Supporting information for:

A generic protocol to immobilize lipopolysaccharides on microbeads for multiplex analysis

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Detailed procedures for the chemical synthesis of alkylated DEDA derivatives

***Tert*-butyl (2-(diethylamino)ethyl)carbamate (1):** Di-*tert*-butyl dicarbonate (47g, 0.22mol) was dissolved in diethyl ether (100mL) and stirred on an ice bath. *N,N*-Diethylethylenediamine (DEDA, 30g, 36 mL, 0,26 mol) was added dropwise. After complete addition, the mixture stirred for 16 hr. at room temperature (RT). Diethyl ether (100mL) was added to the mixture and the mixture was washed with H_2O (3x200mL) and with brine (50mL). Ninhydrin test was performed on two drops of the diethyl ether solution and showed negative (colorless/bright yellow). The diethyl ether solution was dried with sodium sulfate and concentrated in vacuo to yield 41.5 g (89%) of the product as a clear oil. ^{1}H NMR (300 MHz, d_6-DMSO), δ: 0.9 (t, *J* 7.1 Hz, 6H $(CH_2CH_3)_2$), 1.39 (s, 9H, $C(CH_3)_3$), 2.42 (m, 6H, N-$(CH_2$-$CH_3)_2$,N-CH_2-CH_2), 2.96 (q, *J* 6.6 Hz, 2H, NH-CH_2), 6.44 (s,1H,NH). ^{13}C NMR (75 MHz, $CDCl_3$), δ: 155.45(C=O), 77.20 (O-C-$(CH_3)_3$), 51.96 (N-CH_2-CH_2), 46.69 (N-CH_2-CH_3), 38.24 (NH-CH_2), 28.1 (C-$(CH_3)_3$), 11.78 $(CH_2$-$CH_3)_2$. FT-IR (ATR), ν in cm^{-1} 2970.38 (CH_3), 1693.5 (C=O carbamate), 1496.76 (tertiary amine), 1365.5 (CH_3). TLC R_f: 0.6 (ethyl acetate/methanol (6:4)). HPLC-MS: Not obtained due to low UV absorbance of the product.

2-((tert-butoxycarbonyl)amino)-*N,N*-diethyl-*N*-methylethan-1-aminium iodide (2): To a stirred solution of **1** (5.00 g, 24 mmol) dissolved in Acetonitrile (50 mL), methyl iodide (11.75 mL, 190 mmol) was added. The reaction mixture was heated at 60 °C for 24 h. It was then concentrated in vacuo and precipitated by adding diethyl ether (50mL) while stirring. The supernatant was

removed, diethyl ether (30 mL) was added and the mixture was stirred on an ice bath leading to precipitation of the product. The supernatant was removed and the residue dried in vacuo. Yield 7.47g (89%). ^{1}H NMR (300 MHz, $CDCl_3$), δ in ppm 1.39 (s, $C(CH_3)_3$), 1.40 (t, *J* 4.4 Hz, 6H, N^+-$(CH_2$-$CH_3)_2$), 3.24 (s, 3H, CH_3-N^+), 3.58 (m, 8H, N-$(CH_2)_3$, NH-CH_2), 5.94 (s, 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$), δ:156.34(C=O), 80.30 (O-C-$(CH_3)_3$), 59.12 (N^+-CH_2-CH_2), 57.82 (N^+-CH_2-CH_3), 48.7 (N^+-CH_3), 34.89 (NH-CH_2), 28.44 (C-$(CH_3)_3$), 8.5 $(CH_2$-$CH_3)_2$. FT-IR (ATR), ν in cm^{-1} 3277 (NH), 2978 (CH_3), 1697 (C=O carbamate), 1448 (tertiary amine), 1359 (CH_3), TLC R_f (ethyl acetate/methanol 6:4): 0.18. HPLC: t_R: 0.6 min, MS (calc. for $C_{24}H_{54}IN_4O_4^+$): 589.32, found: 589.38.

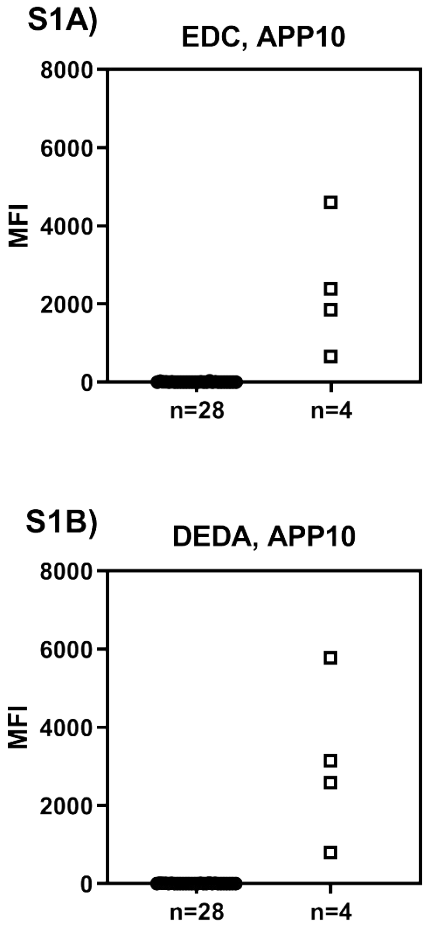
2-((tert-butoxycarbonyl)amino)-*N,N,N*-triethylethan-1-aminium iodide (3): To a stirred solution of **1** (3 g, 14.2 mmol) dissolved in Acetonitrile (100 mL), Iodoethane (42.5 mmol, 3.5 mL) was added and the mixture was heated at 60 °C for 24 h. It was then concentrated in vacuo and precipitated in diethyl ether (50mL). The supernatant was removed, diethyl ether (30 mL) was added and the mixture was stirred on an ice bath to precipitate the product. The Supernatant was removed, and the residue dried in vacuo. Yield 5.2 g (88 %). ^{1}H NMR (300 MHz, d_6-DMSO), δ:1.18 (t, *J* 7.1 Hz, 9H (CH_3-CH_2), 1.38 (s, 9H, $C(CH_3)_3$), 3.15 (q, *J* 3.8 Hz, 2H, N^+-$(CH_2$-$CH_2)$, 3.28 (m, 8H, N^+-$(CH_2)_3$, NH-CH_2), 7.07 (s, 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$), δ: 155.56 (C=O), 78.50 (O-C-$(CH_3)_3$), 54.06 (N^+-CH_2-CH_2), 52.32 (N^+-$(CH_2$-$CH_3)_3$), 33.29 (NH-CH_2), 28.04 (C-$(CH_3)_3$), 7.04 $(CH_2$-$CH_3)_3$. FT-IR (ATR), ν in cm^{-1} 3292 (NH), 2978 (CH_3), 1693 (C=O carbamate), 1456 (tertiary amine). TLC R_f: 0.17 (ethyl acetate/methanol(6:4)). HPLC: t_R: 0.6 min, MS (calc. for $C_{26}H_{58}IN_4O_4^+$): 617.35, found: 617.41.

***N,N*-diethyl-N-methylethane-1,2-diaminium chloride iodide (4):** Compound 2 (3.2g, 8.9 mmol) was added to a mixture of absolute ethanol (23.6 mL) and 36% hydrochloric acid (1.4mL) in a

round-bottomed flask. It was refluxed for 5 min, and then stirred for 45 min. cooling to RT. The mixture was then dropwise added to a round-bottomed flask with diethyl ether (100mL) resulting in precipitation of the product. The supernatant was then removed and diethyl ether (50mL) added. The mixture was stirred for 30 min on an ice bath, the supernatant removed, drying in vacuo gave 1.71 g (65%) of the product as an off-white crystalline. ^{1}H-NMR (300 MHz, d_6-DMSO), δ:1.23 (t, *J* 7.2 Hz, 6H, N^{+}-(CH_2-CH_3)$_2$), 3.02 (s, 3H, $^{+}$N-CH_3), 3.22 (m, 2H, CH_2-NH_3^{+}), 3.39 (q, *J* 7.1 Hz, 4H, N^{+}-(CH_2)$_2$), 3.50 (t, *J* 3.7 Hz, 2H, $^{+}$N-CH_2, 8.55 (s, 3H, NH_3). ^{13}C NMR (75 MHz, $CDCl_3$), δ: 56.5 (N^{+}-CH_2-CH_2), 54.82 (N^{+}-(CH_2-CH_3)$_2$), 47.5 ($^{+}$N-CH_3), 32.01 ($^{+}NH_3$-CH_2), 7.55 (CH_2-CH_3)$_2$, FT-IR (ATR), ν in cm^{-1} 2970 (NH), 2914(NH). TLC R_f: 0.15(ethyl acetate/methanol(6:4)). HPLC: t_R: 0.5 min, MS (calc. for $C_{14}H_{38}IN_4^{+}$): 389.21, found: 389.25.

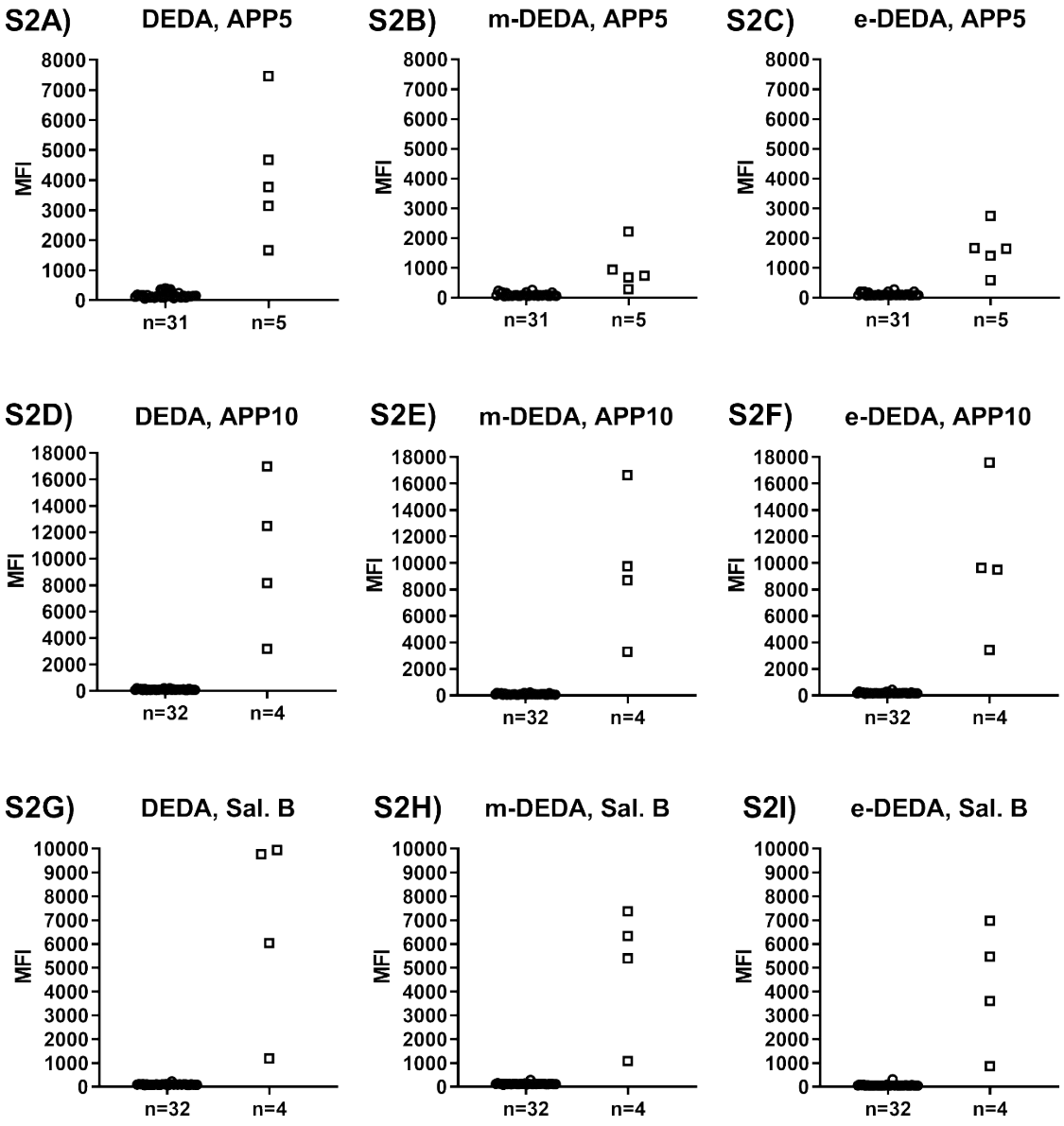
***N,N,N*-triethylethane-1,2-diaminium chloride iodide (5):** Compound 3 (2.0 g, 5.4 mmol) was added to a mixture of absolute ethanol (24.16 mL) and 36% hydrochloric acid (0.84mL) and treated as described for compound **4**. Yield: 0.98 g (59%) ^{1}H-NMR (300 MHz, d_6-DMSO), δ:1.21 (t, *J* 7.2 Hz, 9H, N^{+}-(CH_2-CH_3)$_3$), 3.16 (m, 2H, CH_2-NH_3^{+}), 3.32 (q, *J* 7.2 Hz, 6H, N^{+}-(CH_2)$_3$), 3.45 (m, 2H, N^{+}-CH_2-CH_2, 8.55 (s, 3H, NH_3). ^{13}C NMR (75 MHz, $CDCl_3$), δ: 52.38 (N^{+}-(CH_2-CH_3)$_3$), 51.36 (N^{+}-CH_2-CH_2), 31.55 (NH_3^{+}-CH_2), 6.85 (CH_2-CH_3)$_3$, FT-IR (ATR), ν in cm^{-1} 2976 (NH), 2864 (NH). TLC R_f: 0.046 (ethyl acetate/methanol (6:4)) HPLC: t_R: 0.5 min, MS (calc. for $C_{16}H_{42}IN_4^{+}$): 417.24, found: 417.29.

Supplemental Figure 1



Supplemental Figure 1. Dot plots showing the MFI-results for microbeads coupled with APP10 antigen using the A) three-step EDC-coupling method or the B) DEDA-coupling method. Reactivity of APP10 coupled microbeads with serum samples from 28 ELISA-negative pigs (left) and 4 ELISA-positive pigs (right).

Comparison of serological reactions with a QC serum panel of DEDA, m-DEDA and e-DEDA microbeads coupled with APP5, APP10 or Sal. B antigens: DEDA beads coupled with APP5 antigen gave the highest MFI read-out values with APP5 positive sera (median: 3772) compared to m-DEDA (median: 738) and e-DEDA (median: 1645) relative to the APP5-negative samples showing MFI of DEDA (median: 140), m-DEDA (median: 77) and e-DEDA (median: 94) (Supplemental Figure 2, A-C). The three types of DEDA-microbeads coupled with APP10 antigen showed comparable MFI read out signals for APP10-positive samples (DEDA (median: 10318), m-DEDA (median: 9226), e-DEDA (median: 9573)) relative to the read out signals for APP10-negative samples (DEDA (median: 74.5), m-DEDA (median: 77) and e-DEDA (median: 174,5)) (Supplemental Figure 2, D-F). Compared to m-DEDA and e-DEDA, DEDA-microbeads coupled with Sal. B antigen showed marginally higher MFI read out signals for Sal. B positive samples (DEDA (median: 7904), m-DEDA (median: 5867) and e-DEDA (median: 4538) relative to Sal. B negative samples (DEDA (median: 99.5), m-DEDA (median: 123) and e-DEDA (median: 59.5)) (Supplemental Figure 2, G-I).



Supplemental Figure 2: Dot plots for DEDA-, m-DEDA- and e-DEDA-microbeads coupled with A-C) APP5 LPS, D-F) APP10 LPS and G-I) *Salmonella enterica* serogroup B (Sal. B) LPS. The dot plots show reactivity in Median Fluorescence Intensity (MFI) with 32 ELISA-negative pigs (left) and 4 ELISA-positive pigs (right).

Supplementary Table 1. Swine serum samples included in the validation and statistical analysis of the multiplex analysis. Samples were from swine herds (Danish high-health, conventional and foreign herds) that were positive or negative for antibodies to APP5, APP10 or Sal. B.

Serovar /serogroup	**High-health**		**Conventional**		**Foreign**		**Total**	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
APP5	0	148	48	0	30	62	78	210
APP10	0	171	4	17	30	70	34	258
Salmonella (B)	18	570	113	10	0	0	131	580

The results were compared to results obtained with existing routine analyses (ELISAs) that have been used for decades as Danish National Standards (DN standards) for herd classification in the Danish Specific Pathogen Free (SPF) system (Supplementary Table 2).

Supplementary Table 2. Herd level specificity and sensitivity of the Danish National standard ELISAs.

Serovar/serogroup	Assay type	Specificity (%) (95% CI*)	Sensitivity (%) (95% CI)
APP5	Indirect ELISA	98 (94-100)	100 (54-100)[24]
APP10	Indirect ELISA	99.8 (99.7-99.8)	100 (88.8-100)[†]
Salmonella	Mix-ELISA ‡	99.9 (99.6-100)	73.5 (65.1-81)[1,2, †]

* CI: Confidence Interval

† The specificity and sensitivity of the indirect ELISA for antibodies to *A. pleuropneumoniae* serovar 10 was established as part of quality assurance at the National Veterinary Institute, Denmark, which follow DS/EN ISO/IEC 17025.

‡ *Salmonella* Mix-ELISA detects antibodies in serum against serogroup B and C_1

Data analysis: The ratio between the sample MFI (S) and the positive sample MFI (P) (S/P ratio) was calculated for each sample by:

$$S/P\ ratio = \frac{(MFI_{\ sample} - MFI_{\ negative\ control})}{(MFI_{\ positive\ control} - MFI_{\ negative\ control})} \times 100$$

Receiver Operator Characteristic (ROC) curve analysis was used for the determination of test analysis quality compared to existing in-house analyses (gold standards). Sensitivity was calculated as the percentage of true positive samples found in the gold standard that were also positive in multiplex analysis at a given cutoff. Specificity was calculated as the percentage of true negative samples found in the gold standard that were also negative in multiplex analysis at a given cutoff. Differential positive rates (DPR) (defined as sensitivity + 1- specificity) were used together with dot plot correlation curves to determine optimal cutoffs for the individual analytes in the multiplex analysis assay.[3 4]

In the data analysis, if a herd was found APP-positive in the DN-standards, multiplex data was included for the positive serum samples, while samples in the same herd that tested negative in the DN-standards were excluded in order to minimize the influence of animals undergoing seroconversion that would give borderline reactions.

References

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