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Multianalyte lateral flow immunoassay for simultaneous detection of protein-based inflammation biomarkers and pathogen DNA

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ABSTRACT

The detection of multiple biomolecule classes in one go is highly desirable for a wide variety of areas, and in particular for point-of-care diagnostics. For example, wound infections are a major problem for patient's health and cause huge efforts in our healthcare system. In this regard, monitoring infected wounds through simultaneous detection of pathogens via nucleic acid analysis and detection of local inflammation biomarkers is key in order to enable a personalized therapy, improve the clinical outcome and thus, leading to a reduction of overall healthcare costs. In this regard, wound exudate offers an attractive sample material which can be collected in a non-invasive manner. Here, we report the development of a Multianalyte-Assay detecting inflammation biomarkers and pathogen DNA simultaneously from one sample within 35 min. Protein-compatible amplification and labeling transforms nucleic acid information into the measurement principle for protein detection. The combination with rapid detection via lateral flow immunoassay enables a fast and straightforward analysis of multiple biomolecule classes using identical assay conditions. To demonstrate the feasibility of the Multianalyte-Assay, the proinflammatory cytokine interleukin-6 (IL-6) and gDNA of the opportunistic pathogen *Pseudomonas aeruginosa* (*P. aeruginosa*) are used. The detection limits of 4 ng/mL IL-6 and 70 copies/reaction *P. aeruginosa* gDNA meet the clinically relevant range and thus, having tremendous potential to improve the wound management at the point-of-care.

1. Introduction

The development of new molecular sensors that can detect multiple classes of biomolecules (e.g. proteins and nucleic acids) is highly desirable and will revolutionize a wide variety of areas including medical diagnostics, food safety testing, environmental monitoring, and pharmaceutical product testing. Currently, biomolecules are detected by their class-specific molecular bioassays (e.g. PCR for nucleic acid detection and ELISA for protein detection). In contrast, biosensors for multianalyte analysis allow the simultaneous detection of different biomolecule classes from a single sample using identical assay conditions [1–4]. Thus, they have several notable advantages: they (1) increase the density of information per sample volume, (2) allow the detection of multiple parameters also from samples with limited

availability, (3) decrease the number of specialized instrumentation, and importantly, (4) save time and resources [1,2,4]. These undisputed advantages reflect the huge potential of multianalyte analysis especially for point-of-care (POC) diagnostics.

We have chosen wound infections as one prominent example where a simple and rapid Multianalyte-Assay is indispensable. Surgical site infections, for example, are a major problem for patient's health and cause huge effort in the healthcare system. About 160,000–300,000 surgical site infections are estimated per year only in the United States alone [5]. In this regard, monitoring infected wounds through simultaneous detection of pathogens via nucleic acid analysis and detection of local host immune response biomarkers is key in order to enable a personalized therapy, and thus, can significantly improve the clinical outcome, reduce unwanted side effects and the overall healthcare costs. Only this

combinatorial analysis can give insights into the progression of an infection and allows a personalized (antibiotic) therapy [6,7]. A rapid test monitoring inflammation markers and the presence of pathogens (1) allows to discriminate between an active and inactive infection, and (2) provides a decision tool for a rapid target-oriented therapy based on specific antibiotics. In this regard, wound exudate offers an attractive sample material which can be collected in a non-invasive manner to monitor inflammation biomarkers and pathogens [8,9]. We used *Pseudomonas aeruginosa* (*P. aeruginosa*) genomic DNA (gDNA) and interleukin-6 (IL-6) as model markers. *P. aeruginosa* is an opportunistic bacterium and one of the most common pathogens that cause nosocomial infections and delay healing in wounds [10–12]. IL-6 plays a major role in acute inflammatory response and is one of the earliest biomarkers for innate host immune response in wound infections [13,14]. Thus, the Multianalyte-Assay facilitates rapid wound monitoring and has tremendous potential to improve POC diagnostics.

However, the development of generic platforms detecting different biomolecule classes is very challenging. One needs to consider concentration differences between target nucleic acids and proteins in biological samples. Furthermore, optimal assay conditions and detection methods typically differ or are incompatible for each biomolecule [2,3,15].

In the recent years, a number of multianalyte approaches have been developed for the simultaneous detection of nucleic acids, proteins, and/or small molecules and/or cells. One concept includes the combination of probe hybridization for nucleic acid detection and antigen-antibody interaction for protein detection [1,2,7,16–23]. However, the combination of different measurement principles can lead to suboptimal reaction conditions (e.g. buffer composition, salt concentration, pH, or temperature) and thus, suffers e.g. from a low signal-to-noise ratio or require sequential analysis of the different biomolecule classes. Other approaches use protein-binding molecules (e.g. antibodies or aptamers) linked to a reporter oligonucleotide and subsequently detect proteins and nucleic acids simultaneously using the same measurement principle (e.g. amplification or sequencing) [3,24–31]. In general, these approaches require sophisticated design of several primers and/or probes and rely on complex devices, which adds to the analysis costs and hinders their usage for POC testing.

To address the need for a rapid test capable of simultaneously detecting pathogens and inflammation markers in wounds, we developed a novel Multianalyte-Assay that detects different biomolecule classes within 35 min using the same measurement principle. In this study, we show multianalyte detection using isothermal protein-compatible amplification combined with lateral flow detection. During amplification, primers and probes introduce antigenic labels, resulting in double-labeled target DNA amplicons. This allows us to use the same measurement principle – a sandwich immunoassay – for the simultaneous detection of the target proteins and labeled target amplicons. Hence, nucleic acid information is transformed into the measurement principle for protein detection and thus we called this step "nucleic acid-to-protein transformation". Furthermore, the combination of isothermal amplification and lateral flow immunoassay (LFIA) enabled a fast test result within 35 min. Therefore, we present a rapid and straightforward analysis of different biomolecule classes from a single sample, overcoming the limitations of the above-described approaches.

2. Material and methods

2.1. Reagents

The recombinase polymerase amplification kit (TwistAmp® nfo kit) was obtained from TwistDx Limited (Cambridge, United Kingdom). Primers and probes were obtained from Biomers (Ulm, Germany) and the internal amplification control (IAC)-DNA was purchased from Bio-Cat GmbH (Heidelberg, Germany). The sheep anti-digoxigenin antibody was obtained from Bio-Rad AbD Serotec GmbH (Puchheim, Germany)

and Polystreptavidin (Polystrept R) was obtained from BioTeZ Berlin Buch GmbH (Berlin, Germany). The rat anti-IL6 antibody (MQ2–39C3), rat anti-IL6 antibody (MQ2–13A5), rabbit anti-DNP antibody, donkey anti-sheep IgG antibody and the carboxylate-modified red fluorescent microspheres (FluoSpheres™, 0.2 μm, red fluorescent (580/605)) were purchased from Life Technologies GmbH (Darmstadt, Germany). The carboxylate-modified yellow fluorescent microspheres (Estapor® F1-XC 030, 0.3 μm, yellow fluorescent (470/525) were purchased from Merck Chimie SAS (Fontenay-sous-Bois Cedex, France). The donkey anti-rat IgG antibody was purchased from Novus biologicals (Littleton, Colorado, USA). Recombinant human IL-6 protein was obtained from R&D Systems, Inc. (Minneapolis, Minnesota, USA). *P. aeruginosa* (DSM 1117) gDNA was obtained from DSMZ GmbH (Braunschweig, Germany). The lateral flow dipstick material (Backing card, nitrocellulose membrane CN140 (backed) and absorption pad CF5) was obtained from Kenosha (Amstelveen, The Netherlands), Sartorius AG (Goettingen, Germany), and GE Healthcare Life Science (Freiburg, Germany), respectively.

2.2. Design of primers, probes and internal amplification control

The details regarding primer, probe and IAC-DNA design have been described by us elsewhere [32]. Briefly, the target sequence was a highly conserved region of the *lasB* gene of *P. aeruginosa* [33]. Primers and probes (listed in Table S1) were designed according to the instruction manual from TwistDx Limited (Cambridge, UK). The *lasB*-rev primer was modified at the 5′ end with a digoxigenin, whereas the *lasB*-fwd primer was not modified. The *lasB* probe was modified at the 5′ end with a biotin, an internal tetrahydrofuran (THF) residue, and at the 3′ end with a polymerase extension blocking group (C3 spacer). The RPA reaction produced a single-labeled 161 bp product which is labeled with a digoxigenin tag from the *lasB*-rev primer. The probe hybridizes to this single-labeled product and the nfo nuclease cleaves the THF residue. This leads to the removal of the blocking group and the probe can act as new primer. Thus, a second double-labeled 123 bp product was generated that is labelled with both digoxigenin and biotin. The formation of single- and double-labeled amplification products during the amplification reaction is well described in literature [32,34,35].

To exclude false negative amplification results, a competitive IAC was designed. Hoorfar et al. [36] described and discussed the practical consideration for the IAC design. The IAC-DNA consisted of a 61 bp fragment of fish virus DNA (coding region of the hemorrhagic septicemia virus from rainbow trout, accession no. X66134), flanked by *lasB* primer binding sites. Thus, the same set of primers was used for the target DNA and IAC-DNA. A specific IAC-probe was designed to hybridize to the fish virus DNA sequence. The IAC-probe was modified at the 5′ end with a dinitrophenyl, an internal THF residue, and at the 3′ end with a C3 spacer. The set of *lasB* primers and IAC-DNA probe produced a single-labeled (digoxigenin) 192 bp product and a double-labeled (digoxigenin and dinitrophenyl) 138 bp product. The sequences of the IAC-DNA and IAC-probe are listed in Table S1.

Only the double-labeled target DNA and IAC-DNA amplification products were detected via LFIA. Furthermore, previous results showed no cross-reaction of primers and probes with other pathogens (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, and *Proteus mirabilis*) associated with wound infection [32].

2.3. Synthesis of antibody-conjugated fluorescence microspheres

For the synthesis of antibody-conjugated fluorescence microspheres, we used EDC/NHS chemistry. The sheep anti-digoxigenin antibody was conjugated to 0.2 μm sized carboxylate-modified red fluorescent microspheres (580/605 nm). Therefore, 7 μg anti-digoxigenin antibody was dissolved in MES buffer (50 mM, pH 5.5). The anti-IL6 antibody (MQ2–13A5) was conjugated to 0.3 μm sized carboxylate-modified yellow microspheres (470/525 nm). Therefore, 5 μg anti-IL6 antibody

was dissolved in MES buffer (50 mM, pH 5.7). Next, 100 μg of the carboxylate-modified fluorescent microspheres and 2 μg EDC and 2 μg NHS were added. The suspension was adjusted to a final volume of 400 μL and incubated on a rotary mixer at room temperature for 2 h. To quench the reaction, 300 μg glycine was added, and the reaction mix was incubated for another 30 min. After centrifugation for 8 min at 14,000 rpm the antibody-conjugated fluorescent microspheres were washed once with storage buffer (1x PBS containing 0.05% (v/v) Tween 20, 0.5% (w/v) biotin-free BSA). Finally, the functionalized beads were resuspended in 100 μL storage buffer (to yield 0.1% bead solid) and stored in the dark at 4 °C.

2.4. Assembly of the multianalyte lateral flow dipstick

For the fabrication of lateral flow dipsticks, the backing card (6 ×30 cm), nitrocellulose membrane (4 ×30 cm) and absorbent pad (2.2 ×30 cm) were assembled accordingly. Anti-IL6 antibody (MQ2–39C3, 300 μg/mL), polystreptavidin (75 μg/mL), anti-DNP antibody (300 μg/mL), donkey anti-rat IgG antibody (500 μg/mL) and donkey anti-sheep IgG antibody (500 μg/mL) were diluted in PBS containing 0.1% (w/v) biotin-free BSA and 1% (w/v) trehalose. The solutions were printed (1 μL/cm) onto the nitrocellulose membrane with a line-to-line distance of 2.5 mm using a line printer (AD3220™ Aspirate/Dispense Platform, BioDot Limited, Chichester, United Kingdom). Anti-IL6 antibody and polystreptavidin were used for the protein test line (Protein-TL) and DNA test line (DNA-TL), respectively. The anti-DNP antibody was used for the IAC control line. For the two flow control lines (FC-1 and FC-2) we used secondary antibodies against rat IgGs and sheep IgGs, respectively. The lateral flow dipstick sheets were dried for at least 24 h at room temperature (RT) and subsequently cut into lateral flow dipsticks with a width of 4.4 mm using a guillotine cutter (A-Point Guillotine Cutter, Arista Biologicals Inc, Allentown, Pennsylvania, USA). The lateral flow dipsticks were stored at RT in a box containing silica gel until use.

2.5. DNA reference assay procedure

For the detection of *P. aeruginosa* gDNA via DNA reference assay, the gDNA was added directly to the recombinase polymerase amplification (RPA) reaction. The reaction was performed in a 50 μL volume using the TwistAmp® nfo kit (TwistDx limited, Cambridge, UK). Briefly, 29.5 μL 1x rehydration buffer was mixed with 1.25 μL *lasB*-fwd primer (10 μM), 1.25 μL *lasB*-rev primer (10 μM), 1.2 μL *lasB* probe (10 μM), 1 μL of IAC-DNA (230 copies) and 11.1 μL ddH₂O. Subsequently, 1 μL of *P. aeruginosa* gDNA (final concentration: 10^0-10^6 copies/reaction) were added to the RPA reaction. Next, the RPA reaction pellet and 2.5 μL of magnesium acetate (280 nM) were added. Subsequently, the reaction was incubated for 20 min at 37 °C. The RPA reaction was diluted 1:10 in 50 μL running buffer (10 mM carbonate/bicarbonate buffer, pH 9.6) containing 180 μg/mL anti-digoxigenin-conjugated microspheres, 0.5% (w/v) biotin-free BSA, and 0.1% (v/v) Tween 20. The multianalyte lateral flow strip was dipped into the solution, incubated for 15 min at RT and was imaged in a fluorescence microscope (Lionheart LX Automated Microscope, BioTek Instruments Inc., Bad Friedrichshall, Germany). The fluorescence intensity of DNA-TL, IAC, and FC-2 was determined by image analysis using ImageJ (Fiji is just ImageJ).

2.6. IL-6 reference assay procedure

For the IL-6 reference assay no RPA reaction was performed. Instead, 45 μL PBS was mix with 5 μL IL-6 (final concentration: 1–200 ng/mL). Subsequently the solution was diluted 1:10 in running buffer (10 mM carbonate/bicarbonate buffer, pH 9.6) containing 30 μg/mL anti-IL6-conjugated microspheres, 0.5% (w/v) biotin-free BSA, and 0.1% (v/v) Tween 20. Again, the multianalyte lateral flow strip was dipped into the solution, incubated for 15 min at RT and was imaged in a fluorescence

microscope (Lionheart LX Automated Microscope, BioTek Instruments Inc., Bad Friedrichshall, Germany). The fluorescence intensity of Protein-TL and FC-1 was determined by image analysis using ImageJ (Fiji is just ImageJ).

2.7. Multianalyte-Assay procedure

For simultaneous detection of *P. aeruginosa* gDNA and IL-6 via Multianalyte-Assay both biomarkers were added directly to the RPA reaction, which was performed as described above, but with the following changes. The amount of ddH₂O was decreased to 6.1 μL. Furthermore, 5 μL of IL-6 (final concentration: 1–200 ng/mL) were added to the RPA reaction. Subsequently the reaction was diluted 1:10 in 50 μL running buffer (10 mM carbonate/bicarbonate buffer, pH 9.6) containing 180 μg/mL anti-digoxigenin-conjugated microspheres, 30 μg/mL anti-IL6-conjugated microspheres, 0.5% (w/v) biotin-free BSA, and 0.1% (v/v) Tween 20. Again, the multianalyte lateral flow strip was dipped into the solution, incubated for 15 min at RT and was imaged in a fluorescence microscope (Lionheart LX Automated Microscope, BioTek Instruments Inc., Bad Friedrichshall, Germany). The fluorescence intensity of Protein-TL, DNA-TL, IAC, FC-1 and FC-2 was determined by image analysis using ImageJ (Fiji is just ImageJ).

2.8. Statistical analysis

All experiments were performed in triplicates and all measurements were conducted three times per experiment. Statistical analysis was performed using Origin (OriginLab Corporation, Northampton, Massachusetts, USA). For curve fitting analysis a four parameter logistic non-linear regression model (PL4) was used. The LOD was calculated for each biomarker (IL-6 and *P. aeruginosa* gDNA) from the mean fluorescence intensity (y) and standard deviation (SD) of the blank and of a low concentration sample [37] (see Eq. 1).

$$y_{LOD} = (y_{blank} + 1.645 * SD_{blank}) + 1.645 * SD_{low\ concentration\ sample} \tag{1}$$

By interpolating the calculated fluorescence intensity of the LOD (y_{LOD}) into the sigmoidal fit curve equation (see Table S4 and Table S5), the corresponding concentration and confidence interval was calculated.

3. Results and discussion

3.1. Principle of the Multianalyte-Assay for the simultaneous detection of DNA and protein biomarkers

We are aiming to develop a Multianalyte-Assay for wound monitoring. The assay should enable the simultaneous detection of pathogens via nucleic acid analysis and the local host immune response from one sample. Only this combinatorial analysis allows to differentiate between active and inactive infection and offers a decision tool for a target-oriented therapy. However, the development of generic platforms capable of detecting nucleic acid and protein biomarkers is very challenging. For example, the reaction conditions typically used for the detection of proteins and nucleic acids are not compatible. Furthermore, the multianalyte analysis should be carried out at the POC to offer the patient a fast and personalized therapy. Thus, a rapid and straightforward assay needs to be designed that can be integrated into a POC test.

To mind the different concentration ranges of nucleic acids (varying from under 100 bacterial genomes at the normal skin to up to 10^{10} bacteria genomes/swab in wounds, thus raging from fg/mL to ng/mL [38]) and proteins (typically ranging from pg/mL to ng/mL for pro- and anti-inflammatory cytokines [39]), DNA amplification is required. However, thermal cycling – which is typically used to amplify DNA – would denature the target protein. Therefore, we used isothermal amplification that can be carried out at lower temperatures (37–42 °C).

We selected the RPA as a sensitive and fast isothermal amplification method, which is able to operate at 37 °C [40]. The RPA enables amplification of the target DNA within 20 min, without denaturing the target protein. During amplification, primer and probes introduced antigenic labels, resulting in double-labeled (digoxigenin and biotin) target DNA amplicons (see Fig. 1A). In this way, nucleic acid information was transformed into the measurement principle for protein detection. Therefore, we called this step "nucleic acid-to-protein transformation". This allowed us to use the same measurement principle – a sandwich immunoassay – for the simultaneous detection of the target proteins and labeled target amplicons. Thus, overcoming limitations like incompatible reaction and buffer conditions that typically occur when different measurement principles are used.

The combination of isothermal amplification and LFIA is the ideal choice for a rapid test at the POC and eliminates the requirement of complex devices, and thus, reduces the analysis costs. We designed a LFIA comprising two test lines (TL) and three control lines (see Fig. 1 B and C). Using antibody-conjugated fluorescent microspheres, a fluorescence signal was generated at the corresponding test and control lines. In the presence of target protein, a signal was generated at the Protein-TL, which represents a positive test result for the target protein. Whereas, in the presence of double-labeled target DNA amplicons, a signal was generated at the DNA-TL, which represents a positive test result for the target DNA. Furthermore, we integrated mandatory controls. To exclude false negative amplification results, a competitive IAC was designed that was co-amplified with the target DNA. This means, the same set of primers was used to amplify IAC- and target DNA. A specific IAC-probe allowed the separate detection of the IAC-DNA amplicons. Since, IAC- and target DNA compete for the same primers, the IAC-DNA concentration needs to be held at the lowest concentration leading to reproducible IAC-DNA amplification [36]. A minimum of 230 copies IAC-DNA/reaction was required for a clear IAC signal (see Fig. S1). The double-labeled (digoxigenin and dinitrophenyl) IAC-DNA amplicons were binding to a separate control line, excluding false negative amplification results. Flow controls (FC-1 and FC-2) ensured the functionality of the different antibody-conjugated fluorescent microspheres and showed whether the sample was processed appropriately.

To proof the principle, we added IL-6 and/or *P. aeruginosa* gDNA directly to the RPA reaction. After "nucleic acid-to-protein transformation", the biomolecules were detected via LFIA. In Fig. 1C (bottom) representative fluorescence images of the lateral flow strips are shown. For samples containing IL-6 (200 ng/mL) we observed a

fluorescence signal (8,965,210 RFU) at the Protein-TL, whereas for samples without IL-6 a background of 159,896 RFU was determined at the Protein-TL. Samples containing gDNA (10^6 copies/reaction) generated a fluorescence signal (17,619,909 RFU) at the DNA-TL. For samples without gDNA a background signal of 2,537,105 RFU was observed at the DNA-TL. These results confirm the successful development of a Multianalyte-Assay simultaneously detecting different biomolecules classes from one sample in 35 min.

Taken together, we are using "nucleic acid-to-protein transformation" to subsequently detect protein and DNA biomarkers with the same measurement principle, overcoming the limitations of for the detection of different biomolecule classes. By combining isothermal protein-compatible amplification (20 min) and lateral flow detection (15 min), we developed a rapid (35 min) and straightforward Multianalyte-Assay. Thus, our Multianalyte-Assay fulfills all requirements for a rapid test that can be used to simultaneously detect the local host immune response and pathogens in wound exudate.

3.2. Characterization of the Multianalyte-Assay

After successfully demonstrating the ability to perform simultaneous detection of nucleic acid and protein biomarkers in principle, further characterization of the Multianalyte-Assay was conducted. First, the multianalyte detection of IL-6 and *P. aeruginosa* gDNA was compared to the single-plex detection via corresponding reference assay. In addition, we added IL-6 after the amplification reaction directly to the LFIA ("IL-6 added after RPA" assay) and compared the results to the Multianalyte-Assay and reference assays. The corresponding results and representative lateral flow strips are shown in Fig. 2. To compare Multianalyte-Assay, reference assays, and "IL-6 added after RPA" assay, we calculated the signal difference between the different assays (see Table S3) from the determined fluorescence intensity at the Protein- and DNA-TL (see Table S2 and Table S5).

For single-plex detection of the target protein via IL-6 reference assay, the sample (containing 0, 10, 50 or 200 ng/mL IL-6) was applied to the test strip. Subsequently, the fluorescence intensity of the captured IL-6 at the Protein-TL was determined and compared with the Multianalyte-Assay (samples contained 0, 10, 50 or 200 ng/mL IL-6 combined with 10^6 copies/reaction gDNA). For both assays, the fluorescence signal at the Protein-TL was increasing with the amount of IL-6. Furthermore, the fluorescence signals were distinguishable from the background (0 ng/mL IL-6), confirming again the successful detection of IL-6 via Multianalyte-Assay. In general, the background signal at the
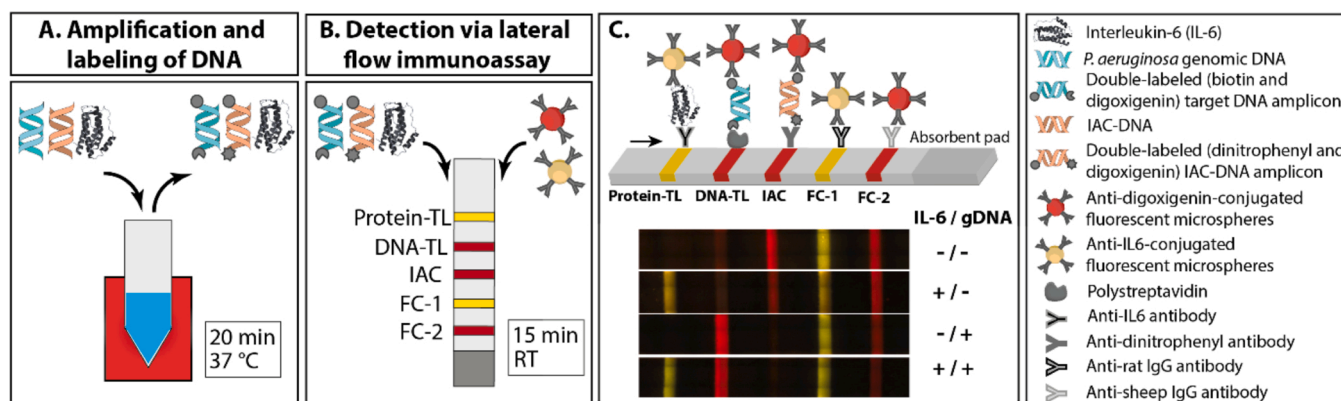

Fig. 1. Principle of the Multianalyte-Assay for the simultaneous detection of DNA and protein biomarkers. (A) Isothermal protein-compatible amplification and labeling of target DNA and IAC-DNA at 37 °C for 20 min (B) Simultaneous detection of protein and DNA biomarkers via LFIA using antibody-conjugated fluorescent microspheres. **(C)** Schematic drawing of the LFIA for multianalyte detection including representative fluorescence images of the respective results for samples with (+) or without (-) IL-6 and/or *P. aeruginosa* gDNA. The IL-6 is binding to the Protein-TL (yellow), whereas the labeled target amplicons bind to the DNA-TL (red). The labeled IAC-DNA amplicons bind to a separate control line (IAC, red) and exclude false negative amplification results. Two FCs (yellow and red) ensure the functionality of the antibody-conjugated fluorescent microspheres and show whether the sample was processed appropriately. TL, test line; IAC, internal amplification control; FC, flow control.
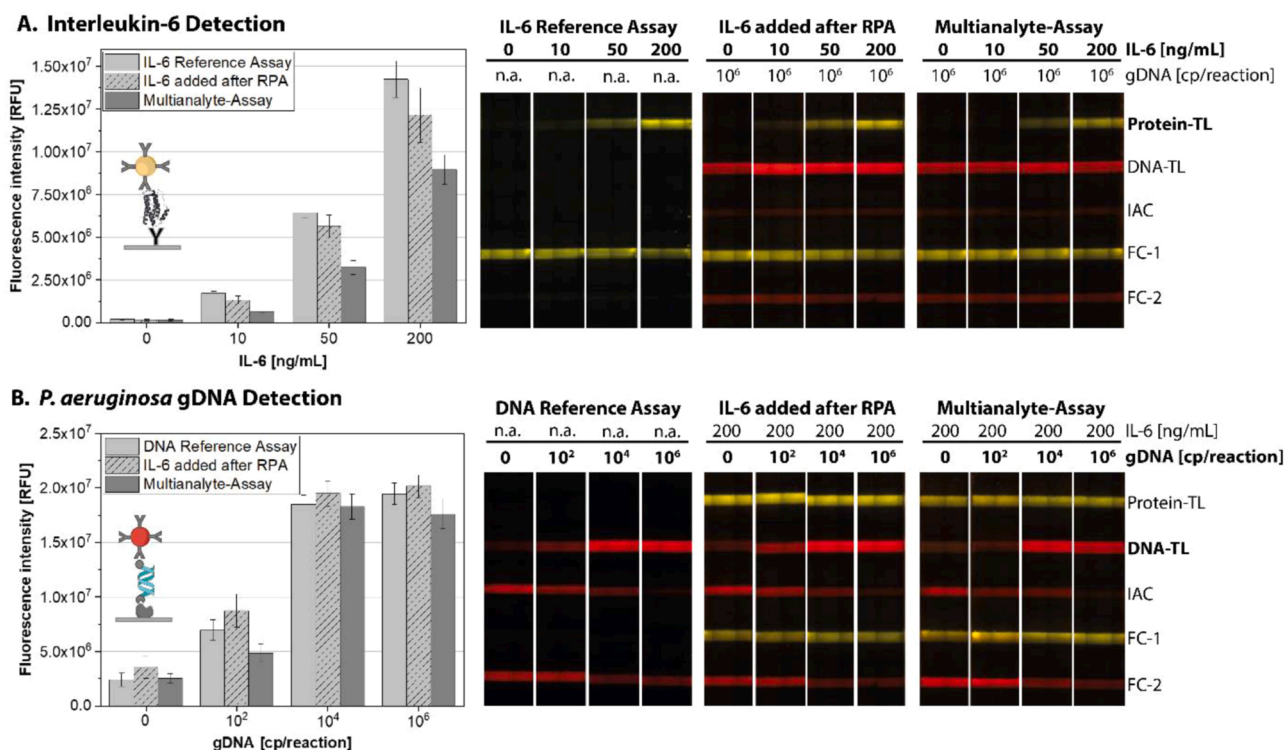
Fig. 2. Compatibility of simultaneous protein and nucleic acid detection. (A) IL-6 (0, 10, 50, and 200 ng/mL) was added to the RPA reaction (Multianalyte-Assay) or after RPA reaction directly to the LFIA ("IL-6 added after RPA" assay). The fluorescence intensity of the Protein-TL (yellow) was compared with the IL-6 reference assay. Representative lateral flow strips are illustrated on the right side. (B) Influence of the IL-6 on the amplification and detection of *P. aeruginosa* gDNA (0, 10^2, 20^4, and 10^4 copies/reaction). IL-6 (200 ng/mL) was added to the RPA reaction (Multianalyte-Assay of after the RPA reaction directly to the LFIA ("IL-6 added after RPA" assay). The fluorescence intensity of the DNA-TL (red) was compared with the DNA reference assay. Representative lateral flow strips are illustrated on the right side. The IAC (red) excluded false negative results and together with the FCs (yellow and red) ensured the validity of the test result. The experiments were conducted three times in triplicates. The error bars indicate one standard deviation. cp, copies; TL, test line; IAC, internal amplification control; FC, flow control.

Protein-TL was lower for the Multianalyte-Assay compared to the IL-6 reference assay (16% signal difference). For samples containing IL-6 (10, 50, 200 ng/mL) we observed a signal difference of 37–63% between IL-6 reference assay versus Multianalyte-Assay. In general, cytokines are known for their short *in vivo* half-life time [41]. Thus, we speculate that the signal difference might originate from degraded IL-6 due to the longer process time (35 min Multianalyte-Assay vs. 15 min IL-6 reference assay). To investigate if the components of the RPA influence the IL-6 detection we compared IL-6 reference assay and "IL-6 added after RPA" assay. For the background signal, we observed a signal difference of 16%. Similar signal differences (12–23%) were observed for samples containing IL-6 (10, 50, 200 ng/mL). Since the background of lateral flow assays depends on the sample matrix, this signal difference is not unusual and was expected. Thus, we concluded that IL-6 detection was not influenced by the RPA components per se, but the increased process time might degrade a portion of the instable cytokine. The addition of protein stabilizers (such as sugars, polyols, surfactants or amino acids [42]) might be an option to reduce the denaturation and thus help to improve the Multianalyte-Assay further. Nevertheless, we show the successful detection of IL-6 in presence of gDNA, thereby proofing our concept for multianalyte detection.

For single-plex detection of the *P. aeruginosa* gDNA via DNA reference assay, samples (containing 0, 10^2, 10^4 or 10^6 copies/reaction *P. aeruginosa* gDNA) were amplified and labeled via RPA. Subsequently, the amplification product was applied to the test strip and the fluorescence intensity of the captured amplicons at the DNA-TL was determined and compared with the Multianalyte-Assay (samples contained 0, 10^2, 10^4 or 10^6 copies/reaction gDNA combined with 200 ng/mL IL-6). For both assays, the fluorescence signal was increasing with the amount of gDNA whereby the fluorescence signals for 10^4 and 10^6 copies/

reaction were comparable. Fluorescence signals at the DNA-TL were distinguishable from the background (0 copies/reaction), confirming the successful detection of gDNA via Multianalyte-Assay. The background signal of the DNA reference assay was similar to the one of the Multianalyte-Assay (6% signal difference). Furthermore, for samples containing 10^4 and 10^6 copies/reaction, the fluorescence intensities were similar for both assays (1% and 9% signal difference). Only for samples containing 10^2 copies/reaction gDNA a signal difference of 30% was observed. The addition of IL-6 to the RPA is increasing the complexity of the sample, which might affect the amplification efficiency. In general, most of the known amplification inhibitors are organic compounds like urea, phenol, melanin as well as different proteins like myoglobin, hemoglobin or immunoglobulin G [43]. Substances like betaine, BSA, trehalose or pullulan are reported to enhance isothermal amplification and thus could help to improve the Multianalyte-Assay further [44]. To further investigate the influence of IL-6 on the amplification reaction, we compared the results of the Multianalyte-Assay to the "IL-6 added after RPA" assay. For the background signal, we observed a signal difference of 13% and for samples containing 10^4 and 10^6 copies/reaction gDNA, a signal difference of 6% and 13% was determined, respectively. Again, for samples containing 10^2 copies/reaction a fluorescence signal difference of 44% was observed. Thus, supporting the considerations, that the addition of IL-6 increases the complexity of the sample and thereby, reducing the amplification efficiency. This observation is further proofed by the comparison of DNA reference assay and "IL-6 added after RPA" assay. The addition of IL-6 after the amplification reaction did not lead to a reduction of the fluorescence signal for samples containing 10^2 copies/reaction after background subtraction. Nevertheless, it is possible to distinguish 10^2 copies/reaction gDNA from the background

for both DNA reference assay and Multianalyte-Assay. Therefore, we show the successful detection of *P. aeruginosa* gDNA in presence of IL-6 via Multianalyte-Assay.

3.3. Analytical performance of the Multianalyte-Assay

To evaluate the analytical performance of the Multianalyte-Assay, we calculated the LOD for each biomarker in presence of the other biomarker. Therefore, a serial dilution of IL-6 or gDNA was combined with a high concentration of the other biomarker. From this combination, one would expect to observe the highest influence of the biomarker on the performance of the assay. This means, 1–200 ng/mL IL-6 were mixed with 10^6 copies/reaction gDNA, whereas, 1–10^6 copies *P. aeruginosa* gDNA were mixed with 200 ng/mL IL-6. The corresponding response curves and representative lateral flow strips are shown in Fig. 3. The LODs for IL-6 and *P. aeruginosa* gDNA are 4 ng/mL and 70 copies/reaction (1.4×10^3 copies/mL), respectively. The detection limits were compared to those of the reference assays (1 ng/mL for IL-6 and 13 copies/reaction for *P. aeruginosa* gDNA). The specificity of primers and probes was tested previously by us [32]. We observed no cross-reaction with other pathogens associated with wound infection (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus aga-lactiae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, and

Proteus mirabilis).

As expected, the LOD of the Multianalyte-Assay for the detection of IL-6 increased, which is in agreement with the characterization experiments. We speculated that the increased process time of the Multianalyte-Assay might lead to the degradation of IL-6. The addition of protein stabilizers might help to improve the Multianalyte-Assay further. Since IL-6 is a pro- and anti-inflammatory cytokine which is released at the site of e.g. infection, the IL-6 levels in wound exudate are several fold higher compared to serum [39,45]. Holzheimer and Stein-metz [39] for example measured IL-6 levels up to 271.7 ng/mL in wound exudate of patients undergoing operative surgery. 54 h postoperatively they observed a decreased to 53.75 ng/mL. Thus, we assume a LOD of 4 ng/mL is sufficient for a rapid test detecting IL-6 in wound exudate. However, further investigations are required regarding the correlation of inflammation marker levels in wound exudate and the progression of infected wounds. Furthermore, we also determined the fluorescence intensity for 10^6 copies/reaction gDNA (DNA-TL) that were added to each sample. As shown in Fig. S2A the signal stayed constant regardless the amount of IL-6 (1–200 ng/mL). Thus, the detection of *P. aeruginosa* gDNA via Multianalyte-Assay is not influenced by the amount of IL-6.

Comparing the DNA reference assay and Multianalyte-Assay (see Fig. 3B) we observed, as expected, a 5.4-fold increase of the LOD for the Multianalyte-Assay. As discussed above, the addition of IL-6 is
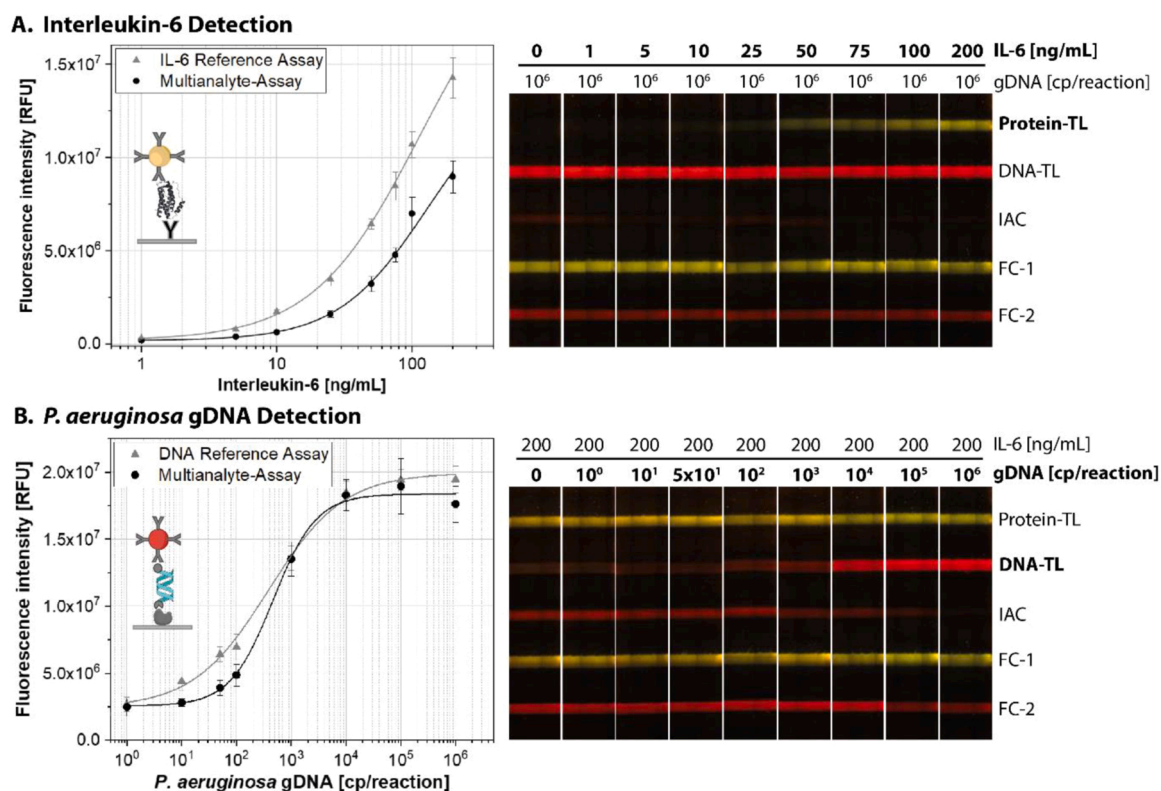

Fig. 3. Analytical sensitivity of the Multianalyte-Assay for the simultaneous detection of IL-6 and *P. aeruginosa* gDNA and comparison with the corresponding reference assays. (A) Response curves for the detection of IL-6 via IL-6 reference assay (grey) and Multianalyte-Assay (black). For the IL-6 reference assay, samples containing 1–200 ng/mL IL-6 were directly analyzed via LFIA. For the detection via Multianalyte-Assay, samples containing 1–200 ng/mL IL-6 and 10^6 copies/reaction gDNA were added to the RPA reaction. Subsequently, IL-6 and the labeled amplicons were detected via LFIA (see fluorescence images of the lateral flow strips on the right side). The LOD was determined using a sigmoidal fit for the fluorescence intensities measured at the Protein-TL (yellow). The analysis of the DNA-TL (red) – 10^6 copies/reaction gDNA were added to each sample – is shown in Fig. S2A. (B) Response curves for the detection of *P. aeruginosa* gDNA via DNA reference assay (grey) and Multianalyte-assay (black). For the DNA reference assay, samples containing 10^0-10^6 copies/reaction gDNA were added to the ampli-fication reaction. Subsequently the labeled amplicons were detected via LFIA. For the detection via Multianalyte-Assay, samples containing 10^0-10^6 copies/reaction gDNA and 200 ng/mL IL-6 were added to the RPA reaction and subsequently both biomolecules were detected via LFIA (see fluorescence images of lateral flow strips on the right side). The LOD was determined using a sigmoidal fit for the fluorescence intensities measured at the DNA-TL (red). The analysis of the Protein-TL (yellow) – 200 ng/mL were added to each sample – is shown in Fig. S2B. The IAC (red) excluded false negative results and together with the FCs (yellow and red) ensured the validity of the test result. The experiments were conducted three times in triplicates. The error bars indicate one standard deviation. For sigmoidal fit curve analysis see Table S4. cp, copies; TL, test line; IAC, internal amplification control; FC, flow control.

increasing the complexity of the sample and thereby decreased the amplification efficiency. In general it is believed that a microbial load of $> 10^5$ CFU per mL wound exudate or g of tissue is required to reach a stage of local infection [46–48]. Thus, our Multianalyte-Assay (LOD of 1.4×10^3 copies/mL) meets the requirements of a rapid test for the detection of pathogens. Furthermore, we also determined the fluorescence intensity for 200 ng/mL IL-6 (Protein-TL) that were added to each sample. As shown in Fig. S2B the signal stayed constant regardless the amount of *P. aeruginosa* gDNA ($1–10^6$ copies/reaction). Thus, the detection of IL-6 via Multianalyte-Assay is not influenced by the amount of gDNA.

4. Conclusion

We successfully developed a rapid lateral flow based Multianalyte-Assay that is capable of detecting *P. aeruginosa* gDNA and IL-6 simultaneously within 35 min. Using "nucleic acid-to-protein transformation" protein and DNA biomarkers can be detected simultaneously with the same measurement principle, thereby overcoming the limitations for the detection of different biomolecule classes. Thus, our work significantly exceeds the current state of the art by (1) implementing a rapid and easy workflow for the simultaneous detection of pathogen DNA and local inflammation biomarkers that can be integrated into a single lateral flow strip, and (2) using for the first time "nucleic acid-to-protein transformation" combined with rapid detection via LFIA to simultaneously analyze different biomolecule classes using identical assay conditions. Thus, our strategy facilitates fast therapy decisions in time-critical wound infection by (1) providing a decision tool for a rapid target-oriented therapy by detecting the gDNA of the pathogen, and (2) allows the discrimination between active and inactive infection by detecting the local immune response. The LODs of the Multianalyte-Assay are meeting the clinical relevant range and controls ensure the validity of the test result. The combination of non-invasive sampling and "sample-in-answer-out" diagnostic devices is the key to enable a rapid and personalized therapy at the POC [8,49]. In this regard, wound exudate offers an attractive sample that can be collected in a non-invasive manner to monitor pathogens and the local immune response in the wound. To realize an integrated diagnostic device for wound monitoring, further research work is required regarding multianalyte-compatible sample preparation. Furthermore, we will focus on integrating all fundamental operations steps into one single paper-based device as shown previously by Lafleur and Bishop et al. [50] for a nucleic acid amplification test detecting methicillin-resistant *Staphylococcus aureus*. Moreover, our assay concept paves the way towards multianalyte detection not only at the POC but could also be used for the simultaneous detection of various biomolecules in other applications areas such as food safety and environmental monitoring.

CRediT authorship contribution statement

Anna Klebes: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization, Project administration. **Anna-Sophia Kittel:** Formal analysis, Investigation, Writing – review & editing, Visualization. **René D. Verboket:** Conceptualization, Formal analysis, Writing – review & editing. **Felix von Stetten:** Methodology, Formal analysis, Writing – review & editing, Supervision. **Susanna M. Früh:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

Anna Klebes and Susanna M. Früh have filed a patent application based on this work (European provisional patent application EP20189814.5).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2021.131283.

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