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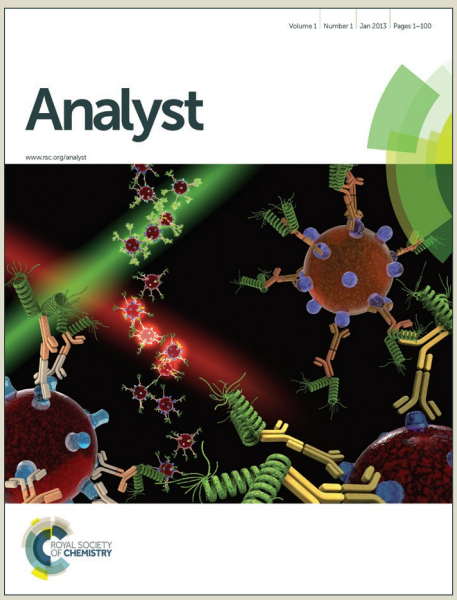

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Integrated closed-tube 2-plex PCR amplification and hybridization assay with switchable lanthanide luminescence based spatial detection

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ABSTRACT

Switchable lanthanide luminescence is a binary probe technology that inherently enables a high signal modulation in a separation-free detection of DNA targets. A luminescent lanthanide complex is formed only when the two probes hybridize adjacently to their target DNA. We have now further adapted this technology for the first time in the integration of a 2-plex polymerase chain reaction (PCR) amplification and hybridization-based solid-phase detection of the amplification products of the *Staphylococcus aureus gyrB* gene and an internal amplification control (IAC). The assay was performed in a sealed polypropylene PCR chip containing a flat-bottom reaction chamber with two immobilized capture probe spots. The surface of the reaction chamber was functionalized with NHS-PEG-azide and alkyne-modified capture probes for each amplicon, labeled with a light harvesting antenna ligand, were covalently attached as spots to the azide-modified reaction chamber using a copper(I)-catalyzed azide-alkyne cycloaddition. Asymmetric duplex-PCR was then performed with either no template, one template or both templates present and with a europium ion carrier chelate labeled probe for each amplicon in the reaction. After amplification europium fluorescence was measured by scanning the reaction chamber as a 10 × 10 raster with 0.6 mm resolution in time-resolved mode. With this assay we were able to co-amplify and detect the amplification products of the *gyrB*

target from 100, 1000 and 10000 copies of isolated *S. aureus* DNA together with the amplification products from initial 5000 copies of the synthetic IAC template in the same sealed reaction chamber. Addition of 10000 copies of isolated non-target *Escherichia coli* DNA in the same reaction with 5000 copies of the synthetic IAC template did not interfere with the amplification or detection of the IAC. The dynamic range of the assay for the synthetic *S. aureus gyrB* target was three orders of magnitude and the limit of detection of 8 pM was obtained. This proof-of-concept study shows that the switchable lanthanide luminescence probes enable a separation-free array-based multiplexed detection of the amplification products in a closed-tube PCR which can enable a higher degree of multiplexing than is currently feasible by using different spectrally separated fluorescent probes.

1 INTRODUCTION

In molecular diagnostics there has been a constant strive to move from heterogeneous, mostly manual low-throughput technologies to closed-tube methods transferable to automation[1]. In addition there has emerged a growing interest in efficient, straightforward and robust nucleic acid amplification based point-of-care tests[2]. These trends have directed the focus towards multiplexed lab-on-a-chip devices, where several nucleic acid targets can be amplified and characterized in one sealed reaction unit a in separation-free manner[3]. Integrated multiplexed processing and handling of small sample and reagent volumes in a disposable reaction unit would make the biological analysis more cost-efficient and user friendly and less prone to human error and contamination. Thus far multiplexed nucleic acid assays have mostly been used in gene expression profiling and genotyping applications, but their importance in microbial diagnostics is growing fast[4] especially in pathogen identification and multidrug resistance screening[3].

Numerous efforts have been made to develop a relatively fast multiplexed nucleic acid amplification test in a closed-tube format for microbial point-of-care testing. Polymerase chain reaction (PCR) is still the most widespread nucleic acid amplification method due to its sensitivity, effectiveness and capacity despite the requirement of a special equipment[5]. Since the introduction of PCR several multiplexed PCR assays have been developed and reached the market. So far most methods need separate post-PCR steps, such as agarose gel electrophoresis[3, 6, 7], restriction enzyme analysis or hybridization assay for analyzing the amplification products[8-12] which remarkably increases the risk of contaminating the

testing area with the amplification products. The importance of a multiplexed test lies in its ability to detect and identify a broad spectrum of pathogens at a time, which is important when a bacterial group is being screened instead of a single specific pathogen[3]. Several hybridization assays with relatively high multiplexing capacity have been developed, such as the commercially available QuantiGene DNA Multiplex Assay by Affymetrix® (http://www.affymetrix.com) which can multiplex up to 33 different DNA targets, the Prove-it Sepsis assay from Mobidiag (http://www.mobidiag.com) which identifies more than 73 pathogens, Check-points' (http://www.check-points.com) Check-MDR series detecting and differentiating up to 40 antibiotic resistance causing β-lactamase genes and Tan *et al*'s[13] integrated lab-on-a-chip DNA assay with which it is possible to identify 26 different pathogens. Despite the high multiplexing capacity all of these assays need separate post-PCR processing which makes them both time-consuming and contamination-prone.

To overcome the contamination risk due to the opening of the PCR reaction unit after amplification of the target(s) nucleic acid amplification closed-tube assays have been developed, which either measure the product accumulation in real-time[14-17] or at the end of the amplification reaction[18-20]. Common detection methods in PCR based closed-tube assays utilize either non-specific fluorescent dyes that intercalate with any double-stranded DNA[21-23] or sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary sequence[1, 14, 16, 24-35] or mixtures thereof[36, 37]. Both intercalating dyes combined with melting curve analysis and the sequence-specific DNA probes allow multiplexing to a certain extent[17, 32, 38, 39]. With current state-of-the-art fluorescence technologies the degree of multiplexing is limited to 4 - 7 parameters due to a restricted number of appropriate fluorescent moieties and filter sets available[40-42]. When combining melting curve analysis, with which up to four different sequences can be differentiated, and the six distinguishable fluorophores it is theoretically possible to identify up to 24 different DNA sequences[43]. Some of the limitations with fluorophores can be diminished by using large Stokes-shift fluorophores[44], double dye identification like in Luminex® xMAP® (http://www.luminexcorp.com) technology or spatial identification of the targets[17]. Spatial identification has already been extensively used in gene expression profiling but is also getting more widely spread into the diagnostic field[4].

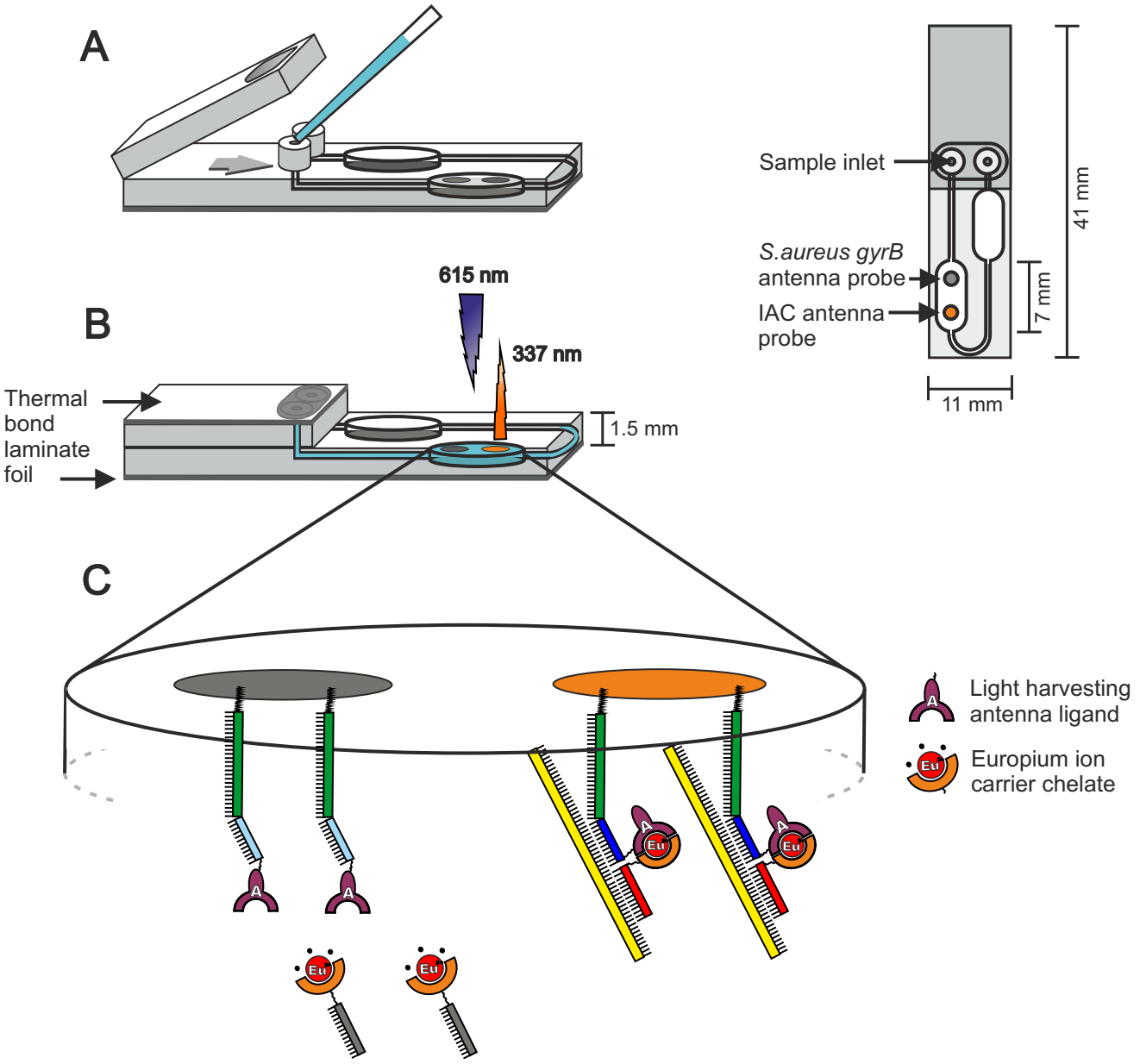
Lanthanide chelates are widely used labels in bioanalytical applications due to their exceptional luminescence properties such as long emission lifetime, sharp emission peaks and large Stokes' shift

enhancing their detectability compared to traditional organic fluorophores[45]. Due to the long emission lifetime, the luminescence can be measured in a time-resolved manner after the short-lived background fluorescence from biological materials has attenuated which remarkably increases the sensitivity[46]. In switchable lanthanide luminescence[16, 25, 45, 47, 48] the intrinsically luminescent lanthanide chelate is splitted into two nonluminescent moieties: a lanthanide ion carrier chelate and a light harvesting antenna ligand, a chromophore that absorbs and transfers the excitation light to the lanthanide ion. By enforcing these moieties into a close proximity they self-assemble to form a luminescent lanthanide chelate complex[49]. In hybridization-directed complex formation these moieties have been conjugated into two short oligonucleotides which upon simultaneous hybridization adjacently into their target sequence bring the moieties in such a close proximity that a luminescent complex is formed.

There are various methods available for immobilizing pre-synthesized biomolecules onto solid supports. Such methods include for example simple adsorption, ultraviolet crosslinking and covalent attachment. In adsorption and ultraviolet crosslinking the attachment of molecules onto the solid support occurs randomly[50]. By using covalent immobilization it is possible to control the orientation and placement of the immobilized molecules although pre-activated solid supports, crosslinkers and/or modified oligonucleotides are often required[11]. Covalent linkage between biomolecules and the solid support is utilized in a variety of molecular biology applications like affinity chromatography, peptide and oligonucleotide synthesis, and biosensor and DNA-microchip technologies and is considered relatively thermostable[51].

The integrated 2-plex amplification and hybridization closed-tube assay (Scheme 1) was performed in a sealed reaction chamber on a primary amine activated (plasma polymerized diaminocyclohexane, DACH) polypropylene PCR chip. DACH was chosen due its ability to form primary amines on the surface in higher extent than for example allylamine, ammonia or nitrogen/hydrogen treatments[52]. Primary amines were further azide-functionalized with NHS-PEG_4-azide. Polyethylene glycol (PEG) reduces non-specific adsorption of proteins and it has been shown to improve DNA hybridization in microarrays[53]. It also renders the surface more hydrophilic which is especially important in the case of hydrophobic supports such as polypropylene[54] which are still rather solvent repellant despite amination[55]. Light harvesting antenna ligand labeled, alkyne-modified capture probes (antenna probes) were covalently attached as spots to the reaction chamber of the azide-functionalized PCR chip using a fast and site-specific copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC), so called click-

chemistry[56-60]. To improve the hybridization efficiency of the targets into the surface bound capture probes the target specific hybridization sequence of the capture probe was separated from the solid support with a 25 thymidine spacer[61] and the polyethylene glycol linker (NHS-PEG_4-azide) used in the azide-functionalization of the surface. Covalent attachment of the capture probes and the high thermal stability of the polypropylene substrate enabled the usage of high temperatures required in PCR. Spatial identification of the amplification products enabled the integration of 2-plex amplification and separation-free detection of the two different amplification products in the closed reaction chamber in this proof-of-concept study.



Scheme 1 Schematic representation of the principle of the integrated 2-plex amplification and hybridization closed-tube assay. (A) Amplification and hybridization reaction mixture was transferred into the reaction chamber spotted with the *gyrB* and IAC antenna probes (B) and the chamber bottom was permanently sealed with a thermal bond laminate foil before the genomic *gyrB* and IAC templates, if present, were amplified with an asymmetric PCR. (C) When the temperature was decreased after the PCR the IAC amplification products (yellow) hybridized both with the solution based Eu-carrier probe (red) and the immobilized antenna probe (dark blue), separated from the solid support with a 25 thymidine spacer (green). The luminescence of the formed lanthanide complex was measured at RT in

time-resolved mode (laser excitation at 337 nm, emission measured at 615 nm) as a 10 × 10 (5.4 mm × 5.4 mm) raster (orange spot). In the absence of *S. aureus* genomes, no *gyrB* target amplification occurred and the solution based Eu-carrier probe (grey) and the immobilized antenna probe (light blue) did not have any amplification product to hybridize into so no luminescent lanthanide complex was formed and there was no signal (grey spot).

2 EXPERIMENTAL

2.1 Materials

Probe oligonucleotides (Table 1) for *gyrB* and IAC[61] amplification and detection were from Biomers.net (http://www.biomers.net, Ulm, Germany), primers from Thermo Fisher Scientific GmbH (http://www.thermofisher.com, Ulm, Germany) and the synthetic IAC template was from TAG Copenhagen (http://www.tagc.dk, Frederiksberg, Denmark). Primers and probes for *gyrB* were designed based on the genomic sequence of *Staphylococcus aureus* (ATCC® Number 29213™) gyrase B gene *gyrB* originally obtained from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov, gene accession number D10489). *S. aureus* and *Escherichia coli* (ATCC® Number 25922™) were originally from American Type Culture Collection (http://www.lgcstandards-atcc.org, Rockville, MD), plate cultured and the genomic DNA was isolated using NucleoSpin® Tissue kit from Macherey-Nagel GmbH & Co. KG (http://www.mn-net.com, Düren, Germany). The concentration of the genomic DNA was determined with a Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen Ltd., Paisley, UK). Polypropylene chips (Abacus Diagnostica Oy, http://www.abacusdiagnostica.com, Turku, Finland) were activated by plasma polymerizing diaminocyclohexane (DACH) on the surface (SP Technical Research Institute of Sweden, http://www.sp.se, Stockholm, Sweden). Thermal bond laminate foil (4titude, http://www.4ti.co.uk, Surrey, UK) was used for chip sealing. Polypropylene chips were designed for liquid phase PCR applications, but offered a suitable heat-resistant platform to be utilized also with immobilized probes and spatial detection on the surface. The thermal stability of the switchable lanthanide luminescence probes has already been demonstrated in homogeneous liquid phase PCR assay by Lehmusvuori *et al.*[16]. All reagents were ACS laboratory reagents and from Sigma-Aldrich (http://www.sigmaaldrich.com, St. Louis, MO) unless stated otherwise. Succinimidyl- and azido-

functionalized tetraethyleneglycol (NHS-PEG_4-azide) was from Thermo Fisher Scientific Inc. (http://www.thermofisher.com, Rockford, IL). Europium ion carrier chelate probes (Eu-carrier probes), primers, synthetic *gyrB* target and synthetic IAC template were stored in oligo storage buffer consisting of 10 mM Tris–HCl (pH 7.5), 50 mM NaCl and 10 μM ethylenediaminetetraacetic acid (EDTA). CuAAC reaction solution contained 50 mM $CuSO_4$, 100 mM L(+)-ascorbic acid (AppliChem GmbH, http://www.applichem.com, Darmstadt, Germany), 100 mM Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) and 10 % (v/v) glycerol. In the functionalization of the polypropylene chips and spotting 100 mM Tris–HCl (pH 8.0) and 10 mM PBS (pH 7.4) with and without 1 mM EDTA were used. The combined amplification and hybridization buffer contained 10 mM Tris–HCl (pH 8.3), 100 mM KCl, 0.2 % BSA (Gemini Bio-Products, http://www.gembio.com, West Sacramento, CA), 1.5 mM $MgCl_2$ and 30 μM diethylenetriaminepentaacetic acid (DTPA) (Merck, http://www.merckgroup.com, Darmstadt, Germany). Hybridization without preceding PCR was done in 50 mM Tris–HCl (pH 7.75), 600 mM NaCl, 0.1 % (v/v) Tween® 20, 0.05 % (w/v) NaN_3 and 30 μM DTPA.

Table 1 Synthetic oligonucleotides with modifications for the amplification and detection of *gyrB* and IAC.

Probe	Label[a]	5′ Modification	Sequence 5′→ 3′	3′ Modification
gyrB antenna	Antenna	Alkyne[b]	$(T)_{25}$CA CAA GAC TTA GAA GTA TAT G	Aminolink C6
gyrB Eu-carrier	DOTA-Eu^{III}	Aminolink C6	CAC AGA AAT GAG ACT ATA TAT C	Phosphate
IAC antenna	Antenna	Alkyne[b]	$(T)_{25}$GG TTC TAG TAC GAC AT	Aminolink C6
IAC Eu-carrier	DOTA-Eu^{III}	Aminolink C6	CAG AGA CAT TCT TTA GA	Phosphate
Oligonucleotide		Sequence 5′→ 3′		3′ Modification
gyrB forward primer		GGT TCA TCA GTT GTA AAC GCA T		
gyrB reverse primer		TAC CTG TCT TAT CAG TTG TGC C		
IAC forward primer		CGA CTT CAG GAC CAA CAT CAG AC		
IAC reverse primer		GTG TGC GCC GAC GTC C		
Synthetic *gyrB* target		ATG ATA TAT AGT CTC ATT TCT GTG TAC ATA TAC TTC TAA GTC TTG TGA CA		Phosphate
Synthetic IAC template		CCG ACT TCA CGA CCA ACA TCA GAC CCT GCT AAG TTC TAA AGA ATG TCT CTG TAT GTC GTA CTA GAA CCT GCG		Phosphate

GTG GAT GGA CGT CGG CGC ACA CAG ATT

[a] Light harvesting antenna ligand (antenna) and europium ion carrier chelate (DOTA-Eu^{III}) were attached from their isothiocyanate groups to the amino groups (aminolink C6) of the oligonucleotides.
[b] 5´-alkylacetylen-cyclohexyl

2.2 Instrumentation

PCR was done with GenomEra™ nucleic acid analyzer prototype (Abacus Diagnostica Oy, http://www.abacusdiagnostica.com, Turku, Finland)[62]. EnVision® Multilabel Plate Reader (PerkinElmer, http://www.perkinelmer.com, Waltham, MA) was used to measure the europium luminescence in time-resolved fluorescence mode.

2.3 Procedures

2.3.1 Labeling of oligonucleotide probes. Antenna and Eu-carrier probes were prepared by labeling the probe oligonucleotides with isothiocyanate-activated form of either the light harvesting antenna ligand (4-((4-isothiocyanatophenyl)ethynyl)pyridine-2,6-dicarboxylic acid)[45] or the europium ion carrier chelate, DOTA-Eu^{III}, ((2,2´,2´´-(10-(3-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)tri(acetate)europium(III))[47] using published methods[25, 61], respectively.

2.3.2 Surface functionalization and spotting. The reaction chamber of the primary amine-activated polypropylene chip was functionalized with NHS-PEG_4-azide and the alkyne-containing antenna probes were covalently immobilized on the bottom of the reaction chamber as a spot format using CuAAC. To each reaction chamber 30 µL of 2 mM NHS-PEG_4-azide in PBS (pH 7.4) was added, incubated for 60 min at 37 °C in a humid atmosphere, washed once with 100 mM Tris-HCl (pH 8.0) (5 min, RT, slow shake) to quench unreacted NHS-PEG_4-azide followed by three washes with PBS (pH 7.4) (5 min, RT, slow shake) after which the reaction chambers were dried (30 min, 37 °C). Antenna probe with an alkyne at the 5´ end was diluted in 10 % glycerol solution to a final concentration of 5 µM, mixed (1:2) with the CuAAC reaction solution and spotted manually (0.75 µL/spot) on the reaction chamber. Spotted reaction chambers were incubated 4 hours at 37 °C in a humid atmosphere

after which they were washed three times with PBS (pH 7.4) containing 1 mM EDTA (5 min, RT, slow shake) and once with MQ-water. Thereafter the chips were dried (30 min, 37 °C) and the bottom of the reaction chamber was sealed with the thermal bond laminate foil. The spotted reaction chambers were used directly in the assay or stored at 4 °C in an aluminium foil bag with a desiccant.

2.3.3 Hybridization assay. The switchable lanthanide luminescence based solid-phase hybridization assay[61] was implemented into a new closed-tube assay platform with covalently immobilized antenna probes and surface detection through the plastic bottom in a closed-tube polypropylene PCR chip with aluminium foil backing. To compare the new assay platform with the conventional microtiter well based assay all parameters other than the antenna probe immobilization chemistry and assay platform were kept as similar as possible. The *gyrB* Eu-carrier probe (50 nM) and synthetic *gyrB* target were diluted in hybridization buffer and added onto the *gyrB* antenna probe spotted reaction chamber as duplicates in a total volume of 60 µL. The inlets leading to the reaction chamber were sealed and the reaction chambers were incubated for 20 min at 50 °C without shaking followed by 15 min incubation at RT with shaking. After incubations the PCR chips were placed into a microtiter plate-sized measurement tray manufactured in-house and the luminescence was measured in time-resolved mode with an EnVision® Multilabel Plate Reader (laser excitation at 337 nm, emission measured at 615 nm, 10 flashes, 2 mm measurement height) through the transparent plastic top of the reaction chamber as a 10 × 10 (5.4 × 5.4 mm) raster (0.6 mm between the measurement points). The highest luminescence signal of the spot area was considered as the spot signal. To find the optimal spotting concentration 0.17, 0.33, 1.67, 3.33 and 6.67 µM *gyrB* antenna probes were spotted on the reaction chamber of the PCR chips resulting in a theoretical maximum of 0.13, 0.25, 1.25, 2.5 and 5 pmol *gyrB* antenna probes per spot. In hybridization assay 1 and 10 nM synthetic *gyrB* targets were used. To define the limit of detection (LOD) and dynamic range of the hybridization assay 1.67 µM (spotting concentration) *gyrB* antenna probe spots and 0–100 nM synthetic *gyrB* target (n = 2) were used. The LOD was calculated from the mean of the blank samples (0 nM target, n = 4) plus 3 standard deviations and compared with the LOD of the microtiter well based hybridization assay[61].

2.3.4 Integrated amplification and detection. Each asymmetric amplification and hybridization reaction (30 µL) was done in the sealed reaction chamber on the PCR chip containing *gyrB* and IAC antenna probe spots (1.67 µM spotting concentration), 50 nM *gyrB* and 50 nM IAC Eu-carrier probes, optimized concentrations (results not shown) of primers (0.2 µM *gyrB* forward/2 µM *gyrB* reverse, 1 µM IAC forward/0.1 µM IAC

reverse) and 1×10^2, 1×10^3 or 1×10^4 copies of *S. aureus* genome together with 5000 copies of synthetic IAC template diluted in the amplification and hybridization buffer containing 0.4 mM dNTP (LAROVA GmbH http://www.larova.com, Jena, Germany) and 1 U Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific Inc., http://www.thermofisher.com, Waltham, MA). For testing the unspecific amplification and hybridization 1×10^4 copies of *E. coli* genome were added to the amplification and hybridization reaction together with 5000 copies of synthetic IAC template. The PCR chips (4 chips / tray) were cycled between heated blocks. The thermal cycling consisted of 180 s initial denaturation at 100 °C followed by 44 cycles of 27 °C for 1.7 s, 60 °C for 15 s, 75 °C for 15 s and 100 °C for 17 s. The temperatures refer to the temperatures of the heated blocks, not to the temperature inside the reaction chamber on the PCR chip. After PCR the reaction chamber was incubated 15 min at RT after which luminescence was measured as described above. The integrated amplification and detection was completed in less than 1.5 hours (preparation of PCR solutions 20 min, PCR 40 min, detection 20 min).

3 RESULTS AND DISCUSSION

3.1 Hybridization assay. The luminescence signals were affected by the spotted antenna probe concentration (Figure 1). With higher spotting concentrations of 3.33 and 6.67 μM (2.5 and 5 pmol/spot, respectively) the spots spread and the background signals in the reaction chamber increased. Even though the spotting droplets were removed after the incubation there might have remained unbound antenna probe that spread outside the actual spot area during washings. Due to the spot spreading the signal-to-background (S/B) ratios were calculated as a ratio between the highest and the lowest luminescence signals of the vertical measurement points (line profiles) going across the peak top in a measured 10 × 10 raster. Even though the S/B ratios were slightly better with the spotting concentration of 0.33 μM (0.25 pmol/spot) the spotting concentration of 1.67 μM (1.25 pmol/spot) gave higher specific luminescence signals with less variation and more defined peak morphology.

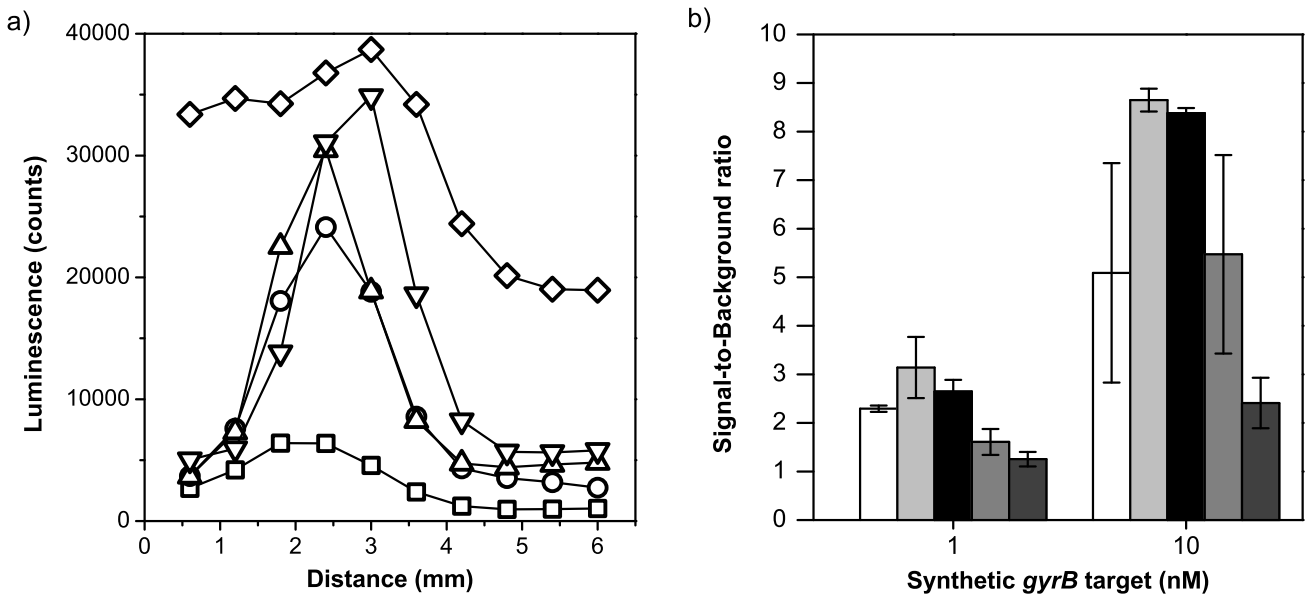


Figure 1 The effect of *gyrB* antenna probe spotting concentration on (a) luminescence signals and (b) S/B ratios. Luminescence signals presented as line profiles from the vertical measurement points in a 10 × 10 raster going across the peak tops with 10 nM *gyrB* target concentrations. The signal-to-background (S/B) ratios were calculated as a ratio between the highest and the lowest luminescence signals of the line profiles. The *gyrB* antenna spotting concentrations were 0.17 μM (squares alt. white bars), 0.33 μM (circles alt. light grey bars), 1.67 μM (up triangles alt. black bars), 3.33 μM (down triangles alt. grey bars) and 6.67 μM (diamonds alt. dark grey bars). The corresponding molar amounts of spotted *gyrB* antenna probes were 0.13, 0.25, 1.25, 2.5 and 5 pmol per spot, respectively. Error bars (b) indicate the standard deviation (n = 2).

The LOD of the hybridization assay was 8 pM (background + 3SD, n = 4) (Figure 2) and the dynamic range three orders of magnitude which equaled well with the LOD (18 pM) of the previously developed solid-phase hybridization assay in conventional microtiter well format utilizing biotin-streptavidin antenna probe immobilization chemistry[61]. With high target concentrations (> 10 nM) the spot signal started to saturate and the concentration of target approached the concentration of Eu-carrier probe.

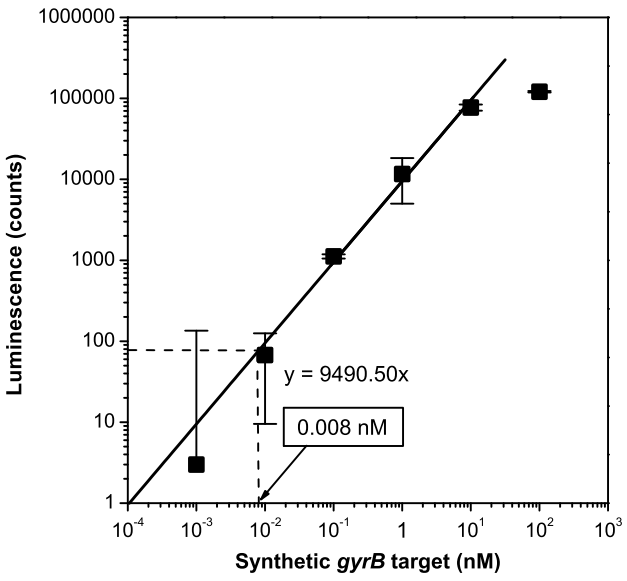


Figure 2 Titration of the synthetic *gyrB* target. The LOD of 0.008 nM (background + 3SD, n = 4) is presented as dotted line. The dynamic range of the optimized assay was three decades. Error bars indicate the standard deviation (n = 2).

3.2 Integrated amplification and detection. In integrated amplification and hybridization closed-tube assay the *gyrB* and IAC targets were co-amplified and successfully detected in the same reaction chamber (Figure 3). The 1×10^4 copies of non-target *E. coli* genomic DNA did not interfere with the amplification of the synthetic IAC template neither did we observe any remarkable unspecific binding into the immobilized *gyrB* antenna probe. The 1×10^2, 1×10^3 and 1×10^4 copies of *S. aureus* genome were co-amplified with 5000 copies of synthetic IAC template and amplification products were successfully co-detected. This was an end-point PCR assay so the amount of DNA at the end of the amplification reaction was independent of the starting template concentration[63] and the luminescence signals were not expected to correspond with the initial amount of the templates in the reaction. The co-amplification of two different targets in one reaction also affected the final amounts of the amplification products because the two reactions competed for the same amplification reagents. Theoretically a single initial copy of *S. aureus* genome should be enough to produce a detectable amount of single-stranded amplification product at the end of the exponential PCR amplification, but in practise the actual yield varies and thus detection limits below 10 copies are seldom reliably obtained[64]. By being able to detect at least 100 initial copies of *S. aureus* genome (which was the lowest amount tested) the integrated assay would be sensitive enough for pathogen detection and identification from

blood culture enriched samples[65-67]. This is in the upper end of the clinically significant area (1–100 colony forming units (CFUs) / mL of blood) for detection of *S. aureus* directly from patients' blood after the state-of-the-art enrichment and DNA isolation[68-70], but there are indications that efficient direct detection of *S. aureus* would require an analytical sensitivity less than 10 CFU / mL of patients' blood[68, 71].

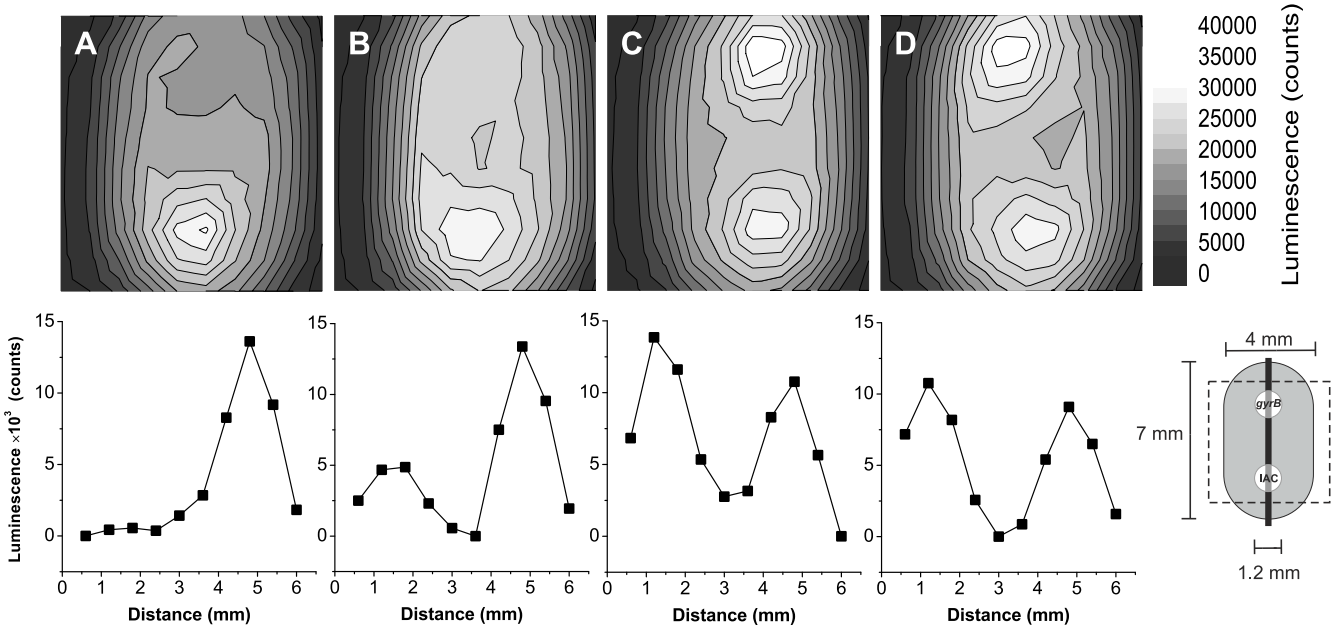


Figure 3 Luminescence images and signal line profiles across the peak top(s) with different amplification reactions (luminescence images without background subtraction, line profiles normalized). In the integrated 2-plex amplification and hybridization assay 5000 copies of synthetic IAC template was amplified together with (A) 1×10^4 copies of non-target *E. coli* genome, (B) 1×10^2, (C) 1×10^3 and (D) 1×10^4 copies of *S. aureus* genome. Line profile luminescence signals were from the vertical measurement points in a 10×10 raster (the measurement area shown as a dashed line in the schematic presentation of the reaction chamber) going across the peak tops shown as a solid black line in the schematic chip image.

4 CONCLUSIONS

In this study we showed an initial proof-of-concept of a truly closed-tube 2-plex assay with integrated target amplification and separation-free array-based detection using switchable luminescence probes.

Array-based detection of the different targets can potentially enable a higher level of multiplexing than is currently possible with spectrally separated fluorescent probes. The spatial resolution of the measurement instrument is currently somewhat limiting higher multiplexing in this assay setup. In the hybridization assay we got a linear response with different amounts of synthetic target but the integrated assay cannot quantitate the initial copy number of *S. aureus* genome due to the end-point detection combined with multiplex application. As a point-of-care test for microbial detection and/or identification a fast and reliable qualitative assay would in most cases be sufficient.

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REFERENCES

1. D. Whitcombe, J. Theaker, S. P. Guy, T. Brown and S. Little, *Nat Biotech*, 1999, **17**, 804-807.
2. T. Klamp, M. Camps, B. Nieto, F. Guasch, R. T. Ranasinghe, J. Wiedemann, Z. Petrasek, P. Schwille, D. Klenerman and M. Sauer, *Scientific reports*, 2013, **3**, 1852.
3. T. Sandle, D. Babenko, A. Lavrinenko, I. Azizov and A. Cheşcă, *European Journal of Parenteral and Pharmaceutical Sciences*, 2014, **19**, 153-164.
4. A. K. Jarvinen, S. Laakso, P. Piiparinen, A. Aittakorpi, M. Lindfors, L. Huopaniemi, H. Piiparinen and M. Maki, *BMC microbiology*, 2009, **9**, 161.
5. C.-M. Chang, W.-H. Chang, C.-H. Wang, J.-H. Wang, J. D. Mai and G.-B. Lee, *Lab on a chip*, 2013, **13**, 1225-1242.
6. T. Gosiewski, M. Brzychczy-Wloch and P. B. Heczko, *Folia microbiologica*, 2012, **57**, 163-167.
7. N. Wellinghausen, A. J. Kochem, C. Disque, H. Muhl, S. Gebert, J. Winter, J. Matten and S. G. Sakka, *Journal of clinical microbiology*, 2009, **47**, 2759-2765.
8. P. Tissari, A. Zumla, E. Tarkka, S. Mero, L. Savolainen, M. Vaara, A. Aittakorpi, S. Laakso, M. Lindfors, H. Piiparinen, M. Mäki, C. Carder, J. Huggett and V. Gant, *The Lancet*, **375**, 224-230.
9. G. A. Cannon, M. J. Carr, Z. Yandle, K. Schaffer, R. Kidney, G. Hosny, A. Doyle, J. Ryan, R. Gunson, T. Collins, W. F. Carman, J. Connell and W. W. Hall, *J Virol Methods*, 2010, **163**, 17-24.
10. L. J. Kricka, *Clin Chem*, 1999, **45**, 453-458.
11. M. Sjoroos, J. Ilonen and T. Lovgren, *Clin Chem*, 2001, **47**, 498-504.

12. L. D. Girard, K. Boissinot, R. Peytavi, M. Boissinot and M. G. Bergeron, *The Analyst*, 2015, **140**, 912-921.
13. J. J. Tan, M. Capozzoli, M. Sato, W. Watthanaworawit, C. L. Ling, M. Mauduit, B. Malleret, A. C. Gruner, R. Tan, F. H. Nosten, G. Snounou, L. Renia and L. F. Ng, *PLoS neglected tropical diseases*, 2014, **8**, e3043.
14. J. Nurmi, T. Wikman, M. Karp and T. Lövgren, *Analytical chemistry*, 2002, **74**, 3525-3532.
15. S. Tyagi and F. R. Kramer, *Nature biotechnology*, 1996, **14**, 303-308.
16. A. Lehmusvuori, U. Karhunen, A. H. Tapio, U. Lamminmaki and T. Soukka, *Analytica chimica acta*, 2012, **731**, 88-92.
17. H. Liu, H. Wang, Z. Shi, H. Wang, C. Yang, S. Silke, W. Tan and Z. Lu, *Nucleic acids research*, 2006, **34**, e4-e4.
18. J. Nurmi, M. Kiviniemi, M. Kujanpaa, M. Sjoroos, J. Ilonen and T. Lovgren, *Analytical biochemistry*, 2001, **299**, 211-217.
19. A. Lehmusvuori, E. Juntunen, A. H. Tapio, K. Rantakokko-Jalava, T. Soukka and T. Lovgren, *Journal of microbiological methods*, 2010, **83**, 302-306.
20. M. Drigo, G. Franzo, I. Belfanti, M. Martini, A. Mondin and L. Ceglie, *J Virol Methods*, 2014, **201**, 79-85.
21. Y. S. Jin X., Wells K.S., Singer V.L., Biophys J 1994.
22. R. Higuchi, G. Dollinger, P. S. Walsh and R. Griffith, *Nat Biotech*, 1992, **10**, 413-417.
23. W. T. Ong, A. R. Omar, A. Ideris and S. S. Hassan, *Journal of Virological Methods*, 2007, **144**, 57-64.
24. V. V. Didenko, *BioTechniques*, 2001, **31**, 1106-1121.
25. U. Karhunen, M. Soikkeli, S. Lahdenpera and T. Soukka, *Analytica chimica acta*, 2013, **772**, 87-92.
26. Q. Li, G. Luan, Q. Guo and J. Liang, *Nucleic acids research*, 2002, **30**, E5.
27. J. Cheng, Y. Zhang and Q. Li, *Nucleic acids research*, 2004, **32**, e61.
28. J. Isacsson, H. Cao, L. Ohlsson, S. Nordgren, N. Svanvik, G. Westman, M. Kubista, R. Sjoback and U. Sehlstedt, *Molecular and cellular probes*, 2000, **14**, 321-328.
29. N. Svanvik, G. Westman, D. Wang and M. Kubista, *Analytical biochemistry*, 2000, **281**, 26-35.
30. D. J. French, C. L. Archard, T. Brown and D. G. McDowell, *Molecular and cellular probes*, 2001, **15**, 363-374.
31. D. J. French, R. L. Howard, N. Gale, T. Brown, D. G. McDowell and P. G. Debenham, *Forensic Science International: Genetics*, 2008, **2**, 333-339.
32. S. Tyagi, D. P. Bratu and F. R. Kramer, *Nature biotechnology*, 1998, **16**, 49-53.
33. P. M. Holland, R. D. Abramson, R. Watson and D. H. Gelfand, *Proceedings of the National Academy of Sciences of the United States of America*, 1991, **88**, 7276-7280.
34. L. E. Lehmann, K.-P. Hunfeld, T. Emrich, G. Haberhausen, H. Wissing, A. Hoeft and F. Stüber, *Med Microbiol Immunol*, 2008, **197**, 313-324.
35. Y. Zhao, S. Park, B. N. Kreiswirth, C. C. Ginocchio, R. Veyret, A. Laayoun, A. Troesch and D. S. Perlin, *Journal of clinical microbiology*, 2009, **47**, 2067-2078.
36. A. Horvath, Z. Peto, E. Urban, C. Vagvolgyi and F. Somogyvari, *BMC microbiology*, 2013, **13**, 300.
37. I. A. Afonina, M. W. Reed, E. Lusby, I. G. Shishkina and Y. S. Belousov, *BioTechniques*, 2002, **32**, 940-944, 946-949.
38. M. L. Wong and J. F. Medrano, *BioTechniques*, 2005, **39**, 75-85.
39. M. Book, L. E. Lehmann, X. Zhang and F. Stuber, *Best practice & research. Clinical anaesthesiology*, 2013, **27**, 279-288.
40. L. G. Lee, K. J. Livak, B. Mullah, R. J. Graham, R. S. Vinayak and T. M. Woudenberg, *BioTechniques*, 1999, **27**, 342-349.
41. R. Köppel, F. Zimmerli and A. Breitenmoser, *Eur Food Res Technol*, 2009, **230**, 125-133.
42. J. A. Richardson, M. Gerowska, M. Shelbourne, D. French and T. Brown, *Chembiochem*, 2010, **11**, 2530-2533.
43. P. S. Bernard and C. T. Wittwer, *Clin Chem*, 2000, **46**, 147-148.
44. J. A. Richardson, T. Morgan, M. Andreou and T. Brown, *The Analyst*, 2013, **138**, 3626-3628.

45. U. Karhunen, L. Jaakkola, Q. Wang, U. Lamminmaki and T. Soukka, *Analytical chemistry*, 2010, **82**, 751-754.
46. I. Hemmilá and V.-M. Mukkala, *Critical Reviews in Clinical Laboratory Sciences*, 2001, **38**, 441-519.
47. U. Karhunen, J. Rosenberg, U. Lamminmaki and T. Soukka, *Analytical chemistry*, 2011, **83**, 9011-9016.
48. A. Oser and G. Valet, *Angewandte Chemie International Edition in English*, 1990, **29**, 1167-1169.
49. S. H. Hsu, M. D. Yilmaz, C. Blum, V. Subramaniam, D. N. Reinhoudt, A. H. Velders and J. Huskens, *Journal of the American Chemical Society*, 2009, **131**, 12567-12569.
50. J. M. Goddard and J. H. Hotchkiss, *Progress in Polymer Science*, 2007, **32**, 698-725.
51. M. Beier and J. D. Hoheisel, *Nucleic acids research*, 1999, **27**, 1970-1977.
52. M. Müller and C. Oehr, *Surface and Coatings Technology*, 1999, **116–119**, 802-807.
53. A. del Campo and I. Bruce, in *Immobilisation of DNA on Chips I*, ed. C. Wittmann, Springer Berlin Heidelberg, 2005, vol. 260, pp. 77-111.
54. R. S. Matson, J. B. Rampal and P. J. Coassin, *Analytical biochemistry*, 1994, **217**, 306-310.
55. M. S. Shchepinov, S. C. Case-Green and E. M. Southern, *Nucleic acids research*, 1997, **25**, 1155-1161.
56. V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, *Angewandte Chemie International Edition*, 2002, **41**, 2596-2599.
57. H. C. Kolb and K. B. Sharpless, *Drug discovery today*, 2003, **8**, 1128-1137.
58. R. Huisgen, *P Chem Soc London*, 1961, 357-&.
59. C. W. Tornoe, C. Christensen and M. Meldal, *The Journal of organic chemistry*, 2002, **67**, 3057-3064.
60. B. Uszczynska, T. Ratajczak, E. Frydrych, H. Maciejewski, M. Figlerowicz, W. T. Markiewicz and M. K. Chmielewski, *Lab on a chip*, 2012, **12**, 1151-1156.
61. S. Lahdenpera, J. Manninen, L. Joki, U. Karhunen and T. Soukka, *Analytical Methods*, 2014, **6**, 5360-5368.
62. V. Hagren, P. von Lode, A. Syrjala, T. Soukka, T. Lovgren, H. Kojola and J. Nurmi, *Analytical biochemistry*, 2008, **374**, 411-416.
63. M. Mirasoli, M. Guardigli, E. Michelini and A. Roda, *Journal of pharmaceutical and biomedical analysis*, 2014, **87**, 36-52.
64. S. A. Bustin, V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele and C. T. Wittwer, *Clin Chem*, 2009, **55**, 611-622.
65. Y. Haimi-Cohen, E. M. Vellozzi and L. G. Rubin, *Journal of clinical microbiology*, 2002, **40**, 898-901.
66. N. Mancini, S. Carletti, N. Ghidoli, P. Cichero, R. Burioni and M. Clementi, *Clinical microbiology reviews*, 2010, **23**, 235-251.
67. G. Domingue, J. W. Costerton and M. R. Brown, *FEMS immunology and medical microbiology*, 1996, **16**, 223-228.
68. P. P. Banada, S. Chakravorty, D. Shah, M. Burday, F. M. Mazzella and D. Alland, *PloS one*, 2012, **7**, e31126.
69. A. J. Loonen, M. P. Bos, B. van Meerbergen, S. Neerken, A. Catsburg, I. Dobbelaer, R. Penterman, G. Maertens, P. van de Wiel, P. Savelkoul and A. J. van den Brule, *PloS one*, 2013, **8**, e72349.
70. A. J. Loonen, P. F. Wolffs, C. A. Bruggeman and A. J. van den Brule, *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology*, 2014, **33**, 1687-1702.
71. B. Jonsson, A. Nyberg and C. Henning, *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica*, 1993, **101**, 595-601.