Selection and characterisation of bioreceptors to develop nanoparticle-based lateral-flow immunoassays in the context of SARS-CoV-2 outbreak

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Materials and Methods

Reagents and devices

17 commercial anti-SARS-CoV-2 nucleoprotein antibodies are listed in Table S1. For further information, contact the authors. Artificial saliva for medical and dental research (part number 1700-0305) was purchased from LCTech GmbH (Obertaufkirchen, Germany) and it was used as received. Tetrachloroauric acid trihydrate 99%, sodium citrate, phosphate buffer saline (PBS) tablets, disodium hydrogen phosphate heptahydrate, monosodium phosphate, sodium bicarbonate, sodium carbonate anhydrous, boric acid, sodium tetraborate decahydrate, hydrochloric acid, sodium hydroxide, bovine serum albumin (BSA), Tween-20, 3,3′,5,5′-Tetramethylbenzidine (TMB, cat no. T0440), and sulfuric acid were purchased from Sigma-Aldrich (Spain). Secondary antibodies labelled with horse radish peroxidase (HRP) chicken anti-rabbit IgG (ab6829) and chicken anti-mouse IgG (ab6814) was purchased from Abcam (Cambridge, United Kingdom). SARS-CoV-2 nucleoprotein-his recombinant protein (40588-V08B) was purchased from Sino Biological. Transparent immuno nonsterile 96-well microplates (10777621) was purchased from Thermo Fisher (Spain). Nitrocellulose membrane CN150 was purchased from Sartorius Stediem (Göttingen, Germany). Cellulose membrane (CFSP001700) and glass fibre were purchased from Merck Millipore (Billerica, MA, USA). Supporting adhesive cards were purchased from Kenosha (Amstelveen, The Netherlands). TEM grids (Ted Pella carbon film 300 MESH Copper grids CF300-CU 01843-F) were purchased from Monocomp Instrumentación (Spain).

AuNPs were synthesised on an IKA® RCT basic IKAMAG™ hot plate magnetic stirrer (Merck Millipore, USA). The conjugates of AuNPs with antibodies was performed in a PCMT ThermoShaker (Grant Instruments, UK). Nanoparticles were centrifuged in an Allegra 64 R centrifuge from Beckman Coulter (USA). A lateral flow reagent dispenser was used to deposit the antibodies on the nitrocellulose membrane (IsoFlow Bioreagent, Imagene Technology, Germany). The SkanMulti reader was provided by Skannex (Oslo, Norway). Colorimetric signal from ELISA assays was acquired by the spectrophotometer SpectraMax iD3 from Molecular Devices (San José, CA, USA). TEM analysis was performed using HR TEM Tecnic G2-F20 from FEI (Hillsboro, OR, USA). DLS and Z-potential measurements were performed using ZetaSizer Nano ZS (Malvern, United Kingdom).

ELISA assay for selection of antibodies.

A solution of anti-nucleoprotein monoclonal antibodies (anti-nucleoprotein mAbs, capture antibodies M1, M2, M3, M4, M5, H1, and H2) was prepared in carbonate-bicarbonate buffer (CBS, 0.05M, pH 9.6) at a concentration of 5 μg/mL, and 100 μL was used to coat the ELISA wells overnight at 4 °C. Then, the antibodies solution was removed and the wells were washed three times using 250 μL of washing buffer PBST (0.01M PBS, 0.1% Tween-20, pH 7.4). Next, 200 μL of a solution of 3% BSA in PBS (0.01 M, pH 7.4) were added in the wells for blocking free sites for 1 h at 37 °C and the BSA solution was removed and the wells were washed just like previous washing steps. The solutions of nucleoprotein were prepared in PBS at concentrations of 0 ng/mL (blank/negative) and 100 ng/mL (positive), and 100 μL of the solutions were added into the wells and incubated for 1h at 25 °C. Then 100 μL of 0.5 μg/mL anti-nucleoprotein Abs (detection antibodies R1, R2, R3, R4, R5, R6, R7, R8, R9, R10 or M1-M5) were added into the wells and incubated for 1h at 25 °C. Next, 100 μL of secondary antibody modified with HRP (0.1 μg/mL) were added into the wells and incubated for another 1h at 25 °C. After every incubation process (incubation with nucleoprotein, detection antibodies, and secondary antibodies), three to five washing steps were followed. 100 μL of TMB (substrate solution) were added into the wells and incubated for 30 min at 25 °C and 50 μL of 1M H_2SO_4 (stop solution) were mixed in the wells. The plates immediately were measured by a spectrophotometer at 450 nm and 620 nm.

Similarly, in order to select the best antibody pairs, we also performed three nucleoprotein calibration curves based on three different pairs of anti-nucleoprotein antibodies. For the experimental work, we followed sandwich ELISA protocol reported in the previous paragraph. In particular, we coated the wells with the rabbit anti-nucleoprotein pAb antibody (R8). Then, we used a series of nucleoprotein standard solutions (0, 1, 3, 10, 30, 100, 300 and 1000 ng/mL in PBS), and 0.5 μg/mL in PBS of M2, M4, and M5 antibody solutions (used as detection antibodies). Next, 100 μL of an HRP-modified anti-mouse pAb (0.1 μg/mL) were added. Finally the enzymatic reaction took place as described in the previous paragraph.

Gold nanoparticle synthesis. Following the Turkevich method [1,2] AuNPs of approximately 20 nm diameter were synthesised by citrate reduction of tetrachloroauric acid ($HAuCl_4$). Specifically, 200 mL of a 0.01 % (w/v) solution of $HAuCl_4$ prepared in Milli-Q water was taken to boiling point. Then, 5 mL of 1 % (w/v) sodium citrate solution was added under continuous vigorous stirring with 600 rpm. The solution was kept boiling for 10 more minutes and then allowed to cool down to room temperature taking about 2 hours. And the AuNPs solution was stored at 4 °C away from light for further usage.

Conjugation of AuNPs with anti-nucleoprotein antibodies (M5)

The conjugates AuNPs&anti-nucleoprotein Abs were prepared by following our protocol [2]. The optimal pH and optimised amount of anti-nucleoprotein antibodies were pH 7.0 and 5 μg/mL (Abs amount/ AuNPs voulme) according to gold aggregation test (not shown). In the final step, the pellet of AuNPs&anti-nucleoprotein conjugates was resuspended in 150 μL of 1% BSA solution in PBS (0.01 M, pH 7.4) instead of 500 μL of the conjugated pad buffer.

Fabrication of the half-stick lateral flow strips (Dot test)

CN150 nitrocellulose membrane was adhered to a laminated card. Absorbent pad (cellulose membrane) was also assembled on the laminated card overlapping 2 mm with the membrane. Then the card was cut into 3 mm-wide lateral flow strips. Anti-nucleoprotein Abs of R1, R7, R8, and R9 were manually dropped (0.3 μL) onto nitrocellulose half-sticks as test dots (T-dot).

Additionally, anti-mouse secondary antibodies were as well dropped (0.3 µL) in the same fashion to obtain the control dots (C-dots). The half-stick strips were dried for 1 h at 37 °C. The AuNPs conjugates were first incubated with PBS buffer in presence of nucleoprotein (100 ng/mL) and absence of nucleoprotein (0 ng/mL) for 10 minutes at room temperature (RT). Then, the half-stick dotted strips were immersed vertically in 20 µL of pre-incubated AuNPs conjugates solutions (2 µL of 10X concentrated AuNPs + 18 µL of sample). After 10 mins of flowing, the half-stick dotted strips were scanned with a SkanMulti reader and the images analysed with ImageJ.

Calibration curves of nucleoprotein detection based on half-stick LFA.

In the Dot test, several pairs of antibodies showed similar performance. In order to decide which one was the best performing couple, preliminary calibration curves were obtained based on half-stick LFA. Here anti-nucleoprotein antibodies R1 or R8 were used as test line and anti-nucleoprotein antibodies M4 or M5 were conjugated with AuNPs, obtaining the following combinations: R1/M5, R8/M4 and R8/M5. A series of concentrations of nucleoprotein (0, 3, 10, 30, 100, 300, 1000 and 3000 ng/mL) were prepared in PBS. The process of preparation of nitrocellulose membrane, deposition of antibody Test and Control dots, AuNPs conjugation and detection of nucleoprotein was as described in the previous section.

For the final calibration curve, Test and Control lines were prepared using an automatic reagent dispenser. 1 mg/mL of anti-nucleoprotein Abs (R8) and 0.75 mg/mL of anti-mouse secondary antibodies were dispensed onto CN150 membrane as test line and control line, respectively. The anti-nucleoprotein mAbs (M5) were conjugated with AuNPs. A series of concentrations of nucleoprotein (0, 1, 3, 6, 10, 20, 30, 60, 100, 200, 300, 600, 1000, 2000, 3000, 6000 and 10000 ng/mL) were spiked in artificial saliva and incubated with AuNPs conjugates for 10 min at RT. Afterwards, half-stick test strips were immersed vertically in 20 µL of pre-incubated AuNPs conjugates solutions (2 µL of 10X concentrated AuNPs + 18 µL of sample). After 10 min of flowing, the half-stick LFA strips were scanned with a SkanMulti reader and the images of LFA strips analysed with ImageJ, correcting the dot area with an equally sized background area.[2]

Data analysis.

Fiji ImageJ software was used to analyse all the images from SkanMulti reader. Origin 2018 software was used for drawing all the bar charts and fitting four-parameters logistics curves [2].

Extended Data

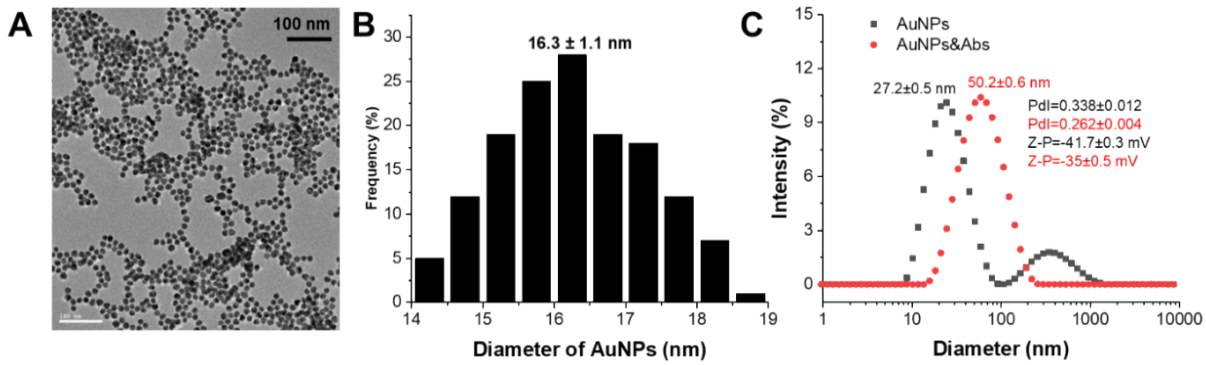


Figure S1. Characterisation of AuNPs before and after conjugation with anti-nucleoprotein Abs. All the characterisation results support the successful conjugation of the antibodies to the AuNPs and the stability of the conjugate particles. (A) TEM images of AuNPs with uniform shape distribution; (B) Histogram of AuNPs (Average size of AuNPs: 16.3 ± 1.1 nm, 150 particles); (C) The change of average hydrodynamic diameters from 27.2 ± 0.5 nm (naked AuNPs) to 50.2 ± 0.6 nm (AuNPs-Abs conjugate) proved AuNPs were covered after conjugation and hence successfully conjugated to anti-nucleoprotein Abs. The change in zeta potential values (-41.7±0.3 mV before and -35±0.5 mV after conjugation) further demonstrated a change on the AuNPs surface attributed to the conjugation. The obtained values indicate that the conjugates remained stable in colloidal solution (absolute Zeta potential value between 30 and 60 mV) [2]. Besides, particle dispersion is better after (polydispersity index (PdI) = 0.262±0.004) than before conjugation with antibodies (PdI = 0.338±0.012).

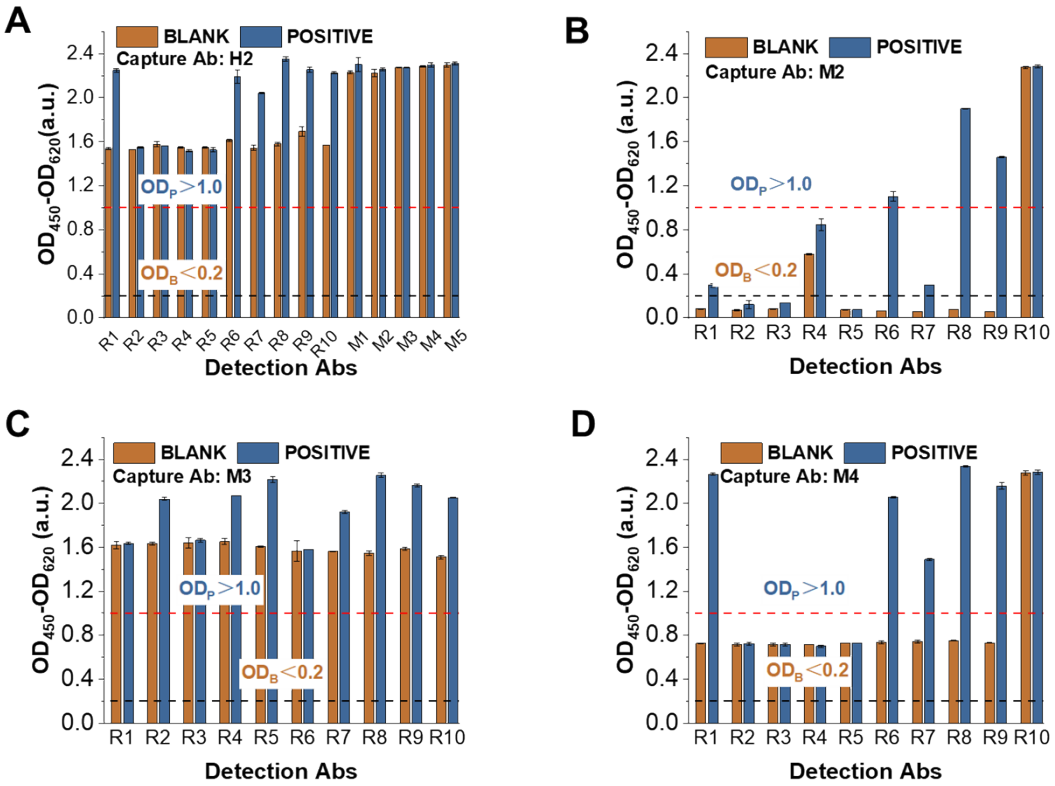
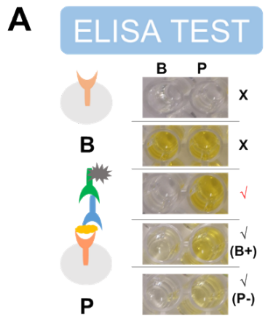
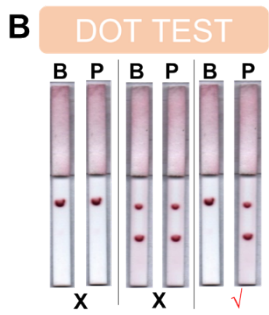


Figure S2. ELISA test results of all possible antibody groups for detection of nucleoprotein. Each individual panel shows the ELISA test results of same capture antibody (coated on ELISA plate wells) combined with various detection antibodies. The antibody combination that meets the performance requirements should have the following conditions: the OD value of blank nucleoprotein solution is less than 0.2 a.u. and the OD value of positive nucleoprotein solution is greater than 1.0 a.u. (A) ELISA results using H2 as capture Ab with different detection Abs (R1-R10 and M1-M5). The results show that there is cross-reactivity between all of the antibody couples, as the OD values of blank samples are far greater than 0.2 a.u. and the blank signal is comparable to that of positive samples. (B) ELISA results using M2 as capture Ab with different detection Abs (R1-R10). The results show that there is high specificity and high affinity towards target between antibody couples of M2/R6, M2/R8 and M2/R9, as the blank signal is less than 0.2 a.u. and the positive signal is more than 1.0 a.u. Antibody couples M2/R4 and M2/R10 show cross-reactivity, as seen by the blank OD values greater than 0.2 a.u. The OD values of positive samples are comparable to that of the blank samples, thus indicating that there is low affinity towards the target between antibody couples M2/R1, M2/R2, M2/R3, M2/R5 and M2/R7. (C) ELISA results using M3 as capture Ab with different detection Abs (R1-R10). The results show that there is cross-reactivity between all of the antibody couples, as the OD values of blank samples are far greater than 0.2 a.u. and the blank signal is comparable to that of positive samples. (D) ELISA results using M4 as capture Ab with different detection Abs (R1-R10). The results show that there is cross-reactivity between all of the antibody couples, as the OD values of blank are greater than 0.2 a.u.



Capture Abs	Detection Abs (ELISA TEST)														
	R1	*R2*	*R3*	*R4*	*R5*	*R6*	*R7*	*R8*	*R9*	*R10*	*M1*	*M2*	*M3*	*M4*	*M5*
M1	√(P-)	X	X	X	X	√	X	√	√(P-)	X	N/A (Not applicable antibody groups)				
M2	X	X	X	X	X	√	X	√	√	X					
M3	X	X	X	X	X	X	X	X	X	X					
M4	√(B+)	X	X	X	X	√(B+)	X	√(B+)	√(B+)	X					
M5	√	X	X	X	X	√	√	√	√	X					
H1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
H2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X



Capture Abs	Detection Abs (Half-stick dot test)			
	M1	*M2*	*M4*	*M5*
R1	X	N/A	X	√
R6	X	X	X	X
R7	N/A	N/A	X	√
R8	X	X	X	√
R9	X	X	X	√

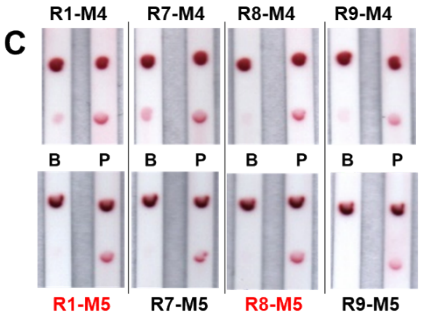


Figure S3. Results of ELISA test and half-stick dot test. (A) Schematic representation of possible ELISA outcomes (left) and all the results of antibody combinations in the table (right); (B) Images of possible outcomes of half-stick dot test (left) and all the results of half-stick dot test (right); (C) Images of half-stick dot test (R1/M5 and R8/M5 are the best two combinations by naked eye). *'X' indicates a negative outcome (lacking in specificity and/or sensitivity). 'V' indicates satisfactory outcome* $OD_{Blank} < 0.2$ *and* $OD_{Positive} > 1.0$*), 'V(P-)' and 'V(B+)' indicates less satisfactory results* ($OD_{Positive}$ *less than a little bit 1.0 and* OD_{Blank} *more than 0.2, respectively) (Figure S3A-left), and 'N/A' means not applicable antibodies groups; B and P indicate blank (0 ng/mL of nucleoprotein) and positive (100 ng/mL of nucleoprotein).*

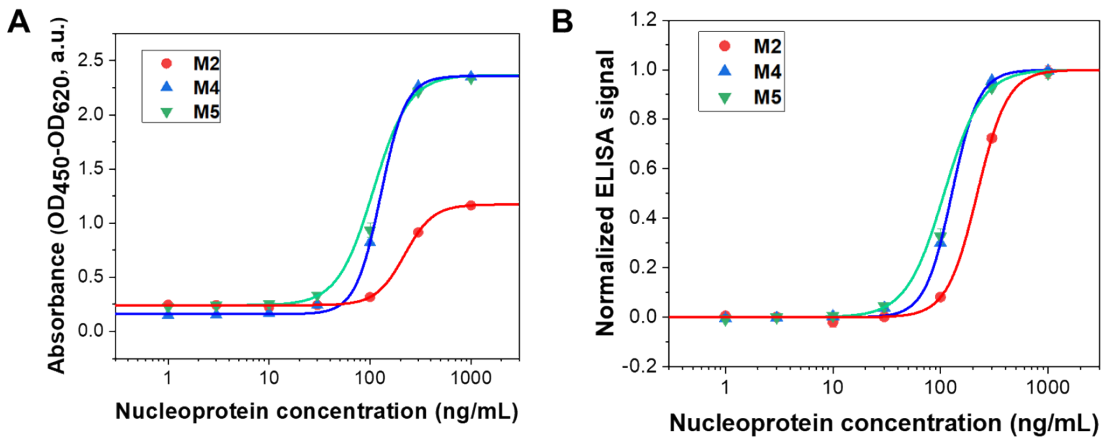


Figure S4. ELISA calibration curves of three antibody pairs for detection of nucleoprotein. ELISA calibration curve using R8 as capture Ab with different detection Abs (M2, M4 and M5). R8/M2 had previously worked in ELISA but not in half-stick, while R8/M4 and R8/M5 had worked in both assays. (A) Calibration curves fitted by using the original absorbance value and (B) calibration curves fitted by using normalised ELISA signal. The fitted curve (obtained using Origin 2018 32-bit and presented as value ± standard error) corresponds to a four parameter logistic equation (sigmoidal curve): y = start + (end – start) × x^n / (k^n + x^n). And the normalised signal was calculated by the equation of (OD value – start) / (end - start) and the normalised calibration curve was fitted by the same mode. All parameters of calibration curve equation are showed in Table S3. The k in the equation corresponds to the EC50. Nonetheless, it can be used to study the affinity of the antibody couple for the antigen. The k values of three antibody couples, R8/M2, R8/M4 and R8/M5, for detection of nucleoprotein are 219.54 ± 4.20, 127.53 ± 4.85 and 107.77 ± 11.12 ng/mL, respectively (Table S3). The k of R8/M5 is the smallest and also showed the best affinity with the nucleoprotein.

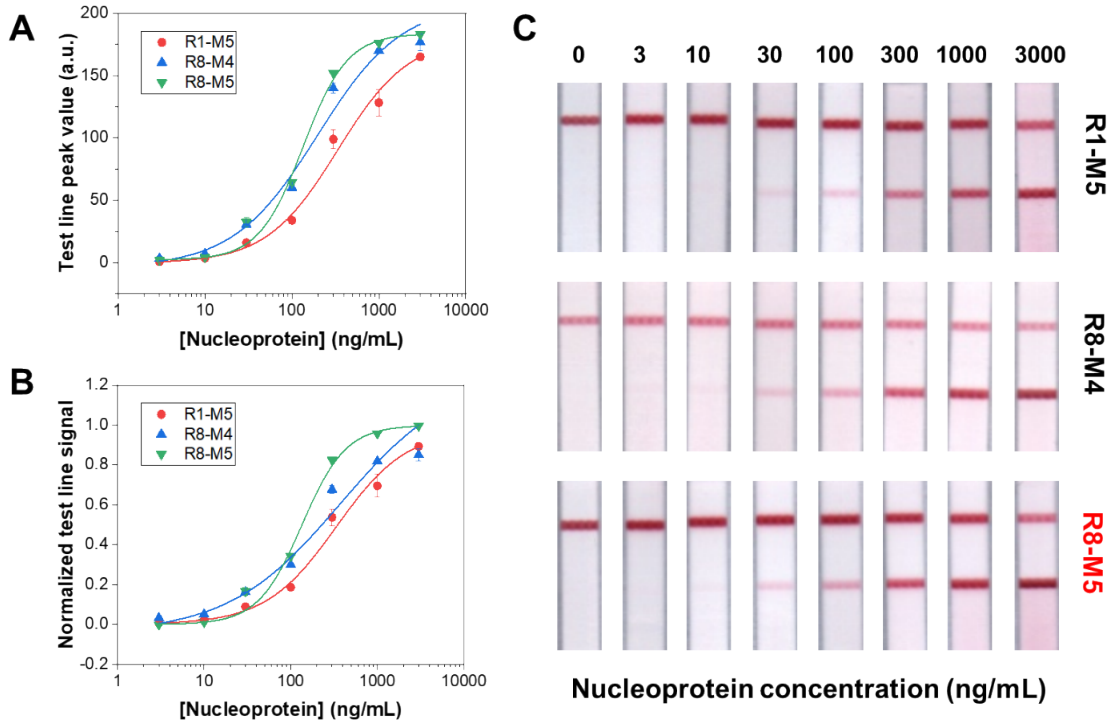


Figure S5. Preliminary calibration curve for screening out the best antibody for detection of nucleoprotein based on half-stick LFA. Preliminary half-stick LFA calibration curves with R1/M5, R8/M4 and R8/M5 antibody couples for nucleoprotein (0, 3, 10, 30, 100, 300, 1000 and 3000 ng/mL): (A) Calibration curves fitted by using the original test line peak value and (B) Calibration curves fitted by using normalised test line signal. The fitted curve (obtained using Origin 2018 32-bit and presented as value ± standard error corresponds to a four parameter logistic equation (sigmoidal curve): y = start + (end – start) × x^n / (k^n + x^n). And the normalised signal was calculated by the equation of (test line peak value – start) / (end - start) and the normalised calibration curve was fitted by the same mode. All parameters of calibration curve equation are shown in Table S4.

206 **Table S1.** Information of all commercial anti-nucleoprotein antibodies.

Code	Host	Antibody Type	Company	Price	Isotype	Proven application
R1	Rabbit	Polyclonal	A	310€/100μg	IgG	WB, ELISA
R2	Rabbit	Polyclonal	B	258€/100μg	IgG	WB, ELISA
R3	Rabbit	Polyclonal	B	331€/100μg	IgG	WB, Simple Western, ELISA, ICC, IF, IHC, IHC-P, Dual RNAscope ISH-IHC
R4	Rabbit	Monoclonal	A	310€/100μg	IgG	WB, ELISA , IHC-P
R5	Rabbit	Polyclonal	C	585€/50μg	IgG	WB
R6	Rabbit	Monoclonal	D	345€/100μg	IgG	WB, ELISA, FC, IHC-P, IP
R7	Rabbit	Monoclonal	A	540€/100μg	IgG	WB, ELISA, IHC-P
R8	Rabbit	Polyclonal	A	310€/100μg	IgG	ELISA
R9	Rabbit	Monoclonal	A	310€/100μg	IgG	WB,ELISA, IHC-P, ICC/IF
R10	Rabbit	Monoclonal	E	303€/100μg	IgG	WB, ELISA, FC, IHC, IF, IP
M1	Mouse	Monoclonal	D	391€/100μg	IgG	WB, ELISA, IHC
M2	Mouse	Monoclonal	D	390€/100μg	IgG	WB, ELISA, ICC, IHC, IF, IP
M3	Mouse	Monoclonal	C	715€/100μg	IgG1	WB,ELISA
M4	Mouse	Monoclonal	A	250€/100μg	IgG1	WB, ELISA, IHC-P, FCM
M5	Mouse	Monoclonal	A	430€/100μg	IgG1	WB, ELISA, IHC-P, FCM
H1	Human	Monoclonal	D	371€/100μg	IgG1, kappa	WB, ELISA
H2	Human	Monoclonal	D	590€/100μg	IgG	WB, ELISA

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Table S2. Recovery of nucleoprotein detection based on half-stick LFA.

Spiked (ng/mL)	Calculated (ng/mL)	Recovery (%)[a]
6	7.0 ± 1.0	116.7 ± 16.7
20	16.6 ± 0.4	83.0 ± 2.0
60	51.6 ± 3.0	86.0 ± 5.0
200	214.3 ± 9.1	107.2 ± 4.6
600	557.3 ± 4.7	92.9 ± 0.8

[a] Recovery (%) = (concentration of calculated nucleoprotein / concentration of spiked nucleoprotein) *100%

Table S3. The equation parameters of ELISA calibration curves (original OD a.u.) for detection of nucleoprotein based on three different pairs of antibodies (R8 as the capture antibody, and M2, M4 and M5 as detection antibodies, respectively).

	M2	M4	M5
START	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
END	1.2 ± 0.0	2.4 ± 0.0	2.4 ± 0.1
k	219.5 ± 4.2	127.5 ± 4.8	107.8 ± 11.1
n	3.1 ± 0.1	3.5 ± 0.5	2.5 ± 0.2
RSS	0.4	43.0	13.0
Adj. R^2	0.9998	0.9990	0.9992

Table S4. The equation parameters of half-stick LFA calibration curves (original OD a.u.) for detection of nucleoprotein based on three different pairs of antibodies (R1, R8 as the capture antibody, and M4 and M5 as detection antibodies).

	R1-M5	R8-M4	R8-M5
START	0.5 ± 2.1	-3.6 ± 10.8	2.3 ± 1.5
END	181.3 ± 11.4	208.3 ± 43.6	183.8 ± 1.6
k	337.1 ± 92.5	197.9 ± 116.0	134.7 ± 11.4
n	1.1 ± 0.2	0.9 ± 0.3	1.7 ± 0.2
RSS	2.4	57.4	11
Adj. R2	0.999	0.9841	0.9994

References

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2 C. Parolo, A. Sena-Torralba, J. F. Bergua, E. Calucho, C. Fuentes-Chust, L. Hu, L. Rivas, R. Álvarez-Diduk, E. P. Nguyen, S. Cinti, D. Quesada-González and A. Merkoçi, *Nat. Protoc.*, 2020, **15**, 3788–3816.