Electronic Supplementary Material (ESI) for Lab on a Chip.

Supplementary Information

Multiplex lateral flow detection and binary encoding enables a molecular colorimetric 7-segment display

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1. Experimental Section

1.1 Oligonucleotides and antibodies

Nucleic acid lateral flow detection was performed on single-stranded synthetic dual-labeled DNA encoding a segment of the Rift Valley Fever virus (RVFV) L gene (5' tgctaggctaagaccagtaagcaaagtcaggcttagatttaggga 3' (Genbank accession number NC_014397.1, nucleotides 6287-6331). This same sequence was used to generate all twelve dual-labeled DNA products to ensure consistency and preclude that individual differences in binding would not be due to subtle changes in DNA sequence. DNA was dual labeled with 6-carboxyfluorescein (6-FAM) at the 5' end, and at the 3' end with either Biotin, CY5Sp, Digoxigenin_N, 6-TAMRASp, or Texas Red-XN, and synthesized and HPLC purified by Integrated DNA Technologies, Inc (IDT., Coralville, USA). Similarly, dual-labeled DNA with a 3' end of either Alexa 488 C6-NH, BODIPY FL C5 C6-NH, Cascade Blue C6-NH, DNP-X C6-NH, or Dansyl-X C6-NH were synthesized by TriLink BioTechnologies (TriLink BioTechnologies, San Diego, USA) and PAGE purified. Finally, dual-labeled DNA with a 3' end of either Lucifer Yellow or Benzopyrene were synthesized by Bio-Synthesis (Bio-Synthesis, Inc., Lewisville, USA) and dual HPLC purified.

A monoclonal anti-fluorescein antibody (Roche Diagnostics Australia Pty. Ltd., Castle Hill, NSW, Australia) was used for AuNP conjugation. Antibodies corresponding to the 3'-labeled DNA were: (i) streptavidin (New England Biolabs, Arundel, QLD, Australia); (ii) monoclonal anti-Cy5 antibody (Sapphire Bioscience Pty. Ltd., Waterloo, NSW, Australia); (iii) polyclonal anti-Digoxigenin antibody (Roche Diagnostics Australia Pty. Ltd., Castle Hill, NSW, Australia); (iv) monoclonal anti-TAMRA antibody (Thermo Fisher Scientific, Scoresby, VIC, Australia); (v) monoclonal anti-Texas Red antibody (Invitrogen Corporation, Carlsbad, CA, USA); (vi) polyclonal anti-Alexa Fluor® 488 antibody (Life Technologies Australia Pty Ltd., Mulgrave, VIC, Australia); (vii) polyclonal anti-BODIPY® FL antibody (Life Technologies Australia Pty Ltd., Mulgrave, VIC, Australia); (viii) polyclonal anti-Alexa Fluor® 405/Cascade Blue® antibody (Life Technologies Australia Pty Ltd., Mulgrave, VIC, Australia); (ix) polyclonal anti-Dinitrophenyl-KLH antibody (Life Technologies Australia Pty Ltd., Mulgrave, VIC, Australia); (x) polyclonal anti-Dansyl antibody (Life Technologies Australia Pty Ltd., Mulgrave, VIC, Australia); (xi) polyclonal Lucifer Yellow antibody (Life Technologies Australia Pty Ltd., Mulgrave, VIC, Australia); (xii) and monoclonal anti-Benzo(a)pyrene antibody (Biorbyt Ltd., Cambridge, Cambridgeshire, United Kingdom). These antibodies were deposited at the test zone of the nitrocellulose membrane. In addition, a polyclonal rabbit anti-mouse antibody (Sapphire Bioscience Pty. Ltd., Waterloo, NSW, Australia) was deposited at the control zone.

1.2 Preparation of AuNPs conjugates

Anti-fluorescein antibody was coupled to AuNPs, which served as the signaling molecule (red in color) to allow the visualization of the immuno-sandwich complex by eye. Coupling to AuNPs (40 nm, 20 OD) was performed using the InnovaCoat® GOLD 10x Multi Explorer labeling kit (BioNovus Life Sciences, Cherrybrook, NSW, Australia). Briefly, reagents were thawed to 25 °C, and 12 µL antibody (diluted to 0.1 mg/mL using the diluent provided) was mixed with 42 µL reaction buffer. The mixture (45 µL) was used to resuspend a vial of InnovaCoat GOLD nanoparticles, which was incubated for 10 min before addition of 5 µL Quencher, resulting in a final 20 OD solution (50 µL) of anti-fluorescein/AuNP. The conjugates were washed twice with the borate running buffer by centrifuging at 7000 x g for 6 min, before resuspension to the original (50 µL) volume. Conjugates were stored at 4 °C.

1.3 Preparation of single-plex nucleic acid lateral flow strip and multiplexed nucleic acid lateral flow strip

Conjugate and sample pads (Millipore, Billerica, MA, USA) were blocked with blocking solution (1% polyvinyl alcohol, 20 mM Tris base, pH 7.4) for 30 min and dried at room temperature for 2 h. The two pads were then impregnated in borate running buffer (100 mM H_3BO_3, 100 mM $Na_2B_4O_7$, 1% BSA, 0.05% Tween 20, pH 8.8) by soaking in buffer for 30 min before drying at 25 °C for 6 hours.

Assembled devices (6.1 cm x 0.3 cm) for single-plex lateral flow detection comprised treated sample pad (1.5 cm), treated conjugate pad (0.6 cm), a nitrocellulose membrane (2.5 cm; Hi-Flow Plus HF135), and an absorbent pad (1.5 cm) (Millipore, Billerica, MA, USA) combined on an adhesive backing card (Lohmann Corporation, Hebron, KY, USA), with a 0.1 cm overlap between components. The multiplexed lateral flow strips were assembled using the same components and procedure, using treated sample pad (0.5 cm), treated conjugate pad (0.6 cm), a nitrocellulose membrane (3.5 cm), and an absorbent pad (2.5 cm).

For single-plex lateral flow detection, detection ligand or antibodies [either streptavidin (1.0 mg/mL), anti-Cy5 antibody (2.0 mg/mL), anti-Digoxiginin (0.75 U/ μL), anti-TAMRA (1.0 mg/mL), anti-Texas Red antibody (1.0 mg/mL), anti-Alexa Fluor® 488 (1.0 mg/mL), anti-BODIPY® FL (3.0 mg/mL), anti-Alexa Fluor® 405/Cascade Blue® (3.0 mg/mL), anti-Dinitrophenyl-KLH (2.0 mg/mL), anti-Dansyl (1.0 mg/mL), anti-Lucifer Yellow (3.0 mg/mL), or anti-Benzo(a)pyrene (1.0 mg/mL)] were pipetted (0.4 μL) onto the test zone of the nitrocellulose membrane. Rabbit anti-mouse antibody (1 mg/mL in 50% glycerol) was pipetted (0.4 μL) at the control zone. Test and control antibodies were spotted 0.5 cm apart and dried at 25 °C for 45 min. For multiplexed lateral flow detection, twelve or seven detection ligands or antibodies (0.2 μL) were deposited and as a control, rabbit anti-mouse antibody was pipetted (0.2 μL) in triplicate at the end of each array.

1.4 Single-plex lateral flow test and multiplexed lateral flow test procedure

Single-plex lateral flow detection was performed as described previously.[1] Briefly, anti-fluorescein/AuNP conjugate (1 μL) was pipetted onto the conjugate pad and the strip was dipped into a mixture containing 100 μL running buffer and 1 μL dual-labeled RVFV DNA (1 to 0.0005 μM). An additional 1 μL anti-fluorescein/AuNP conjugate was pipetted onto the conjugate pad once the running buffer reached the bottom of the absorbent pad as this double-run method has been demonstrated to be effective for developing high signal intensity with reduced anti-fluorescein/AuNP consumption.[1] The strip was developed for 15 min. Negative control strips (no DNA) were run in parallel. All experiments were performed in duplicate and repeated at least twice to demonstrate consistency of results.

For the multiplexed lateral flow detection, the strip was run using mixtures of single-stranded synthetic RVFV DNAs (1 μM; 0.3 μL of 5'FAM/3'Benzopyrene, 5'6-FAM/3'CY5Sp, 5'6-FAM/3'Digoxigenin_N, and 5'FAM/3' DNP-X C6-NH; 0.4 μL of 5'FAM/3'Alexa 488 C6-NH, 5'6-FAM/3'Biotin, and 5'6-FAM/3'BODIPY FL C5 C6-NH; 0.5 μL of 5'FAM/3'Cascade Blue C6-NH, 5'6-FAM/3'6-TAMRASp, and 5'6-FAM/3'Texas Red-XN; 0.6 μL of 5'FAM/3'Dansyl-X C6-NH; 1.0 μL 5'FAM/3'Lucifer Yellow) and 150 μL running buffer firstly, and then anti-fluorescein/AuNP conjugate (5 μL) were pipetted onto the conjugate pad and finished developing with another 100 μL running buffer for 25 min. All experiments were repeated at least three times.

1.5 7-segment lateral flow test procedure

The 7-segment display of numbers 0-9 were tested by adding different dual-labeled DNA mixtures (out of seven: 5'6-FAM/3'Digoxigenin_N, 5'6-FAM/3'Texas Red-XN, 5'6-FAM/3'6-TAMRASp, 5'FAM/3'Cascade Blue C6-NH, 5'FAM/3' DNP-X C6-NH, 5'6-FAM/3'Biotin, and/or 5'FAM/3'Dansyl-X C6-NH) with the same concentration mentioned above) in the borate running buffer:

- **Number "0"**: 5'6-FAM/3'Digoxigenin_N, 5'6-FAM/3'Texas Red-XN, 5'6-FAM/3'6-TAMRASp, 5'FAM/3' DNP-X C6-NH, 5'6-FAM/3'Biotin, and 5'FAM/3'Dansyl-X C6-NH.
- **Number "1"**: 5'6-FAM/3'6-TAMRASp and 5'6-FAM/3'Biotin.
- **Number "2"**: 5'6-FAM/3'Digoxigenin_N, 5'6-FAM/3'6-TAMRASp, 5'FAM/3'Cascade Blue C6-NH, 5'FAM/3' DNP-X C6-NH, and 5'FAM/3'Dansyl-X C6-NH.
- **Number "3"**: 5'6-FAM/3'Digoxigenin_N, 5'6-FAM/3'6-TAMRASp, 5'FAM/3'Cascade Blue C6-NH, 5'6-FAM/3'Biotin, and 5'FAM/3'Dansyl-X C6-NH.
- **Number "4"**: 5'6-FAM/3'Texas Red-XN, 5'6-FAM/3'6-TAMRASp, 5'FAM/3'Cascade Blue C6-NH, and 5'6-FAM/3'Biotin.
- **Number "5"**: 5'6-FAM/3'Digoxigenin_N, 5'6-FAM/3'Texas Red-XN, 5'FAM/3'Cascade Blue C6-NH, 5'6-FAM/3'Biotin, and 5'FAM/3'Dansyl-X C6-NH.
- **Number "6"**: 5'6-FAM/3'Digoxigenin_N, 5'6-FAM/3'Texas Red-XN, 5'FAM/3'Cascade Blue C6-NH, 5'6-FAM/3'Biotin, 5'FAM/3' DNP-X C6-NH, and 5'FAM/3'Dansyl-X C6-NH.
- **Number "7"**: 5'6-FAM/3'Digoxigenin_N, 5'6-FAM/3'6-TAMRASp, and 5'6-FAM/3'Biotin.
- **Number "8"**: 5'6-FAM/3'Digoxigenin_N, 5'6-FAM/3'Texas Red-XN, 5'6-FAM/3'6-TAMRASp, 5'FAM/3'Cascade Blue C6-NH, 5'FAM/3' DNP-X C6-NH, 5'6-FAM/3'Biotin, and 5'FAM/3'Dansyl-X C6-NH.
- **Number "9"**: 5'6-FAM/3'Digoxigenin_N, 5'6-FAM/3'Texas Red-XN, 5'6-FAM/3'6-TAMRASp, 5'FAM/3'Cascade Blue C6-NH, 5'6-FAM/3'Biotin, and 5'FAM/3'Dansyl-X C6-NH.

All the seven corresponding antibodies (at the same concentration and amount mentioned in preparation of single-plex nucleic acid lateral flow strip and multiplexed nucleic acid lateral flow strip section) were pre-deposited on the nitrocellulose membrane. The running procedure of the multiplexed lateral flow test was performed as for the 7-segment lateral flow test.

1.6 Image analysis

Reacted lateral flow strips were dried, imaged using the MultiDoc-It™ Digital Imaging System (Upland, CA, USA), and analyzed using ImageJ software (National Institutes of Health, MD, USA). Image brightness/contrast and color balance were auto-adjusted. The background was subtracted against negative-control strips and the spot intensity was reported as mean gray value. The lowest detection limits were determined by the test dot intensity values that were below the average plus three standard deviations. Statistical differences between test dot intensities were analyzed by t-test using PRISM (Graphpad software Inc. version 6.0 Mac, San Diego, California).

2. Supplementary Figures

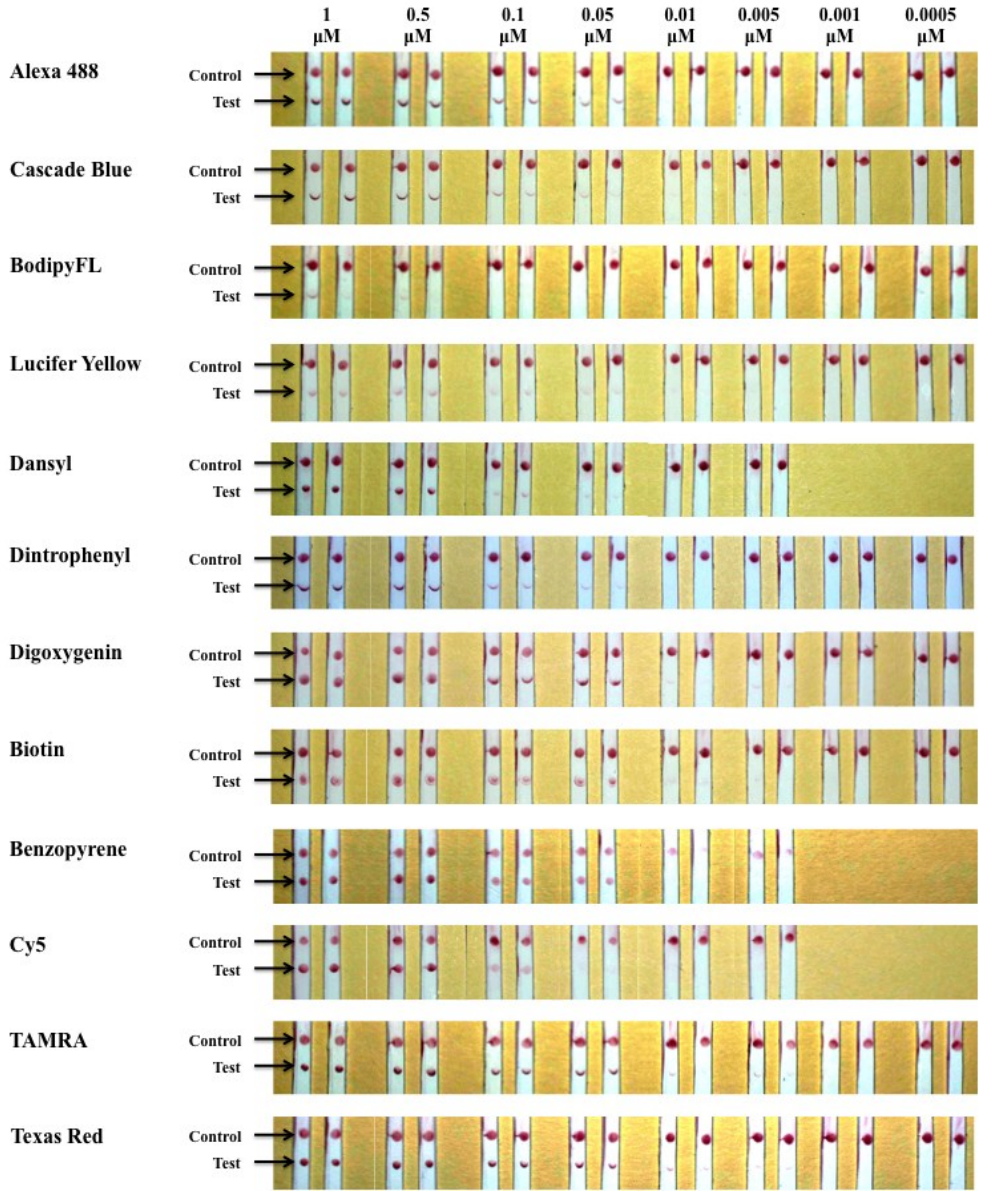

Fig. S1 Single-plex lateral flow detection results for twelve antigen-antibody pairs. Detection antibody (0.4 µL) corresponding to each antigen was deposited on the nitrocellulose membrane and dried at room temperature for 45 min. Control rabbit anti-mouse antibody (0.4 µL) was similarly added 0.5 cm above each test dot for assay validation. Each strip was tested using our pre-developed double-run method,[1] which involves dipping the lateral flow dipstick into a mixture containing 100 µL running buffer and 1 µL dual-labeled analyte (RVFV DNA), followed

by pipetting an additional 1 µL anti-fluorescein/AuNP conjugate onto the conjugate pad once the running buffer reached the bottom of the absorbent pad. Analyte concentrations tested were from 1 to 0.0005 µM. The strip was developed for 15 min. Reacted lateral flow strips were dried overnight, imaged using the MultiDoc-ItTM Digital Imaging System, and analyzed using ImageJ software. Labels Alexa488, Cascade Blue, Lucifer Yellow, Benzopyrene, BodipyFL, and Dansyl, which have not previously been applied in lateral flow detection. Antigen-antibody pairs Digoxigenin/anti-Digoxigenin, TAMRA/anti-TAMRA, and Texas Red/anti-Texas Red had the lowest detection limits (0.005 µM of DNA), while Cy5/anti-Cy5 (0.1 µM of DNA), and BodipyFL/anti-BODIPY® FL (0.5 µM of DNA) pairs had the highest detection limits. Successful implementation of Cyanine5 (Cy5), Carboxytetramethylrhodamine (TAMRA), Texas Red, Biotin, Dinitrophenyl (DNP), and Digoxigenin has been previously reported.[2, 3] Interestingly, the dot morphology and intensity varied among different antigen-antibody pairs. Only Biotin/Streptavidin, Benzopyrene/anti-Benzo(a)pyrene, and Cy5/anti-Cy5 pairs did not show a "coffee ring effect" as the DNA concentration decreased. However, this effect was different to that described by Deegan[4] who suggested the uneven staining is due to capillary flow from different evaporation rates across the drop (where liquid evaporating from the edge is replenished by liquid from the interior). The "coffee ring effect" we observed was dependent on the analyte (DNA concentration), and this phenomenon has not previously been reported. Additionally, the label pairs Biotin/Streptavidin, Lucifer Yellow/anti-Lucifer Yellow, and BodipyFL/anti-BODIPY® FL showed less intense test dots compared to the other pairs (Fig. S1).

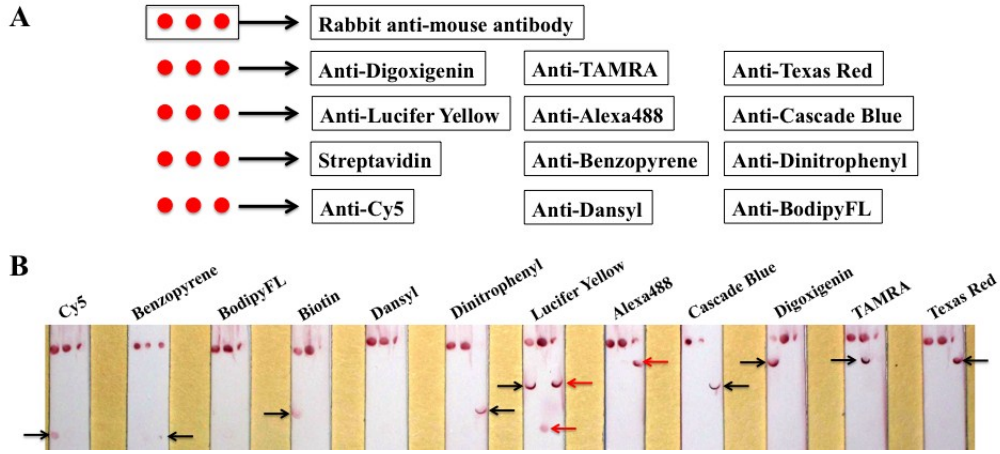

Fig. S2 Specificity test results of all the twelve antigen-detection antibody pairs. A: Positioning of the detection antibodies in the multiplex LFD 3x4 array. B: Dual-labeled DNA sample containing FAM/X (where X = Cy5, Benzopyrene, BodipyFL, Biotin, Dansyl, Dinitrophenyl, Lucifer Yellow, Alexa488, Cascade Blue, Digoxigenin, TAMRA, or Texas Red) was added as shown. The black arrow denotes the correct test dot while the red arrow denotes the incorrect test dot. The assay was independently performed three times with similar results and a representative photograph from one test is shown. Three non-specific reactions were observed: (1) Benzopyrene bound non-specifically with anti-BODIPY® FL antibody; (2) Lucifer Yellow bound non-specifically with anti-Alexa Fluor® 405/Cascade Blue® and anti-Dansyl antibodies; and (3) Alexa488 bound non-specifically with anti-Texas Red antibody (Fig. S2). Curiously, the Alexa488 bound only to the anti-Texas Red antibody and not to its corresponding anti-Alexa Fluor® 488 antibody in this particular configuration (Fig. S2).

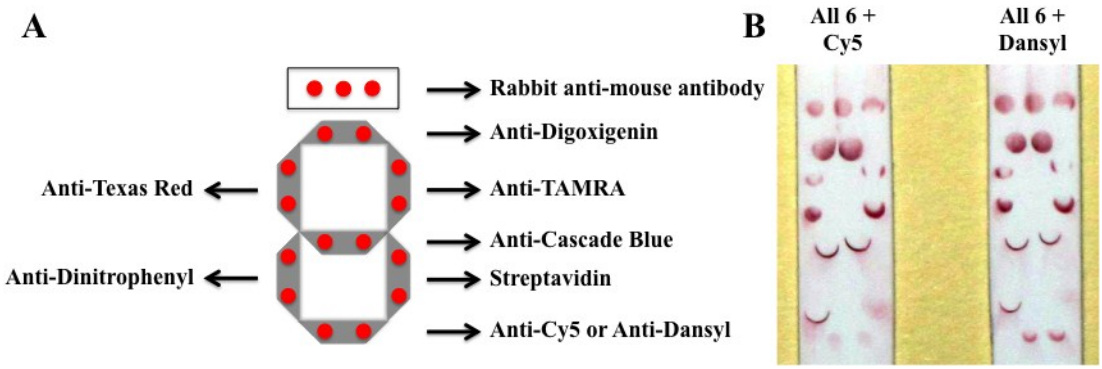

Fig. S3 7-segment display results. A: Positioning of the detection antibodies, where each segment of the display was represented by duplicate detection antibody deposition to create two test dots. B: The numerical number

“8” appeared by the addition of a analytes labeled with a molecular antigen signature consisting of Biotin, Cascade Blue, Digoxigenin, Dintrophenyl, TAMRA, and Texas Red combined with either Cy5 or Dansyl.

References

1. J. Li, D. McMillan and J. Macdonald, *Sensor. Mater.*, 2015, **27**, 549-561.

2. P. Noguera, G. A. Posthuma-Trumpie, M. van Tuil, F. J. van der Wal, A. de Boer, A. P. H. A. Moers and A. van Amerongen, *Anal Bioanal Chem*, 2011, **399**, 831-838.

3. J. A. Tomlinson, S. Ostoja-Starzewska, I. P. Adams, D. W. Miano, P. Abidrabo, Z. Kinyua, T. Alicai, M. J. Dickinson, D. Peters, N. Boonham and J. Smith, *J. Virol. Methods.*, 2013, **191**, 148-154.

4. R. D. Deegan, O. Bakajin, T. F. Dupont, G. Huber, S. R. Nagel and T. A. Witten, *Nature.*, 1997, **389**, 827-829.