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Supporting Information

Two-layer cascaded catalytic hairpin assemblies based on locked nucleic acids for one-step and highly sensitive ctDNA detection

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Table S1 The oligonucleotide sequences in the two-layer cascade LNA-assisted CHA circuits.

Name	Sequence(5'-3')
PIK3CA E542K (Target ctDNA)	CTCAGTGA(3*) TTTTAGA(2*) GAGAGGAT(1*)
DNA-H_1	ATCCTCTC(1) TCTAAAA(2) TCACTGAG(3) CCATGTGTAGA(4*) CTCAGTGA(3*) TTTTAGA(2*) CCTTGTCA(5*) TAGAGCAC(6*)
H_1	ATCCTCTC(1) TCTAAAA(2) TCACTGAG(3) CCATGTGTAGA(4*) CTCAGTGA(3*) TTTTAGA(2*) CCTTGTCA(5*) TAGAGCAC(6*)
$H_{1\text{-}S}$	ATCCTCTC(1) TCTAAAA(2) TCACTGAG(3) CCATGTGTAGA(4*) CTCAGTGA(3*) TTTTAGA(2*) CCTTGTCA(5*) TAGAGCAC(6*)
$H_{1\text{-}R}$	ATCCTCTC(1) TCTAAAA(2) TCACTGAG(3) CCATGTGTAGA(4*) CTCAGTGA(3*) TTTTAGA(2) CCTTGTCA(5*) TAGAGCAC(6*)
DNA-H_2	TCACTGAG(3) TCTACACATGG(4) CTCAGTGA(3*) TTTTAGA(2*) CCATGTGTAGA(4*)
H_2	TCACTGAG(3) TCTACACATGG(4) CTCAGTGA(3*) TTTTAGA(2*) CCATGTGTAGA(4*)
DNA-H_3	GAAATCGG(9) GTGTAGTC(8) CCTTGTCA(5*) TAGAGCAC(6*) GCACCTCCTATATCG(7*) GTGCTCTA(6) TGACAAGG(5) TCTAAAA(2)
H_3	GAAATCGG(9) GTGTAGTC(8) CCTTGTCA(5*) TAGAGCAC(6*) GCACCTCCTATATCG(7*) GTGCTCTA(6) TGACAAGG(5) TCTAAAA(2)
DNA-H_4	GCACCTCCTATATCG(7*) CCTTGTCA(5*) TAGAGCAC(6*) CGATATAGGAGGTGC(7) GTGCTCTA(6)
H_4	GCACCTCCTATATCG(7*) CCTTGTCA(5*) TAGAGCAC(6*) CGATATAGGAGGTGC(7) GTGCTCTA(6)
Rep-F	TGACAAGG(5) GACTACAC(8*) CCGATTTCCCAT(9*)-FAM
Regular Rep-F	TGACAAGG(5) GACTACAC(8*) CCGATTTCCCAT(9*)-FAM
Rep-Q	BHQ-1-ATGGGAAATCGG(9) GTGTAGTC(8)
KRAS G12DM	ACT CTT GCC TAC GCC ATC AGC TCC AAC TAC CAC AAG TTT
EGFR L858R	CAG CAG TTT GGC CAG CCC AAA ATC TGT GAT CTT GAC ATG
BRAF V600E	GAT TTT GGT CTA GCT ACA GAG AAA TCT CGA TGG AGT GGG
1-Mut	CTC AGT GAT TTA AGA GAG AGG AT
2-Mut	CTC AGT GAT TTA AGA GTG AGG AT
3-Mut	CTC AGA GAT TTA AGA GTG AGG AT
Rep-F (one-layer)	FAM-CGA GTGCTCTA TGACAAGG TCTAAAA
Rep-Q (one-layer)	CCTTGTCA TAGAGCAC TCG-BHQ-1

All the designed oligonucleotide sequences were analyzed and optimized using a NUPACK software.[1] Red letters represented the locked nucleic acid (LNA) base.

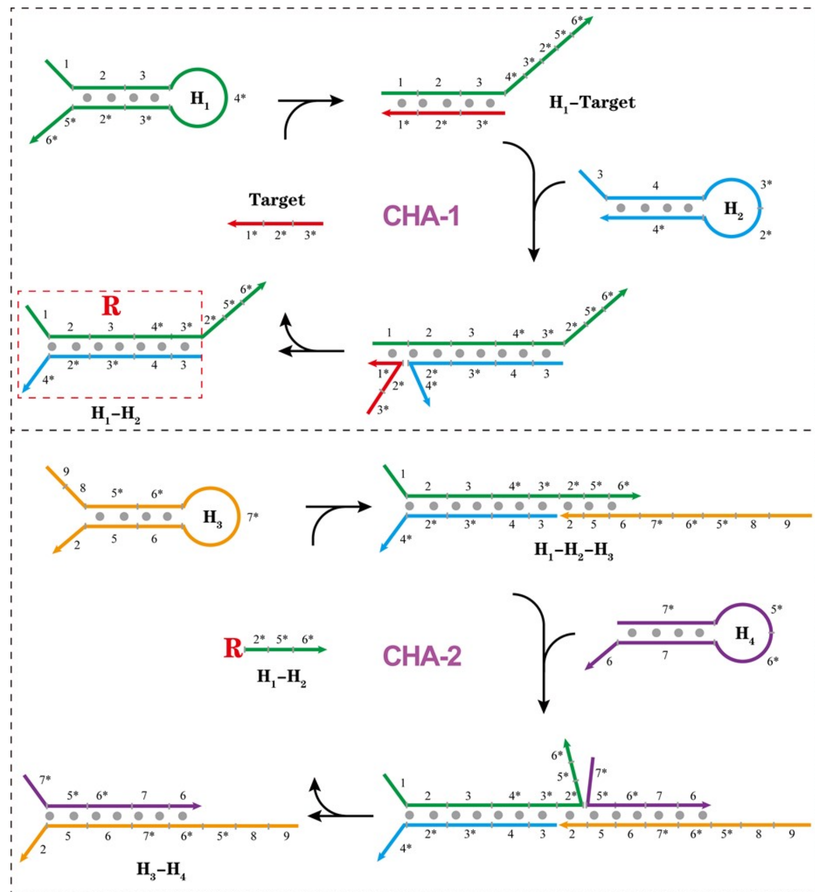

Fig. S1 Proposed response process of the two-layer cascaded LNA-assisted CHA circuits to target ctDNA. Arrows represented the 3'-termini of strands. Gray circles represented base-pairing of hybridized duplex. The numbers of domains and the corresponding asterisks were complementary. Short gray dashes represented the junctions between domains.

The circuits included four hairpins (H_1, H_2 H_3 and H_4) and the fluorophore (FAM)/quencher (BHQ-1) pair labelled reporter, and these hairpins and reporter were respectively modified two pairs of LNAs near its stem-end. The target ctDNA firstly hybridized with the toehold domain 1 of H_1 to initiate a toehold-mediated strand displacement, leading to the formation of H_1-target hybrid through domain hybridization (1-2-3 and 3*-2*-1*). Then, the exposed sequence 3* of H_1-target complex hybridized with the toehold 3 of hairpin H_2 to initiate a branch-migration, continuously generating a H_1-H_2 duplex as another catalyst for the second layer CHA reaction and releasing the target ctDNA for multiple cycles in first layer CHA reaction. Therefore, the exposed domain 2* of H_1-H_2 complex hybridized with the toehold 2 of hairpin H_3 to initiate a toehold-mediated strand displacement, leading to the formation of H_1-H_2-H_3 hybrid. And the newly exposed sequence 6* of H_1-H_2-H_3 complex docked to the toehold 6 of hairpin H_4 via branch-migration, continuously generating a H_3-H_4 duplex and releasing the H_1-H_2 complex for multiple cycles in second layer CHA reaction.

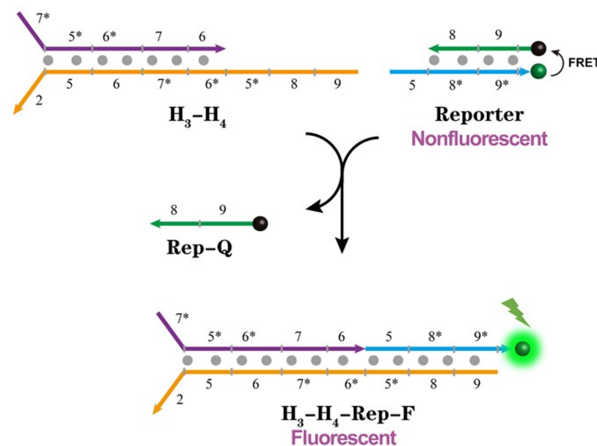

Fig. S2 Fluorescence response process of reporter in the two-layer LNA-assisted CHA circuits. The reporter was consisted of four LNAs-incorporated duplex, and respectively labeled with FAM (Rep-F) and BHQ-1 (Rep-Q). The FAM denoted 6-carboxy fluorescein, and BHQ-1 denoted Black Hole Quencher-1. The newly exposed domain 5* of H_3-H_4 complex further hybridized with the domain 5 of reporter, resulting the separation of Rep-Q and fluorescence recovery.

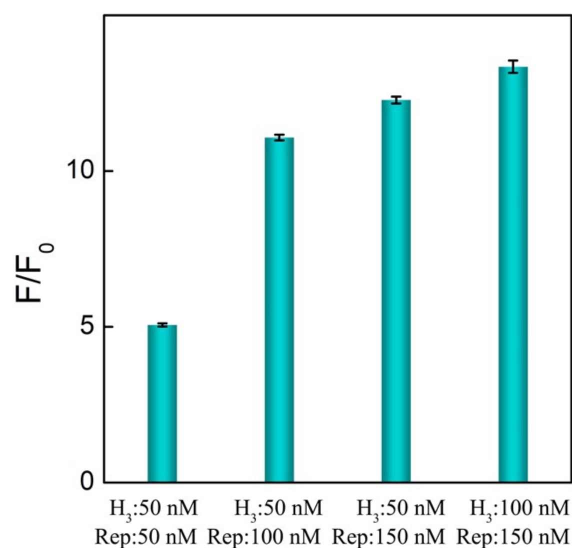

Fig. S3 Fluorescence response of the two-layer LNA-assisted CHA circuits to target ctDNA in the different combinations of hairpin concentrations for 80 min at 25℃. The used concentration included target ctDNA (0.5 nM), H_1 (50 nM), H_2 (100 nM), H_3 (50 nM, 100 nM), H_4 (200 nM) and reporter (50 nM, 100 nM, 150 nM). F and F_0 represented the fluorescence intensity with and without target ctDNA, respectively. Results are presented as means ± standard deviation (SD) (n=3).

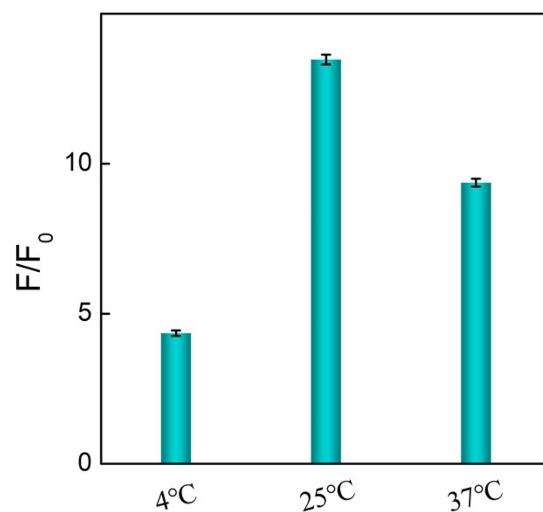

Fig. S4 Fluorescence response of the two-layer LNA-assisted CHA circuits to target ctDNA for 80 min under different temperature conditions. The used concentration included target ctDNA (0.5 nM), H_1 (50 nM), H_2 (100 nM), H_3 (100 nM), H_4 (200 nM) and reporter (150 nM). F and F_0 represented the fluorescence intensity with and without target ctDNA, respectively. Results are presented as means ± standard deviation (SD) (n=3).

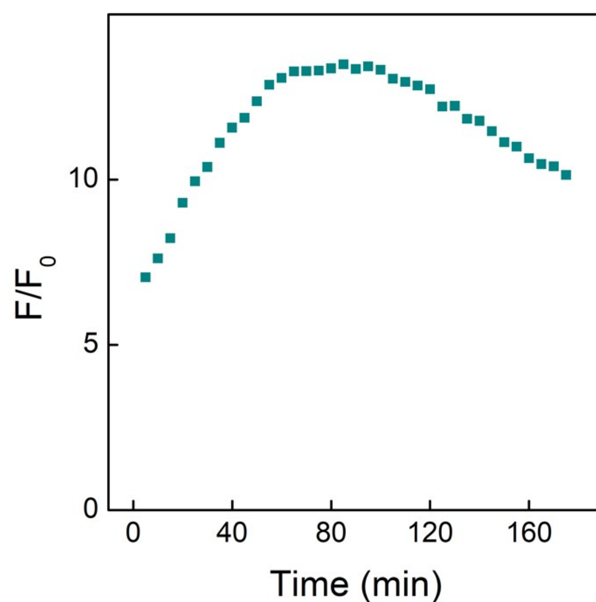

Fig. S5 Fluorescence kinetics of the two-layer LNA-assisted CHA circuits in the presence of target ctDNA at 25℃. The used concentrations included target ctDNA (0.5 nM), H_1 (50 nM), H_2 (100 nM), H_3 (100 nM), H_4 (200 nM), and reporter (150 nM). F and F_0 represented the fluorescence intensity with and without target ctDNA, respectively.

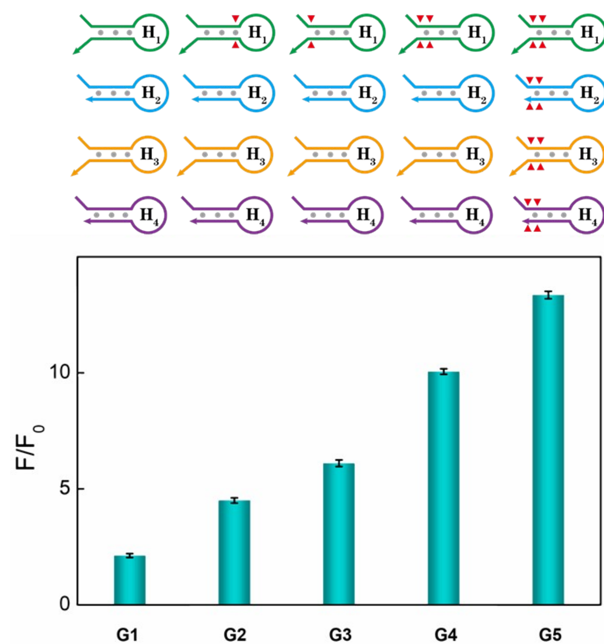

Fig. S6 Fluorescence response of the two-layer LNA-assisted CHA circuits to target ctDNA after introducing different groups of LNA-incorporated hairpins for 80 min at 25℃. The used concentration included target ctDNA (0.5 nM), H_1 (50 nM), H_2 (100 nM), H_3 (100 nM), H_4 (200 nM) and reporter (150 nM). F and F_0 represented the fluorescence intensity with and without target ctDNA, respectively. Results are presented as means ± standard deviation (SD) (n=3).

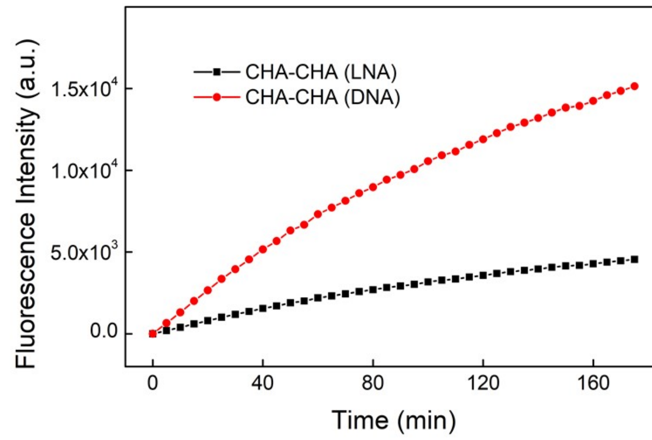

Fig. S7 Fluorescence kinetics of the two-layer LNA-assisted CHA circuits and the two-layer regular CHA circuits in MCF-7 cell lysate at 25℃. The used concentration included H_1 (50 nM), H_2 (100 nM), H_3 (100 nM), H_4 (200 nM) and reporter (150 nM).

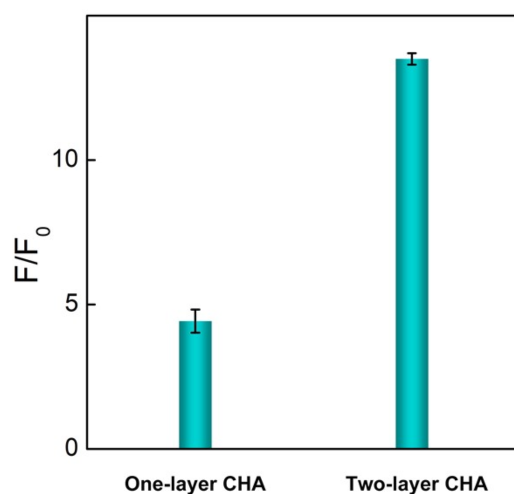

Fig. S8 Fluorescence response of one-layer and two-layer LNA-assisted CHA circuits to target ctDNA for 80 min at 25°C. F and F_0 represented the fluorescence intensity with and without target ctDNA, respectively. The used concentration included target ctDNA (0.5 nM), H_1 (50 nM), H_2 (100 nM), H_3 (100 nM), H_4 (200 nM) and reporter (150 nM). Results are presented as means ± standard deviation (SD) (n=3).

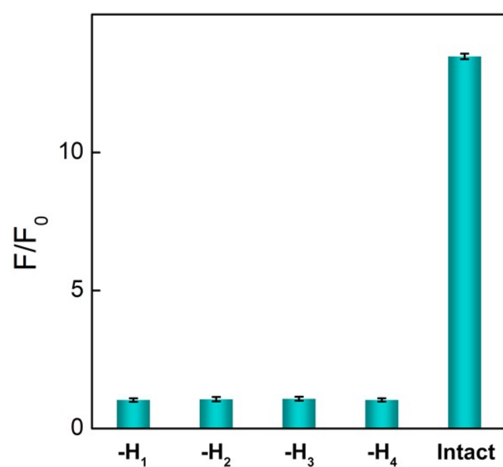

Fig. S9 (a) Fluorescence response of the two-layer LNA-assisted CHA circuits subtracted by H_1 alone, H_2 alone, H_3 alone, or H_4 alone, and the intact system for 80 min at 25°C. The used concentration included target ctDNA (0.5 nM), H_1 (50 nM), H_2 (100 nM), H_3 (100 nM), H_4 (200 nM) and reporter (150 nM). F and F_0 represented the fluorescence intensity with and without target ctDNA, respectively. Results are presented as means ± standard deviation (SD) (n=3).

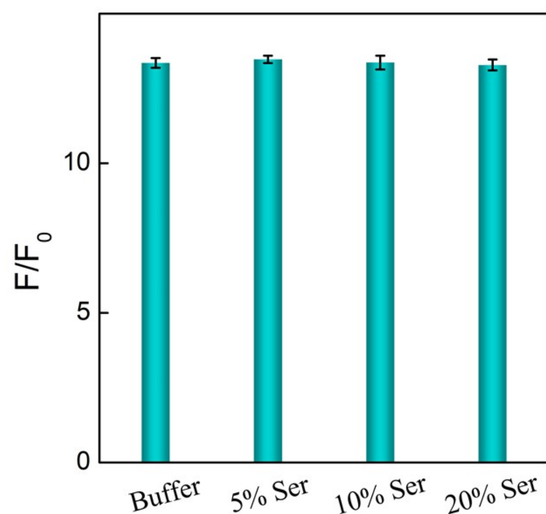

Fig. S10 Fluorescence response of the two-layer LNA-assisted CHA circuits to target ctDNA in different ratios of fetal bovine serums (buffer, 5% serum, 10% serum, and 20% serum) for 80 min at 25℃. The used concentration included target ctDNA (0.5 nM), H_1 (50 nM), H_2 (100 nM), H_3 (100 nM), H_4 (200 nM) and reporter (150 nM). F and F_0 represented the fluorescence intensity with and without target ctDNA, respectively. Results are presented as means ± standard deviation (SD) (n=3).

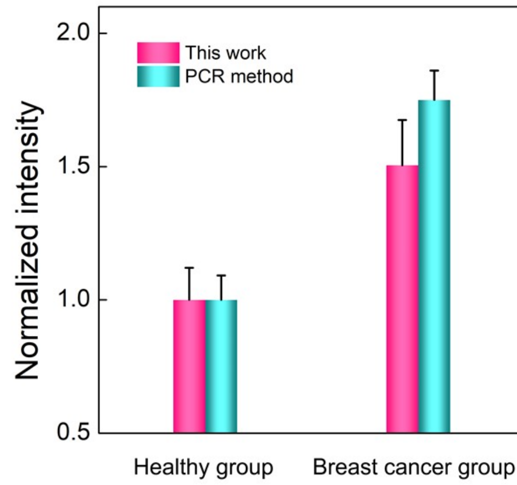

Fig. S11 Clinical samples ctDNA analysis of the two-layer LNA-assisted CHA circuits and commercial PCR method. Normalization is performed against healthy group. Results are presented as means ± standard deviation (SD) (n=3).

Table S2. The test results of 15 healthy donors and 15 breast cancer patients detected by two-layer LNA-assisted CHA circuits and PCR method.

Samples	Proposed method	PCR method
Serum samples from healthy donors	Normalized intensity (F/F_0)	Normalized intensity (F/F_0)
1	0.95	0.99
2	1.05	1.03
3	1.01	1.02
4	0.95	0.97
5	1.02	1.01
6	0.92	0.98
7	1.12	1.06
8	1.01	1.04
9	1.04	1.02
10	0.89	0.97
11	0.93	0.96
12	1.01	1.02
13	0.97	1.01
14	0.91	0.99
15	0.96	1.03
Serum samples from breast cancer patients	Normalized intensity (F/F_0)	Normalized intensity (F/F_0)
1	1.15	1.54
2	1.25	1.61
3	1.45	1.76
4	1.75	1.85
5	1.55	1.69
6	1.55	1.73
7	1.23	1.82
8	2.45	2.68
9	1.74	1.85
10	1.25	1.66
11	1.95	2.12
12	1.24	1.73
13	1.03	1.46
14	1.33	1.58
15	1.65	1.83

References

1. J. N. Zadeh, C. D. Steenberg, J. S. Bois, B. R. Wolfe, M. B. Pierce, A. R. Khan, R. M. Dirks and N. A. Pierce, *J. Comput. Chem.*, 2011, **32**, 170-173.