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Oligonucleotide-templated Lateral Flow Assays for Amplification-free Sensing of Circulating microRNAs

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Supporting Information

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S1. Experimental information

Chemicals and materials

Tris-borate-EDTA (TBE) 10X buffer was purchased from Thermoscientific, U.K. Fmoc-Arg(Pbf)-Wang resin (0.65 mmol/g loading) and 4-aminobutanethiol-4-methoxytrityl (0.76 mmol/g loading) were purchased from Merck Millipore, U.K. PNA monomers were sourced from LGC Genomics Ltd., U.K. All other chemicals and solvents were purchased from sigma Aldrich, U.K. DNA oligonucleotides were purchased from Invitrogen, U.K. The lateral flow assay strips were made from CN95 nitrocellulose paper LFA with a streptavidin printed test line and a grade 142 sample loading pad (Mologic Ltd, U.K.).

Oligonucleotide-templated reactions on lateral flow strips

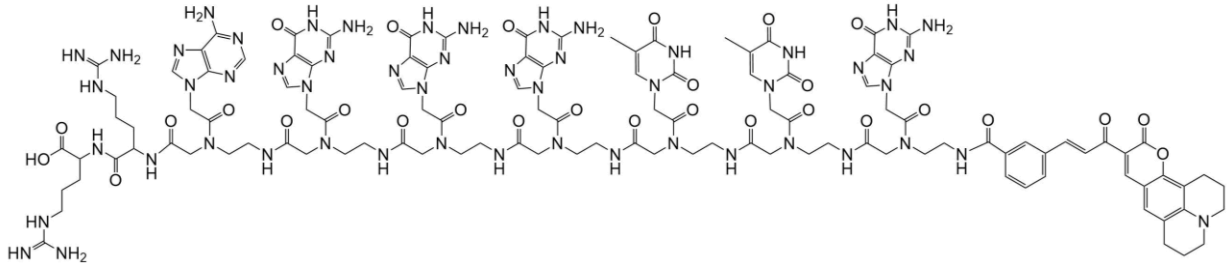
In a typical experiment, the test line was first saturated with 25 µL of a 5 µM solution of biotinylated PNA-thiol complementary to the 3'-end of the target miRNA, containing 0.1% tween to minimise probe adsorption and assist flow (Scheme 1, panel 2, main text). After 5 mins, 30 µL of distilled water was added to wash away any unbound PNA. Simultaneously, the target DNA (0 - 1 µM) was mixed with the PNA coumarin (1 µM) and 0.1% tween up to 25 µL. After 10 mins incubation at room temperature (to allow hybridisation and duplex formation between the coumarin and DNA probes), this mixture was added to the loading pad (Scheme 1, panels 1 and 3, main text). After 5 mins, the sample was washed through addition of 1×TBE buffer (30 µL) also containing 0.1% tween. Once dry, the strips were imaged with either a fluorescence scanner (Typhoon FLA9500, GE Healthcare, Scheme 1 - panel 4 main text) or a small benchtop reader (ESEQuant LR3, Qiagen, Fig. 1 main text).

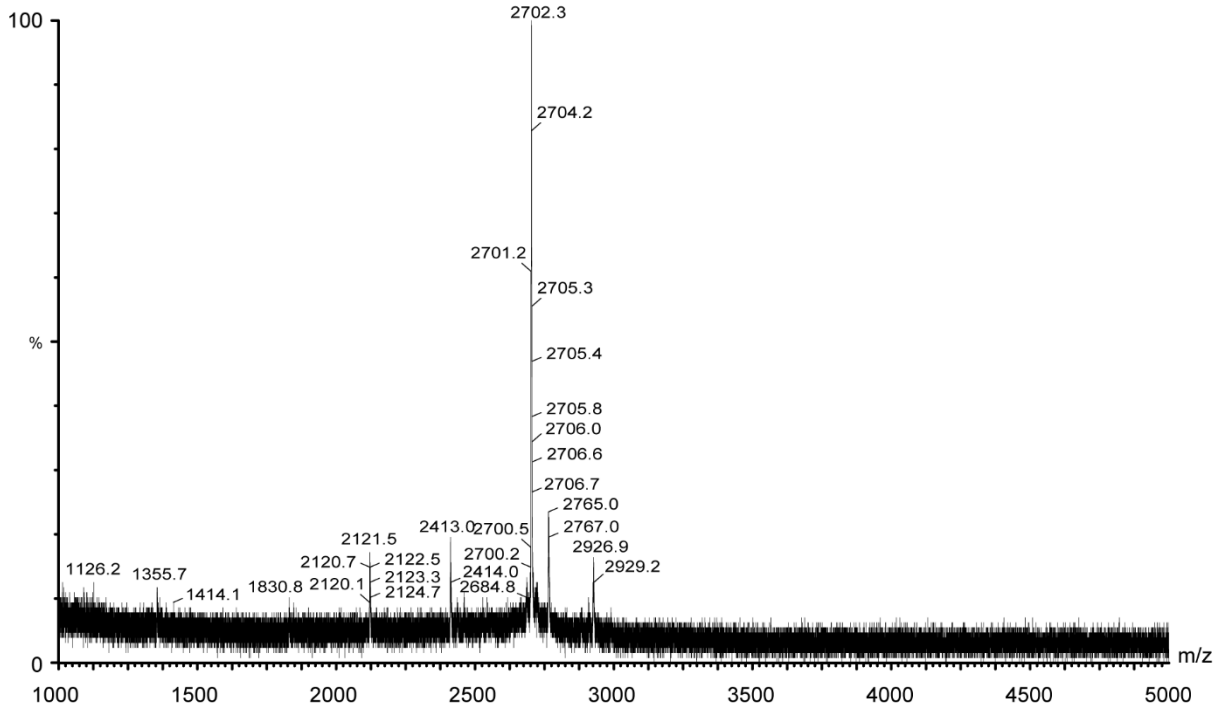
S2. PNA probes synthesis and chemical characterization

Two 7-mer PNA probes were synthesised in house via solid phase peptide synthesis using standard Fmoc chemistry. Briefly, the PNA coumarin probe ("sensing probe") was synthesised on a 0.03 mmol scale from preloaded Fmoc-Arg(Pbf)-Wang resin and functionalised at the N-terminus with a coumarin probehead as previously reported by us.[12] The PNA thiol probe ("catching probe") was synthesised on a 0.03 mmol scale from 4-amminobutanethiol 4-methoxytrityl resin and functionalised with biotin at its N-terminus and a butane thiol at its C terminus. The synthesised PNA probes were purified by HPLC and characterised using Matrix Assisted Laser Desorption and Ionization Time of Flight analysis (MALDI-TOF), using a MALDI microMX instrument with sinapinic acid as the matrix. The probe sequences and corresponding MALDI-TOF spectra are provided below.

PNA coumarin 'sensing' probe

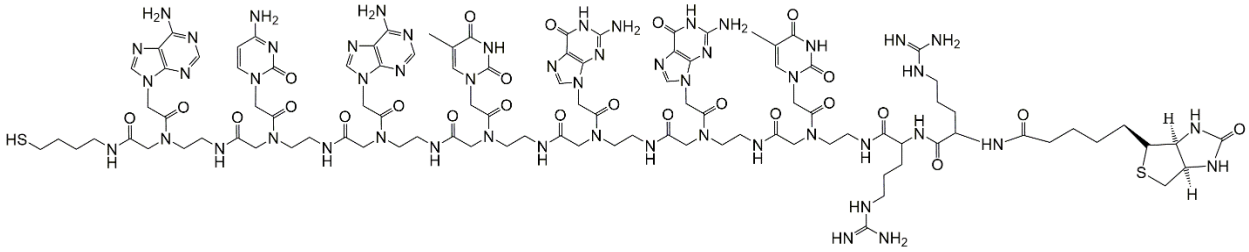
Arg-Arg-AGGGTTG-Coumarin, $C_{114}H_{138}N_{52}O_{29}$, Calculated Molecular Weight = 2700.69 Da

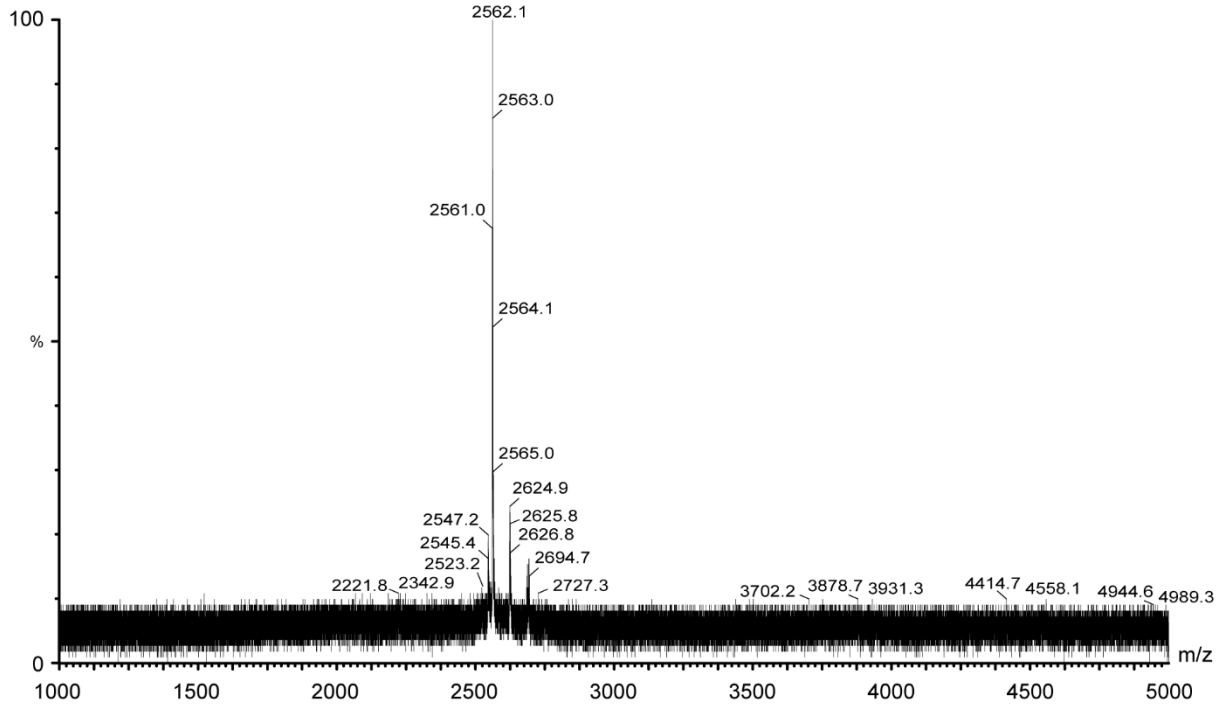




PNA thiol 'capture' probe

Thiol-ACATGGT-Arg-Arg-Biotin, $C_{102}H_{442}N_{52}O_{25}S_2$, Calculated Molecular Weight = 2560.72 Da





S3. Effect of sensing probe concentration on signal-to-noise ratio

The concentration of PNA sensing probe was optimized to minimize background fluorescence and increase the signal-to-noise ratio (SNR). As commonly observed for solution phase oligo-templated reactions (OTRs), increasing concentrations of sensing probes resulted in a decrease in SNR (mainly caused by an increase in background noise). The highest SNR was obtained with 1 μM of PNA coumarin (10× or more excess of coumarin concentration to target DNA concentration), which was then used for all further lateral flow assay (LFA) experiments. Note, lower coumarin concentrations than 1 μM were tested in initial experiments (not shown), where significantly lower SNRs were observed (SNR = 1.1 for 0.1 μM coumarin).

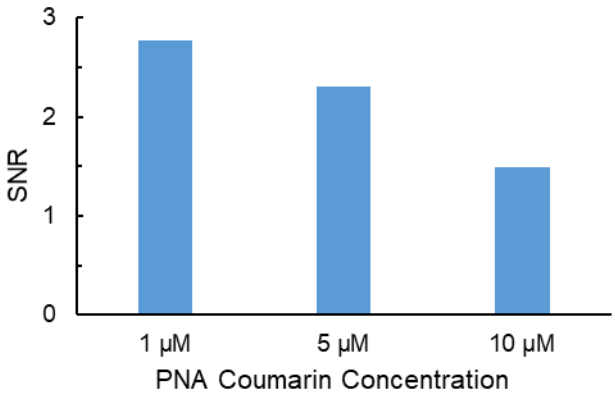


Fig. S1 Effect of varying PNA coumarin concentration on LFA signal-to-noise ratio (SNR is here defined as the ratio of the fluorescence intensity at the test line for an LFA OTR using 0.1 μM of miR-150 DNA target and the fluorescence intensity at the test line for a similar LFA experiment in the absence of DNA target).

S4. Investigation of drying time and kinetics on OTR fluorescence

As shown in Fig. S2a, a time dependent increase in OTR fluorescence was observed at the test line, (orange triangles) until a plateau was reached at 150 min (time chosen for the analysis of all our LFA experiments). Interestingly, the same effect was observed when immobilising a dual-labelled biotin and FITC DNA oligonucleotide (black circles) onto the test line (sequence: 3'-FITC-TCTCCCAACCCTTGTACCAGTG- biotin-5'). This suggests that the time-dependent increase in fluorescence is at least in part due to optical effects on wet paper[13] and also to slow kinetics of the templated reaction on paper (as previously observed in solution[12a]). Importantly, similar fluorescence intensities were achieved when actively drying the LFA strips with a hair dryer for 7 mins, compared to drying in air for 150 minutes, reducing the time-to-analysis to within 10 min (Fig. S2b).

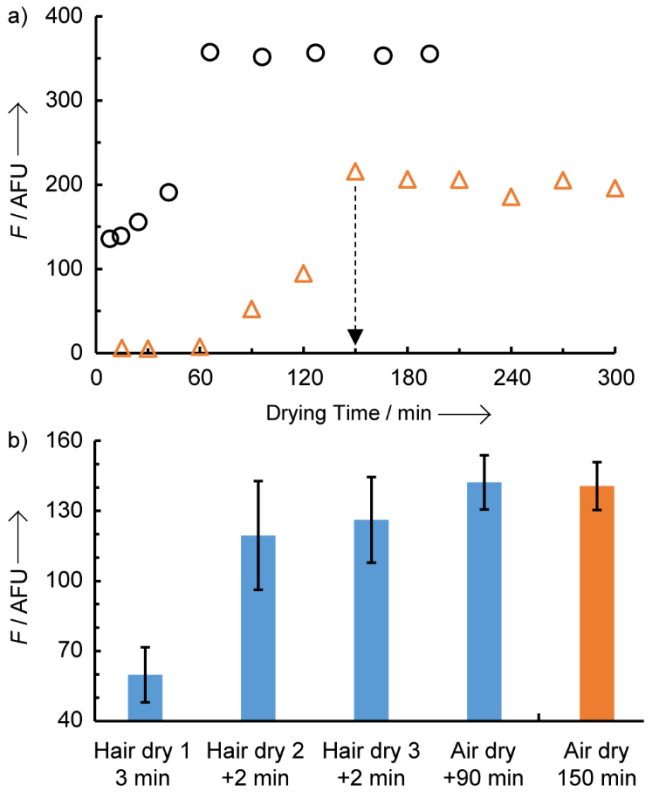


Fig. S2 a) Measurement of fluorescence during drying for the miR-150-5p OTR (orange triangles) and a Biotin-FITC labelled DNA (black circles, sequence: 3'-FITC-TCTCCCAACCCTTGTACCAGTG- biotin-5'), where the drying time is since the 30 µL washing aliquot was added b) Comparison of air drying for 150 mins (orange bar) versus drying with a hair dryer (blue bars 1-3) after three successive hair drying sessions, then leaving for a further 90 mins to dry in air (4th blue bar).

S5. Typhoon scanned images of LFAs from the miR-150 DNA calibration curve

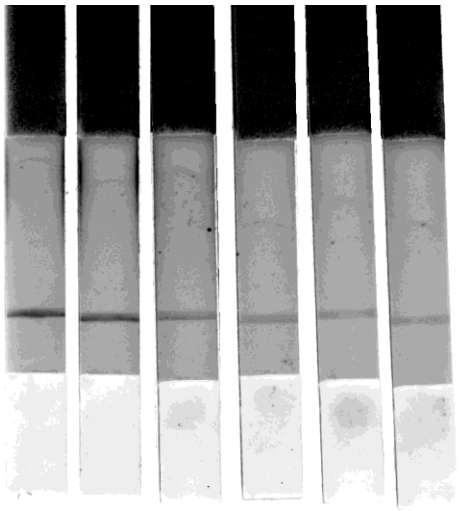


Fig. S3 Representative scanned images (Typhoon FLA9500, GE Healthcare) of the LFA strips corresponding to the calibration curve in Figure 2 (main text), i.e. 1, 0.5, 0.1, 0.05. 0.01 and 0 µM miR-150-5p DNA from left to right.

S6. Investigation of test line saturation using Biotin-FITC

To test the saturation limit of the streptavidin coated on the test line, the dual-labelled 5'-Biotin, 3'-FITC DNA oligonucleotide was added to LFA strips. As shown in Figure S4, a linear increase in fluorescence intensity was observed at the test line when increasing DNA concentration, with a tail off effect at concentrations above 0.2 nM.

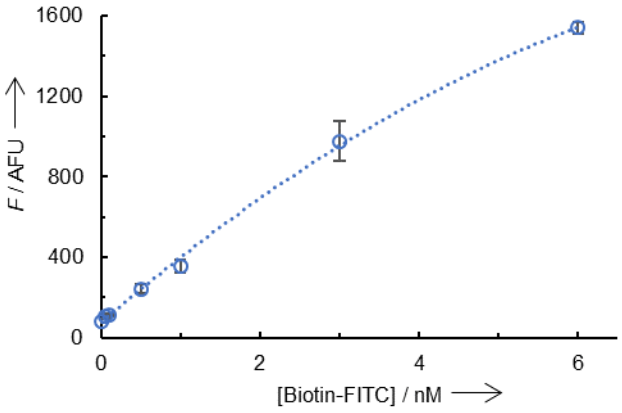


Fig. S4 Fluorescence intensity as a function of Biotin-FITC concentration (6, 3, 1, 0.5, 0.1, 0.05, 0.01 and 0 nM), best fit polynomial curve equation: intensity = -16.0 × (biotin concentration)^2 + 342 × (biotin concentration) + 74.7, R^2 = 0.9986.

S7. Clinical patient samples collection and analysis

All experiments were performed in compliance with the relevant laws and institutional guidelines. Ethical approval was granted by the Hertfordshire Research Ethics Committee (22/02/2011-REC reference number 11/H0311/6) and informed written consent was received from all participants.

Extraction of miRNA from clinical samples

Plasma samples of patients known to have delivered either at term or pre-term, were previously collected and stored at -80°C as part of a clinical trial at the Hammersmith Hospital, London (REC reference for the clinical study: 11/H0311/6), as described in our previous study (Table S1).[3] Plasma samples were processed further to extract miRNAs using the Plasma/Serum Circulating and Exosomal RNA Purification Mini Kit from Norgen Biotek Corp. In short, 1 mL of plasma sample was centrifuged at 3000 RPM for 10 mins. To 0.75 mL of this solution was added 1.3 mL of a lysis buffer, 13 µL of β-Mercaptoethanol and 0.2 mL of a slurry, followed by vortexing for 15 s and incubation at 60°C for 10 mins. Next, 2.25 mL of ethanol was added, followed by vortexing for 15 s and centrifugation at 3000 RPM for 30s. The supernatant was discarded, then 0.3 mL of a lysis buffer was added followed by vortexing for 15 s and incubation at 60°C for 10 mins. Next, 0.3 mL ethanol was added, followed by vortexing for 15 s. 0.65 mL of this mixture was transferred to a spin column and spun at 14,000 RPM for 1 min and the flow through discarded. This step was repeated until all the reaction mixture had been transferred. Then, 0.4 mL of a wash solution was added, the column was spun at 14,000 RPM for 1 min, then the flow through was discarded. After 3 washes, the column was spun empty at 14,000 RPM for 3 mins, then 0.1 mL of an elution solution (A) was added and the column spun at 2000 RPM for 2 mins then 14,000 RPM for 3 mins. This elution was repeated with a further 50 µL of the elution solution. To concentrate and clean the resulting 150 µL of extracted sample, 270 µL of RNAse free water was added, then the mixture was loaded into an Amicon Ultra YM-3 column. The column was centrifuged at 14,000 g for 80 mins, followed by inversion of the column and collection through centrifugation at 4000 g for 2 mins. Finally, the resulting solution was made up to 25 µL through addition of RNAse free water and stored at -80°C between extraction and sensing. 5 µL of this sample was added to each LFA strip for sensing (in a 25 µL solution containing 1 µM coumarin).
The clinical samples were tested in two batches (when the samples were received) and these values were normalized to each other through comparison against 100 nM miR150 DNA and a non-templated reaction.

Table S1 Analysis of clinical samples via reference method (nCounter probe count) and LFA fluorescence (n = 2 per sample), corresponding with Fig. 5. Note, the sample designations w and d correspond to weeks and days into the pregnancy respectively, also these sample identities were validated by PCR in the previous study that collected the clinical samples.[3]

ID and patient outcome	Sample collected	Cervical shortening	nCounter probe count	LFA fluorescence
1 Term	17w 1d	No short	13	70.8
2 Term	12w 6d	No short	30	69.3
3 Term	12w 1d	No short	40	73.3
4 Term	12w 3d	No short	43	68.2
5 Term	14w 0d	No short	49	69.7
6 Term	12w 2d	No short	143	52.7
7 Term	13w 1d	No short	153	46.6
8 Term	12w 5d	No short	206	70.7
9 Term	12w 0d	No short	208	70.2
10 Term	17w 5d	Short	954	69.3
1 Preterm	13w 2d	Short	306	121
2 Preterm	16w 5d	Short	373	91.5
3 Preterm	12w 2d	Short	389	86.7
4 Preterm	12w 2d	Short	419	115
5 Preterm	19w 3d	Short	524	69.7
6 Preterm	14w 2d	Short	655	92.0
7 Preterm	11w 6d	Short	655	79.7
8 Preterm	13w 6d	Short	661	79.4

References:

1. a) G. A. D. Metcalf, A. Shibakawa, H. Patel, A. Sita-Lumsden, A. Zivi, N. Rama, C. L. Bevan, S. Ladame, *Anal. Chem.* 2016, **88**, 8091 – 8098; b) D. Al Sulaiman, J. Y. H. Chang, S. Ladame, *Angew. Chem. Int. Ed.* 2017, **56**, 5247 – 5251.
2. a) K. Weber, M. Lokar, *Trans. Faraday Soc.* 1955, **51**, 1362– 1366; b) K. G. Shah, P. Yager, *Anal. Chem.* 2017, **89**, 12023– 12029.
3. J. Cook, P.R. Bennett, S.H. Kim, T.G. Teoh, L. Sykes, L.M. Kindinger, A. Grant, R. Binkhamis, D.A. MacIntyre, V. Terzidou, *Sci, Rep.* 2019, **9**, 5861.