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Experimental design for the development of a multiplex antigen lateral flow immunoassay detecting the Southern African Territory (SAT) serotypes of Foot-and-mouth disease virus.

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Abstract

Antigenic Lateral Flow immunoassays (LFIA), rely on the non-competitive sandwich format, including a detection (labelled) antibody and a capture antibody immobilized onto the analytical membrane. When the same antibody is used for the capture and the detection (single epitope immunoassay), the saturation of analyte epitopes by the probe compromises the capture and lowers the sensitivity. Hence, several factors, including the amount of the probe, the antibody-to-label ratio, and the contact time between the probe and the analyte before reaching the capture antibody, must be adjusted. We explored different designs of experiments (full-factorial, optimal, sub-optimal models) to optimize a multiplex sandwich-type LFIA for the diagnosis and serotyping of two Southern African Territory (SAT) serotypes of the Foot-and-mouth disease virus, and to evaluate the reduction of the number of experiments in the development. Both assays employed single epitope sandwich, so most influencing variables on the sensitivity were studied and individuated. We upgraded a previous device increasing the sensitivity by a factor of two and reached a the visual limit of detection of $10^{3.7}$ and $10^{4.0}$ (TCID/mL) for SAT 1 and SAT 2, respectively. The positioning of the capture region along the LFIA strip was the most influent variable to increase the detectability. Furthermore, we confirmed that the 13-optimal DoE was the most convenient approach for designing the device.

Introduction

Foot-and-Mouth Diseases (FMD) is an impactful viral disease widespread throughout the world, particularly in Asia, Africa and the Middle East[1, 2]. WOA/FAO endorsed a Global FMD Control Strategy to detect rapidly and promptly the disease, and to differentiate circulating serotypes, which include O, A, Asia1, SAT 1 and SAT 2 types. Laboratory-based analysis for virus detection in clinical samples includes Virus Isolation (VI), enzyme-linked immunosorbent assay (ELISA) and nucleic acid detection methods, while “pen side tests” exploiting the Lateral Flow Immunoassays (LFIA) technique have been developed as point-of-care testing (POCT) for the rapid, simple and on-field detection of the virus[3]. Viral and bacterial antigens are early infection biomarkers, and their detection allows for an efficient control of the outbreaks and is essential for monitoring and limiting the spread on the disease[4] compared with serological tests. Also, for FMD diagnosis, the identification of the serotype involved in an outbreak is mandatory to accelerate the

41 confinement of the infection by implementing vaccination campaigns. This considered, in a previous work,
42 we developed two multiplex LFIA for the detection and serotyping of the FMD virus: one device aimed at
43 identifying O, A and Asia-1 serotypes (*Eurasia* LFIA currently named LFD1), and the other was developed for
44 the serotyping of SAT 1 and SAT 2 serotypes (*Africa* currently named LFD2). Both devices include PAN-FMDV
45 line and enable the detection of all known FMD serotypes[5]. . The format we used was the sandwich-type
46 immunoassay, in which on monoclonal antibody (mAb) was anchored onto the analytical membrane (capture
47 antibody) and another mAb was labelled with gold nanoparticles (detection antibody, mAb_AuNPs) The LFD1
48 employed the serotype specific monoclonal antibodies as the capture antibodies and a PAN-reactive mAb as
49 the detector, while the LFD2 used serotype specific mAbs and PAN-reactive mAb coated onto the membrane
50 and a pool of the same mAbs as detection antibodies. While almost equivalent analytical performances to
51 those of a reference antigen-ELISA kit were shown by all the diagnostic lines, an exception for the SAT 2
52 rendered the LFD2 sub-performing for this serotype[6]. In the previous work, we also encountered for the
53 first time an antigen saturating hook effect (asHE), which occurs when sandwich assays are realized in the
54 lateral flow immunoassay platform using the same antibodies as capture and detection ligand (single-epitope
55 sandwich assay), as in the case of the LFD2[5]. The typical "hook effect" is described as a signal decrease due
56 to the simultaneous reaction of an excess target antigens with both immobilized and labelled antibodies,
57 preventing the formation of a sandwich complex, which in turn leads to a loss of signal intensity[7]. Therefore,
58 the traditional hook effect is explained by the excess of the antigen compared to antibodies (**Fig.1**). On the
59 contrary, the asHE occurs when the excess of the detection antibody masks the analyte inhibiting the binding
60 to the capture antibody.

61 The asHE was then observed in other studies involving single-epitope sandwich assays for the detection of
62 viral antigens[8][9]. The saturation of the antigen seems to be peculiar of the LFIA format, because the sample
63 encounters the detection antibody first, instead of reacting with the capture antibody as happens usually in
64 ELISA. In addition, the LFIA probe is composed of several antibodies linked to the same-coloured
65 nanoparticles whereas tracers used in ELISA typically or ideally show an antibody-to-label ratio of 1:1. We
66 proposed a possible model for the interpretation of the asHE (**Fig.1a-c**), based on the competition between
67 capturing and detection antibodies for the same epitope. As such, we suggested three factors that could
68 impact on the asHE. The first factor was the time of contact: the more the time the detection antibody spent
69 with the antigen; the higher the probability to saturate antigen epitopes and to inhibit the binding of the
70 antigen to the capture mAb (**Fig.1a**). The time of contact is directly linked to the distance between the region
71 where the sample is applied and the test line (region where the capture antibody is immobilized). For
72 multiplex LFIA development this aspect is especially critical since a choice must be made between test line
73 orders. The second factor depended on the amount of probe: the higher the number of mAb_AuNPs, the
74 higher the probability to saturate the antigen epitopes, again impairing the binding to the test line (**Fig.1b**).

75 Finally, the third factor considered the impact of the amount of mAb adsorbed on the single AuNPs, (**Fig.1c**).
76 A high number of antibodies adsorbed on the same signal reporter may increase the affinity of the probe
77 towards the antigen so that the antigen saturation was more probable. Provided that different factors should
78 be studied and that their number increased exponentially because we aimed at developing a multiplex LFIA
79 a high number of experiments was needed Therefore, we explored an alternative to the try and error
80 approach, and applied three designs of experiment (DoE) models to optimize the LFIA configuration with
81 the final goal of increasing the diagnostic sensitivity of the LFD2 and, also to identify which of the factors
82 most influenced the sensitivity. To this aim, we investigated the distance of the test line, the mAb-to-AuNP
83 ratio, and the amount of probe and use the maximization of the test line colour as the main figure-of-merit.
84 So, a second aim of the work was to understand if it was possible to limit the number of experiments by the
85 means of reduced DoE designs. Finally, the optimized device was compared with the pristine LFD2, to confirm
86 the effective gain in sensitivity.

87

88 **Materials and Methods**

89 *Chemicals*

90 Au (III) chloride trihydrate (ACS reagent), anti-mouse immunoglobulin G antibody produced in rabbit (7023),
91 casein sodium salt from milk, sucrose, and bovine serum albumin (BSA) were obtained from Merck/Sigma-
92 Aldrich (St. Louis, MO, USA). Tween20 and other chemicals were purchased from VWR International (Milan,
93 Italy). Nitrocellulose membranes (CNPC-SS12) with cellulose adsorbent pad and glass fibre FR-1 sample pads
94 were purchased by MDI membrane technologies (Ambala, India). Glass fibre conjugate pads were obtained
95 from Merck Millipore (Billerica, MA, USA). Statistical calculations were carried out with Chemometric Agile
96 Tool (CAT) free software. Three anti-FMDV specific mAbs (#2H6 anti-FMDV SAT 2, #HD7 anti-FMDV SAT 1 and
97 #1F10 PAN-FMDV) used both as capture mAbs and mAb_AuNP conjugates were provided by the Istituto
98 Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna.

99

100 *Synthesis of mAb_AuNP conjugates*

101 AuNPs with a localized surface plasmon resonance (LSPR) band centred at 525.5 nm and mean diameter of
102 ca. 32 nm were prepared by Au (III) chloride trihydrate reduction with sodium citrate (further detail on the
103 synthesis of AuNPs is reported in the SI)[10, 11]. Mab_AuNPs probes used for the LFIA device development
104 were prepared as described in the previous work with minor modifications for the experimental design and
105 final device. The three levels of amounts of antibody for each mL AuNP optical density 1 (mAb-to-AuNPs)
106 were determined based on a salt-induced aggregation test (further detail on the salt-induced aggregation
107 test is reported in the SI)[12]. Briefly, concentrated sodium chloride was added as the aggregation promoter,
108 to mAb_AuNP conjugates obtained from variable mAb-to-AuNPs. When the mAb_AuNP is sufficiently
109 shielded no salt-induced aggregation occurs. According to the stress test, the stabilizing quantities of mAbs
110 were found to be 4 µg, and 8 µg of #2H6, #1F10 and #HD7 mAb, respectively (**Fig.S1**). Hereafter, the
111 aforementioned amounts are indicated as mAb titre (T). For the experimental design, mAb-to-AuNPs
112 corresponding to T, T/2 and T/4 were used. The Visible spectra of the conjugates were acquired by means of
113 an Agilent Cary 60 spectrophotometer (Agilent, Palo Alto, CA, USA) (**Fig.S2**).

114

115 *Preparation of the mAb_AuNPs mix for the LFD2.*

116 After the experimental design, the best combinations were defined as T/2 (2ug) #2H6-to-AuNPs with OD2
117 and T/4 (2ug) #HD7-to-AuNPs with OD3. The #1F10_AuNP was added for the detection of Eurasian serotypes
118 on the test line 3 (T3) and used as previously described[5]. We then produced a mix of the conjugates
119 including the #2H6_AuNP: #HD7_AuNP: #1F10_AuNP in the ratio 2: 2: 1 (OD=5). The mixture was dispensed
120 on the conjugate pad as previously described[5]. Minor interventions on the conjugation procedure were
121 made to better shield the conjugates and limit false positive results due to non-specific interactions.
122 Modifications included using casein in place of BSA in some of the buffers used to prepare the conjugates
123 (details on the casein tests are **reported in the SI**; results are shown in **Table S1**).

124

125 *Production of the single-test-line LFIA devices for the experimental design and the multiplex LFD2*

126 The various capture antibodies (#2H6, #HD7, #1F10 and rabbit anti-mouse #7023) used for drawing test and
127 control lines of the LFIA devices were diluted in phosphate buffer (20 mM pH 7.4). The concentrations were
128 1.5mg/mL (#2H6), 1.0mg/mL (#HD7), 1.0mg/mL (#1F10) and 0.5mg/mL (rabbit anti-mouse #7023) and
129 applied at 1 µL/cm onto the nitrocellulose membrane by means of a XYZ3050 platform (Biodot, Irvine, CA,

130 USA), equipped with BioJetQuanti™ 3000 Line Dispenser for non-contact dispensing. The configurations of
131 the device used in the experimental design and in the multiplex format steps are depicted in **Fig.2**. In the FF-
132 DoE, both for SAT 1 and for SAT 2 detecting systems, the “near” and “far” devices were produced by drawing
133 the serotype specific mAbs in T1 and T2 lines, respectively, distancing about 5mm from each other (**Fig.2a**).
134 After the information obtained from the DoE, the configuration of the multiplex device was: anti-SAT 2 (#2H6)
135 1.5mg/mL as the T1, anti-SAT 1 (#HD7) 1.0mg/mL as the T2, PAN-FMDV (#1F10) 1.0mg/mL as the T3, and
136 rabbit anti-mouse (7023) 0.3mg/mL as the control lines. that the new device differed from the original one
137 for the reverse disposition of the SAT 1 and SAT 2 test lines (**Fig.2b-c**). Strips were composed by overlapping
138 sample pad, conjugate pad, membrane, and adsorbent pad, and were cut in 4 mm-width by means of a
139 CM4000 guillotine (Biodot, Irvine, CA, USA). Finally, strips were inserted into plastic cassettes (Eximio Biotech,
140 China) to fabricate the ready-to-use LFIA device. Cassettes were stored in the dark in plastic bags containing
141 silica at room temperature until use.

142 *Execution of the two LFD2 devices*

143 For standardization, an inactivated virus suspension was used to fortify the running buffer and was used
144 diluted 10-fold. As the negative control the running buffer was used. The formulation of the running buffer
145 was: 26 mM hydrogen carbonate buffer at pH 7.9 supplemented with 1% v/v tween20, 0.25% w/v casein,
146 0.02% w/v sodium azide. 80uL of the sample was added to the sample well of the device and the mixture of
147 sample and resuspended gold conjugate was left flowing for 15’.

148 *Experimental designs*

149 Given the experimental specifications, the first step in D-optimal design is to create a candidate set of
150 experimental points. The candidate set is a table with one row for each point (experiment) that is eligible for
151 the experimental design (i.e., a full factorial, in this case). For our study, the original candidate set is a full
152 factorial design for 3 factors with 2 and 3 levels, containing $2*3*3 = 18$ possible experiments. The D-optimal
153 design tries to maximize an efficiency parameter, which represents variance of the parameter estimates of
154 the model. The efficiency of the full-factorial DoE is 100% and it depends on the number of points in the
155 experimental design, the number of independent variables in the model, and, if available, the maximum
156 standard error for the prediction over the experimental design points. Therefore, the D-optimal approach
157 aims to define a set of candidate experiments that maximize the efficiency of the DoE, even by reducing the
158 number of experiments. As it is reported in **Fig.S3**, the D-optimal design suggested the preparation of a 13-
159 experiment optimal design (optimal) as it reported the maximum value of efficiency in terms of log
160 (Normalized Determinant). Moreover, a second 9-experiment design(sub-optimal) was performed,
161 representing the minimum number of experiments to be performed in order to obtain consistent evaluations
162 when interpreting the DoE results.

163 The use of the D-optimal function of CAT software allowed us to obtain the list of the experiments and the
164 experimental conditions of the parameters to be employed when building the different experimental
165 schemes (as reported in **Table 1**). Once the 3 different DoE models have been developed, the p-values of the
166 regression coefficients of the developed multiple regression analysis (MLR) model were evaluated to identify
167 the statistically significant parameters and visualize the nature of the relationships of the parameters
168 involved in the DoE. Then, the coefficient of the variable "distance" was evaluated with respect to the
169 maximum signal to decide which of the SAT sandwiches should be placed in the near (T1) or far (T2) position.
170 Finally, once the selected position was determined, the response surfaces of the DoE models were elaborated
171 to find the combinations with maximum intensity (mAb-to-AuNPs; OD).

172
173 *Experiments involved in the full-factorial, optimal, and sub-optimal designs.*

174 To furtherly describe and characterize the behaviour of the single-epitope sandwiches, we explored
 175 reasonable levels of the variables that are supposed to be the most influencing: distance, optical density, and
 176 amount of mAb-to-AuNPs (**Table 1**). The “near” and “far” distance variables were codified as near (T1) =-1
 177 and far (T2) =+1, considering we could draw the test line only in position 1 or 2 in the LFD2. Three levels of
 178 amount of mAb-to-AuNPs, defined basing on the salt-induced aggregation test, where T is the minimal
 179 stabilizing amount, (T=+1, T/2=0, and T/4=-1) were explored.

Table 1: Design of experiments codifications for each of the two mAbs and experiment involved in the different designs

#exp	Distance ^a	mAb-to-AuNP ^b	Optical density ^c	Full factorial	Optimal	Sub-optimal
1	-1	-1	-1	X	X	X
2	-1	-1	0	X	-	-
3	-1	-1	+1	X	X	-
4	-1	0	-1	X	-	-
5	-1	0	0	X	X	-
6	-1	0	+1	X	-	X
7	-1	+1	-1	X	X	X
8	-1	+1	0	X	-	X
9	-1	+1	+1	X	X	-
10	+1	-1	-1	X	X	X
11	+1	-1	0	X	X	-
12	+1	-1	+1	X	X	X
13	+1	0	-1	X	X	-
14	+1	0	0	X	X	X
15	+1	0	+1	X	X	-
16	+1	+1	-1	X	X	X
17	+1	+1	0	X	X	-
18	+1	+1	+1	X	X	X

^adistance levels codification: T1=-1, T2=+1

^bmAb-to-AuNP levels codification: T/4=-1, T/2=0, T=+1

^coptical density levels codification: 1=-1, 2=0, 3=+1

180 Similarly, the third variable, the optical density, was explored with three levels (OD3=+1, OD2=0, and OD1=-
 181 1). Then, truncated DoE were extracted by means of the D-optimal tool of the CAT software, including 13 for
 182 the optimal and 9 for the sub-optimal experiments. The DoE were repeated for each of the two mAbs (anti-
 183 SAT 1 and anti-SAT 2) and the experiments were carried in two replicates. As selected factors encompassed
 184 both 2 and 3 levels, we needed an approach that could efficiently handle factors with different levels. We
 185 opted for a D-optimal design to strike a balance between the number of experiments conducted and the
 186 amount of information obtained. D-optimal designs are specifically tailored to handle situations where
 187 factors have varying levels, thus allowed us to optimize the allocation of experimental runs, ensuring that we
 188 obtained the most information with the fewest experiments. Criteria used to judge the results were: no signal
 189 appearing at test lines for the negative control antigen and the more intense colour observed at each line for
 190 the specific FMDV type. To compare colour intensity, images of the strips were acquired by a benchtop
 191 scanner (OpticSlim 550 scanner, Plustek Technology GmbH, Norderstedt, Germany) and processed by
 192 QuantiScan 3.0 software (Biosoft, Cambridge, UK). For the detection of the Eurasian serotypes, also the #1F10
 193 was explored fixing the position (T3), so the half of the experiments were needed.

194 Statistical analyses were conducted using the software CAT, developed by the Italian Group of Chemometrics
 195 (by Prof. Riccardo Leardi *et al.*) of the Italian Society of Chemistry (SCI), freely available on internet, within
 196 the R version 3.1.2 version[13][14].

197

198 *Analytical performance of the LFD2*

199 To investigate the analytical performance of the new LFD2 and to compare it towards the one shown by the
200 former LFD2, the intensity of the test line was quantified as the area of the coloured lines quantified by means
201 of the QuantiScan 3.0 software as described above.

202 The visual LOD was estimated by serial dilutions of the virus SAT 1 BOT 1/68 and SAT 2 ZIM 5/81; the initial
203 titre of the virus was $10e6.8$ TCID₅₀/mL for both strains. They were initially 20-fold diluted in the buffer and
204 then 2-fold serial dilutions were prepared and analysed with the two devices (old and new LFD2). The visual
205 LOD was defined as the greater virus dilution at which the colour was still visible at the test line. Colour
206 intensities of the developed strips were quantified from pictures and plotted towards dilution factor.

207 The reproducibility of the new LFD2 was evaluated as follows: three batches of devices were fabricated on
208 different days. Each batch was used to test the inactivated virus suspension (1:10 in buffer) in four replicates.
209 The coefficient of variation (CV%) of replicates ($n=3 \times 4=12$) was calculated.

210 The stability over time was studied by storing some devices at room temperature for 4 months and checking
211 their performance by the application of the inactivated viral suspension as described above. In addition, an
212 accelerated stability study was also conducted, in which devices were stored at 37°C for one week and tested
213 as above.

214 A series of 9 positive samples, i.e., homogenates of epithelium from infected cattle (**Table S2**) were analysed
215 by the old and new LFD2 and results were visually inspected to confirm the attribution of positivity and the
216 absence of cross-reactivity among lines. The colour intensity of the positive lines was recorded and pairwise
217 compared. Differences were statistically evaluated by applying a pairwise T-test and were considered as
218 significant for $p < 0,05$ (95% confidence level). Calculations were carried out by SigmaPlot 12.0 (Inpixon, CA,
219 USA).

220 **Results and Discussion**

221 *Results from the FF, 13-optimal, and 9-suboptimal DoEs*

222 Given the experimental specifications, the first step in D-optimal design is to create a candidate set of
 223 experimental points. The candidate set is a table with one row for each point (experiment) that is eligible for
 224 the experimental design (i.e., a full factorial, in this case). For our study, the original candidate set is a full
 225 factorial design for 3 factors with 2 and 3 levels, containing $2 \times 3 \times 3 = 18$ possible experiments. The D-optimal
 226 design tries to maximize an efficiency parameter, which represents variance of the parameter estimates of
 227 the model. The efficiency of the full-factorial DoE is 100% and it depends on the number of points in the
 228 experimental design, the number of independent variables in the model, and, if available, the maximum
 229 standard error for the prediction over the experimental design points. Therefore, the D-optimal approach
 230 aims to define a set of candidate experiments that maximize the efficiency of the DoE, even by reducing the
 231 number of experiments. As it is reported in **Fig.S3**, the D-optimal design suggested the preparation of a 13-
 232 experiment optimal design (optimal) as it reported the maximum value of efficiency in terms of log
 233 (Normalized Determinant). Moreover, a second 9-experiment design (sub-optimal) was performed,
 234 representing the minimum number of experiments to be performed in order to obtain consistent evaluations
 235 when interpreting the DoE results.

236 Accordingly, six mAb_AuNPs were synthesized, which were characterised by a red-shift in the LSPR due to
 237 the coating with antibodies and passivating proteins[9]. The former AuNPs showed a maximum absorption
 238 (λ_{max}) due to the LSPR at 525 nm. After the conjugation process, the #HD7_AuNPs gold conjugates showed a
 239 red-shift of the λ_{max} of 6 nm independently on the amounts of mAb added, while the #2H6_AuNPs ones
 240 showed a 6, 6.5, and 7 nm red-shift for the T/4, T/2, and T amounts of mAb, respectively. The gold conjugates
 241 were inserted into the device in three different concentrations (measured as the optical density of the

Table 2: The ratio between the intensity of the more intense signal from each data set compared to the coefficient of the distance

	Data set	intensity at the top of the surface (a.u.)	coefficient of the distance from the model	saturation effect (%)
#HD7 (anti-SAT 1)	FF	720	-65.2	9
	13-optimal	700	-63.7	9
	9-suboptimal	760	-73.8	10
#2H6 (anti-SAT 2)	FF	200	-21.0	11
	13-optimal	210	-22.3	11
	9-suboptimal	200	-22.2	11

242 solution used to saturate the conjugate pad). All the experiments were carried by testing 80uL of a 10-fold
 243 dilution in the running buffer of the inactivated virus (reference FMDV strains grown in cell cultures) as the
 244 positive control. The signals developed were then acquired with the OpticSlim scanner and processed
 245 recording the intensity of the colour.

246 The data were reported in the **Table S3**. The graphs in **Fig.S4-6** show the trends of the increase of the mAb-
 247 to-AuNPs ratio on respect to the OD and evidence the decrease of the signal as a function of the optical
 248 density for high mAb-to-AuNPs, as a confirmation of the asHE. In addition, as we previously hypothesized,
 249 the distance is a parameter that largely affects the asHE, since all the experimental designs showed a
 250 coefficient <0 for this parameter (**Fig.S7-8**). By calculating the impact of the saturation, we normalized the
 251 coefficient for the maximum level in the response surface (**Fig.S4-6**). The use of the whole set of experiments
 252 (FF) allows us understanding that SAT 1 suffered less the impact of the asHE (9%) on respect to SAT 2 (11%).
 253 The same conclusion could be extracted from the 13 data set, while the difference was much slighter in the
 254 9-suboptimal data set (10% instead of 11%) (**Table 2**).

255 Then, considering the sole set in which the SAT 2 assay was carried out as the first test line (T1) and the one
 256 in which the SAT 1 assay was carried out as the second test line (T2), we selected the best performing

257 combination in terms of signal intensity. The description of the response curve (**Fig.S4-6**) suggested that the
258 best approach is using probes with low mAb-to-AuNPs ratio with higher optical density. We hypothesized a
259 behaviour analogous to competitive tests, where the amount of the labelled antibody should be carefully
260 tuned to reach high sensitivity and a compromise between the signal (binding to the competitor antigen) and
261 the binding to the analyte. Moreover, once the sandwich has taken place, the rest of the antibodies on the
262 AuNP surface is probably useless and does not participate to generating the signal through other binding
263 events.

264 In the different DoE investigated, we found a very diverse description of the experimental space: the FF
265 design and the 13-experiment D-optimal DoE allowed us to compute the quadratic and the interaction terms
266 for the factors under evaluation. On the other hand, the use of the 9-experiment design did not allow us to
267 manage a proper number of degrees of freedom (considering the quadratic and the interaction terms).
268 Therefore, in order to obtain diagnostic plots and compute the variance explained also for this model, no
269 interactions and no quadratic terms were considered for the 9-suboptimal design. A decreasing amount of
270 explained variance was justified by the different models for both the SAT 1 and SAT 2 detecting systems. The
271 results in terms of Explained Variance in Cross-Validation (CV) are reported in the in **Table S4**The FF and the
272 13-experiment D-optimal designs were capable of describing a larger amount of variance, thus indicating that
273 they can model and interpret the variability of the collected data in a robust way, especially when compared
274 with the 9-experiment models, which did not include the quadratic and the interaction terms in their
275 computation. Moreover, the amount of CV Explained Variance (%) described by SAT 1 system is, on average,
276 higher than the one expressed by the DoE designs of SAT 2 system. This result might suggest that the whole
277 variability of SAT 2 system is not entirely managed by the DoE models as the collected results showed a higher
278 number of random effects. Finally, the evaluation of the response surfaces, explained variance and
279 coefficients of the MLR models allowed us to observe that lowering excessively the number of experiments
280 increased the risk of misleading the configuration so, following the indication from the FF DoE and the 13-
281 optimal DoE, SAT 1 was assigned to the T2 line in the multiplex configuration (**Fig.2c**).

282

283 *Gold conjugate mix including #HD7_AuNP, #2H6_AuNP, and #1F10_AuNP.*

284 The gold conjugate mix including the three labelled mAbs at the top of their performing conditions, as
285 established by the above-described DoE, were incorporated into the LFIA device and the reactive zones were
286 aligned as follows: SAT 2, SAT 1, and PAN-selective assays. T1 and T2 were decided upon DoE described in
287 the previous section. The DoE to optimize the PAN-selective gold conjugate was carried out by evaluating it
288 in the sole T3 position. The top performing #2H6_AuNP (anti-SAT 2) for the T1 position, was characterised by
289 a mAb-to-AuNPs of 2 μ g (T/2) per mL of AuNP (OD 1) and an optical density of 2, while for #HD7_AuNP (anti-
290 SAT 1), detected in the T2 position, the optimal was recorded for 4 μ g (T/2) per mL AuNP (OD 1)) #HD7-to-
291 AuNPs and OD equal to 2. The PAN reactive probe, #1F10_AuNP, was characterised by a mAb-to-AuNPs of
292 4 μ g (T) per mL AuNP (OD 1) and an optical density of 1. We then mixed up the three gold conjugates
293 #2H6_AuNP + #HD7_AuNP + #1F10_AuNP in the ratio 2 + 2 + 1. Adjustments of the conjugation procedure
294 were made to better shield the gold nanoparticles and to prevent undesired signals on the negative control,
295 including using casein in place of BSA in some of the buffers (details on studies utilizing casein are **reported**
296 **in the SI** and results are shown in **Table S1**). The procedure that allowed for completely avoid the false
297 positivity while maintaining a sufficiently appreciable specific signal was the inclusion of 1mg/mL of casein in
298 the second washing, in the dilution, and in the pre-saturation, buffers used in the conjugation process. The
299 use of casein in other conjugation steps, such as, for example, in the overcoating cleaned up the non-specific
300 binding, as well, but significantly reduced the signal of the positive control, therefore, was discarded.

301

302 *Comparison of the performance of the new LFD2 towards the old one*

303

304 The optimised high-sensitive LFD2 (new *LFD2*) was employed for testing serial dilution of reference strains of
305 FMDV SAT 1 and FMDV SAT 2 cultured on susceptible cell lines, namely SAT 1/III (WZ)/ZIM/68 and SAT
306 2/II/ZIM/81 both titrated as TCID₅₀/mL=10^{6.8}. The visual LOD (vLOD) was established as the highest dilution
307 which was undoubtedly attributed as positive by observing the strip by the unaided eye by three independent
308 operators (**Figure 4**). The old LFD2 was characterised by a visual LOD of 10^{4.0} TCID₅₀/mL and 10^{4.3} TCID₅₀/mL
309 for FMDV SAT 1 and FMDV SAT 2 detection, respectively, while the new LFD2 showed lower vLOD for both
310 the serotypes (10^{3.7} TCID₅₀/mL and 10^{4.0} TCID₅₀/mL for serotypes SAT 1 and SAT 2, respectively). Accordingly,
311 the improvement reached by the optimization process was confirmed.

312 To investigate the reproducibility of the new LFD2, three independent batches were fabricated and used to
313 analyse the inactivated viral suspension (positive control). The CV% of replicate measurements (n=4) within
314 batch and between batches (n=12) were both below 20%.

315 Stability over time of the LFD2 was verified by testing the same samples after storing devices for 4 months at
316 room temperature (mean signal variation compared to t=0 was 30%). Moreover, some devices were also
317 stored for 7 days at 37°C, which is considered as an accelerated predictive stability assessment[15]. Also in
318 this study, the colour intensities of the test lines decreased less than 30% (**Table S5**). These values were
319 considered acceptable and promising for the future validation of the new optimised LFD2.

320

321 The new LFD2 was compared with the previous device also by testing nine tissue homogenates from three
322 cattle infected with FMDV SAT 1 and six with FMDV SAT 2 (**Fig.4**). All samples were positive to the PAN FMD
323 real-time RT-PCR (Ct value ranging from 11.66 to 19.48) and reference Antigen-ELISA (OD value ranging from
324 0.97 to 2.89)[16].

325 The results were visually evaluated, and the quantitative differences were estimated as colour intensity
326 measured at the test lines. The improvement of the detectability was generally confirmed. Three samples
327 showed statistically significant increment of the signal for the new LFD2 compared to the former one. One
328 sample (SAT1_#1) instead showed a lower signal. It should be noted that the new device was designed with
329 the inversion of the order of type-specific lines. As discussed above, the increasing of the distance between
330 the line and the sample application point decreased the detectability, therefore the slight decrement of the
331 SAT 1-specific detection was expected. The SAT 2_#6 sample showed signals barely detectable by both
332 devices. The result may be explained by the low concentration of the virus, measured also by the reference
333 Antigen-ELISA (**Table S2**). Remaining four samples showed a general increased signal for the new LFD2,
334 though not statistically significant. The intensity of the signals from the PAN-FMD test line (T3) was also
335 compared (**Fig.S9**). Comparing the new LFD2 with the old LFD2, in the first we observed an increase of colour
336 intensity was shown for FMDV SAT 1-positive samples, while all the FMDV SAT 2 positive samples are
337 confirmed to be negative, in accordance with reference Antigen-ELISA results.

338 A few examples of LFD have been reported previously for the detection and differentiating of SAT FMD virus
339 (**Table 3**). The visual detection exploiting gold nanoparticles as colorimetric labels, has been the preferred
340 choice to enable the fabrication of affordable analytical tools. Our previous test showed several advantages,
341 such as the versatility (one device was able to diagnosis the infection and to discriminate SAT 1, SAT 2
342 serotypes), the diagnostic sensitivity (more than 90.5%) and specificity (no-cross reaction detected), and the
343 rapidity (10' for completing the assay).

344 **Table 3.** An overview on recently reported rapid methods for detecting Southern African Territory (SAT) FMDV
345 serotypes.

Biosensor	Detection (signal reporter)	Serotype detected	Serotype differentiated	LOD (LOG TCID ₅₀ /mL)	Time for completing the assay	Ref.
LFD	Colorimetric (gold nanoparticles)	O, A, Asia 1, C, (most SAT 1 and SAT 3, some SAT 2)	No	n.a. ^a	60'	[17]
LFD	Colorimetric (gold nanoparticles)	SAT 2	SAT 2 specific	n.a. ^a	120'	[18]
LFD	Colorimetric (gold nanoparticles)	SAT 2	SAT 2 specific	SAT 2 3.8	30'	[19]
LFD	Colorimetric (gold nanoparticles)	SAT 1, SAT 3	SAT 1 and SAT 3 (SAT 1 LFD cross-react with SAT 2)	SAT 3.1 SAT 3 1.8	30'	[20]
Multiplex LFD	Colorimetric (gold nanoparticles)	Other serotypes, SAT 1, SAT 2	SAT 1, SAT 2	SAT 1 4.9 SAT 2 5.3	10'	[5] ^b
Multiplex LFD	Colorimetric (gold nanoparticles)	Other serotypes, SAT 1, SAT 2	SAT 1, SAT 2	SAT 1 3.7 SAT 2 4.0	10'	This work

346 ^a not available

347 ^b only the parameters of the device for the SAT serotypes detection is reported here

348

349 We intervened on the same test device, aiming at keeping the same advantages, but upgrading the device in
350 order to increase the analytical sensitivity. In fact, the new LFD2 was able to correctly classify 8 out of the 9
351 positive epithelial homogenates (as the previous LFD2), however providing more intense colouring of the lines,
352 which facilitated the unaided eye evaluation of the result and limit the occurrence of uncertain attribution
353 (and false negativity). At the same time, no false positivity was observed, and the samples were always
354 attributed to the pertinent serotype. A significant improvement of the vLOD was also obtained. This result
355 preliminary also suggests an improved diagnostic sensitivity of the new LFD2, which need to be verified by
356 analysing a larger set of samples.

357

358 Conclusions

359 This work aimed at optimizing a single epitope multiplex LFIA to enhance the sensitivity of a previously
360 reported device for detecting and differentiating two FMDV serotypes (LFD2). The multiplexing complicated
361 further the optimization process, by introducing an additional variable (i.e., the order of alignment of the
362 reactive zones). As such, we applied a DoE approach and compared three different cuts of the DoE, in order
363 to investigate the possibility of limiting the number of experiments, without excluding any relevant variables
364 from the study. The D-optimal cut was able to individuate the most influential variable (line position) and find
365 the top of the response surface as well as the FF-DoE, demonstrating that a reduced number of experiments
366 could be carried out saving time and costs, while maintaining the predictive ability. The optimized device
367 (new LFD2) showed improved analytical performance with a decrease of about 1 order of magnitude in the

368 visual LOD. The increased detectability was further confirmed by revealing SAT 1 and SAT 2-type FMDV in
369 epithelial tissues from infected cattle. Full diagnostic validation of the new LFD2 is ongoing, to also investigate
370 the on-field applicability.

371
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373 **References**

- 374 1. Poonsuk K, Giménez-Lirola L, Zimmerman JJ (2018) A review of foot-and-mouth disease virus (fmdv)
375 testing in livestock with an emphasis on the use of alternative diagnostic specimens. *Anim Heal Res*
376 *Rev* 19:100–112. <https://doi.org/10.1017/S1466252318000063>
- 377 2. Admassu B, Getnet K, Shite A, Mohammed S (2015) Review on Foot and Mouth Disease: Distribution
378 and Economic Significance. *Acad J Anim Dis* 4:160–169.
379 <https://doi.org/10.5829/idosi.ajad.2015.4.3.95165>
- 380 3. Wong CL, Yong CY, Ong HK, et al (2020) Advances in the Diagnosis of Foot-and-Mouth Disease. *Front*
381 *Vet Sci* 7:477. <https://doi.org/10.3389/fvets.2020.00477>
- 382 4. Wu X, Chen Q, Li J, Liu Z (2022) Diagnostic techniques for COVID-19: A mini-review. *J Virol Methods*
383 301:114437. <https://doi.org/10.1016/J.JVIROMET.2021.114437>
- 384 5. Cavallera S, Russo A, Foglia EA, et al (2022) Design of multiplexing lateral flow immunoassay for
385 detection and typing of foot-and-mouth disease virus using pan-reactive and serotype-specific
386 monoclonal antibodies: Evidence of a new hook effect. *Talanta* 240:123–155.
387 <https://doi.org/10.1016/J.TALANTA.2021.123155>
- 388 6. Grazioli S, Ferris NP, Dho G, et al (2020) Development and validation of a simplified serotyping ELISA
389 based on monoclonal antibodies for the diagnosis of foot-and-mouth disease virus serotypes O, A, C
390 and Asia 1. *Transbound Emerg Dis* 67:3005–3015. <https://doi.org/10.1111/TBED.13677>
- 391 7. Ross GMS, Filippini D, Nielen MWF, Salentijn GIJ (2020) Unraveling the Hook Effect: A
392 Comprehensive Study of High Antigen Concentration Effects in Sandwich Lateral Flow
393 Immunoassays. *Anal Chem* 92:15587–15595. <https://doi.org/10.1021/ACS.ANALCHEM.0C03740>
- 394 8. Cavallera S, Pezzoni G, Grazioli S, et al (2022) Investigation of the “Antigen Hook Effect” in Lateral
395 Flow Sandwich Immunoassay: The Case of Lumpy Skin Disease Virus Detection. *Biosensors* 12:739.
396 <https://doi.org/10.3390/BIOS12090739>
- 397 9. Cavallera S, Colitti B, De Mia GM, et al (2023) Development of molecular and antigenic-based rapid
398 tests for the identification of African swine fever virus in different tissues. *Talanta* 258:.
399 <https://doi.org/10.1016/J.TALANTA.2023.124443>
- 400 10. Turkevich J, Stevenson PC, Hillier J (1951) A study of the nucleation and growth processes in the
401 synthesis of colloidal gold. *Discuss Faraday Soc* 11:55–75. <https://doi.org/10.1039/DF9511100055>
- 402 11. Kimling J, Maier M, Okenve B, et al (2006) Turkevich method for gold nanoparticle synthesis
403 revisited. *J Phys Chem B* 110:15700–15707. <https://doi.org/10.1021/JP061667W>
- 404 12. Guo YR, Liu SY, Gui WJ, Zhu GN (2009) Gold immunochromatographic assay for simultaneous
405 detection of carbofuran and triazophos in water samples. *Anal Biochem* 389:32–39.
406 <https://doi.org/10.1016/J.AB.2009.03.020>
- 407 13. Gruppo di Chemiometria della Società Chimica Italiana (SCI).
408 <http://gruppochemiometria.it/index.php/software>
- 409 14. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical
410 Computing, 2014 Vienna, Austria. <http://www.r-project.org/>.

- 411 15. Anfossi L, Di Nardo F, Profiti M, et al (2018) A versatile and sensitive lateral flow immunoassay for
412 the rapid diagnosis of visceral leishmaniasis. *Anal Bioanal Chem* 410:4123–4134.
413 <https://doi.org/10.1007/s00216-018-1067-x>
- 414 16. Callahan JD, Brown F, Osorio FA, et al (2002) Use of a portable real-time reverse transcriptase-
415 polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. *J Am Vet Med*
416 *Assoc* 220:1636–1642. <https://doi.org/10.2460/JAVMA.2002.220.1636>
- 417 17. Ferris NP, Nordengrahn A, Hutchings GH, et al (2009) Development and laboratory validation of a
418 lateral flow device for the detection of foot-and-mouth disease virus in clinical samples. *J Virol*
419 *Methods* 155:10–17. <https://doi.org/https://doi.org/10.1016/j.jviromet.2008.09.009>
- 420 18. T J, Z LL, W R, et al (2011) Development and validation of a lateral flow immunoassay using colloidal
421 gold for the identification of serotype-specific foot-and-mouth disease virus O, A and Asia 1. *J Virol*
422 *Methods* 171:74–80. <https://doi.org/https://doi.org/10.1016/j.jviromet.2010.10.002>
- 423 19. Yang M, Mudabuka B, Quizon K, Nfon C (2019) Generation of monoclonal antibodies against foot-
424 and-mouth disease virus SAT 2 and the development of a lateral flow strip test for virus detection.
425 *Transbound Emerg Dis* 66:1158–1166. <https://doi.org/10.1111/tbed.13076>
- 426 20. Yang M, Mudabuka B, Dueck C, et al (2021) Development of two rapid lateral flow test strips for
427 detection of foot-and-mouth disease virus SAT 1 and SAT 3. *J Virol Methods* 291:113967

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