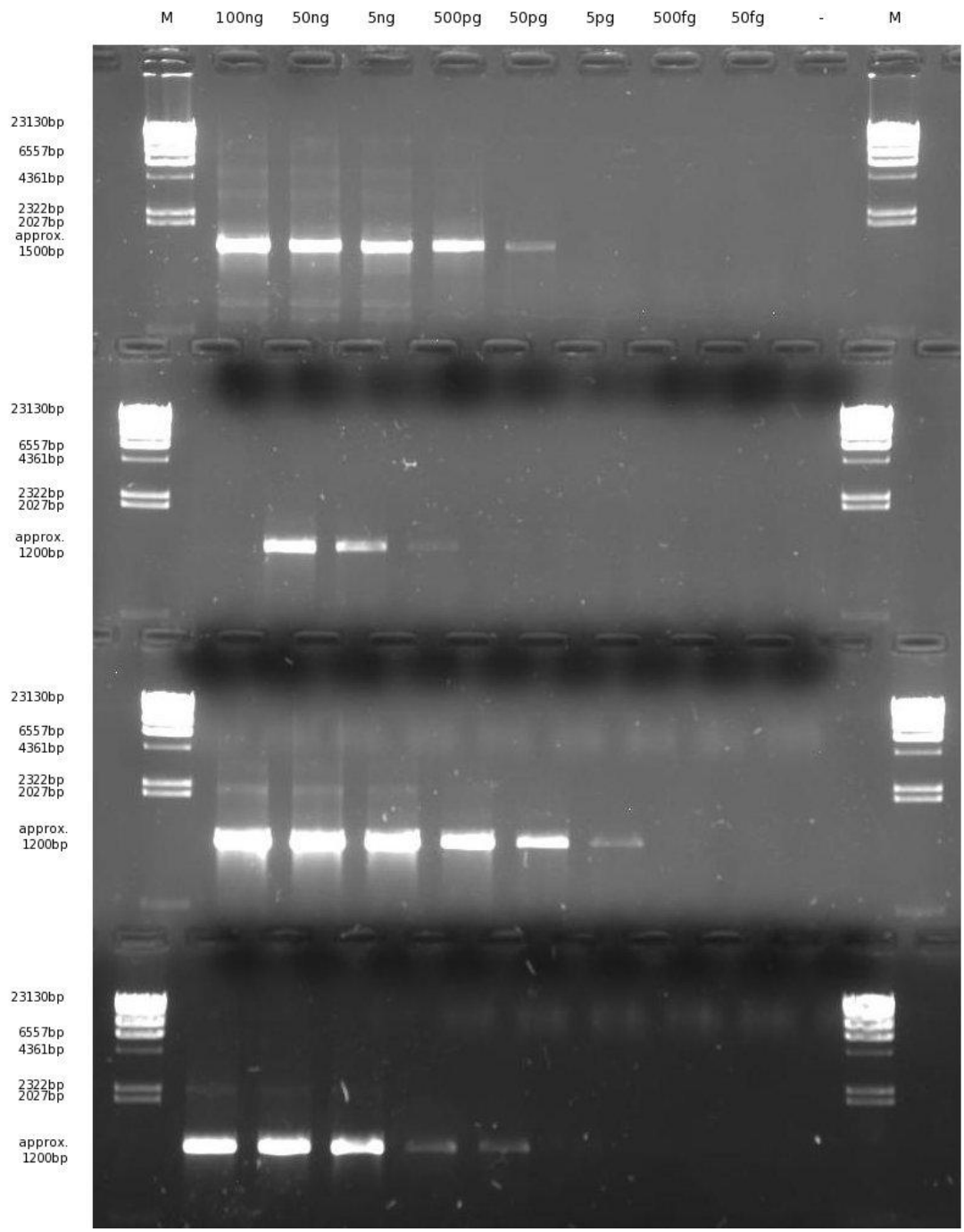

Figure 14: Sensitivity test with *E. coli*. PCR products (5µl + 5µl loading dye) on 1% agarose gel. Electrophoresis was performed at 120V for 50 minutes. Top to bottom: *16S* PCR, *gyrB* PCR with old primers, *gyrB* PCR with new specific primers, *gyrB* PCR with new primer mix (1:10). M: Lambda/HindIII Marker . -: negative control.

For *16S* PCR the LOD is 50pg, for *gyrB* PCR with old primers 500pg, for *gyrB* PCR with new single primers 5pg and for *gyrB* PCR with the new primer mix (1:10) 50pg, which is the same as for *16S* PCR and one log step higher than for the *gyrB* PCR with the old primers.

4.5 Hybridization

The new and old primers were also compared regarding the effect on the hybridization on the microarray. *gyrB* PCR was performed with both old and new primer mix (1:10) and PCR products

were loaded to a 1% agarose gel to check the size of the fragment. Samples were hybridized with the array after alkaline phosphatase treatment and SSELO.

The signals were normalized to the internal control (probe Msi_294). Normalized signals greater than 25% are considered positive. More than 50% of the probes giving a positive signal are required for unambiguous detection.

The complete list of results can be found in the annex.

4.5.1 *E. coli*

The PCR products from the PCR sensitivity tests with 50ng, 50pg, 5pg and 500fg template DNA were used for this experiment. Amplicons were purified with a PCR Purification Kit (Quiagen).

The normalized results of the hybridization with *E. coli* gDNA are shown in Table 22:

Table 22: Hybridization results of the *E. coli* dilution series (only relevant probes shown). Green: Positive controls. Yellow: Blanks. Positive results are shaded in black. Values between 10 and 25 (cut-off value) are shaded in gray.

	Msi_294	Msi_294	Msi_294	Msi_294	Eco_1402	Eco_1472	Eco_1521	blank	Salm_1457	Salm_1950	Salm_1620
E. coli 50ng old purified	105	95	106	94	138	146	146	0	0	0	1
E. coli 50ng new (mix 1:10) purified	102	98	105	95	133	177	157	1	0	0	1
E. coli 50pg old purified	104	96	99	101	4	6	2	0	0	0	0
E. coli 50pg new (mix 1:10) purified	107	93	92	108	27	59	25	6	10	12	17
E. coli 5pg old purified	96	104	91	109	1	4	1	0	0	0	0
E. coli 5pg new (mix 1:10) purified	106	94	97	103	15	16	10	0	0	0	0
E. coli 500fg old purified	105	95	97	103	0	0	0	0	0	0	0
E. coli 500fg new (mix 1:10) purified	82	118	95	105	2	3	1	0	0	1	0
neg. control	97	103	99	101	0	0	0	0	0	0	0

The limit of detection is greater than 50pg with the old *gyrB* PCR, while it is 50pg with the new *gyrB* PCR. On one slide, a low-level cross-hybridization with *Salmonella* spp. probes was observed; however, this was attributed to technical error.

4.5.2 S. Typhimurium

The PCR products from the PCR sensitivity tests with 50ng, 50pg, 5pg and 500fg template DNA were used for this experiment. Figure 15 shows the scan of the sample with 5pg template DNA:

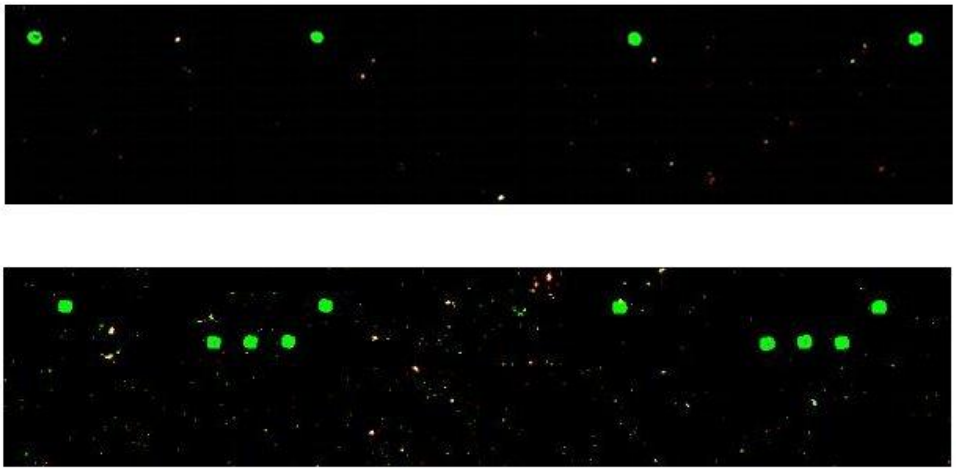

Figure 15: Hybridization results of *S.* Typhimurium (5pg). Top: old *gyrB* PCR. Bottom: new *gyrB* PCR. Images scanned at 600V PMT. Brightness set to 84%, contrast set to 92%.

The normalized results of the hybridization experiment with *S.* Typhimurium gDNA are shown in Table 23:

Table 23: Hybridization results of the *S. Typhimurium* dilution series (only relevant probes shown). Green: Positive controls. Yellow: Blanks. Positive results are shaded in black. Values between 10 and 25 (cut-off value) are shaded in gray.

	Msi_294	Msi_294	Msi_294	Msi_294	Eco_1402	Eco_1472	Eco_1521	blank	Salm_1457	Salm_1950	Salm_1620
S. Typhimurium 50ng old	103	97	110	90	1	1	0	0	128	101	117
S. Typhimurium 50ng new	108	92	102	98	1	0	0	0	92	110	90
S. Typhimurium 50 pg old	98	102	101	99	10	13	7	0	7	7	6
S. Typhimurium 50pg new	103	97	104	96	1	1	0	0	125	99	114
S. Typhimurium 5pg old	109	91	111	89	8	9	5	0	2	2	1
S. Typhimurium 5pg new	118	82	91	109	1	1	1	0	53	46	46
S. Typhimurium 500fg old	111	89	109	91	0	1	0	0	0	0	0
S. Typhimurium 500fg new	105	95	102	98	3	4	4	0	16	9	55
neg. control	92	108	90	110	0	0	0	0	0	0	0
neg. control	108	92	101	99	0	0	0	0	0	0	0

The limit of detection for *S.* Typhimurium is greater than 50pg gDNA with the old *gyrB* PCR and 5pg with the new *gyrB* PCR which represents a difference of at least two log steps in the limit of detection.

4.5.3 gDNA Mix

A mix containing gDNA of *E. coli*, *S.* Typhimurium, *S. aureus* and *C. perfringens* (50ng/µl each) was prepared and diluted from 1:10 to 1:1000 (in tenfold steps). *gyrB* PCR was performed with the old primers as well as with the new primer mix (1:10). Figure 16 shows the scan of the 1:10 dilution:

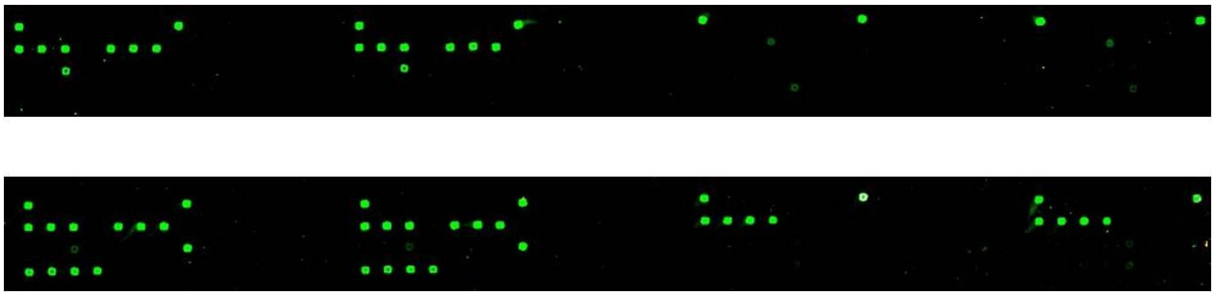

Figure 16: Hybridization results of the gDNA mix (1:10). Top: old *gyrB* PCR. Bottom: new *gyrB* PCR. Images scanned at 600V PMT. Brightness set to 75%, contrast set to 93%.

It is clearly visible that more spots can be seen after amplification with the new *gyrB* primers. These spots correspond to the probes for *C. perfringens* and *S. aureus*. The normalized results are listed in Table 24:

Table 24: Hybridization results of the mix containing gDNA from *E. coli*, *S.* Typhimurium, *S. aureus* and *C. perfringens* (only relevant probes shown). Green: Positive controls. Yellow: Blanks. Positive results are shaded in black. Values between 10 and 25 (cut-off value) are shaded in gray.

	Msi_294	Msi_294	Msi_294	Msi_294	Eco_1402	Eco_1472	Eco_1521	blank	Salm_1457	Salm_1950	Salm_1620	blank	Cperf_2341	Cperf_1477	Cperf_1755	Cperf_1832	Cperf_1499	blank	Msi_294	Msi_294	Msi_294	Msi_294	Saur_2106	Saur_2320	Saur_2033	Saur_1648	blank
gDNA mix old	112	88	88	112	89	103	100	0	87	106	103	0	1	1	1	1	1	0	92	108	111	89	7	5	4	6	0
gDNA mix new	109	91	96	104	94	121	119	0	85	112	103	0	80	50	20	52	46	0	91	109	97	103	85	90	85	8	0
gDNA mix 1:10 old	105	95	100	100	47	77	44	0	48	44	44	0	0	1	1	0	0	0	96	104	103	97	1	1	1	7	0
gDNA mix 1:10 new	106	94	100	100	75	104	88	0	65	88	90	0	34	20	17	22	20	0	92	108	100	100	72	67	74	12	0
gDNA mix 1:100 old	94	106	100	100	15	28	15	0	10	8	22	0	0	11	1	0	1	0	89	111	115	85	1	0	0	6	0
gDNA mix 1:100 new	121	79	92	108	70	96	70	0	83	74	55	0	11	7	4	6	6	0	93	107	113	87	40	32	26	6	0
gDNA mix 1:1000 old	113	87	95	105	14	20	10	0	6	5	5	0	0	0	1	0	0	0	88	112	106	94	0	0	0	7	0
gDNA mix 1:1000 new	107	93	104	96	15	29	11	0	36	30	27	0	2	1	1	0	1	0	84	116	108	92	6	5	4	6	0
neg. control	105	95	96	104	0	0	0	0	0	0	0	0	0	0	0	0	0	0	83	117	76	124	0	0	0	8	0

As can be seen in Table 24, using the old *gyrB* PCR only *E. coli* and *S.* Typhimurium can be detected in the undiluted mix and the 1:10 dilution. In the 1:100 dilution, none of the organisms can clearly be detected with the signals of the *E. coli* probes being below the cut-off value.

Using the new primers, all organisms are detected in the mix. In the 1:10 and 1:100 dilution *E. coli*, *S.* Typhimurium and *S. aureus* give positive results. Only one probe is positive for *C. perfringens* in the 1:10 dilution while the other four are ambiguous. In the 1:100 dilution, results for *C. perfringens* are clearly negative. In the 1:1000 dilution, only *S.* Typhimurium gives positive results; however, there is a clear tendency showing the better performance of the new *gyrB* PCR.

4.5.4 *S.* Typhimurium in *E. coli*

gDNA from *S.* Typhimurium (50ng/µl) was added to gDNA from *E. coli* (50ng/µl) in concentrations of 10%, 1% and 0.1% and *gyrB* PCR performed with both the old and new primer mix (1:10). The scan of the microarray hybridized with 10% *S.* Typhimurium in *E. coli* is shown in Figure 17:

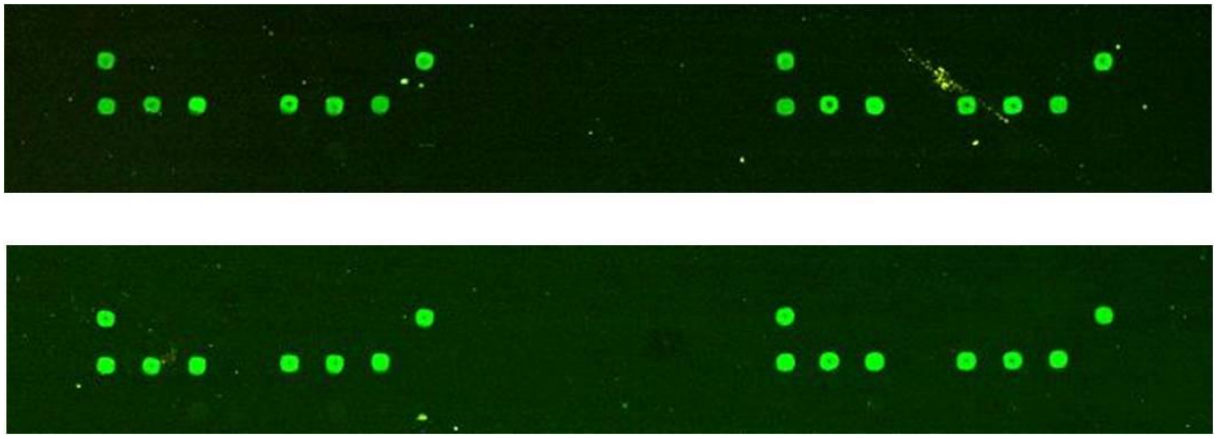

Figure 17: Hybridization results of *S.* Typhimurium in *E. coli*. Relative abundance of *S.* Typhimurium was 10%. Top: old *gyrB* PCR. Bottom: new *gyrB* PCR. Images scanned at 600V PMT. Brightness set to 78%, contrast set to 82%.

The results are listed in Table 25:

Table 25: Hybridization results of gDNA from *S.* Typhimurium in gDNA from *E. coli* (only relevant probes shown). Green: Positive controls. Yellow: Blanks. Positive results are shaded in black. Values between 10 and 25 (cut-off value) are shaded in gray.

	Msi_294	Msi_294	Msi_294	Msi_294	Eco_1402	Eco_1472	Eco_1521	blank	Salm_1457	Salm_1950	Salm_1620
E. coli + 10% *S.* Typhimurium old	83	117	89	111	87	100	154	0	109	114	89
E. coli + 10% *S.* Typhimurium new	102	98	108	92	138	124	181	0	143	94	119
E. coli + 1% *S.* Typhimurium old	101	99	84	116	93	102	120	0	56	40	36
E. coli + 1% *S.* Typhimurium new	100	100	110	90	117	117	124	0	58	39	40
E. coli + 0.1% *S.* Typhimurium old	98	102	105	95	91	107	113	0	9	6	6
E. coli + 0.1% *S.* Typhimurium new	97	103	97	103	98	106	117	0	9	7	7
neg. control	102	98	96	104	0	87	0	0	0	0	0

S. Typhimurium can be detected in concentrations of 10% and 1%. Although spots are slightly visible for 0.1% *S.* Typhimurium as well, the normalized values are below the cut-off value of 25 and thus not considered positive. Both the old and the new *gyrB* PCR give the same results.

4.5.5 *C. perfringens* in *E. coli*

gDNA from *C. perfringens* (50ng/µl) was added to gDNA from *E. coli* (50ng/µl) in concentrations of 10%, 1% and 0.1% and *gyrB* PCR performed with both the old primers and new primer mix (1:10). The scan of the microarray hybridized with 10% *C. perfringens* in *E. coli* is shown in Figure 18. The slide hybridized with samples amplified with old *gyrB* PCR had to be scanned at a lower PMT value, because spots were saturated at 700V PMT.

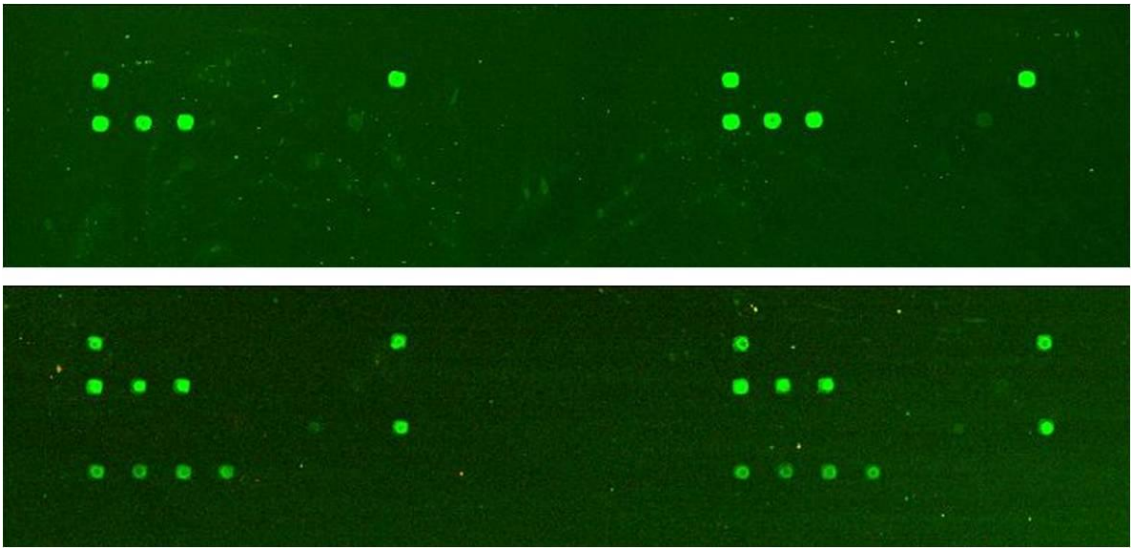

Figure 18: Hybridization results of *C. perfringens* in *E. coli*. Relative abundance of *C. perfringens* was 10%. Top: old *gyrB* PCR. Bottom: new *gyrB* PCR. Images scanned at 550V and 700V PMT respectively. Brightness set to 78%, contrast set to 82%.

The results are listed in Table 26:

Table 26: Results of the hybridization of *C. perfringens* in *E. coli* (only relevant probes shown). Green: Positive controls. Yellow: Blanks. Positive results are shaded in black. Values between 10 and 25 (cut-off value) are shaded in gray.

	Msi_294	Msi_294	Msi_294	Msi_294	Eco_1402	Eco_1472	Eco_1521	blank	Cperf_2341	Cperf_1477	Cperf_1755	Cperf_1832	Cperf_1499
E. coli + 10% *C. perfringens* old	91	109	103	97	113	111	162	0	1	1	0	0	0
E. coli + 10% *C. perfringens* new	100	100	93	107	167	117	193	1	109	55	35	55	48
E. coli + 1% *C. perfringens* old	98	102	100	100	88	106	110	0	0	0	0	0	0
E. coli + 1% *C. perfringens* new	93	107	92	108	134	105	151	0	28	8	6	8	6
E. coli + 0.1% *C. perfringens* old	104	96	101	99	89	115	120	0	0	0	0	0	0
E. coli + 0.1% *C. perfringens* new	86	114	88	112	134	98	155	0	4	1	3	1	1
neg. control	96	104	106	94	0	72	1	0	0	0	0	0	0

Using the old *gyrB* primers, *C. perfringens* could not be detected at all. The new primers enabled the detection of *C. perfringens* at 10%; however, it could not be detected at lower concentrations.

4.5.6 Spiked Food Samples

DNA from previous spike experiments was used for these experiments. The experimental setup was the same as described above, comparing the performance of both the old and new *gyrB* PCR (using primer mix [1:10]) on the microarray.

4.5.6.1 *Salmonella* spp.

Chicken meat was spiked in triplicates with *S.* Typhimurium DSM 554 at different levels:

- Samples 140-142: 0 cfu/25g food
- Samples 143-145: 1-10 cfu/25g food
- Samples 146-147: 10-100 cfu/25g food

Biological enrichment was performed in buffered peptone water and RVS medium according to ISO standard 6579:2002 and extracted DNA was stored at -20°C.

The scan of the microarrays hybridized with sample 143 is shown in Figure 19:

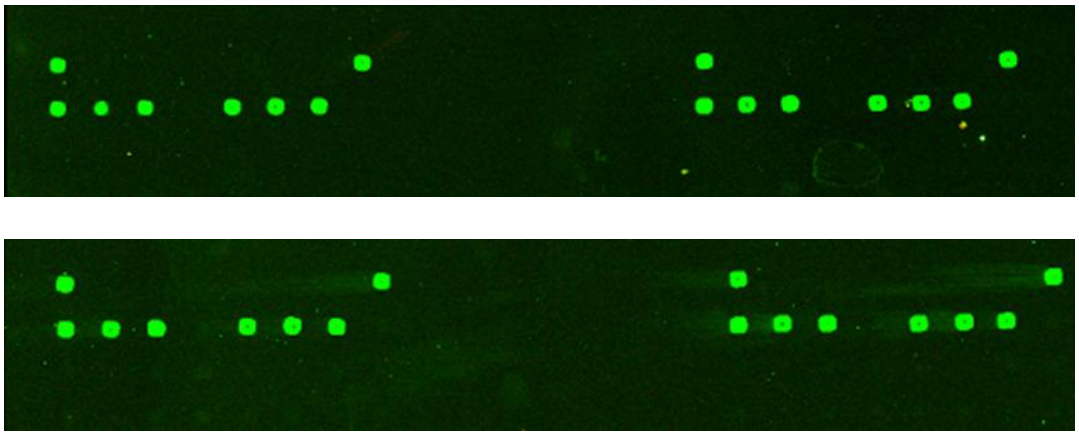

Figure 19: Hybridization results of spiked food sample 143 (chicken meat spiked with *S.* Typhimurium [1-10cfu/25g food]). Top: old *gyrB* PCR. Bottom: new *gyrB* PCR. Images scanned at 600V PMT; Brightness set to 78%, contrast set to 82%.

The results of the hybridization are shown in Table 27:

Table 27: Hybridization results of spiked food sample (chicken meat spiked with *S.* Typhimurium (only relevant probes shown). Green: Positive controls. Yellow: Blanks. Positive results are shaded in black. Values between 10 and 25 (cut-off value) are shaded in gray.

	Msi_294	Msi_294	Msi_294	Msi_294	Eco_1402	Eco_1472	Eco_1521	blank	Salm_1457	Salm_1950	Salm_1620	blank
140 old	99	101	110	90	84	85	111	0	0	1	3	1
140 new	102	98	97	103	87	87	110	0	0	1	3	0
141 old	86	114	98	102	76	74	83	0	0	1	2	1
141 new	83	117	96	104	85	65	73	0	0	0	1	1
142 old	90	110	98	102	73	75	100	0	0	1	18	1
142 new	74	126	93	107	65	101	84	0	0	1	3	1
143 old	95	105	91	109	95	67	108	0	88	77	78	0
143 new	99	101	92	108	118	92	118	0	86	68	58	0
144 old	83	117	88	112	77	76	99	0	81	65	57	1
144 new	92	108	88	112	88	65	113	0	85	69	60	0

	Msi_294	Msi_294	Msi_294	Msi_294	Eco_1402	Eco_1472	Eco_1521	blank	Salm_1457	Salm_1950	Salm_1620	blank
145 old	120	80	95	105	96	87	121	0	89	71	71	0
145 new	91	109	81	119	72	74	94	0	72	85	66	0
146 old	106	94	108	92	112	78	116	1	98	89	88	0
146 new	98	102	95	105	85	75	107	0	92	91	77	0
147 old	82	118	79	121	64	68	85	0	69	68	51	0
147 new	90	110	100	100	107	84	135	0	96	87	101	0
neg. control	95	105	101	99	0	0	0	0	0	0	0	0

Salmonella spp. could be detected at spiking levels of 1-10cfu/25g food and 10-100cfu/25 g food.

4.5.6.2 C. coli

Pork meat was spiked with *C. coli* DSM4689 at different levels:

- Samples 63-65: 0 cfu/25g food
- Samples 66-68: 1-10 cfu/25g food
- Samples 69-71: 10-100 cfu/25g food

Biological enrichment was performed in Bolton broth according to ISO standard 10272-1:2006 and DNA was stored at -20°C.

The scan of the microarrays hybridized with sample 67 is shown in Figure 20:

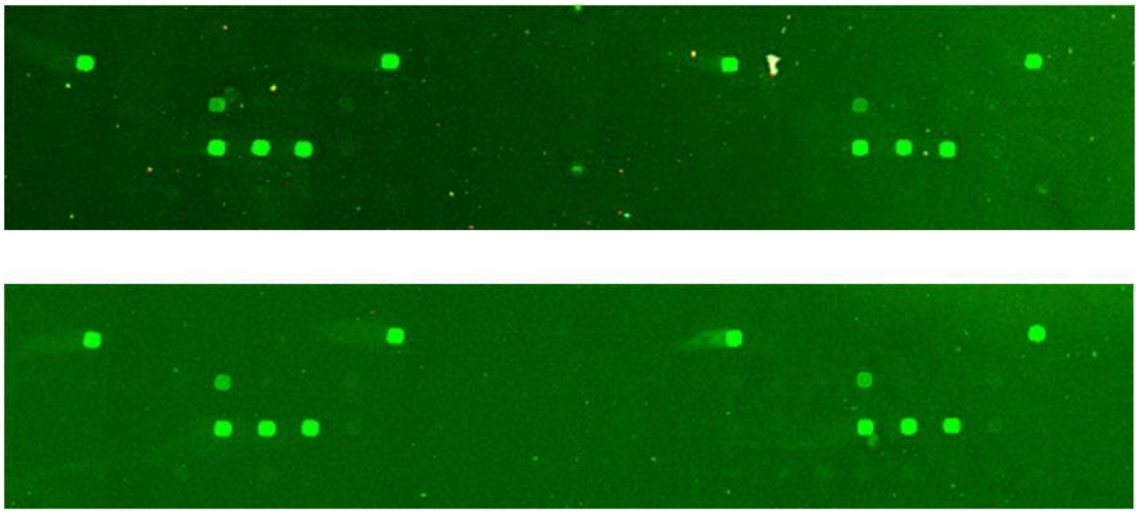

Figure 20: Hybridization results of spiked food sample 67 (pork meat spiked with *C. coli* [1-10cfu/25g food]). Top: old *gyrB* PCR. Bottom: new *gyrB* PCR. Images scanned at 600V PMT;. Brightness set to 79%, contrast set to 87%.

The results of the hybridization are shown in Table 28:

Table 28: Results of the hybridization of *C. coli* from spiked pork meat after biological enrichment (only relevant probes shown). Green: Positive controls. Yellow: Blanks. Positive results are shaded in black. Values between 10 and 25 (cut-off value) are shaded in gray.

	Msi_294	Msi_294	Msi_294	Msi_294	Cje_2000	Cje_2016	Cje_1615	Clari_2018	Clari_2272	Clari_2132	Ccoli_1820	Ccoli_1934	Ccoli_2011	Cups_1444	Cups_1849	Cups_1689
63 old	110	90	98	102	3	0	0	0	0	1	15	2	1	1	1	0
63 new	97	103	117	83	22	1	0	3	1	1	12	4	4	2	4	1
64 old	82	118	111	89	0	0	0	0	0	0	1	3	0	0	0	0
64 new	101	99	104	96	0	0	0	0	0	0	4	3	0	0	0	0
65 old	85	115	107	93	0	0	0	0	0	0	0	1	0	0	0	0
65 new	98	102	175	25	2	0	1	0	0	0	20	3	0	0	0	0
66 old	90	110	124	76	0	1	0	0	0	0	100	80	84	1	0	0
66 new	89	111	109	91	1	1	0	1	0	0	112	97	95	1	0	0
67 old	91	109	111	89	0	1	1	0	0	0	101	76	79	0	0	0
67 new	81	119	105	95	0	1	0	0	0	0	86	81	66	1	0	0
68 old	90	110	111	89	0	1	1	0	0	0	133	109	94	3	0	0
68 new	90	110	100	100	0	1	0	0	0	0	115	113	100	1	0	0
69 old	101	99	114	86	0	0	0	0	0	0	43	25	32	0	0	0
69 new	100	100	108	92	0	0	0	0	0	0	91	71	73	0	0	0
70 old	125	75	131	69	0	1	0	1	0	1	139	65	49	2	0	1
70 new	91	109	96	104	0	0	0	1	0	0	117	88	80	0	0	0
71 old	100	100	106	94	0	0	0	0	0	1	162	142	115	1	0	0
71 new	105	95	119	81	0	0	0	1	0	0	131	102	98	1	0	0
neg. control	121	79	101	99	0	1	0	0	0	0	0	4	1	1	0	0

C. perfringens could be detected at spiking levels of 1-10cfu/25g food and 10-100cfu/25 g food.

4.5.6.3 Y. enterocolitica

Pork meat was spiked with *Y. enterocolitica* NCTC 10460 at different levels:

- Samples 207-209: 0 cfu/25g food
- Samples 210-212: 1-10 cfu/25g food
- Samples 213-215: 10-100 cfu/25g food

Biological enrichment was performed in ITC according to ISO standard 10273:2003 and DNA was stored at -20°C.

The scan of the microarrays hybridized with sample 215 is shown in Figure 21:

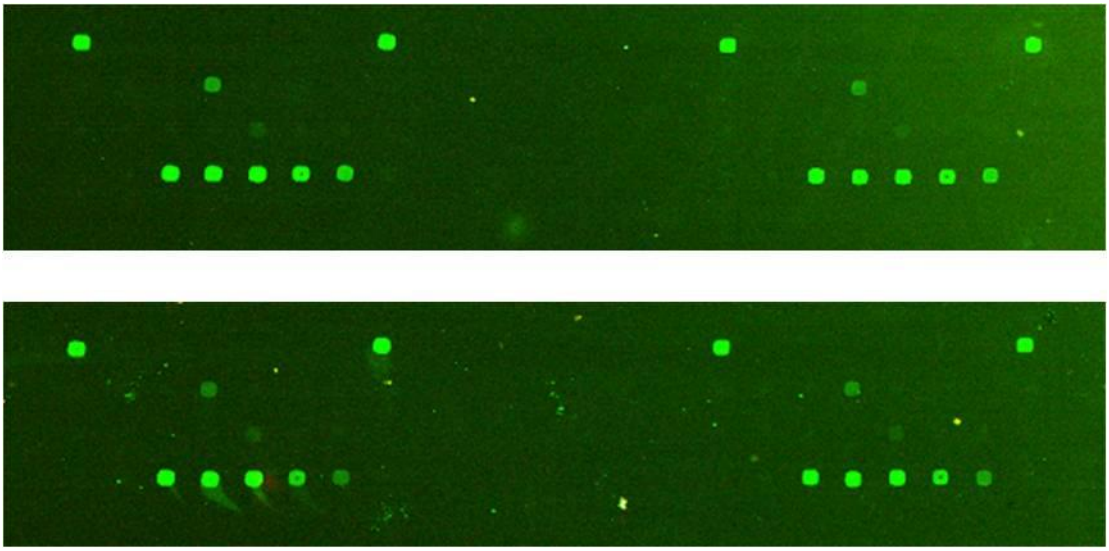

Figure 21: Hybridization results of spiked food sample 215 (pork meat spiked with *Y. enterocolitica* [10-100cfu/25g food]). Top: old *gyrB* PCR. Bottom: new *gyrB* PCR. Images scanned at 600V PMT. Brightness set to 84%, contrast set to 87%.

The results of the hybridization are shown in Table 29:

Table 29: Results of the hybridization of *Y. enterocolitica* from spiked pork meat after biological enrichment (only relevant probes shown). Green: Positive controls. Yellow: Blanks. Positive results are shaded in black. Values between 10 and 25 (cut-off value) are shaded in gray.

	Msi_294	Msi_294	Msi_294	Msi_294	blank	Yen_1215	Yen_1451	Yen_1508	Ypse_1467	Ypse_1675	Ypse_1483
207 old	111	89	90	110	0	1	0	7	0	0	0
207 new	100	100	95	105	0	1	0	1	0	0	0
208 old	101	99	99	101	0	2	0	12	0	0	0
208 new	103	97	113	87	0	0	0	0	0	0	0
209 old	92	108	78	122	1	14	2	88	20	22	1
209 new	107	93	94	106	0	0	0	2	0	0	0
210 old	86	114	83	117	0	0	0	0	0	0	0
210 new	106	94	94	106	0	2	1	11	0	0	0
211 old	99	101	86	114	1	8	1	56	3	5	2
211 new	97	103	100	100	0	1	0	15	0	0	0
212 old	93	107	85	115	0	1	0	8	0	0	0
212 new	95	105	87	113	0	1	0	1	0	0	0
213 old	106	94	108	92	2	21	3	70	2	2	2
213 new	106	94	99	101	0	1	0	4	0	0	0
214 old	94	106	101	99	0	1	0	2	0	0	0
214 new	100	100	89	111	0	64	76	76	35	13	0

	Msi_294	Msi_294	Msi_294	Msi_294	blank	Yen_1215	Yen_1451	Yen_1508	Ypse_1467	Ypse_1675	Ypse_1483
215 old	99	101	104	96	0	56	60	66	30	14	0
215 new	80	120	76	124	1	72	67	65	15	7	0
neg. control	88	112	99	101	0	0	0	0	0	0	0

Y. enterocolitica could not be detected at spiking levels of 1-10cfu/25g food. At the spiking level of 10-100cfu/25g food, two of the three samples gave positive results with the new primers and one with the old primers. However, cross-hybridization with *Y. pseudocolitica* probes (Ypse_1467, Ypse_1675) can be observed.

5 Discussion

The microbial diagnostic microarray developed by Kostić *et al.* (2007, 2010) allows highly specific detection of food- and water-borne pathogens at species level due to the use of the *gyrB* gene as a marker gene. However, the sensitivity of the detection was limited by the low efficiency of the *gyrB* amplification. Different approaches were used for the optimization of the *gyrB* PCR, i.e. nested PCR and eventually the design and testing of new primers.

The primers UP1, UP1G, UP2r and UP2Ar for *gyrB* amplification introduced by Yamamoto and Harayama (1995) have a 23bp sequencing tag which was used as target region for the nested primers UP1_S and UP2r_S. Since the two primers had a difference of ca. 7°C in their melting temperatures, they were tested in gradient PCRs, but mostly only smears could be observed when PCR products were checked in electrophoresis. Possible reasons for the observation of smears include amplification with too many cycles or suboptimal reaction conditions with regard to the reaction buffer, especially $MgCl_2$ concentration. Exact data on $MgCl_2$ concentration in FailSafe PCR 2x PreMix E (Epicentre) is not provided by the manufacturer, but it ranges from 3 to 7mM.

Another reason might be the use of too much starting template. However, this cannot have been the case as the *gyrB* PCR products generated from 100ng template DNA produced defined lanes on 1% agarose gel after the first nested *gyrB* PCR (see Figure 8).

An enzyme concentration too high can also lead to formation of smears. The manufacturer (Invitrogen) recommends a concentration of 1.0 to 2.5U/100µl reaction. For the previously described experiment 4U/100µl reaction were used; however, this is probably not the cause for smears since the same concentration was used in conventional *gyrB* PCR where no smears were observed.

Primer degradation could also result in smears; therefore, experiments were performed with fresh dilutions made from the primer stock, but there was no difference in the results. Most likely, the primer design was not optimal. Usually, nested primers would target a region lying between the first primers, but in this approach, the tail region of the first primers was targeted. The use of shorter primers targeting a region five bases from the end of the PCR products was intended to facilitate the binding of the primers, considering that the quality of PCR products may be inferior at the end. However, this attempt did not lead to an improvement. A possible explanation is that the binding site was still too close to the end of the PCR product, but another factor is that there was also a big difference between the T_m of UP1_S_k and UP2r_S_k (about 7°C; compare Table 17), which exacerbates the annealing of the primers.

The establishment of the sequence database for the design of new primers proved difficult due to the fact that only few complete sequences of the *gyrB* gene were available. Those that were available often lacked sequence information in the primer region. Nevertheless, ten new primer pairs with reduced degeneracy could be designed; resulting in increased sensitivity of the *gyrB* PCR.

The LOD of the old and new *gyrB* PCRs were determined by the amplification of gDNA dilution series of different organisms, using both individual primers as well as the multiplex approach. It was noted that the amplification with the individual primers was more sensitive than in the multiplex reaction (compare 4.4.3). Preferential amplification has been reported to be an issue in multiplex PCR, which is favored by using primers with different T_m and the amplification of PCR products of different lengths (You *et al.*, 2008). Both these conditions do not apply to the experimental setup that was

used for this work. It is more likely that the decrease in sensitivity as compared to single primer reactions is due to unspecific binding of primers. Anyway, in order to enable fast parallel detection of pathogens, the use of multiplex PCR cannot be circumvented. Besides, it could be shown that the LOD using multiplex PCR is one log step lower than with the old primers.

The hybridization results also showed a definitive improvement in the sensitivity. For *S.* Typhimurium, for instance, the sensitivity could be improved by minimum two log steps; the LOD being greater than 50pg for the old *gyrB* PCR and 5pg for the new *gyrB* PCR. The new *gyrB* PCR also showed a better performance in the analysis of a gDNA mix containing *E. coli*, *S.* Typhimurium, *S. aureus* and *C. perfringens*, where all four pathogens were detected at a concentration of 50ng/µl each and three out of four in 1:10 and 1:100 dilutions of the gDNA mix. Using the old *gyrB* PCR, only *E. coli* and *S.* Typhimurium could be detected and only at the two highest concentrations.

A more realistic setting was simulated by testing gDNA extracted from three spiked food samples after biological enrichment. Using the new primer mix, it was possible to detect *S.* Typhimurium and *C. coli* at spiking levels of 1-10cfu/25g food. The sample containing *Y. enterocolitica* did not give unanimously positive results at any spiking level. This is not necessarily due to a fault in the amplification reaction since it was shown by Kostić and co-workers (2011) that the enrichment protocols currently considered as "gold standards" for food safety assessments are questionable with regard to specificity. Especially in the presence of high levels of background flora enrichment efficiency of low levels of *Y. enterocolitica* is poor.

Nevertheless, the results show that amplification with new *gyrB* primers is suitable for the detection of pathogens in food and that the limitation in the sensitivity caused by PCR efficiency could be overcome.

Abbreviations

ddNTP	dideoxynucleotide
DMSO	Dimethyl sulfoxide
DNA	deoxyribonuleic acid
dNTP	deoxyribonucleotide
ds	double-stranded
EFSA	European Food Safety Authority
EU	European Union
gDNA	genomic DNA
LB	lysogeny broth
LOD	limit of detection
LSU	large-subunit
MDM	microbial diagnostic microarray
NCBI	National Center for Biotechnology Information
ONC	overnight culture
PBS	phosphate buffered saline
PCR	Polymerase Chain Reaction
RC	reverse complement
RNA	ribonucleic acid
rRNA	ribosomal RNA
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulfate
ss	single-stranded
SSC	saline sodium citrate
SSELO	sequence-specific end-labeling of oligonucleotides
SSU-LSU	small-subunit-large-subunit
T_a	annealing temperature
TBE	Tris-Borat-EDTA
T_m	melting temperature
TMAC	tetramethylammonium chloride
tmRNA	transfer-messenger RNA
Tris	*N*-Tris(hydroxymethyl)aminomethane
WHO	World Health Organization

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6 Annex

	Msi_294	Msi_294	Msi_294	Msi_294	Eco_1402	Eco_1472	Eco_1521	blank	Salm_1457	Salm_1950	Salm_1620	blank	Lmono_2172	Lmono_2396	Lmono_2460	Lseel_1346	Lwelsh_1458	Linn_1882	blank	Cperf_2341	Cperf_1477	Cperf_1755	Cperf_1832	Cperf_1499	Cdiff_2224	Cdiff_2131	Cdiff_2318	blank	Msi_294	Msi_294	Msi_294	Msi_294	blank	Yen_1215	Yen_1451	Yen_1508	Ypse_1467	Ypse_1675	Ypse_1483
E. coli 50ng new (single primers) purified	113	87	106	94	158	91	105	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	129	71	143	57	0	2	0	0	0	0	0
E. coli 5ng new (single primers) NOT purified	123	77	121	79	194	113	117	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	110	90	142	58	0	2	0	1	0	0	0
E. coli 500pg new (single primers) purified	109	91	93	107	46	29	18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	118	82	129	71	0	1	0	0	0	0	0
E. coli 50pg new (single primers) purified	90	110	91	109	62	27	45	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	123	77	124	76	0	6	3	1	1	0	0
E. coli 5pg new (single primers) purified	84	116	101	99	17	15	11	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	120	80	139	61	0	4	3	3	2	1	0
PCR neg. control new (single primers) purified	82	118	99	101	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	124	76	121	79	0	2	2	1	1	1	0
neg. control	85	115	87	113	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	130	70	116	84	0	2	0	0	0	0	0
E. coli 50ng old purified	105	95	106	94	138	146	146	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	121	79	104	96	0	2	0	0	0	0	0
E. coli 50ng new (mix 1:10) purified	102	98	105	95	133	177	157	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	113	87	74	126	0	2	0	0	0	0	0
E. coli 50pg old purified	104	96	99	101	4	6	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	103	97	100	100	0	1	0	0	0	0	0
E. coli 50pg new (mix 1:10) purified	107	93	92	108	27	59	25	6	10	12	17	0	11	11	10	11	9	4	-1	8	9	9	7	3	10	4	7	-2	143	57	144	56	4	15	14	9	9	11	6
E. coli 5pg old purified	96	104	91	109	1	4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	106	94	74	126	0	1	0	0	0	0	0
E. coli 5pg new (mix 1:10) purified	106	94	97	103	15	16	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	102	98	104	96	0	3	2	1	0	0	0
E. coli 500fg old purified	105	95	97	103	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	136	64	108	92	0	1	0	0	0	0	0

	Msi_294	Msi_294	Msi_294	Msi_294	Eco_1402	Eco_1472	Eco_1521	blank	Salm_1457	Salm_1950	Salm_1620	blank	Lmono_2172	Lmono_2396	Lmono_2460	Lseel_1346	Lwelsh_1458	Linn_1882	blank	Cperf_2341	Cperf_1477	Cperf_1755	Cperf_1832	Cperf_1499	Cdiff_2224	Cdiff_2131	Cdiff_2318	blank	Msi_294	Msi_294	Msi_294	Msi_294	blank	Yen_1215	Yen_1451	Yen_1508	Ypse_1467	Ypse_1675	Ypse_1483
E. coli 500fg new (mix 1:10) purified	82	118	95	105	2	3	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	109	91	84	116	0	2	2	1	1	1	0
neg. control	97	103	99	101	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	107	93	98	102	0	1	0	0	0	0	0
Salmonella 50ng old	103	97	110	90	1	1	0	0	128	101	117	0	0	0	8	0	0	0	0	0	4	0	0	0	0	1	0	0	96	104	105	95	0	3	2	4	0	1	0
Salmonella 50ng old purified	103	97	98	102	4	7	4	3	135	85	108	3	4	8	2	1	1	2	2	1	6	2	3	3	2	4	2	2	99	101	89	111	1	2	2	1	1	1	2
Salmonella 50ng new (mix 1:10)	108	92	102	98	1	0	0	0	92	110	90	0	0	0	1	0	0	0	0	0	2	0	0	0	0	0	0	0	98	102	87	113	0	0	0	1	0	0	0
Salmonella 50 ng new (mix 1:10) purified	122	78	100	100	1	1	1	1	114	61	97	1	6	1	0	0	0	0	0	-1	1	0	0	2	0	0	1	1	142	58	88	112	2	1	1	0	0	0	0
Salmonella 50 pg old	98	102	101	99	10	13	7	0	7	7	6	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	106	94	97	103	1	0	0	1	0	0	0
Salmonella 50 pg old purified	106	94	95	105	6	5	3	0	5	3	3	0	2	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	105	95	94	106	1	1	0	0	0	0	0
Salmonella 50pg new (mix 1:10)	103	97	104	96	1	1	0	0	125	99	114	0	0	0	8	0	0	0	0	0	4	0	0	0	0	1	0	0	96	104	105	95	0	3	2	4	0	1	0
Salmonella 50pg new (mix 1:10) purified	100	100	89	111	15	1	1	1	85	47	71	1	1	0	1	0	1	1	1	1	0	0	0	1	0	1	0	1	98	102	81	119	1	5	2	2	1	0	0
Salmonella 5pg old	109	91	111	89	8	9	5	0	2	2	1	1	0	0	14	0	0	0	0	0	0	3	0	0	0	0	0	0	97	103	107	93	0	1	0	2	0	1	1
Salmonella 5pg old purified	109	91	113	87	5	4	3	1	2	2	1	1	0	1	13	2	3	1	2	1	1	1	1	1	1	1	1	1	106	94	85	115	0	2	0	1	1	1	17
Salmonella 5pg new (mix 1:10)	118	82	91	109	1	1	1	0	53	46	46	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	1	0	100	100	95	105	1	3	3	4	1	0	0
Salmonella 5pg new (mix 1:10) purified	114	86	98	102	2	2	1	2	20	12	14	2	1	1	2	1	2	2	1	0	2	0	0	1	2	3	-1	1	92	108	107	93	0	1	1	2	1	4	2
Salmonella 500fg old	111	89	109	91	0	1	0	0	0	0	0	1	0	0	18	0	0	0	0	0	1	0	0	0	0	0	0	0	99	101	88	112	0	1	0	6	0	0	1
Salmonella 500fg old purified	106	94	109	91	1	3	12	2	1	0	1	1	1	1	2	1	2	1	3	1	1	1	2	1	1	1	0	0	102	98	94	106	0	0	3	-1	1	1	3
Salmonella 500fg new (mix 1:10)	105	95	102	98	3	4	4	0	16	9	55	0	2	1	4	1	3	1	0	0	22	1	1	1	2	1	1	0	100	100	97	103	1	15	5	108	3	5	1
Salmonella 500fg new (mix 1:10) purified	108	92	108	92	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	94	106	88	112	0	1	1	1	0	0	0
neg. control	92	108	90	110	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	84	116	87	113	0	0	0	0	0	0	0
neg. control	108	92	101	99	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	2	0	0	93	107	103	97	0	1	0	0	0	0	1

	Msi_294	Msi_294	Msi_294	Msi_294	Eco_1402	Eco_1472	Eco_1521	blank	Salm_1457	Salm_1950	Salm_1620	blank	Lmono_2172	Lmono_2396	Lmono_2460	Lseel_1346	Lwelsh_1458	Linn_1882	blank	Cperf_2341	Cperf_1477	Cperf_1755	Cperf_1832	Cperf_1499	Cdiff_2224	Cdiff_2131	Cdiff_2318	blank	Msi_294	Msi_294	Msi_294	Msi_294	blank	Yen_1215	Yen_1451	Yen_1508	Ypse_1467	Ypse_1675	Ypse_1483
gDNA mix old	112	88	88	112	89	103	100	0	87	106	103	0	0	0	8	0	0	0	0	1	1	1	1	1	0	0	0	0	92	108	111	89	0	1	0	1	0	0	1
gDNA mix new	109	91	96	104	94	121	119	0	85	112	103	0	0	0	1	0	0	0	0	80	50	20	52	46	0	0	0	0	91	109	97	103	0	1	0	0	0	0	0
gDNA mix 1:10 old	105	95	100	100	47	77	44	0	48	44	44	0	0	0	16	0	0	0	0	0	1	1	0	0	0	0	0	0	96	104	103	97	0	1	0	5	0	0	0
gDNA mix 1:10 new	106	94	100	100	75	104	88	0	65	88	90	0	0	0	6	0	0	0	0	34	20	17	22	20	0	0	0	0	92	108	100	100	0	1	0	2	0	0	0
gDNA mix 1:100 old	94	106	100	100	15	28	15	0	10	8	22	0	0	0	4	0	1	0	0	0	11	1	0	1	1	1	0	0	89	111	115	85	0	2	0	19	0	0	1
gDNA mix 1:100 new	121	79	92	108	70	96	70	0	83	74	55	0	0	0	4	0	0	0	0	11	7	4	6	6	0	1	0	0	93	107	113	87	0	1	1	2	0	0	0
gDNA mix 1:1000 old	113	87	95	105	14	20	10	0	6	5	5	0	0	0	26	1	1	0	0	0	0	1	0	0	1	0	15	0	88	112	106	94	0	1	0	5	0	0	0
gDNA mix 1:1000 new	107	93	104	96	15	29	11	0	36	30	27	0	0	0	12	0	0	0	0	2	1	1	0	1	0	0	0	0	84	116	108	92	0	1	1	4	0	0	0
neg- control	105	95	96	104	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	83	117	76	124	0	0	0	0	0	0	0
E. coli & 10% Salmonella (50ng/µl) old	83	117	89	111	87	100	154	0	109	114	89	0	1	0	0	0	0	2	0	1	0	0	0	0	0	0	0	1	92	108	103	97	0	1	0	0	0	0	0
E. coli & 10% Salmonella (50ng/µl) new	102	98	108	92	138	124	181	0	143	94	119	0	1	0	3	0	0	1	0	0	1	0	0	0	0	0	0	0	102	98	94	106	0	1	0	1	0	0	0
E. coli & 1% Salmonella (50ng/µl) old	101	99	84	116	93	102	120	0	56	40	36	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	1	94	106	102	98	0	2	0	1	0	0	0
E. coli & 1% Salmonella (50ng/µl) new	100	100	110	90	117	117	124	0	58	39	40	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	89	111	101	99	0	1	0	0	0	0	0
E. coli & 0.1% Salmonella (50ng/µl) old	98	102	105	95	91	107	113	0	9	6	6	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	92	108	108	92	0	1	0	1	0	0	0
E. coli & 0.1% Salmonella (50ng/µl) new	97	103	97	103	98	106	117	0	9	7	7	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	93	107	98	102	0	2	0	1	0	0	0
Listeria 50ng old	111	89	111	89	8	80	6	0	1	1	1	0	249	122	10	1	5	3	2	0	0	0	0	0	0	0	0	0	90	110	121	79	0	0	0	0	0	0	0
Listeria 50ng new	106	94	100	100	0	65	0	0	0	0	0	0	161	75	9	1	4	2	0	0	0	0	0	0	0	0	0	0	96	104	131	69	0	0	0	0	0	0	0

	Msi_294	Msi_294	Msi_294	Msi_294	Eco_1402	Eco_1472	Eco_1521	blank	Salm_1457	Salm_1950	Salm_1620	blank	Lmono_2172	Lmono_2396	Lmono_2460	Lseel_1346	Lwelsh_1458	Linn_1882	blank	Cperf_2341	Cperf_1477	Cperf_1755	Cperf_1832	Cperf_1499	Cdiff_2224	Cdiff_2131	Cdiff_2318	blank	Msi_294	Msi_294	Msi_294	Msi_294	blank	Yen_1215	Yen_1451	Yen_1508	Ypse_1467	Ypse_1675	Ypse_1483
neg. control	102	98	96	104	0	87	0	0	0	0	0	0	1	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	85	115	111	89	0	0	0	0	0	0	0
E. coli & 10% C. perfringens old	91	109	103	97	113	111	162	0	0	1	3	0	0	0	1	0	0	1	0	1	1	0	0	0	0	0	0	0	103	97	111	89	0	1	0	1	0	0	0
E. coli & 10% C. perfringens new	100	100	93	107	167	117	193	0	1	1	4	1	0	0	1	0	0	7	1	109	55	35	55	48	0	1	0	1	106	94	114	86	0	2	1	0	1	1	1
E. coli & 1% C. perfringens old	98	102	100	100	88	106	110	0	0	0	2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	94	106	108	92	0	0	0	0	0	0	0
E. coli & 1% C. perfringens new	93	107	92	108	134	105	151	4	1	3	5	0	0	0	4	0	1	5	0	28	8	6	8	6	0	1	0	0	94	106	122	78	1	3	0	2	1	0	0
E. coli & 0.1% C. perfringens old	104	96	101	99	89	115	120	0	0	0	2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	90	110	99	101	0	0	0	0	0	0	0
E. coli & 0.1% C. perfringens new	86	114	88	112	134	98	155	1	1	4	5	0	1	15	6	1	1	5	0	4	1	3	1	1	1	1	1	0	98	102	111	89	1	4	1	4	1	1	1
neg. control	96	104	106	94	0	72	1	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	110	90	62	138	0	0	0	0	0	0	0
Salmonella 140 new	112	88	102	98	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	22	14	32	5	72	0	0	93	107	90	110	0	0	0	1	0	0	0
141 new	108	92	84	116	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	1	0	0	0	0	0	95	105	86	114	0	1	0	1	0	0	0
142 new	91	109	104	96	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	95	105	115	85	0	1	0	4	0	0	0
143 new	104	96	88	112	0	0	0	0	0	0	0	0	1	0	12	1	0	1	1	0	0	0	0	0	0	0	0	0	101	99	100	100	0	1	0	6	0	0	0
144 new	102	98	106	94	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	89	111	103	97	0	1	0	5	0	0	0
145 new	105	95	89	111	0	0	0	0	0	0	0	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	90	110	100	100	0	1	0	4	0	0	0
146 new	100	100	95	105	0	0	0	0	0	2	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	103	97	111	89	0	0	0	0	0	0	0
147 new	97	103	104	96	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	99	101	88	112	0	0	0	0	0	0	0
neg. control	76	124	89	111	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	94	106	90	110	0	0	0	1	0	0	0
C. coli 63 old	113	87	90	110	1	1	0	1	0	0	0	0	0	0	3	0	0	0	0	0	5	0	0	0	0	0	0	0	110	90	98	102	0	2	0	18	0	0	1

	Msi_294	Msi_294	Msi_294	Msi_294	Eco_1402	Eco_1472	Eco_1521	blank	Salm_1457	Salm_1950	Salm_1620	blank	Lmono_2172	Lmono_2396	Lmono_2460	Lseel_1346	Lwelsh_1458	Linn_1882	blank	Cperf_2341	Cperf_1477	Cperf_1755	Cperf_1832	Cperf_1499	Cdiff_2224	Cdiff_2131	Cdiff_2318	blank	Msi_294	Msi_294	Msi_294	Msi_294	blank	Yen_1215	Yen_1451	Yen_1508	Ypse_1467	Ypse_1675	Ypse_1483
63 new	85	115	108	92	1	2	1	0	1	1	1	0	0	0	2	0	1	1	0	0	15	1	1	1	1	1	0	1	97	103	117	83	1	6	1	61	1	2	0
64 old	98	102	96	104	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	82	118	111	89	0	1	0	2	0	0	0
64 new	102	98	98	102	1	0	0	0	0	0	0	0	0	0	9	0	0	0	0	0	1	0	0	0	0	0	0	0	101	99	104	96	0	1	0	5	1	0	0
65 old	98	102	101	99	1	1	0	0	0	0	0	0	0	0	4	0	1	0	0	0	0	0	0	0	0	1	0	0	85	115	107	93	0	0	0	1	0	0	0
65 new	86	114	103	97	0	0	0	0	0	0	0	0	0	0	5	0	1	0	0	0	2	0	0	0	0	0	0	0	98	102	175	25	1	2	1	13	0	0	0
66 old	106	94	101	99	2	2	1	0	0	0	0	1	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	90	110	124	76	0	0	1	0	0	0	0
66 new	81	119	98	102	2	3	2	0	1	2	1	0	0	0	4	0	1	0	0	0	0	0	0	0	0	0	0	0	89	111	109	91	0	1	1	2	0	0	0
67 old	92	108	101	99	1	1	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	91	109	111	89	0	0	0	0	0	0	1
67 new	99	101	115	85	1	1	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	81	119	105	95	0	0	0	0	0	0	0
68 old	63	137	96	104	0	0	0	0	0	0	0	1	2	0	0	0	4	0	0	0	0	0	0	0	0	1	2	0	90	110	111	89	0	0	0	0	0	0	0
68 new	87	113	93	107	2	3	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	90	110	100	100	0	1	0	1	0	0	0
69 old	99	101	90	110	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	110	90	132	68	0	0	0	0	0	0	0
69 new	93	107	95	105	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	2	0	0	0	103	97	120	80	0	0	0	0	0	0	0
70 old	98	102	105	95	8	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	92	108	112	88	0	0	0	0	0	0	0
70 new	95	105	87	113	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	119	81	110	90	0	0	0	0	0	0	0
71 old	93	107	100	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	79	121	101	99	0	0	0	1	2	0	1
71 new	93	107	100	100	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	79	121	101	99	0	0	0	1	0	0	0
69 old	82	118	87	113	2	2	1	0	1	1	1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	101	99	114	86	0	1	0	2	0	0	0
69 new	85	115	90	110	2	2	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100	108	92	0	1	1	1	0	0	0
70 old	114	86	80	120	0	0	0	1	0	0	0	0	1	0	5	0	0	2	0	2	0	1	0	1	0	0	0	0	125	75	131	69	1	1	0	4	0	0	0
70 new	111	89	73	127	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	91	109	96	104	0	0	0	0	0	0	0
71 old	81	119	61	139	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	1	0	0	100	100	106	94	0	0	0	0	0	0	0
71 new	84	116	102	98	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	14	0	0	7	0	0	0	0	0	105	95	119	81	0	1	0	1	0	0	0
neg. control	95	105	101	99	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	121	79	101	99	0	0	0	0	0	0	0

	Msi_294	Msi_294	Msi_294	Msi_294	Eco_1402	Eco_1472	Eco_1521	blank	Salm_1457	Salm_1950	Salm_1620	blank	Lmono_2172	Lmono_2396	Lmono_2460	Lseel_1346	Lwelsh_1458	Linn_1882	blank	Cperf_2341	Cperf_1477	Cperf_1755	Cperf_1832	Cperf_1499	Cdiff_2224	Cdiff_2131	Cdiff_2318	blank	Msi_294	Msi_294	Msi_294	Msi_294	blank	Yen_1215	Yen_1451	Yen_1508	Ypse_1467	Ypse_1675	Ypse_1483
Y. enterocolitica 207 old	111	89	90	110	0	0	0	0	0	0	0	0	0	0	7	0	0	0	0	0	1	0	0	0	0	1	0	0	109	91	99	101	0	1	0	7	0	0	0
207 new	100	100	95	105	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	97	103	120	80	0	1	0	1	0	0	0
208 old	101	99	99	101	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	2	0	0	0	0	0	0	0	92	108	104	96	0	2	0	12	0	0	0
208 new	103	97	113	87	0	1	0	0	0	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	119	81	94	106	0	0	0	0	0	0	0
209 old	92	108	78	122	2	7	16	1	2	18	1	1	5	5	1	3	41	2	1	3	51	3	3	8	11	1	12	1	101	99	96	104	1	14	2	88	20	22	1
209 new	107	93	94	106	5	7	5	0	0	1	0	1	0	0	4	0	0	0	1	0	0	0	0	0	0	0	0	0	89	111	84	116	0	0	0	2	0	0	0
210 old	86	114	83	117	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100	100	100	0	0	0	0	0	0	0
210 new	106	94	94	106	1	2	1	0	0	0	1	1	0	0	3	0	0	0	0	0	2	1	0	0	0	0	0	0	106	94	92	108	0	2	1	11	0	0	0
211 old	99	101	86	114	1	2	2	1	1	3	3	1	1	2	2	1	5	2	1	0	21	2	1	2	1	1	1	0	94	106	94	106	1	8	1	56	3	5	2
211 new	97	103	100	100	0	0	0	1	1	1	0	0	0	0	3	0	0	1	1	0	4	0	0	0	0	0	0	1	99	101	106	94	0	1	0	15	0	0	0
212 old	93	107	85	115	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	2	1	0	0	0	0	0	0	85	115	85	115	0	1	0	8	0	0	0
212 new	95	105	87	113	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	60	140	99	101	0	1	0	1	0	0	0
213 old	106	94	108	92	1	1	1	1	1	2	1	2	1	5	13	4	13	3	2	1	22	2	3	5	2	2	2	2	97	103	117	83	2	21	3	70	2	2	2
213 new	106	94	99	101	0	0	0	0	0	0	1	0	0	0	7	0	0	0	0	0	1	0	0	0	0	0	0	0	100	100	91	109	0	1	0	4	0	0	0
214 old	94	106	101	99	0	1	0	0	0	0	0	0	0	0	8	0	3	0	0	0	0	0	0	0	0	0	0	0	100	100	108	92	0	1	0	2	0	0	0
214 new	100	100	89	111	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	85	115	91	109	0	64	76	76	35	13	0
215 old	99	101	104	96	0	1	0	0	0	1	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	103	97	112	88	0	56	60	66	30	14	0
215 new	80	120	76	124	0	1	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	82	118	95	105	1	72	67	65	15	7	0
neg. control	88	112	99	101	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	97	103	93	107	0	0	0	0	0	0	0
140 old	99	101	110	90	84	85	111	0	0	1	3	1	0	0	0	0	1	0	0	0	0	0	0	0	0	30	0	0	94	106	101	99	1	1	0	2	0	0	0
140 new	102	98	97	103	87	87	110	0	0	1	3	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	2	0	92	108	83	117	0	1	0	1	0	0	0
141 old	86	114	98	102	76	74	83	0	0	1	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	95	105	96	104	0	0	0	0	0	0	0
141 new	83	117	96	104	85	65	73	0	0	0	1	1	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	97	103	115	85	0	1	0	0	0	0	0
142 old	90	110	98	102	73	75	100	0	0	1	18	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	99	101	99	101	0	7	0	1	0	0	0

	Msi_294	Msi_294	Msi_294	Msi_294	Eco_1402	Eco_1472	Eco_1521	blank	Salm_1457	Salm_1950	Salm_1620	blank	Lmono_2172	Lmono_2396	Lmono_2460	Lseel_1346	Lwelsh_1458	Linn_1882	blank	Cperf_2341	Cperf_1477	Cperf_1755	Cperf_1832	Cperf_1499	Cdiff_2224	Cdiff_2131	Cdiff_2318	blank	Msi_294	Msi_294	Msi_294	Msi_294	blank	Yen_1215	Yen_1451	Yen_1508	Ypse_1467	Ypse_1675	Ypse_1483
142 new	74	126	93	107	65	101	84	0	0	1	3	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	94	106	99	101	0	0	0	0	0	0	0
143 old	95	105	91	109	95	67	108	0	88	77	78	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	104	96	104	96	0	1	0	0	0	0	0
143 new	99	101	92	108	118	92	118	0	86	68	58	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	102	98	105	95	0	1	0	1	0	0	0
144 old	83	117	88	112	77	76	99	0	81	65	57	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	90	110	102	98	0	0	0	1	0	0	0
144 new	92	108	88	112	88	65	113	0	85	69	60	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	101	99	120	80	0	2	0	1	0	0	0
145 old	120	80	95	105	96	87	121	0	89	71	71	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	94	106	106	94	0	1	0	0	0	0	0
145 new	91	109	81	119	72	74	94	0	72	85	66	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	92	108	108	92	0	1	0	1	0	0	0
146 old	106	94	108	92	112	78	116	1	98	89	88	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	104	96	131	69	0	2	0	1	0	0	0
146 new	98	102	95	105	85	75	107	0	92	91	77	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	97	103	109	91	0	1	0	1	0	0	0
147 old	82	118	79	121	64	68	85	0	69	68	51	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	87	113	120	80	0	0	0	2	0	0	0
147 new	90	110	100	100	107	84	135	0	96	87	101	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	105	95	123	77	0	2	0	6	0	0	0
69 old	82	118	87	113	2	2	1	0	1	1	1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	101	99	114	86	0	1	0	2	0	0	0
69 new	85	115	90	110	2	2	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100	108	92	0	1	1	1	0	0	0
70 old	114	86	80	120	0	0	0	1	0	0	0	0	1	0	5	0	0	2	0	2	0	1	0	1	0	0	0	0	125	75	131	69	1	1	0	4	0	0	0
70 new	111	89	73	127	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	91	109	96	104	0	0	0	0	0	0	0
71 old	81	119	61	139	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	100	100	106	94	0	0	0	0	0	0	0
71 new	84	116	102	98	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	14	0	0	7	0	0	0	0	0	105	95	119	81	0	1	0	1	0	0	0
neg. control	95	105	101	99	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	121	79	101	99	0	0	0	0	0	0	0