Lateral Flow Biosensors for Low Abundance Detection of Brain Natriuretic Peptide with Enzyme-Free Amplification

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Supplementary Notes, Figures and Tables

Signal Analysis

Boltzmann Curve

The Boltzmann curve was chosen as a fit, due to the underlying biological and chemical processes. Specifically, the binding and release of the Initiator strand by the aptamers exhibit cooperative behaviour and a characteristic response to changing concentrations. The functional form of the Boltzmann equation used for fitting is:

$$Y = A_2 + \frac{A_1 - A_2}{1 + e^{(x - x_0)/dx}}$$

Where:

Y is the measured signal (band intensity from nPAGE or fluorescence intensity)
x is the input concentration ([Aptamer 1&2] or [Initiators])
A_1 is the lower asymptote (initial minimum signal value)
A_2 is the upper asymptote (final maximum signal value)
dx is the slope factor that describes the steepness of the curve

x_0 is the inflection point of the curve, representing the concentration at which the signal is halfway between its minimum and maximum.

The CHA circuit exists in two primary states: “OFF” (signal low, Initiators sequestered by aptamers) and “ON” (signal high, Initiators free to trigger amplification). Above a concentration threshold, the system undergoes a transition from the OFF state to the ON state, captured by the Boltzmann curve through the catalytic, switch-like phase transition.[1]

The inflection point (x_0) identifies the critical concentration where the system is most sensitive to changes in the input, allowing us to tune the aptamers and Initiator concentration ratios to achieve the desired clinical sensitivity for BNP detection. The high R^2 values (e.g., 0.992 in Figure 2B) confirm that the model indicates a reliable empirical description of the system’s behaviour, validating the experimental approach and the inherent CHA kinetics.

Hill Equation

The Hill equation is commonly used to model the binding interaction between ligands (here Aptamer 1&2) and receptors (Initiator), particularly when cooperativity is present. The equation is expressed as:

$$Y = \frac{[L]^n}{K_d + [L]^n}$$

Where:

Y is the fraction of bound ligands (aptamers)
[L] is the concentration of the ligands [Aptamer 1&2]
n is the Hill coefficient, indicating cooperativity
K_d is the dissociation constant.

From Figure 3B in the main text, the fitted value of Hill coefficient (n = 2.78, n > 1) strongly suggests positive cooperativity in the binding of the dual aptamers to the single Initiator, that the binding of the first aptamer likely induces a conformational change in the Initiator that enhances the accessibility and affinity for the second aptamer to bind. This is consistent with our Initiator design, where its 5' and 3' halves are complementary to Aptamer 2 and Aptamer 1, respectively. Once one aptamer binds, it may stabilise the conformation of the Initiator strand, making the second binding event more favourable.

The apparent dissociation constant (K_d = 104.5 nM) indicates the concentration of [Aptamer 1&2] is consistent with the stoichiometry of the dual-aptamer system for the Initiator (100 nM). This metric also helps define the dynamic range of our system, as the assay responds most sensitively to changes in [Aptamer 1&2] around this concentration. It is notably higher than the BNP concentration the assay is aimed to detect (0.03 nM), so that only when BNP is present does the Initiator get released and become available for CHA amplification. The Hill plot confirms the cooperative nature of the Initiator-dual aptamer interaction, enhancing the specificity and sharpness of the response in our CHA circuit. This ensures that in the absence of BNP, the Initiator remains bound, and the CHA reaction is suppressed.

Supplementary Discussion on Binding Efficiency

The lack of significant binding of Apt1/Apt2 observed below 14.4 nM of BNP coating, followed by a sharp increase in aptamer bound fraction to over 50% at 14.4 nM, could be explained by several factors, including:

- The binding events between aptamers and immobilised BNP may be contingent on the surface density of BNP. At low coating concentrations (0 – 14.4 nM), the density of BNP molecules on the plate surface is not able to ellicit a measurable binding response. At 14.4 nM, hybridising opportunities for aptamers are increased passed a measurable threshold.
- The aptamer-BNP interaction may exhibit cooperative behavior, where binding at one site enhances binding at neighboring sites, so that at lower BNP densities (0 – 14.4 nM), the lack of cooperative effects results in limited binding. The rapid surge in bound fraction of aptamers at 14.4 nM of BNP coating could suggest that the aptamers hold high affinity for BNP and the system reaches saturation rapidly once the concentration exceeds this K_d.
- Quantification of unbound aptamers via PCR could result in undetectable signal changes at lower BNP coating densities. The unbound aptamer population may remain largely unchanged, generating minimal differences in PCR Ct value. At 14.4 nM of BNP coating, the apparent bound fraction of aptamers becomes significant enough to be detectable, triggering in the signal response to climb abruptly.
- In comparison, existing panel of BNP aptamers reported K_d values stretching from 12.5 to 139 nM,[1] placing our system within a competitive range while offering the added advantages of enzyme-free amplification and LFT compatibility. When coupled with CHA, it is sufficient to achieve a clinically relevant LoD of 0.03 nM, as the reaction amplifies the signal from even a small number of Initiators released from the aptamer binding events, compensating for the modest K_d. Moreover, our dual-aptamer approach enhances specificity by targeting distinct epitopes simultaneously, thereby reducing off-target binding and the likelihood of cross-reactivity with related peptides such as NT-proBNP, which is a common source of false positives in single-probe BNP assays.

1. Wang, Y.; Wu, J.; Chen, Y.; Xue, F.; Teng, J.; Cao, J.; Lu, C.; Chen, W., Magnetic microparticle-based SELEX process for the identification of highly specific aptamers of heart marker--brain natriuretic peptide. *Microchimica Acta* **2015**, 182 (1), 331-339.

Supplementary Figures

A

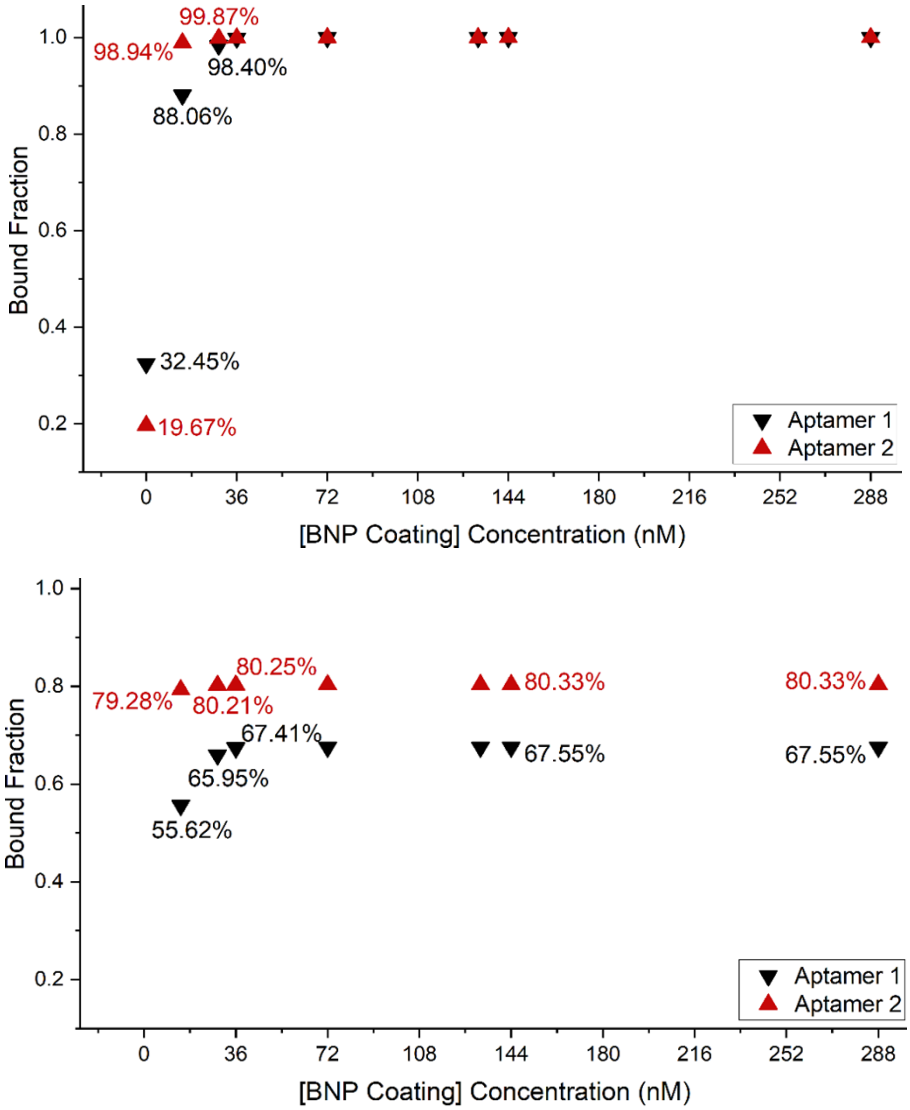


Figure S1. *Expected bound fraction of aptamers remaining in different wells against BNP coating concentrations, where symbols in black denote bound fractions of Apt1, and red for Apt2. (A). Bound fractions from the wells with non-specific binding of aptamers. (B). Bound fractions of aptamers after additional blocking and exclusion of the non-specifically bound portion.*

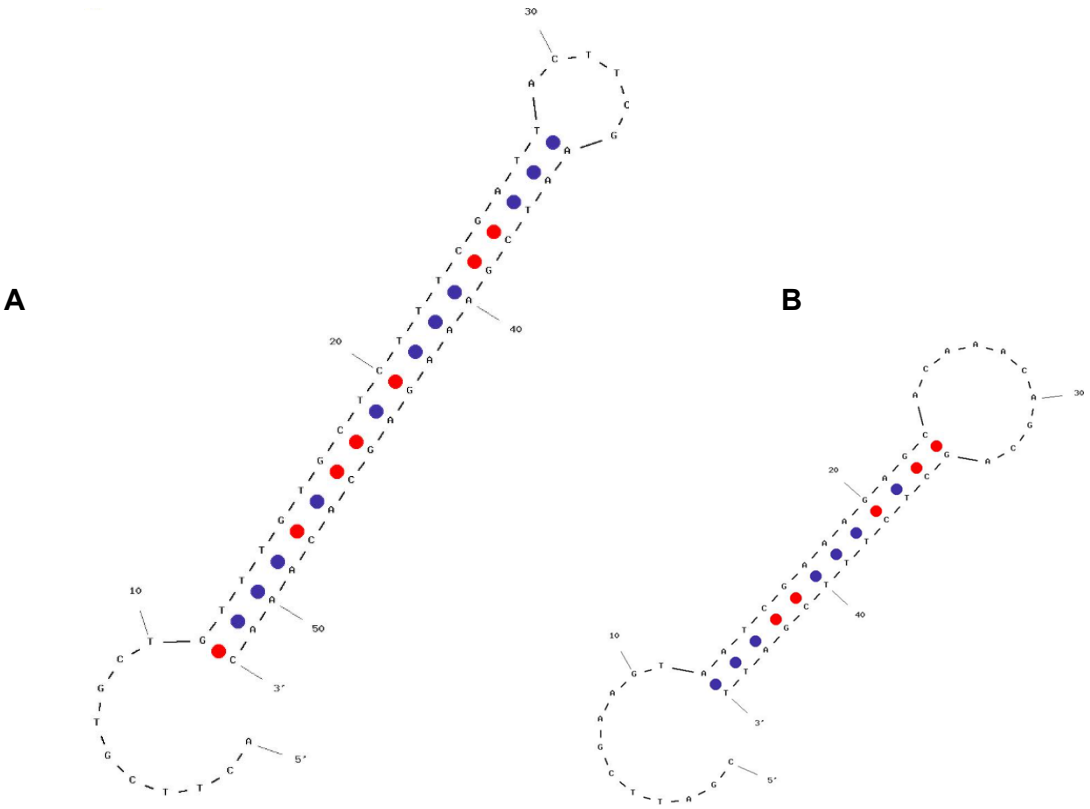


Figure S2. *Prediction of the lowest free energy of hairpin structures. (A) H1_(52), ΔG=-21.81 kcal/mol; (B) H2_(45), ΔG = -13.62 kcal/mol.*

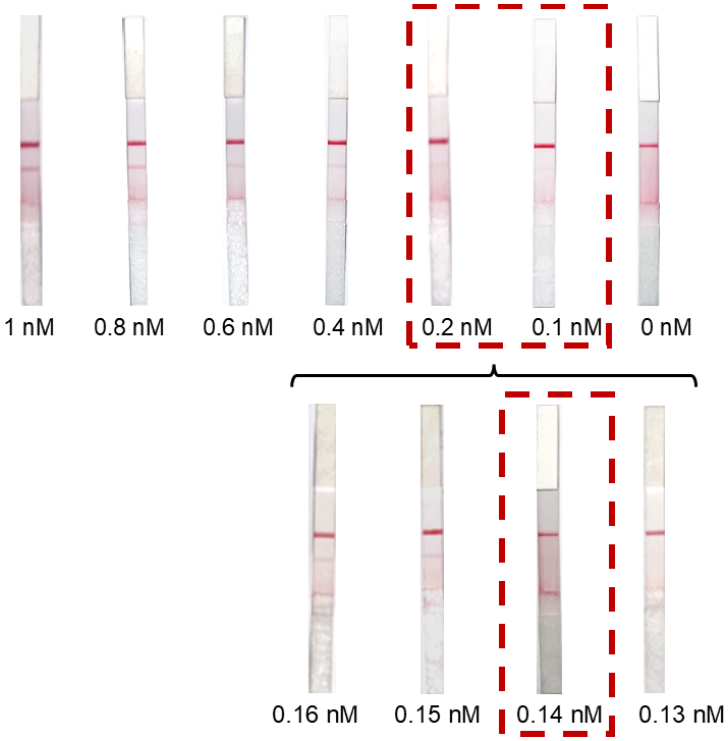


Figure S3. *Optimisations of CHA background signal on LFTs. The CHA leakage reactions are detected at the test line, whenever the concentration of H1 (5' FITC) and H2 (5' Biotin) is above 0.14 nM. The noted concentrations are the combination of both hairpins in a 1:1 ratio, i.e., 0.14 nM of the mixture contains 0.07 nM of H1 and 0.07 nM of H2.*

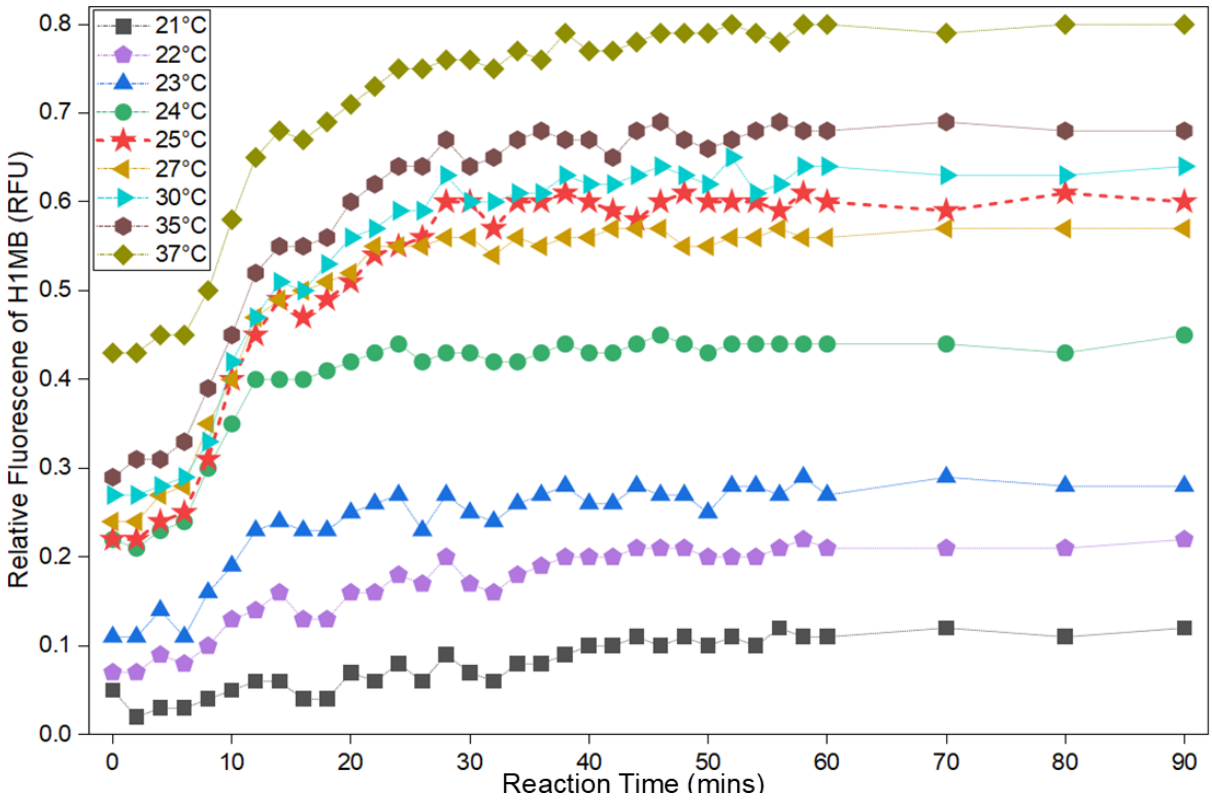
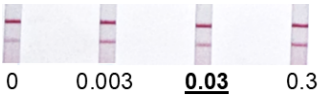


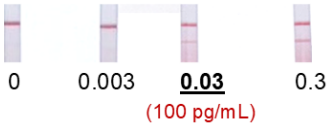
Figure S4. *The fluorescence curves for all reaction temperatures have the characteristic shape with a rapid initial increase followed by a levelling off after 30 min, reflecting the reaction completion when no more signal is being generated. Lower incubation temperatures like 21 °C – 23 °C yield marginal background noise, yet the final signal at the plateau is also much lower, suggesting slow and inefficient reaction. While a higher temperature like 30 °C or 37 °C might reach the plateau slightly faster, they do so at the cost of higher background noise (non-specific amplification or initial fluorescence due to less stable hairpin probes). As the optimal compromise, 25 °C (starred red) results in the best signal-to-noise ratio among the tested conditions. To maintain temperature in POC settings, the system would require active components, which are easily engineered for a simple tube as is required here.*

I

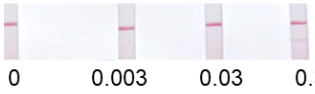
2.7 nM Apt + 1.8 nM Initiator + 4.2 nM (H1 + H2) → 65.25 μL WB (30X dilution)



2.25 nM Apt + 1.5 nM Initiator + 3.5 nM (H1 + H2) → 54 μL WB (**25X** dilution)



1.8 nM Apt + 1.2 nM Initiator + 2.8 nM (H1 + H2) → 42.75 μL WB (20X dilution)



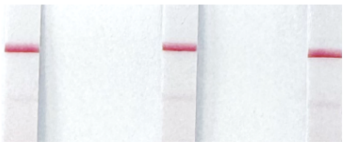
0.9 nM Apt + 0.6 nM Initiator + 1.4 nM (H1 + H2) → 20.25 μL WB (10X dilution)

BNP Concentration (nM)

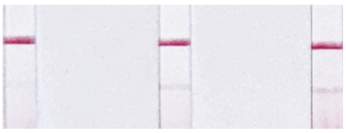
II

2.8 nM H1 + 2.8 nM H2 → 42.75 μL WB (20X dilution) — **A**

1.2 nM Initiator + 2.8 nM (H1 + H2) → 42.75 μL WB (20X dilution)

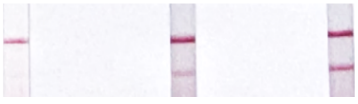
 — **B**

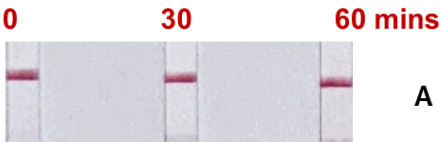
4.2 nM H1 + 4.2 nM H2 → 65.25 μL WB (30X dilution)

 — **C**

III

1.8 nM Initiator + 4.2 nM (H1 + H2) → 65.25 μL WB (30X dilution)

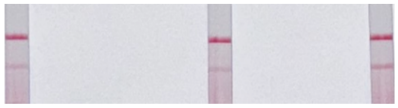
 — **D**



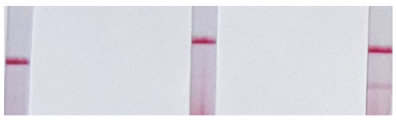
3.5 nM H1_52 (FITC) + 3.5 nM H2_45 (Biotin)

 — **A**

1.5 nM Initiator + 3.5 nM (H1 + H2) → **No** Dilution

 — **B**

3.5 nM H1 + 3.5 nM H2 → 54 μL WB (**25X** dilution)

 — **C**

1.5 nM Initiator + 3.5 nM (H1 + H2) → 54 μL WB (**25X** dilution)

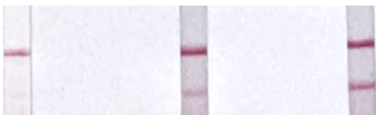
 — **D**

Figure S5. *Wash buffer (WB) optimisation. (**I**) Assays of BNP testing incorporated into the system with pre-optimised concentrations from designated reaction ratio. The optimisation aims to enable the detection of a clinically relevant BNP concentration (cut-off at 0.03nM), avoiding false positives (concentrations below the threshold) and ensuring a strong band signal at this concentration. (**II**) Post-dilution results of the LFT strips, with a constant ratio of [Initiator]:[hairpin] concentrations at 3:7. Provided the hairpin supplies at 2.8 nM, adding 42.75 µL of washing buffer to achieve 20X dilution is necessary for noise control in (A), yet their levels are deficient for signal amplification in (B); when the hairpin concentrations are increased to 4.2 nM, adding 65.25 µL of WB to achieve 30X dilution is no longer impractical for leakage mitigation in (C), but the total signals are strengthened after incubation with 1.8 nM of the Initiators in (D). (**III**) Results of the LFT strips photographed by 30-min intervals of the CHA reaction time. (A) Undiluted negative controls with only H1 and H2 both at 3.5 nM, where the leakage signal is emerging from 30 mins of incubation. (B) Undiluted positive controls with 1.5 nM Initiators and hairpins still at 3.5 nM. (C) Addition of 54 µL of WB for 25X diluted negative controls show that the leakage is persisting after 60 mins of incubation. (D) Diluted positive controls show that the CHA-amplified signal intensifies as the reaction progresses.*

Supplementary Table

Table S1. *Sequence designs of the hairpins in CHA reaction.*

Name	Sequence	Length (nt)	Tm (℃)
H1(52)	5’ ACTTCGTGCTGTTTGTGCTCTTTCGATTACTTCGAATCGAAAGAGCACAAAC [FITC] 3’	52	76.4
H2(45)	5’ CGATTCGAAGTAATCGAAAGAGCACAAACAGCAGCTCTTTCGATT [Biotin] 3’	45	72.6