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Liquid array diagnostics: a novel method for rapid detection of microbial communities in single-tube multiplex reactions

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ABSTRACT

We present a novel liquid array diagnostics (LAD) method, which enables rapid and inexpensive detection of microbial markers in a single-tube multiplex reaction. We evaluated LAD both on pure cultures, and on infant gut microbiota for a 15-plex reaction. LAD showed more than 80% accuracy of classification and a detection limit lower than 2% of the Illumina reads per sample. The results on the clinical dataset showed that there was a rapid decrease of staphylococci from 10-day- to 4-month-old children, a peak of bifidobacteria at 4 months, and a peak of *Bacteroides* in 2-year-old children, which is in accordance with findings described in the literature. Being able to detect up to 50 biomarkers, LAD is a suitable method for assays where high throughput is essential.

METHOD SUMMARY

Liquid array diagnostics use short DNA duplexes, where one of the oligonucleotides is labeled with a fluorophore and the other, upon the presence of target DNA, becomes labeled with a quencher molecule. The novelty of this method lies in the combination of many duplex melting profiles and several channels of detection on a qPCR instrument, to detect multiple events of fluorescence quenching in a single-tube multiplex reaction.

KEYWORDS:

fluorescence • liquid array • microbiota • qPCR instrument • quenching

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The field of gut microbiota analysis has, until now, been dominated by relatively small-scale explorative studies, with several contradicting findings obscuring the truth in literature [1,2]. We are therefore at a stage where high-throughput, low-cost, targeted approaches are needed in order to generalize knowledge, and to evaluate previous findings. Presently, the GA-map® platform (Genetic Analysis AS) is the only clinically validated tool designated for gut microbiota diagnostics. The GA-map method allows for the faster assessment of the abundance of microbial markers in a sample, compared with NGS techniques [1]. However, it is based on solid-phase hybridization, which creates a bottleneck in sample processing and renders the test relatively expensive.

In this article, we present liquid array diagnostics (LAD), a novel approach for detecting bacterial communities using real-time PCR instrumentation. LAD combines single nucleotide primer extension with high-resolution melting (HRM) in the concept of a liquid array. It does not require physical separation of the probes prior to detection, thus avoiding a bottleneck in sample processing and ensuring rapid results at very low running costs. Requiring only a qPCR instrument, it has great potential for use as a routine tool for diagnostics by reporting multiple gut microbial markers in a single-tube multiplex reaction within a working day. A schematic outline of LAD is provided in Figure 1.

We evaluated LAD both on pure cultures, and on infant gut microbiota. The rationale for investigating the infant gut microbiota is that their composition and development are well described by many studies [2–4], and that we can utilize an already designed and validated GA-map probe set [5]. Furthermore, the development of the gut microbiota during infancy is crucial for health later in life. However, large-scale validation studies are required before knowledge about the gut microbiota can be utilized in clinical practice.

We present results demonstrating the sensitivity and specificity of LAD, in addition to exemplifying its utility on a medium-scale clinical cohort.

Taken together, LAD is a promising method, filling the need for large-scale gut microbiota validation tools.

MATERIALS & METHODS

Template generation for labeling probes labeling

We used genomic DNA extracted from 18 different bacterial isolates for PCR amplification. These strains represented targets for one or more labeling probes (LP), thus the purpose was to use them for validation of specificity and reproducibility of our assay. The chosen bacteria were: *Gemella sanguinis, Escherichia coli, Salmonella bongori, Salmonella enterica, Salmonella typhimurium, Klebsiella pneumoniae* subsp. *Pneumonae, Streptococcus pyogenes, Streptococcus pneumoniae, Salmonella enterica* subsp. *Enterica, Bacteroides vulgatus, Bacteroides fragilis, Bacteroides dorei, Staphylococcus aureus* subsp. *Aureus, Staphylococcus aureus, Bifidobacterium breve, Bifidobacterium longum, Enterococcus faecalis* and *Streptococcus sanguinis*.

In addition, DNA extracted from 541 PACT (Prevention of Allergy Among Children in Trondheim) study stool samples was utilized in PCRs to generate the templates for LP labeling. These samples were collected from pregnant mothers and their children at up to several post-birth ages. Their ▶

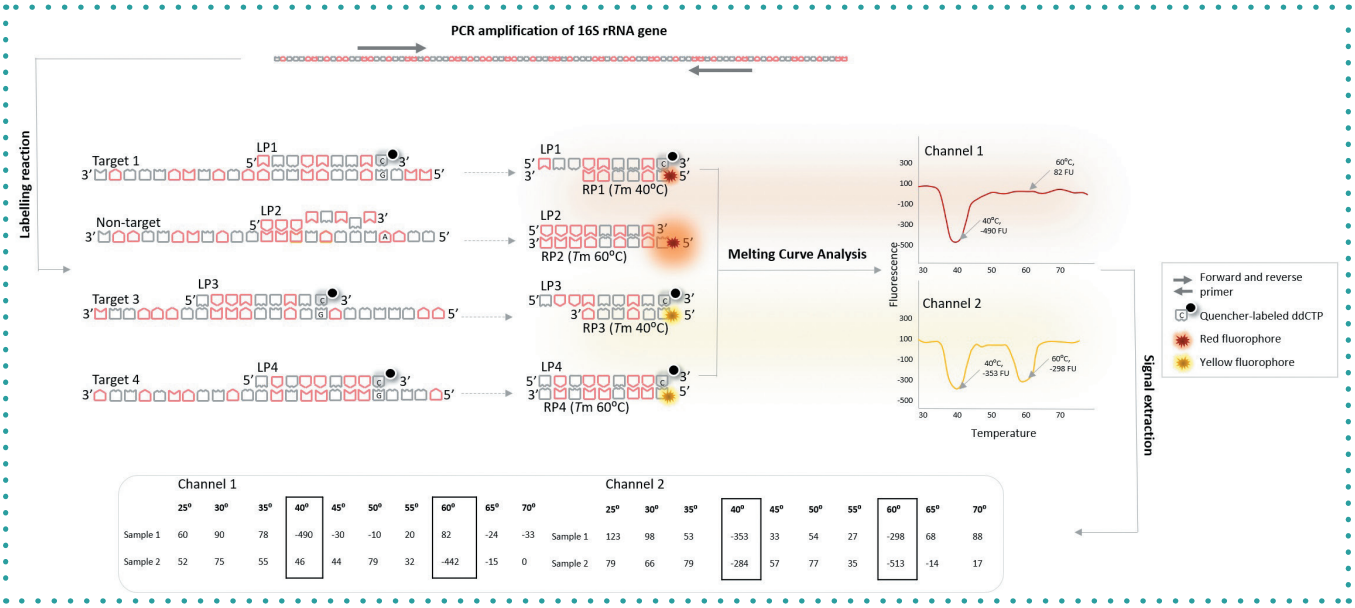

Figure 1. An overview of liquid array diagnostics (LAD) method. The initial step includes PCR amplification of 16S rRNA gene, where each LP is targeted. If the target DNA is present, LPs become labeled with a ddCTP conjugated with a quencher molecule. Subsequently, fluorophore-labeled RPs complementary to LPs are added into the solution mix. Upon duplex formation, at a specific melting temperature, the fluorescence of the reporter decreases abruptly. Multiple targets can be detected in a single-tube reaction by combining different duplex melting temperatures and fluorophore colors. In the last step, the derivative fluorescence units (FU) are extracted from each temperature where signals are expected for further data processing.
LP: Labeling probe; RP: Reporter probe.

▶ distribution was as following: 110 were collected from pregnant mothers, 122 from children up to 10 days old, 126 samples from 4-month-old children, 89 samples from 1-year-old children, and 94 from 2-year-old children. We used gDNA that was already extracted. The extraction protocol can be found in the Materials and Methods section of Vebø et *al.* (2011) [5].

A total reaction volume of 25 µl comprising 1 µl bacterial lysate as a source of DNA template, 0.05 U HOT FIREPol® DNA Polymerase, 1X B1 buffer, 2.5 mM $MgCl_2$ (all from Solis Biodyne, Estonia), 0.2 mM dNTPs (Thermo Fisher Scientific, MA, USA), 0.2 µM sense primer (Mangala F-1, 5′-TCCTACGGGAG-GCAGCAG-3′), and 0.2 µM antisense primer (16SUR, 5′-3′ CGGTTACCTTGTTACGACTT) was designed to amplify a segment of 16S rRNA gene. PCR amplification was initiated with a period of 15 min at 95°C to activate the DNA polymerase, followed by 30 cycles, each consisting of 30 s denaturation at 95°C, 30 s annealing at 55°C and an 80 s elongation at 72°C performed using an Applied Biosystems Veriti™ Thermal Cycler (Life Technologies, CA, USA). A final elongation step of 7 min at 72°C was also included. The amplified products were treated with 2.4 U of Exonuclease I (ExoI, Biolabs Inc., MA, USA) and 6.4 U of shrimp alkaline phosphatase (USB Corporation, OH, USA) prior to incubation at 37°C for 120 min, and at 80°C for 15 min.

Single nucleotide extension of the LPs

A total reaction volume of 15 µl comprising 5 µl Exo-SAP-treated template (or water as 'no template' control), LPs at a final concentration of 0.1 µM, 0.8 µM ddCTP-ATTO612Q (Jena Biosciences, Germany), 20 µM ddTTP, 1 mM $MgCl_2$, 1XC buffer and 0.25 U HOT TERMIPOL® DNA Polymerase (all from Solis Biodyne, Estonia) was prepared. Labeling reactions were performed using an Applied Biosystems Veriti™ Thermal Cycler, employing an activation step at 95°C for 12 min, followed by 40 cycles, each consisting of 96°C denaturation for 20 s and 60°C annealing/elongation for 40 s.

Melting curve analysis

5′ fluorescently labeled reporter probe(s) (RP) were added to the LP labeling reactions at a final concentration of 0.005 µM each, with the exception of RPs 1_1 RP ROX, 1_2_2 RP ROX, 6_2_2 RP HEX, 6_1_4 RP HEX and 2_4_1 RP FAM, which had a final concentration of 0.02 µM each; reagent S, available from INN (Inland Norway University of Applied Sciences, Norway), was also added to a final concentration of 0.1%. The melting curve analysis was performed using a 7500 Fast qPCR instrument (Applied Biosystems, USA) with the following dissociation steps: 95°C for 15 s, 30°C for 1 min, 95°C for 15 s and 60°C for 15 s. Fluorescence was detected and expressed in dissociation curves as the derivative of the fluorescence versus temperature measurements (dF/dT) versus temperature (Temp.). Positive signals were observed as negative peaks, representing the abrupt, temperature-dependent drop of fluorescence.

Extraction of peaks & determination of positive signals for clinical samples

For the sake of simplicity, all data were multiplied by −1 since originally, positive LAD signals have negative values.

Fluorescence values were extracted from temperature measurements where quenching signals were expected (e.g., the fluorescence value at 67.7°C on HEX channel, where UNI probe was designed to quench). In addition, such values were extracted from 5 no template controls (NTC), with the aim of determining the borderline

Table 1. Probes designed by Genetic Analysis AS for GA-map® array, used as labeling probes by liquid array diagnostics.

Probe identifier	Taxonomic group(s) detected	Probe sequence (5′–3′)
1_1	*Bacteroides*	TTGCGGCTCAACCGTAAAATTG
1_2_2	*Bacteroides* (*dorei, fragilis, thetaiotaomicron, vulgatus*)	GCACTCAAGACATCCAGTATCAACTG
2_1_min1b	Gamma-proteobacteria	CAGGTGTAGCGGTGAAATGCGTAGAGAT
2_3_2	Gamma-proteobacteria subgroup	CGGGGATTTCACATCTGA
2_4_1	Gamma-proteobacteria subgroup	TGCCAGTTTCGAATGCAGTT
4_1	Firmicutes (Lactabacillales, *Clostridium perfringens, Staphylococcus*)	CGATCCGAAAACCTTCTTCACT
4_4_2	*Enterococcus, Listeria*	TCCAATGACCCTCCC
4_5_2	*Streptococcus pyogenes*	GATTTTCCACTCCCACCAT
4_6_1	*Streptococcus sanguinis*	CACTCTCACACCCGTT
4_8_1	*Streptococcus pneumoniae, Entrococcus*	CGCGGCGTTGCTCGGTCAGACTT
5_1	Firmucutes (Clostridia, Bacillales, *Enterococcus, Lactobacillus*)	GGACAACGCTTGCCAC
5_1_2	*Staphylococcus*	CGTGGCTTTCTGATTAGGTA
6_1_4	*Bifidobacterium longum*	TGCTTATTCAACGGGTAAACT
6_2	Actinobacteria	CGTAGGCGGTTCGTCGCGT
6_2_2	*Bifidobacterium breve*	CGGTGCTTATTCGAAAGGTACACT
UNI01	16S Universal	CGTATTACCGCGGCTGCTGGCA

separating positive signals from background fluorescence. First, we calculated the distance of the observed positive signals from the mean background fluorescence using a standard Z-score. Following that, the margin separating the signals from the background was assigned to be the mean value of NTC plus two-times its standard deviation ($\mu+2\Sigma$).

However, a different approach was used to assign positive signals for 5_1_2. Considering that there is a tight melting temperature (*T*m) range separating 5_1 from 5_1_2 signals, using the above-mentioned formula would report false-positive signals for *Staphylococcus* (5_1_2 probe) since the fluorescence measurements at 55.8°C, where 5_1_2 is designed to quench, are interferingly high for each sample where 5_1 is truly quenched (50.8°C). Thus, fluorescence values at 55.8°C were extracted from eight random samples where only 5_1 was observed to give signal. The mean value of these samples was added with three standard deviations ($\mu+3\Sigma$), which was used as a margin to separate the bona fide *Staphylococcus* signals. All data values higher than the margins were accepted as positives.

Probe design

The probes, designed by Genetic Analysis AS [5], were used as LPs (Table 1), whereas the RPs were designed to be complementary to the LPs, so that they create duplexes that dissociate at a chosen temperature. Each probe has a code identifier (for example 1_1 for *Bacteroides*), originally used in Vebø *et al.* (2011) [5]. The *T*m of the probes was calculated by the Oligoanalyzer 3.1 web-based bioinformatics tool (Integrated DNA Technologies) and target *T*ms were achieved by varying the length of the RPs.

The reporter probes were designed to anneal to the 3′-end of each respective labeling probe, thus placing the fluorophore, coupled to the terminal 5′nucleotide of the RP, in close physical proximity to the quencher molecule located at the 3′ end of the labeled LP. The list of the reporter probes is presented in Table 2.

Comparison of LAD-based results with Illumina sequencing data

87 random PACT samples (34 samples of children up to 10-days old, 15 of 4-month-olds, 15 of 1-year-olds, 12 of 2-year-olds and 11 of pregnant women) were picked to be sequenced with an Illumina MiSeq System (Illumina, CA, USA). The purpose of this step was to confirm the identities of samples and compare them with the results obtained with the LAD assay, by performing *in silico* labeling of the reads. *In silico* labeling was performed by textual mapping of the 'labeled' LPs to the operational taxonomic unit (OTU) DNA sequences retrieved by Illumina, using the Sequence Manipulation Suite: Primer Map tool [6]. All OTUs that were detected by the same probe were grouped together and their number of reads was summed up for each sample. The total number of such reads was then compared with the LAD signal intensity for the said probe. Prior to doing so, LAD data were normalized so that any number below the cut-off value would be equal to zero.

To calculate the specificity and sensitivity, we performed a receiver operator characteristic (ROC) curve analysis (MedCalc Software, Ostend, Belgium), which plots the true positive signals (as determined with LAD) against the false positives for different cut-off points (the number of Illumina reads). This helped find the optimum copy number of target sequences that can be detected using our method. ▸

Table 2. Reporter probe sequences.

Reporter probe	5′–3′ sequence
1_1 RP ROX	/56-ROXN/TTTCAATTTTACGG
1_2_2 RP ROX	/56-ROXN/TTTCAGTTGATACTGG
2_1_min1b RP ROX	/56-ROXN/TATCTCTACGCATTTCACCGCTACA
2_3_2 RP ROX	/56-ROXN/TTTCAGATGTGAAATCCC
4_1 RP CY5	/5CY5/TTTAGTGAAGAAG
4_5_2 RP CY5	/5CY5/TATGGTGGGAGT
4_8_1_RP2_CY5	/5CY5/TAAGTCTGACCGAGCAACGCCGC
4_6_1 RP CY5	/5CY5/TTAACGGGTGTGAGAGTG
2_4_1 RP FAM	/56-FAM/TAACTGCATTC
4_4_2 RP FAM	/56-FAM/TTTGGGAGGGTCAT
5_1 RP FAM	/56-FAM/TTTGTGGCAAGCGTTG
5_1_2 RP FAM	/56-FAM/TTACCTAATCAGAAAGCCACG
6_2 RP HEX	/5HEX/TTTTACGCGACG
6_2_2 RP HEX	/5HEX/TTAGTGTACCTTTCG
6_1_4 RP HEX	/5HEX/TTAGTTTACCCGTTGAAT
UNI01 RP HEX	/5HEX/TTGCCAGCAGCCGCGGTAATACG

Subsequently, for each probe, the numbers of the Illumina reads lower than LAD detection limit were equated to zero, to test the correlation of the positive signals using Spearman's Rho test.

Statistical analysis

Minitab Release 15.1.1.0 (Minitab Inc. 2007) was used to perform Student's t-test to compare the differences on quenching strength (fluorescence mean value) between cohorts. For the sake of illustration, the data were normalized so that the cut-off value equals zero. In addition, the differences regarding the prevalence of positive signals were analyzed by using Pearson's chi-squared test.

RESULTS & DISCUSSION

Optimization of the LAD-based microbiota detection assay

Based on pure cultures, we first adjusted the level of probes present in the reaction in order to achieve high signal-to-noise ratios. A detailed description of the experimental setup used in the evaluation is provided in Supplementary Figures S1, S2 and S3. This process was performed empirically (see supplement for details), resulting in an assay that was capable of reporting 15 distinguishable signals in a one-tube multiplex reaction, consisting of probes reported in Table 1. The signals for each of our probes, besides 6_2 duplex, were at least two standard deviations above the average value of no target reactions, which represented the background noise (Z>2), with a p-value < 0.02 (Table 3).

The initial evaluation of the assay performance was based on comparisons between experimental and theoretical signals, derived from Vebø *et al.* (2011) [5]. This analysis showed that the accuracy and specificity of probes was very high, reporting only the target strains in reactions holding individual bacteria or defined bacteria mixtures (Figure 2).

Comparison of LAD-based microbiota assay with Illumina sequencing

To compare LAD with the output of Illumina sequencing, we sequenced 87 clinical samples, then performed *in silico* labeling of the retrieved sequences for the nine probes covered by the sequencing amplicon. Subsequently, for each probe we compared LAD signals with the number of sequence copies that acted as a template during *in silico* labeling. Specifically, we performed ROC curve analysis for each probe to determine accuracy of classification, and to determine limit of detection for the LAD assay. For most of the probes the accuracy of detection, i.e. the number of correct predictions, was high (>80%). The detection limit for the probes was between 0.2 and 2%, as determined by the percentage of Illumina sequencing reads detected. Furthermore, there was a significant quantitative correlation between Illumina read counts and LAD signals ($p < 0.05$), with Spearman's rho ranging between 0.45 and 0.86 for all the probes (Table 4).

Use of LAD to genotype clinical samples

The verified assay was used to probe the microbiota composition from 541 PACT study fecal samples from infants and their mothers.

The highest number of positive signals was reported for 5_1 and 6_2_2 probe duplexes, designed to detect Firmicutes and *Bifidobacterium breve*, respectively. Overall, the results showed that in terms of prevalence, there is overrepresentation of gammaproteobacteria and *Enterococcus*/*Listeria* in 4-month-old children, *Bacteroides* at 2-years old, *Bifidobacterium* at 4 months and *Staphylococcus* in 10-day-old children (Figure 3).

Table 3. Probe signal-to-noise ratios.

Probe	Average of positive signals (μ_1)	Average of NT signals (μ_2)	NT standard deviation (Σ)	Z-score((μ_1 - μ_2)/ Σ)	p-value
6_2	−287.5	70.4	87.9	**−4.1**	>0.99
UNI01	1704.1	−331.1	58.2	**34.9**	<0.0002
6_2_2	723.8	−91.2	121.8	**6.7**	<0.0002
6_1_4	2022.9	−7.2	95.9	**21.2**	<0.0002
4_1	−537.4	−2599.6	896.6	**2.3**	0.01
4_5_2	−446.3	−2398.5	826.1	**2.3**	0.01
4_6_1	−364.9	−2013.9	816.4	**2.0**	0.02
4_8_1[†]	N/D			**N/D**	N/D
1_1	2062.9	−569.1	421.3	**6.2**	<0.0002
1_2_2	2010.5	−336.4	106.5	**22.0**	<0.0002
2_3_2	2230.5	−326.5	234.0	**10.9**	<0.0002
2_1_min1b	1865.0	−473.9	83.4	**28.0**	<0.0002
2_4_1	117.9	−836.5	365.8	**2.6**	0.0047
4_4_2	1251.5	−801.7	198.5	**10.3**	<0.0002
5_1	1355.9	−1021.1	109.9	**21.6**	<0.0002
5_1_2	2286.7	−515.5	520.8	**5.4**	<0.0002

[†]The fluorescence values for 4_8_1 probe duplex were not determinedbecause we lacked the DNA template.
N/D: No data; NT: No template.

The signal strength for *S. pyogenes* and *S. sanguinis* had a peak in 1-year-old children, while the strongest signals for *S. pneumoniae* were in 10-day-old children.

There was no significant change in prevalence for the probe detecting a group of species within Firmicutes (5_1 probe, detecting for Clostridia, Bacillales, *Enterococcus* and *Lactobacillus*); however, the signal strength showed an increase parallel with age. The opposite was observed for the other Firmicutes probe, 4_1 (detecting for *Lactobacillus*, *C. perfringens* and *Staphylococcus*), which had a decrease both on prevalence and signal strength in older children.

Use of LAD for rapid detection of microbial communities

Here we present LAD, a novel technique that combines single-nucleotide-extension of the probes with HRM analysis. Compared with existing tools for microbiome testing, LAD-based tests are simpler to perform, are cheaper as they do not require expensive instrumentation and reagents, and yield results faster, within a working day.

Our method does not require a dedicated instrument that would solely be used for LAD-based tests. It requires real-time PCR instrumentation, which is widely and commonly used in most laboratories. In comparison with other real-time PCR-based approaches, it offers a higher level of multiplexity per well, few reagents and short hands-on time, satisfying the actual need of detecting a relatively low number of markers (<50) in a very large number of samples.

LAD represents a highly reproducible method. Initially, the designed probes undergo a process of validation for their specificity, which ensures that all probes become labeled only when their target is present in the reaction. Further on, each labeled probe is tested to ensure it hybridizes only with its corresponding ▶

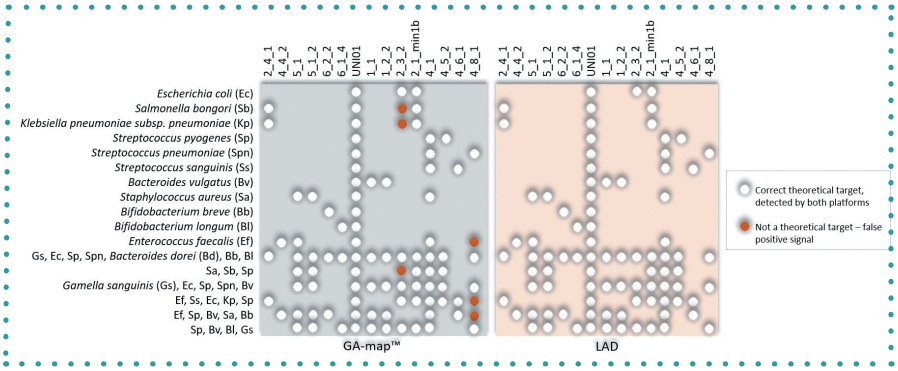

Figure 2. Evaluation of LAD probe accuracy and sensitivity. All signals that were at least two standard deviations away from the background fluorescence were accepted as positives. Tests on individual bacterial strains or defined mixtures of bacteria showed identical results for the correct targets on both platforms, GA-map® (left) and LAD (right). No false-positive signals, reporting nontargets, were registered with LAD.

Figure 3. Signal strength and prevalence of positive signals. Significant differences, that here are depicted with *, were observed between groups for most of the probes.

reporter probe, thus avoiding false-positive signals being generated in the presence of a nontarget probe.

The addition of a synthetic quencher-and-fluorophore-labeled duplex (designated *T*m and detection channel) into the master mix will provide the basis for a well-to-well data normalization, ensuring reproducibility.

For this study, we chose to adopt probes designed by Genetic Analysis AS [5], considering that their platform, GA-map, is an already validated method based on single nucleotide extension. Results obtained with GA-map served as a reference and allowed us to evaluate the overall performance of LAD. We found highly comparable probe specificities using the two technologies, suggesting the transferability of GA-map probes to LAD detection.

Our results on the clinical dataset show that there is a rapid decrease of staphylococci from 10-day- to 4-month-old children, and a peak of bifidobacteria at the age of 4 months, which is in full accordance with the previous findings made with GA-map [5]. However, we identified a peak of *Bacteroides* in 2-year-old children, whereas Vebø *et al.* (2011) [5] found that *Bacteroides* were overrepresented in 4-month-old children. This may be explained by the fact that we did not test an identical set of samples, since an increase of *Bacteroides* in older children has already been described from many other papers in the literature [7,8].

In addition, we compared our assay with the outcome of Illumina MiSeq sequencing, which demonstrated a high classification accuracy and low detection limit for LAD, providing evidence of its sensitivity. The quantitative comparisons, however, showed some more deviations between the two platforms. Unfortunately, we could not Illumina-sequence the ~1200 bp PCR fragment analyzed with LAD due to the 300-bp limitation in Illumina read-length chemistry, which could potentially explain the differences between the two sets of results.

Numerous gut microbial markers that are linked with many disorders such as obesity [9–11], diabetes [11–13], multiple sclerosis [14,15] or irritable bowel syndrome [1,16] have already been described, but these have not yet been clinically validated in large-scale multicenter studies. With its main advantage of being very cheap, rapid

Table 4. Evaluation of the diagnostic ability of liquid array diagnostics-based tests.

Probe	2_4_1	5_1	5_1_2	1_1	1_1_2	2_3_2	2_1_min1b	4_1	4_8_1
Detection limit (%)	0.8	0.4	1.2	2.4	0.1	1.1	0.4	0.022	0.002
Sensitivity (%)	90	91.9	93.3	84.8	82	85	65.5	62.7	69.2
Specificity (%)	92.1	76.9	95.8	91.7	65.5	83	86.2	85.7	68.9
Spearman's Rho	0.74	0.82	0.86	0.81	0.57	0.72	0.66	0.65	0.45

and simple, in addition to being an accurate method, LAD will offer this possibility.

We acknowledge the limitations of our method regarding systems where the microbiome composition is complex, unpredictable and constantly shifting. Building a LAD assay *de novo* is best conducted in systems with relatively low complexity, where the knowledge regarding the microbiome composition is already described, such as is the case with gut microbiota. A well-defined composition is a prerequisite towards designing targeting probes.

Here, we used 15 different probe duplexes, which were designed to utilize four channels of detection and at least three *T*ms per channel. By using a qPCR machine with six channels of detection and exploiting at least six resolvable *T*ms per channel, the multiplex level can be elevated to at least a 36-plex. Thus, the possibility of multiplexing is limited by the instrument, and not by LAD technology in itself.

In conclusion, we believe LAD will fulfill the need for assays able to detect up to 50 biomarkers, where high throughput is essential. This will particularly relate to human gut microbiota markers related to health and disease.

AUTHOR CONTRIBUTIONS

PH, RW and KR conceived and designed the experiments and drafted the work; OS, RJ and TØ helped with the sample collection. All authors contributed equally to analysis and interpretation of data and critical revision of the drafts. All authors approved the final version to be published.

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SUPPLEMENTARY DATA

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2018-0134

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