Electronic Supplementary Material (ESI) for Analytical Methods

Electronic Supplementary Informations - ESI

Real-time PCR based detection of a panel of food borne pathogens on a centrifugal microfluidic LabDisk with on-disk quality controls and standards for quantification

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The supporting material includes:

(1) Photograph of the LabDisk

(2) Primer and probe sequences

(3) Prestorage scheme of primers and hydrolysis probes for different operation modes (qualitative and quantitative)

(4) Concept for integrated sample-to-answer nucleic acid analysis

(5) Coating and laser cutting of the LabDisk

(6) Pathogen count per 10 pg, 1 pg and 0.1 pg genomic pathogen DNA.

(7) PCR results for sample vs. positive control for qualitative mode

(8) Intra-disk variation of PCR performance

1) Photograph of the LabDisk

Figure S1 depicts a microthermoformed LabDisk for real-time PCR based food pathogen detection, fabricated from thin polymer foils. Each LabDisk accommodates two independent microfluidic structures. Each structure features 2 x 7 amplification chambers that can be used for detection of pathogens, for positive or negative controls or for co-amplification of integrated DNA standards.

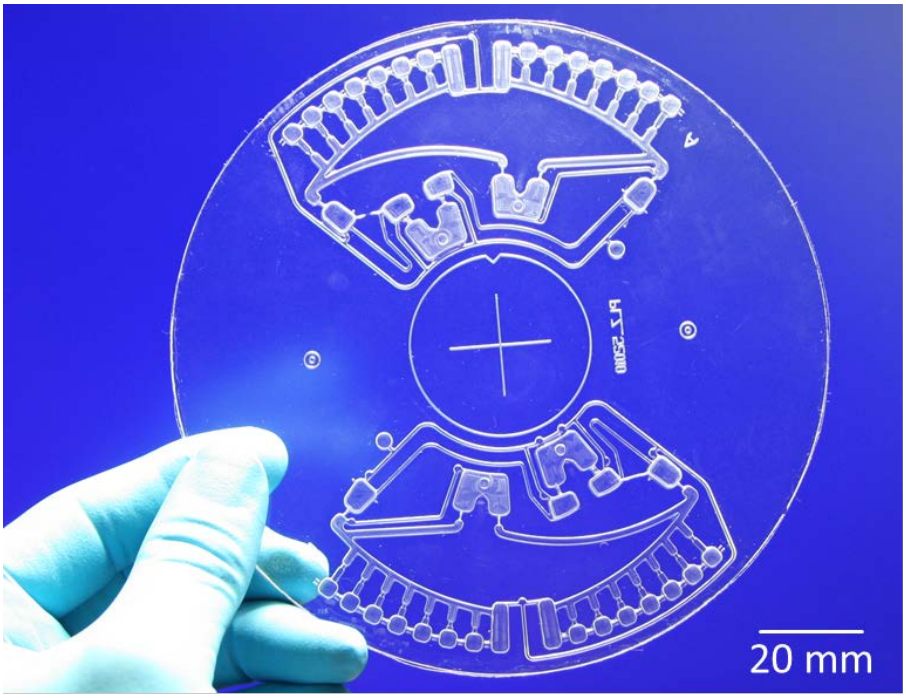

Fig. S1: Photograph of a LabDisk featuring two independent fluidic structures with 2 x 7 separated amplification chambers per microfluidic structure. The LabDisk can be operated in two different modes. A qualitative mode for processing two samples and detection of 6 food borne pathogens per sample and a quantitative mode for detection and quantification of two food borne pathogens from one sample.

2) Primer and Probe Sequences

Table S1: Sequences of the primers and probes. Sequences of *Listeria monocytogenes*, *Citrobacter freundii* and *Staphylococcus aureus* are confidential but can be purchased from Eurofins GeneScan.

Pathogen / #Accession	Type	Nucleotide Sequence 5' --> 3'	T_M [°C]	Source
Listeria monocytogenes FM242711.1		Cat. No. 5423300201	60.3 64.5 66.1	Eurofins GeneScan
Salmonella typhimurium AE006468.1	F_Pr	GTGAAATTATCGCCACGTTCGGGC	73.6	based
	R_Pr	CTTCATCGCACCGTCAAAGGAACC	72.6	on
	Probe	6FAM-TACCGGGCATACCATCCAGAGAA-BBQ	69.1	[37]
Campylobacter ssp. HM007568	F_Pr	CTGCTTAACACAAGTTGAGTAGG	59.3	[38]
	R_Pr	TTCCTTAGGTACCGTCAGAA	58.5	
	Probe	6FAM-TGTCATCCTCCACGCGGCGTTGCTGC-BBQ	82.9	
Citrobacter freundii AJ227962.1		Cat. No. 5423300301	78.4 70.9 77.7	Eurofins GeneScan
Staphylococcus aureus U73374.1		Cat. No. 5423300101	65.9 61.1 69	Eurofins GeneScan
enterohemorrhagic Escherichia coli AB015056.1	F_Pr	GACTGCAAAGACGTATGTAGATTCG	64.3	[39]
	R_Pr	ATCTATCCCTCTGACATCAACTGC	64	
	Probe	6FAM-TGAATGTCATTCGCTCTGCAATAGGTACTC-BBQ	70.7	

Melting Temperature calculated with: http://www.thermoscientificbio.com/webtools/tmc/; F_Pr: Forward Primer; R_Pr: Reverse Primer;

37] K. Rahn, S. de Grandis, R. Clarke, S. McEwen, J. Galán, C. Ginocchio, R. Curtiss and C. Gyles, *Molecular and Cellular Probes*, 1992, **6**, 271–279

[38] M. H. Josefsen, N. R. Jacobsen and J. Hoorfar, *Applied and Environmental Microbiology*, 2004, **70**, 3588–3592

[39] A. M. Ibekwe, P. M. Watt, C. M. Grieve, V. K. Sharma and S. R. Lyons, *Applied and Environmental Microbiology*, 2002, **68**, 4853–4862.

3) Prestorage scheme of primers and probes for different operation modes

Schematic layout of the LabDisk (**Fig. S2**) with two separate microfluidic structures (A and B) for real-time PCR based detection of food pathogens. Each microfluidic structure features a DNA inlet to add DNA to the intermediate chambers and amplification chambers A1 – A14 and B1 – B14. The corresponding prestorage scheme for primers, hydrolysis probes and genomic pathogen DNA, depending on the mode of operation, is depicted in **Table S2**

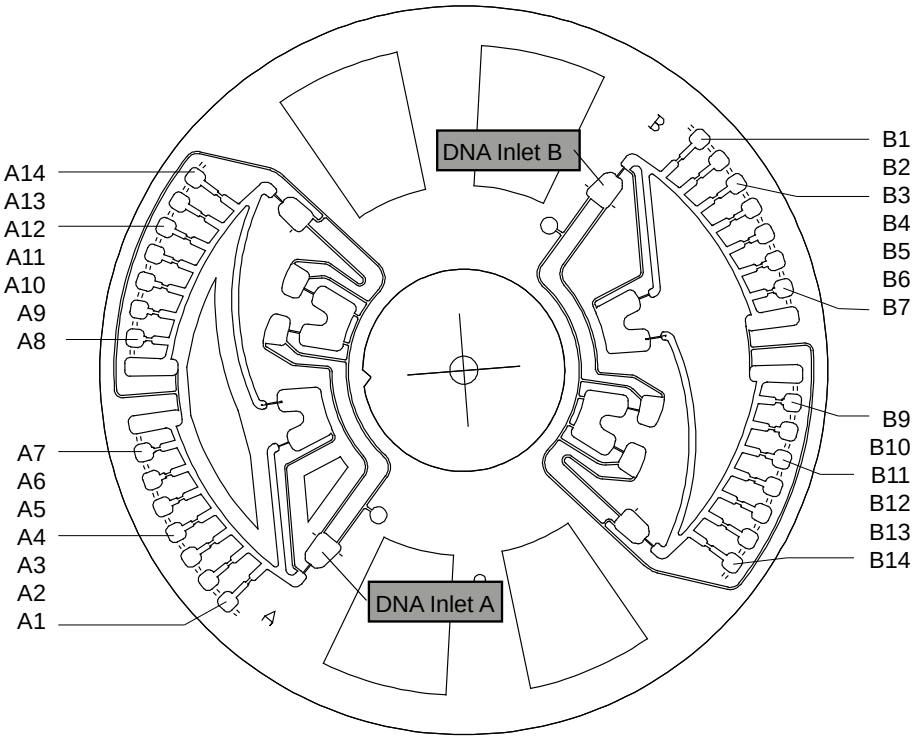

Fig S2: 2D CAD drawing of LabDisk with two independent microfluidic structures "A" and "B". Each structure features 2 x 7 amplification chambers A1 – A 14 and B1 – 14. DNA can be added to the structure via intermediate chambers that will serve as elution chambers in the future.

Table S2: Reagent prestorage scheme for all amplification chambers A1 – A14 and B1 – B14 depending on the mode of operation (qualitative or quantitative). Det (detection): Primers and hydrolysis probes for singleplex detection of one pathogen per amplification chamber are prestored in each of the amplification chambers. PC (positive control): Primers and hydrolysis probes and DNA for amplification of one pathogen are prestored. NTC (No-template control): Primers and hydrolysis probes for all six pathogens are prestored in each reaction chamber to enable unspecific detection of contaminations; STD 1, STD 2 and STD 3 = Standards for quantification; Primers and hydrolysis probes and DNA for amplification of one pathogen are prestored (STD 1 = 1 ng DNA, STD 2 = 100 pg DNA, STD 3 = 10 pg DNA).

	1) Qualitative operation		**2) Quantitative operation**	
	Purpose	Pathogen	Purpose	Pathogen
A1	Det	*C. jejuni*	Det	*L. monocytogenes*
A2	Det	*S. typhimurium*	Det	*L. monocytogenes*
A3	Det	*L. monocytogenes*	Det	*L. monocytogenes*
A4	Det	EHEC	Det	*S. typhimurium*
A5	Det	*C. freundii*	Det	*S. typhimurium*
A6	Det	*S. aureus*	Det	*S. typhimurium*
A7	empty		empty	
A8	PC	*S. aureus*	empty	
A9	PC	*C. freundii*	STD 1	*S. typhimurium*
A10	PC	EHEC	STD 2	*S. typhimurium*
A11	PC	*L. monocytogenes*	STD 3	*S. typhimurium*
A12	PC	*S. typhimurium*	STD 1	*L. monocytogenes*
A13	PC	*C. jejuni*	STD 2	*L. monocytogenes*
A14	NTC	All pathogens	STD 3	*L. monocytogenes*
B1	Det	*C. jejuni*	STD 3	*L. monocytogenes*
B2	Det	*S. typhimurium*	STD 1	*L. monocytogenes*
B3	Det	*L. monocytogenes*	STD 2	*L. monocytogenes*
B4	Det	EHEC	STD 3	*S. typhimurium*
B5	Det	*C. freundii*	STD 1	*S. typhimurium*
B6	Det	*S. aureus*	STD 2	*S. typhimurium*
B7	empty		empty	
B8	PC	*S. aureus*	empty	
B9	PC	*C. freundii*	STD 1	*S. typhimurium*
B10	PC	EHEC	STD 3	*S. typhimurium*
B11	PC	*L. monocytogenes*	STD 2	*S. typhimurium*
B12	PC	*S. typhimurium*	STD 1	*L. monocytogenes*
B13	PC	*C. jejuni*	STD 3	*L. monocytogenes*
B14	NTC	All pathogens	STD 2	*L. monocytogenes*

C. jejuni = Campylobacteri jejuni, S. typhimurium = Salmonella typhimurium, L. monocytogenes = Listeria monocytogenes, EHEC = enterohemorrhagic *Escherichia coli*, *C. freundii = Citrobacter freundii*, *S. aureus = Staphylococcus aureus*

4) Concept for integrated sample-to-answer nucleic acid analysis

The microfluidic structure for real-time PCR based detection of food pathogens presented herein can be concatenated to an upstream unit operation for DNA extraction that was recently published by Strohmeier et al., *Lab Chip*, **2013**, 13, 146–155. Here, the nucleic acids are extracted with silica coated magnetic beads. After integration of both structures (the nucleic acid extraction and the real-time PCR based amplification) into one LabDisk, elution of the nucleic acids from the surface of the silica coated magnetic beads will be conducted in the intermediate chamber. Afterwards, the eluted DNA can be forwarded to the amplification chambers for real-time PCR where pathogen specific primers and probes are prestored as presented herein (**Fig. S3**).

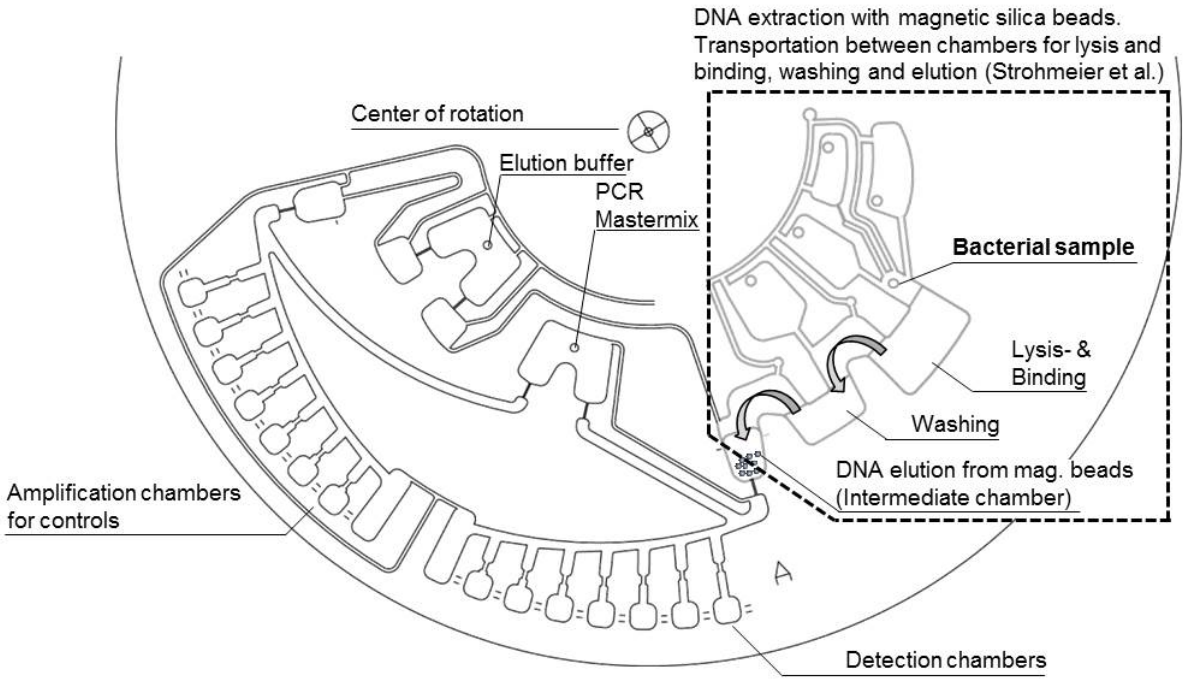

Fig. S3: 2D CAD depiction of a proposed centrifugal microfluidic cartridge for nucleic acid based sample-to-answer analysis of pathogens. A centrifugal microfluidic unit operation for nucleic acid extraction with silica coated magnetic beads in light grey (Strohmeier et al, *Lab Chip*, **2013,** 13, 146 - 155) is concatenated to the herein presented centrifugal microfluidic structure for real-time PCR based pathogen detection by geometric multiplexing including PC, NTC and STD. The elution chamber of the unit operation for DNA extraction is represented by the intermediate chamber in the presented work, linking both microfluidic structures.

5) Coating and lasercutting of the LabDisk

To enhance capillary priming, hydrophilic Vistex II coating was applied and baked at 120 °C. To increase breakthrough frequencies of geometric constrictions, hydrophobic Teflon coatings were applied. To avoid capillary priming of ventilation channels, hydrophobic coatings with Teflon carbon black were applied (**Fig. S4**).

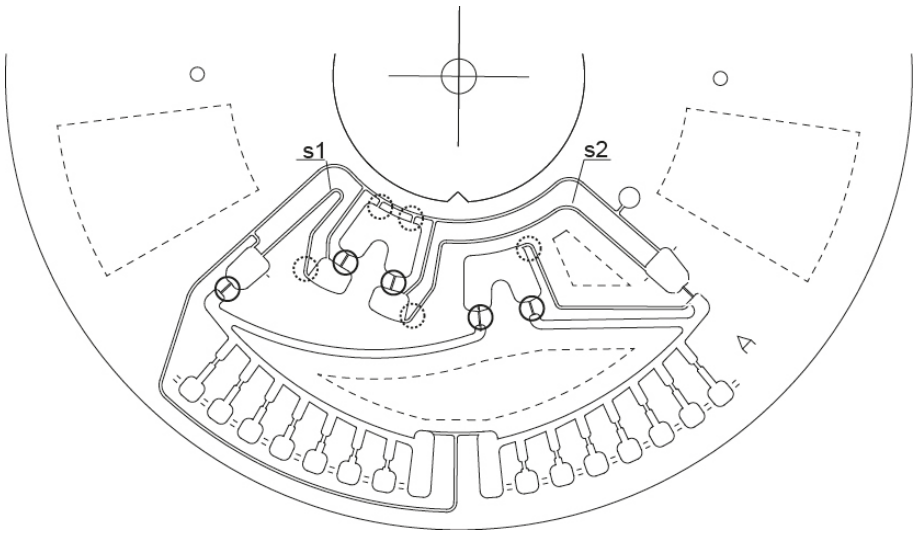

Fig. S4: 2D CAD of one half LabDisk. Dashed areas are cut out by laser cutting to improve air flow through the disk which is required for rapid air mediated thermocycling. Capillary siphons s1 and s2 are coated with Vistex II. Hydrophobic constrictions (solid circles) are coated with Teflon to increase fluidic breakthrough frequency, venting channels are coated with Teflon carbon black (dashed circles) to avoid undesired capillary priming.

6) Pathogen count

Table S3: Pathogen count per 10 pg, 1 pg and 0.1 pg of genomic pathogen DNA

Pathogen	Genomesize	Pathogen count			Source
	[base pairs]	per 10 pg	per 1 pg	per 0.1 pg	
Campylobacter jejuni	1.64×10^6	5650	565	56.5	[1]
Citrobacter freundii	5.11×10^6	1810	181	18.1	[2]
EHEC	5.44×10^6	1700	170	17.0	[1]
Listeria monocytogenes	2.94×10^6	3150	315	31.5	[1]
Salmonella typhimurium	4.90×10^6	1890	189	18.9	[1]
Staphylococcus aureus	2.82×10^6	3290	329	32.9	[3]

Pathogen count was calculated with the webtool “DNA / RNA copy number calculator”: http://www.endmemo.com/bio/dnacopynum.php

[1] http://home.comcast.net/~john.kimball1/BiologyPages/G/GenomeSizes.html; Accessed 22.06.2013; [2] N. K. Pretty et al.,"The Citrobacter rodentium genome sequence reveals convergent evolution with human pathogenic Escherichia coli", *J. Bacteriol*., **2010**, 192:525-538; [3] M.T.G. Holden et al.,"Complete genomes of tow clinical Staphylococcus aureus strains evidence for the rapid evolution of virulence and drug resistance", *PNAS*, **2004**, 101:9786-9791

7) PCR results for sample vs. positive control for qualitative mode

For all experiments in the qualitative operation mode, DNA concentration in positive control (PC) was chosen to be equal to the expected DNA concentration of the sample in the amplification chamber (i.e. for samples with 10 pg per amplification chamber, 10 pg DNA were dryly prestored for PC). The C_q values for 10 pg, 1 pg and 0.1 pg (two experiments A and B) are depicted in **Table S4**. Deviations of C_q values between PC and Sample might be a result of dry prestorage of the DNA into the amplification chambers for PC. Secondly, the sample DNA might be unevenly distributed between the amplification chambers for detection due to insufficient mixing in the intermediate chamber. On the developed LabDisk, all PCR amplifications are conducted with the same thermocycling protocol. Therefore, the herein used PCR assays might require further primer and probe sequence optimization for working at identical thermocycling protocols.

Table S4: Quantification cycles (C_q) for sample amplification and PC for *Campylobacter jejuni.*, *Citrobacter freundii*, enterohemorrhagic *Escherichia coli* (EHEC), *Listeria monocytogenes*, *Salmonella typhimurium* and *Staphylococcus aureus* and genomic pathogen DNA concentrations of 10 pg, 1 pg and 0.1 pg of DNA per amplification cavity.

	10 pg		**1 pg**		**0.1 pg Exp A**		**0.1 pg Exp B**	
	PC [Cq]	**Sample [Cq]**	**PC [Cq]**	**Sample [Cq]**	**PC [Cq]**	**Sample [Cq]**	**PC [Cq]**	**Sample [Cq]**
Campylobacter spp.	24,5	42,8	28,7	26,7	31,5	30,8	31,9	36,30
Citrobacter jejunii	30,8	34,1	33,3	33,9	37,4	37,0	40,3	44,5
EHEC	27,9	30,0	30,9	30,8	34,7	35,3	37,1	38,3
Listeria monocytogenes	27,9	26,8	31,4	30,7	34,8	34,3	37,3	38,6
Salmonella typhimurium	27,4	25,9	30,7	31,0	34,8	35,5	fail	37,1
Staphylococcus aureus	25,7	25,9	31,5	28,9	32,7	33,1	35,4	36,8

8) Intra-disk variation of PCR performance

For all experiments in the quantitative operation mode, three amplification chambers per disk were filled with 10 pg DNA, three with 100 pg DNA and three with 1 ng DNA of *Listeria monocyctogenes* or *Salmonella typhimurium* (Table S1; Prestorage scheme for PCR standard STD 1, STD 2 and STD 3). Additionally, *L. monocytogenes* and *S. typhimurium* DNA samples with expected DNA concentrations of 50 pg and 500 pg were amplified in three different amplification chambers per disk after aliquoting. Intra-disk variation of PCR performance was calculated from the resulting C_q values as depicted in **Table S5**. Intra-disk standard deviation of PCR performance between three different amplification chambers was 0.2 to 0.8 cycles for amplification chambers with prestored DNA what might be attributed to the dry prestorage as explained before in ESI chapter 6. Standard deviation of C_q values for PCR amplifications with aliquoted DNA was 0.1 to 0.5.

Table S5: C_q values of PCRs from experiments in quantitative mode. Each PCR was conducted in triplicates per DNA concentration, pathogen and disk. Therefrom, mean values, standard deviation and the overall R^2 value of the PCR standard curve were calculated.

	***Listeria monocytogenes* [C_q]**				***Salmonella typhimurium* [C_q]**			
	10 pg[1]	100 pg[1]	1 ng[1]		10 pg[1]	100 pg[1]	1 ng[1]	
Disk 1	30,0	25,6	23,3		27,3	23,6	20,3	
	29,9	26,2	23,0		27,6	24,1	20,8	
	29,3	25,9	22,6		27,3	23,7	20,3	
mean	**29,7**	**25,9**	**22,9**		**27,4**	**23,8**	**20,5**	
std. dev	**0,4**	**0,3**	**0,4**		**0,2**	**0,2**	**0,3**	
R^2		**0.98492**				**0.99453**		
				500 pg [2]				50 pg [2]
Disk 2	30,2	27,3	22,9	23,4	28,1	24,7	20,5	25,0
	29,8	26,9	22,4	24,1	27,1	23,6	20,0	25,0
	29,7	25,9	22,4	23,7	27,5	23,5	19,9	25,2
mean	**29,9**	**26,7**	**22,6**	**23,7**	**27,5**	**24,0**	**20,1**	**25,1**
std. dev	**0,3**	**0,7**	**0,3**	**0,4**	**0,5**	**0,7**	**0,3**	**0,1**
R^2		**0.97923**				**0.98192**		
				50 pg [2]				500 pg [2]
Disk 3	30,5	27,4	23,2	26,8	28,3	25,1	21,1	21,5
	29,5	26,2	22,3	26,4	27,9	24,1	20,8	22,1
	29,4	25,9	22,1	26,7	27,6	no PCR	20,5	21,1
mean	**29,8**	**26,5**	**22,6**	**26,6**	**27,9**	**24,6**	**20,8**	**21,5**
std. dev	**0,6**	**0,8**	**0,6**	**0,3**	**0,4**	**0,7**	**0,3**	**0,5**
R^2		**0.96536**				**0.98667**		

[1] DNA prestored in amplification chamber with primers and probes as depicted in **Table S2** for STD 1, STD 2 and STD3
[2] DNA fluidically aliquoted into amplification chambers