

## Supporting Information

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

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#### Table of Content:

**Figure S1.** Optimization of the IAC-DNA concentration

**Figure S2.** Analysis of the fluorescence signal at the DNA- and Protein-TL and representative lateral flow strips of the Multianalyte-Assay.

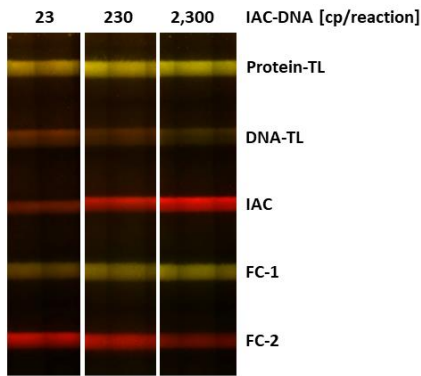
**Table S1.** Primers, probes and internal amplification control-DNA sequences.

**Table S2.** Mean, standard deviation (SD), and coefficient of variation (CV) of the “IL-6 added after RPA” assay.

**Table S3.** Comparing Multianalyte-Assay, reference assays, and “IL-6 added after RPA” assay regarding signal differences at the Protein- and DNA-TL.

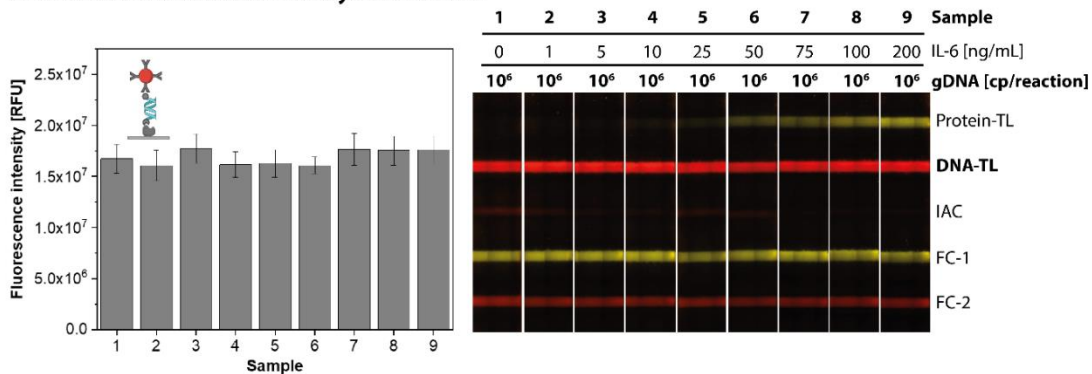
**Table S4.** Sigmoidal fit curve analysis.

**Table S5.** Mean, standard deviation (SD), coefficient of variation (CV) and limit of detection (LOD) of the Multianalyte-Assay and reference assays.

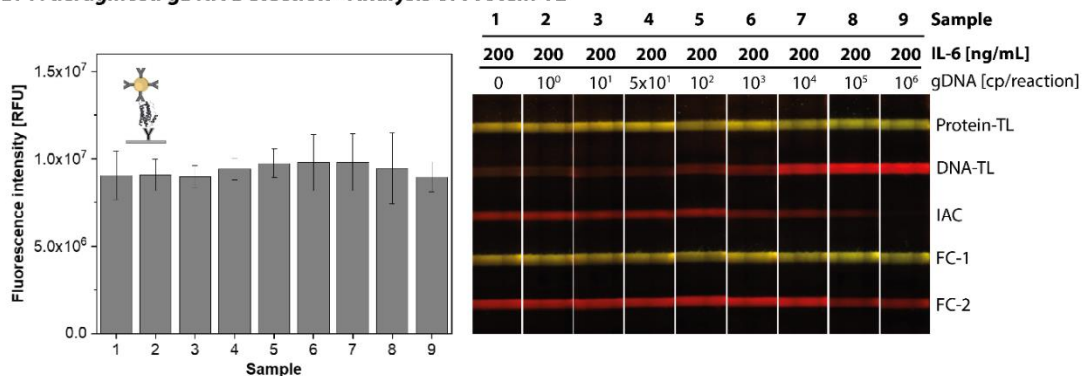


**Figure S1.** Optimization of the IAC-DNA concentration. To exclude false negative amplification results, a competitive IAC was designed that is co-amplified with the target DNA. This means, the same set of primers was used to amplify IAC- and target DNA. A specific IAC-probe allowed the separate detection 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. We added 23 cp/reaction, 230 cp/reaction, or 2,300 cp/reaction of IAC-DNA to the Multianalyte-Assay (200 ng/ml IL-6 and 0 cp/reaction *P. aeruginosa* gDNA). A minimum of 230 cp/reaction was required for a clear IAC signal. cp, copies; TL, test line; IAC, internal amplification control; FC, flow control.

**A. Interleukin-6 Detection - Analysis of DNA-TL**



**B. *P. aeruginosa* gDNA Detection - Analysis of Protein-TL**



**Figure S2.** Analysis of the fluorescence signal at the DNA- and Protein-TL and representative lateral flow strips of the Multianalyte-Assay. **(A)** Analysis of the DNA-TL (red) for samples (Sample 1-9) containing 10<sup>6</sup> copies/reaction *P. aeruginosa* gDNA combined with 1-200 ng/mL IL-6. Since, the same amount of gDNA was added to each sample we observed a constant fluorescence signal at 16,877,612±1,470,312 RFU (CV of 8.7 %). The analysis of the Protein-TL (yellow) – 1-200 ng/mL were

added to each sample – is shown in Figure 3. **(B)** Analysis of the Protein-TL (yellow) for samples (Sample 1-9) containing 200 ng/mL IL-6 combined with  $10^0$ - $10^6$  copies/reaction gDNA. Since, the same amount of IL-6 was added to each sample we observed a constant fluorescence signal at  $9,371,882 \pm 1,242,955$  RFU (CV of 13.3 %). The analysis of the DNA-TL (red) –  $10^0$ - $10^6$  copies/reaction were added to each sample – is shown in Figure 3. 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; CV, coefficient of variation.

**Table S1.** Primers, probes and internal amplification control-DNA sequences.

Name	Sequence (5'-3')
<i>lasB</i> -fwd primer	GAGAATGACAAAGTGGAAGTGGTGTATCCGCCTG
<i>lasB</i> -rev primer	Dig-GCCAGGCCTTCCCAGTATCGAGCACTTCGCCG
<i>lasB</i> probe	Biotin-GAACAACATCGCCCAACTGGTCTA CAACGT[THF]TCCTACCTGATTCCC-C3 spacer
IAC-probe	DNP-CAACTGCAGGGACGATTCCCTTTGTCC CGAT[THF]CGACCAGCTCAACTC-C3 spacer
IAC-DNA	AAGACCGAGAATGACAAAGTGGAACTGGTGTATCCGCCTGGGCGATATAC ACTCATCCCTCCAAGTGCAGGGACGATTCCCTTTGTCCCGATTTCGACCAGC <u>TCAACTCAGGTGTCCTCATGAAGGCGAGGGACTGTCGCGGCCGCATTTCG</u> TCATCGACGCCAAGACCGCGAAGTGCTCGATCAGTGGGAAGGCCTGGC CCACGC

Dig, digoxigenin; THF, tetrahydrofuran; C3 spacer, polymerase extension blocking group; DNP, dinitrophenyl; IAC-DNA, internal amplification control-DNA; underlined sequence, fish virus DNA sequence.

**Table S2.** Mean, standard deviation (SD), and coefficient of variation (CV) of the “IL-6 added after RPA” assay. The IL-6 was added after the RPA reaction directly to the LFIA.

Mean, SD, and CV of “IL-6 added after RPA” Assay (Figure 2)							
Sample (IL-6 was added after RPA to the LFIA)							
IL-6 [ng/mL]	0	10	50	200	200	200	200
gDNA [cp/react.]	$10^6$	$10^6$	$10^6$	$10^6$	$10^4$	$10^2$	0
Analysis of the Protein-TL							
Mean [RFU]	159,896	1,327,839	5,646,520	12,148,815	12,552,290	12,713,553	12,694,232
SD [RFU]	52,154	232,325	677,719	1,599,146	1,884,486	1,633,700	1,695,599
CV [%]	33	17	12	13	15	13	13
Analysis of the DNA-TL							

Mean [RFU]	16,741,322	19,800,875	19,919,971	20,158,056	19,471,840	8,747,130	3,575,449
SD [RFU]	1,418,412	904,239	889,147	1,049,164	1,129,021	1,511,582	1,029,275
CV [%]	8	5	4	5	6	17	29

**Table S3.** Comparing Multianalyte-Assay, reference assays, and “IL-6 added after RPA” assay regarding signal differences at the Protein- and DNA-TL.

	Signal difference: Protein-TL					
	IL-6 Reference Assay vs. Multianalyte-Assay		IL-6 Reference Assay vs. “IL-6 added after RPA” assay		“IL-6 added after RPA” assay vs. Multianalyte-Assay	
IL-6 [ng/mL]	Δ [RFU]	Δ [%]	Δ [RFU]	Δ [%]	Δ [RFU]	Δ [%]
0	30,530	16	30,530	16	0	0
10	1,089,513	63	401,985	23	687,527	52
50	3,207,760	50	789,537	12	2,418,223	43
200	5,291,471	37	2,107,866	15	3,183,605	26
	Signal difference: DNA-TL					
	IL-6 Reference Assay vs. Multianalyte-Assay		IL-6 Reference Assay vs. “IL-6 added after RPA” assay		“IL-6 added after RPA” assay vs. Multianalyte-Assay	
gDNA [cp/reaction]	Δ [RFU]	Δ [%]	Δ [RFU]	Δ [%]	Δ [RFU]	Δ [%]
0	138,362	6	-534,032	22	395,671	13
10 <sup>2</sup>	2,089,428	30	-1,789,996	26	3,879,423	44
10 <sup>4</sup>	194,218	1	-1,013,333	5	1,207,551	6
10 <sup>6</sup>	1,832,884	9	-705,263	-4	2,538,147	13

**Table S4.** Sigmoidal fit curve analysis.

<b>Sigmoidal fit curve analysis (Figure 3a, IL-6 Reference Assay)</b>	
<b>Function</b>	$y = A2 + (A1-A2)/(1 + (x/x0)^p)$ $A1 = 191684.28107 \pm 24356.00949$ $A2 = 2.19379E7 \pm 3074376.6902$ $x0 = 112.58296 \pm 26.19409$ $p = 1.10708 \pm 0.05592$
<b>R<sup>2</sup></b>	0.99768
<b>Sigmoidal fit curve analysis (Figure 3a, Multianalyte-Assay, IL-6 Detection)</b>	
<b>Function</b>	$y = A2 + (A1-A2)/(1 + (x/x0)^p)$ $A1 = 181540.24919 \pm 19802.54785$ $A2 = 1.42862E7 \pm 2207058.25379$ $x0 = 129.15303 \pm 28.70075$ $p = 1.31798 \pm 0.07849$
<b>R<sup>2</sup></b>	0.99624
<b>Sigmoidal fit curve analysis (Figure 3b, DNA Reference Assay)</b>	
<b>Function</b>	$y = A2 + (A1-A2)/(1 + (x/x0)^p)$ $A1 = 2437591.22467 \pm 249738.02004$ $A2 = 1.99388E7 \pm 403727.7639$ $x0 = 382.3198 \pm 65.46922$ $p = 0.63236 \pm 0.05258$
<b>R<sup>2</sup></b>	0.99798
<b>Sigmoidal fit curve analysis (Figure 3b, Multianalyte-Assay, DNA detection)</b>	
<b>Function</b>	$y = A2 + (A1-A2)/(1 + (x/x0)^p)$ $A1 = 2560822.58359 \pm 99063.43614$ $A2 = 1.8367E7 \pm 325327.72593$ $x0 = 466.35795 \pm 50.61403$ $p = 1.10038 \pm 0.07435$
<b>R<sup>2</sup></b>	0.99839

**Table S5.** Mean, standard deviation (SD), coefficient of variation (CV) and limit of detection (LOD) of the Multianalyte-Assay and reference assays.

Mean, SD, CV, and LOD of sigmoidal fit curve analysis (Figure 3a, IL-6 Reference Assay)									
Sample									
IL-6 [ng/mL]	0	1	5	10	25	50	75	100	200
Analysis of the Protein-TL									
Mean [RFU]	190,426	316,137	782,135	1,729,825	3,485,327	6,436,057	8,473,509	10,688,265	14,256,681
SD [RFU]	38,281	41,837	84,614	128,118	355,295	284,760	763,074	673,234	1,080,644
CV [%]	20	13	11	7	10	4	9	6	8
LOD [RFU]	$Y_{LOD} = (190,426 + 1.645 * 38,281) + 1.645 * 41,837 = 322,219$ RFU								
LOD [ng/mL]	1.1 ng/mL (95 % confidence interval: n.a.)								
Mean, SD, CV, and LOD of sigmoidal fit curve analysis (Figure 3a, Multianalyte-Assay)									
Sample									
IL-6 [ng/mL]	0	1	5	10	25	50	75	100	200
gDNA [cp/react.]	10 <sup>6</sup>	10 <sup>6</sup>	10 <sup>6</sup>	10 <sup>6</sup>	10 <sup>6</sup>	10 <sup>6</sup>	10 <sup>6</sup>	10 <sup>6</sup>	10 <sup>6</sup>
Analysis of the Protein-TL									
Mean [RFU]	159,896	212,279	396,569	640,312	1,604,045	3,228,297	4,775,894	6,994,765	8,965,210
SD [RFU]	52,154	41,125	61,961	76,463	169,471	412,994	379,893	896,651	856,333
CV [%]	33	19	16	12	11	13	8	13	10
LOD [RFU]	$Y_{LOD} = (159,896 + 1.645 * 52,154) + 1.645 * 41,125 = 313,339$ RFU								
LOD [ng/mL]	3.8 ng/mL (95 % confidence interval: 2.8 – 4.8 ng/mL)								
Analysis of the DNA-TL									
Mean [RFU]	16,741,322	16,060,913	17,734,881	16,165,793	16,278,912	16,071,960	17,675,860	17,548,963	17,619,909
SD [RFU]	1,418,412	1,509,803	1,423,857	1,209,138	1,337,528	824,342	1,553,131	1,418,774	1,341,056
CV [%]	8	9	8	7	8	5	9	8	8
Mean, SD, CV, and LOD of sigmoidal fit curve analysis (Figure 3b, DNA Reference Assay)									
Sample									
gDNA [cp/react.]	0	10 <sup>0</sup>	10 <sup>1</sup>	5x10 <sup>1</sup>	10 <sup>2</sup>	10 <sup>3</sup>	10 <sup>4</sup>	10 <sup>5</sup>	10 <sup>6</sup>
Analysis of the DNA-TL									

Mean [RFU]	2,398,743	2,703,332	4,407,848	6,416,298	6,957,134	13,593,458	18,458,507	19,419,882	19,452,793
SD [RFU]	606,206	520,165	721,782	556,069	954,023	916,495	868,245	759,026	999,109
CV [%]	25	19	16	9	14	7	5	4	5
LOD [RFU]	$Y_{LOD} = (2,398,743 + 1.645 * 606,206) + 1.645 * 520,165 = 4,251,623$ RFU								
LOD [cp/react.]	12.6 copies/reaction (95 % confidence interval: 7.5 – 20.1 copies/reaction)								
<b>Mean, SD, CV, and LOD of sigmoidal fit curve analysis (Figure 3b, Multianalyte-Assay)</b>									
<b>Sample</b>									
IL-6 [ng/mL]	200	200	200	200	200	200	200	200	200
gDNA [cp/react.]	0	10 <sup>0</sup>	10 <sup>1</sup>	5x10 <sup>1</sup>	10 <sup>2</sup>	10 <sup>3</sup>	10 <sup>4</sup>	10 <sup>5</sup>	10 <sup>6</sup>
<b>Analysis of the Protein-TL</b>									
Mean [RFU]	9,059,397	9,084,745	8,983,850	9,437,570	9,750,352	9,791,736	9,813,015	9,461,062	8,965,210
SD [RFU]	1,375,290	898,075	619,273	622,289	810,591	1,622,034	1,642,591	2,027,554	856,333
CV [%]	15	10	7	7	8	17	17	21	10
<b>Analysis of the DNA-TL</b>									
Mean [RFU]	2,537,105	2,481,734	2,807,802	3,919,639	4,867,706	13,519,583	18,264,289	18,949,951	17,619,909
SD [RFU]	438,398	639,229	298,594	553,610	810,443	1,261,376	1,155,971	2,064,743	1,341,056
CV [%]	17	26	11	14	17	9	6	11	8
LOD [RFU]	$Y_{LOD} = (2,537,105 + 1.645 * 438,398) + 1.645 * 639,229 = 4,309,801$ RFU								
LOD [cp/react.]	70.2 copies/reaction (95 % confidence interval: 55.9 – 90.8 copies/reaction)								