Electronic Supplementary Material (ESI) for Analyst.

Supplementary Information for

A highly specific and flexible detection assay using collaborated actions of DNA-processing enzymes for identifying multiple gene expression signatures in breast cancer

Dain Kim,[a] Jiyoung Lee,[b] Jueun Han,[c] Jaewoo Lim,[d,e] Eun-Kyung Lim[d,e,f*] and Eunjung Kim[a,b*]

[a] Department of Bioengineering & Nano-bioengineering, Research Center for Bio Materials and Process Development, Incheon National University, Incheon 22012, Republic of Korea

[b] Division of Bioengineering, Incheon National University, Incheon 22012, Republic of Korea

[c] Department of Chemistry, Incheon National University, Incheon 22012, Republic of Korea

[d] BioNanotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 34141, Republic of Korea

[e] Department of Nanobiotechnology, KRIBB School of Biotechnology, UST, Daejeon 34113, Republic of Korea

[f] School of Pharmacy, Sungkyunkwan University, Suwon 16419, Republic of Korea

* Corresponding authors.

E-mail addresses: eklim1112@kribb.re.kr (E-K. Lim), e.kim@inu.ac.kr (E. Kim)

NUPACK analysis

Nucleic acid package (NUPACK) algorithms enable a thermodynamic analysis of nucleic acid sequences in a test tube containing arbitrary numbers of interacting strand species at equilibrium. This analysis further allows for predicting equilibrium base-pairing information of the possible ordered complexes in a dilution solution at the given concentrations of each strand species. Therefore, we first predicted secondary structures of each DB template and their free energy at 37 °C. Then, to estimate the hybridization efficiency of DB templates with ProDNA, we computed DB templates with various DNA strand species (ProDNA, Flap DNA, and cProDNA) that are fully or partially complementary to DB template sequences. We set initial concentration of each strand to 200 nM to formulate the complexes, including DB/ProDNA, DB/Flap DNA, DB/cProDNA, and DB/ProDNA/Flap DNA/cProDNA complexes, and ran the NUPACK program at the elevated temperature, here 60 °C.

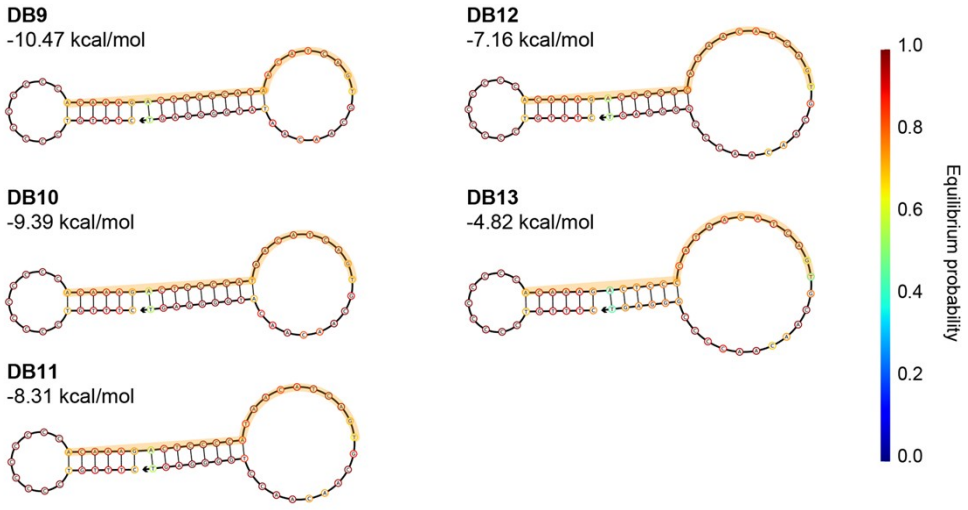

Fig. S1 Secondary structures and free energies of DB templates with various toehold lengths predicted by NUPACK at 37 °C, with the associated depiction of equilibrium base-pairing probabilities. The sequences to which ProDNA binds were highlighted in orange.

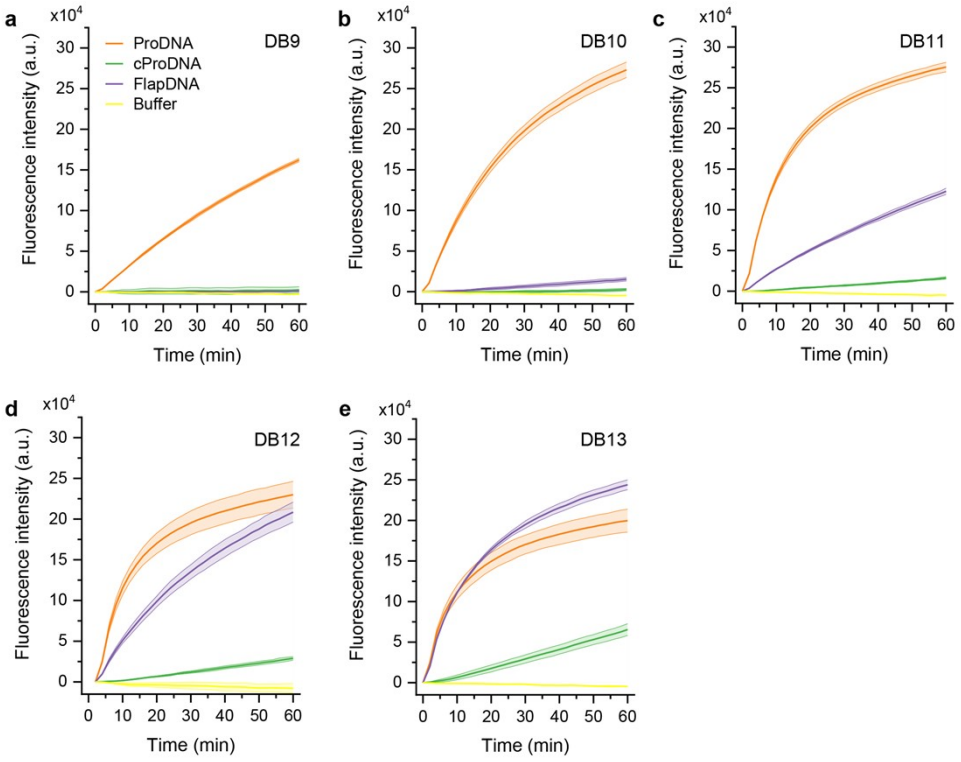

Fig. S2 Real-time fluorescence analysis of tRCA with various DB templates: (a) DB9, (b) DB10, (c) DB11, (d) DB12, and (e) DB13. The reaction was initiated by various DNA strands, including ProDNA, cProDNA, and FlapDNA, at a concentration of 50 nM. As a control, only the DB template was added to the RCA master mix, confirming no noticeable background signals. Data represent mean ± s.d. for three independent experimental replicates, shown as lines and shading, respectively.

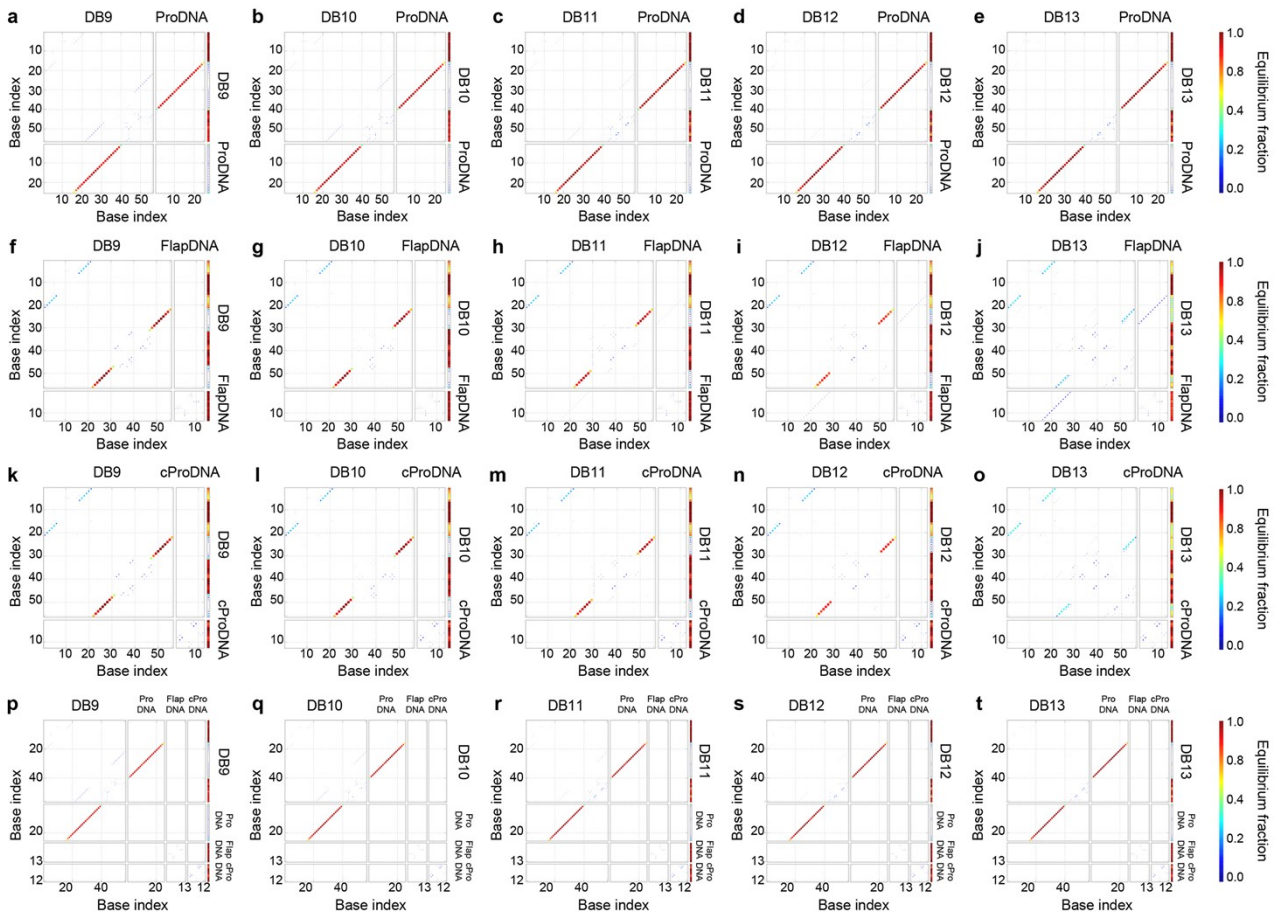

Fig. S3 Ensemble pair fraction plots of the DB template complexed with ProDNA, FlapDNA, and cProDNA in the diluted solution generated by NUPACK. Ensemble pair fraction plots of (a-e) DB/ProDNA, (f-j) DB/FlapDNA, (k-o) DB/cProDNA, and (p-t) DB/ProDNA/FlapDNA/cProDNA complexes simulated at 60 °C with the associated depiction of equilibrium fraction of a particular species of base-pairing. Each dot in the column represents the fraction of strands of a given species with the corresponding base unpaired at equilibrium. The concentration of each DNA strand was set as 200 nM. The DB/ProDNA complexes dominate with a few secondary structures of free DB, ProDNA, FlapDNA, and cProDNA.

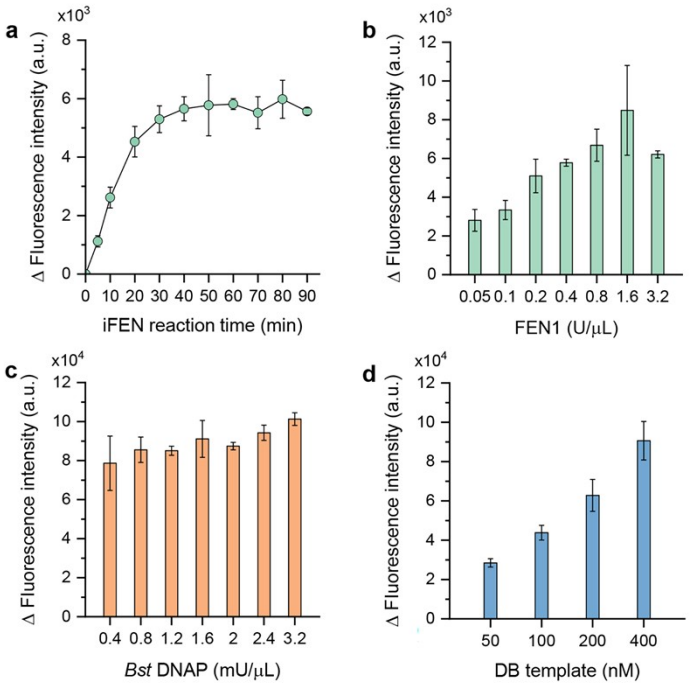

Fig. S4 Optimization of iFEN and tRCA reactions. (a, b) Fluorescence intensity changes of iFEN reaction with various incubation time and FEN1 concentrations at a fixed temperature of 56.2 °C. ΔFluorescence intensity indicates the difference in fluorescence intensities of the iFEN products in the absence or presence of a DNA target (200 nM), respectively. (c, d) Fluorescence intensity changes of tRCA reaction with various *Bst* DNAP and DB template concentrations at a fixed temperature of 50 °C. ΔFluorescence intensity indicates the difference in fluorescence intensities of the tRCA products in the absence or presence of ProDNA (5 nM), respectively. Data represent mean ± s.d. for two or three independent experiments.

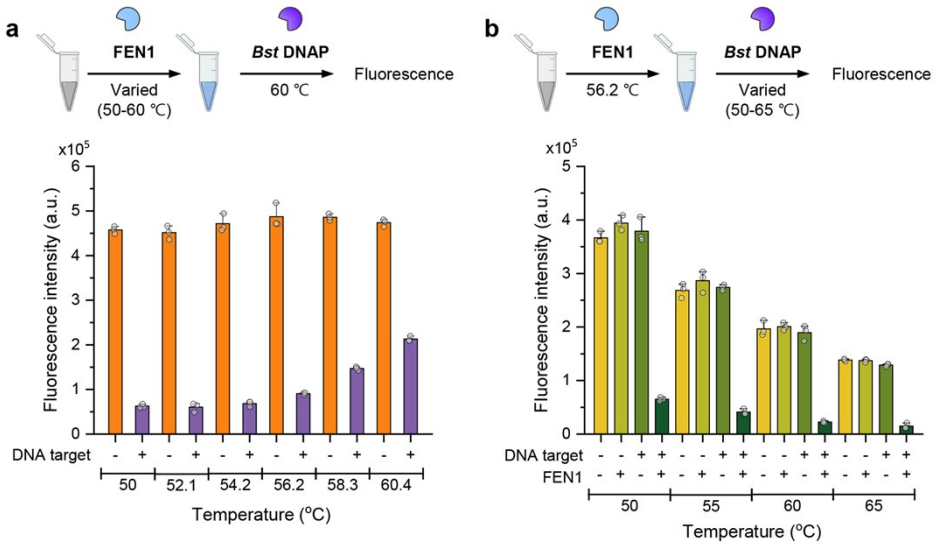

Fig. S5 Optimization of iFEN–tRCA reactions. (a) Fluorescence intensity of the resulting tRCA products in the absence or presence of a DNA target (5 nM) after reaction with FEN1 at temperatures ranging from 50 to 60.4 °C and *Bst* DNAP at a fixed temperature (50 °C). (b) Fluorescence intensities of the tRCA products in the absence or presence of a DNA target (5 nM) and/or FEN1 after reaction with FEN1 at a fixed temperature (56.2 °C) and *Bst* DNAP at increasing temperatures from 50 to 65°C. Data represent mean ± s.d. for three independent experiments.

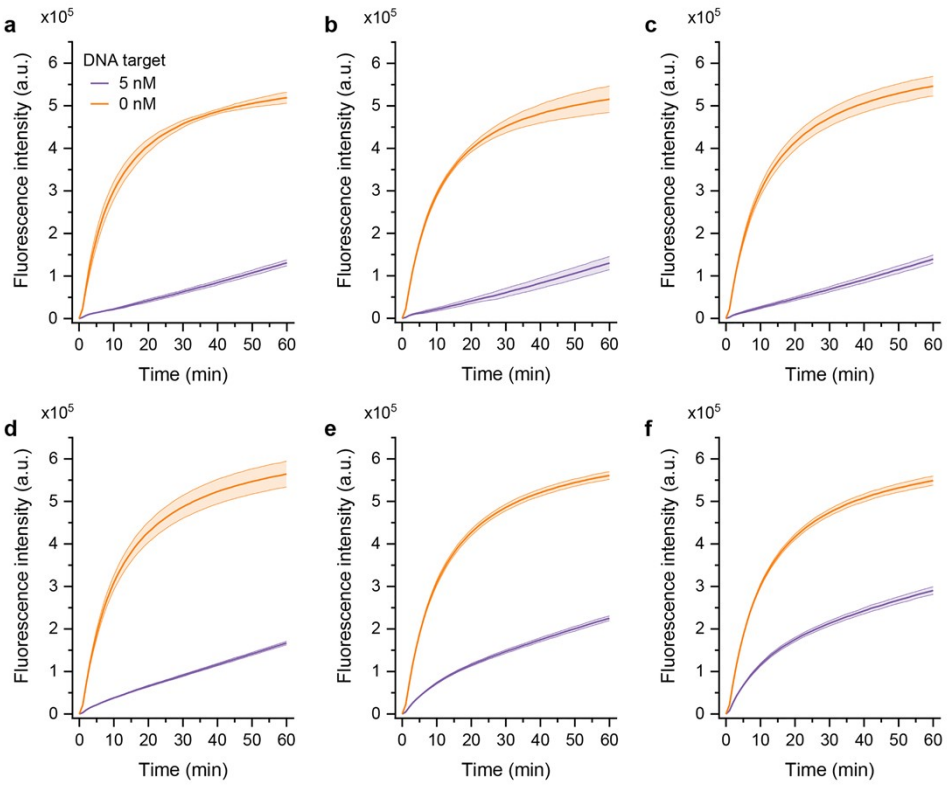

Fig. S6 Real-time fluorescence analysis of iFEN–tRCA at various iFEN reaction temperatures: (a) 50 °C, (b) 52.1 °C, (c) 54.2 °C, (d) 56.2 °C, (e) 58.3 °C, and (f) 60.4 °C. The tRCA was performed at 50 °C. The reaction was performed in the absence or presence of 5 nM of DNA target. Data represent mean ± s.d. for three independent experimental replicates, shown as lines and shading, respectively.

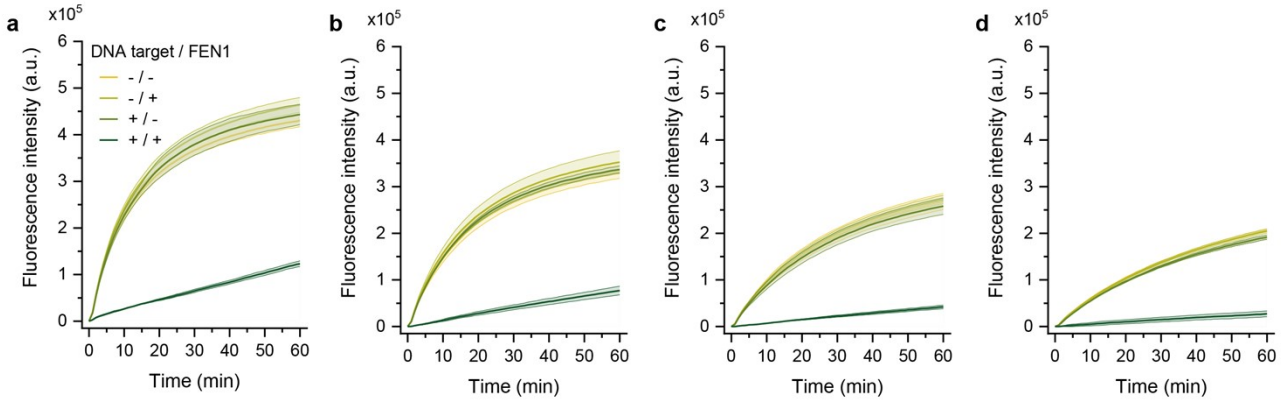

Fig. S7 Real-time fluorescence analysis of iFEN–tRCA at different tRCA reaction temperatures: (a) 50 °C, (b) 55 °C, (c) 60 °C, and (d) 65 °C. The reaction was conducted in the absence or presence of FEN1 and carried out at a fixed temperature of 56.2 °C. The reaction was also performed in the absence or presence of 5 nM of DNA target. Data represent mean ± s.d. for three independent experimental replicates, shown as lines and shading, respectively.

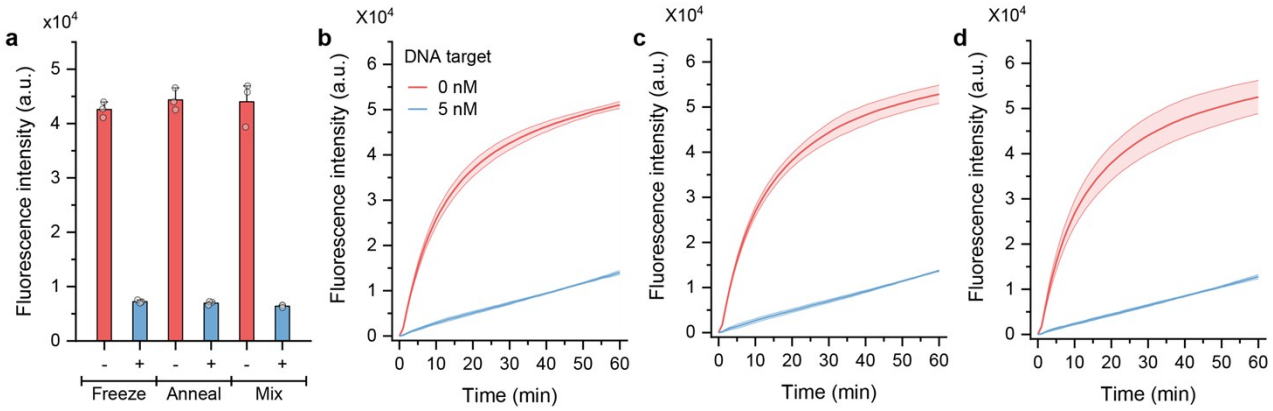

Fig. S8 Comparison of hybridization of ProDNA and InDNA with DNA target in the iFEN reaction. (a) Fluorescence intensity and (b-d) real-time fluorescence intensity changes of iFEN–tRCA without or with 5 nM of DNA target, where the ProDNA and InDNA were hybridized to the target through (b) freeze-thawing, (c) thermal annealing, and (d) simple mixing. Data in (a) represent mean ± s.d. for three independent experimental replicates. Significance was based on two-way ANOVA followed by Tukey's post-hoc test. Data in (b-d) represent mean ± s.d. for three independent experimental replicates, shown as lines and shading, respectively.

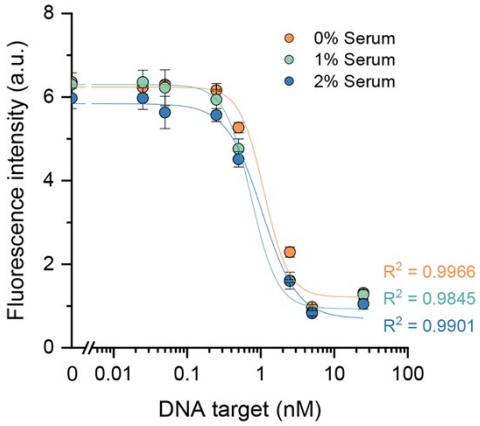

Fig. S9 Fluorescence responses of iFEN–tRCA with various concentrations of DNA target spiked into 0, 1, and 2% (v/v) heat-inactivated fetal bovine serum. Data represent mean ± s.d. for three independent experiments.

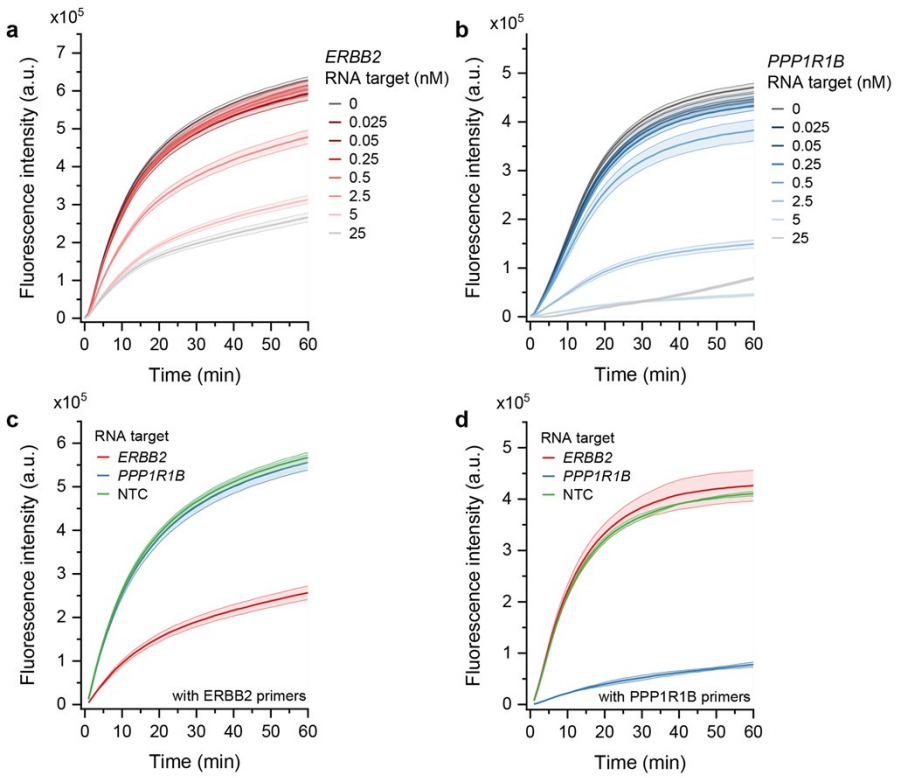

Fig. S10 Real-time fluorescence analysis of iFEN–tRCA with various concentrations of (a) *ERBB2* and (b) *PPP1R1B* RNA targets. Specificity test of iFEN-tRCA against *ERBB2* and *PPP1R1B* RNA targets using (c) *ERBB2* and (d) *PPP1R1B* primers, respectively. No target control (NTC) sample was used as a control. Data represent mean ± s.d. for three independent experimental replicates, shown as lines and shading, respectively.

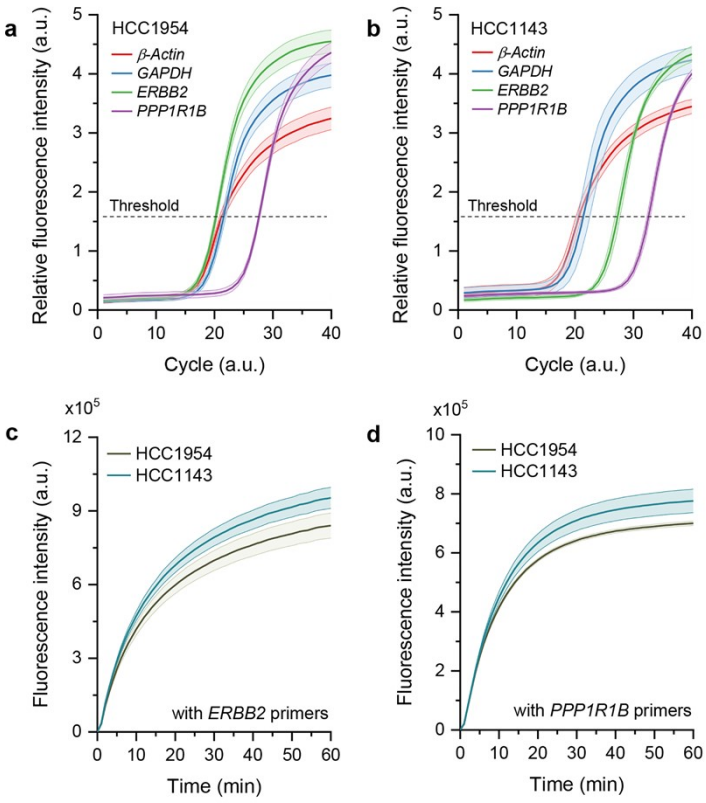

Fig. S11 Amplification curves of qRT-PCR of *β-actin*, *GAPDH*, *ERBB2*, *PPP1R1B* genes in total RNA extracts from (a) HCC1954 (HER2-positive) and (b) HCC1143 (HER2-negative) cells, respectively. Real-time fluorescence analysis of iFEN–tRCA with (c) *ERBB2* and (c) *PPP1R1B* primers in the presence of cellular RNA extracts obtained from HCC1954 and HCC1143 cells, respectively. Data represent mean ± s.d. for three biological replicates and three to six independent experimental replicates, shown as lines and shading, respectively.

Table S1. Summary of synthetic oligonucleotide sequences used in this study.

Name		Sequence (5' to 3')	Modification
DNA target	DNA target	TCAGAATCCACAAAGACTCCCCAGTGCTGTTCCT CTTCCAACGAGGCTGG	-
DNA target with mismatches	DNA target mm1	TCAGAATCCACAAAGAATCCCCAGTGC TGTTCCTCTTCCAACGAGGCTGG	-
	DNA target mm2	TCAGAATCCACAAAGAATCCCCTGTGC TGTTCCTCTTCCAACGAGGCTGG	-
	DNA target mm3	TCAGAATCCACAAAGAATCCCCTGTGC TGTTCCTATTCCAACGAGGCTGG	-
Scrambled DNA target	DNA target scram	ACTCTATCCTCACCCTGTTTTGTGCTCTTCCCCTC GCCTGCTAGGGCTGC	
Invading DNA	InDNA	GCCTCGTTGGAAGAGGAACAGCAC**C**	-
Probe DNA	ProDNA	CACTGATGTTATGGGGAGTCTTTGT	-
Fluorescently labelled probe DNA	FProDNA	CACTGATGTT*ATGGGGAGTCTTTGT	*Fluorescein dT and 3' Iowa Black FQ modification
Cleaved probe DNA	cProDNA	GGGGAGTCTTTGT	-
Flap DNA	Flap DNA	CACTGATGTTAT	-
Dumbbell template DNA with 9 nt toehold	DB9	CTTTGTCCCCCCCCCACAAAGACTCCCCATA*ACAT CAGTG*CAACAATATGGGGAGT	5' Phosphorylation
Dumbbell template DNA with 10 nt toehold	DB10	CTTTGTCCCCCCCCCACAAAGACTCCCCAT*AACAT CAGTG*CAACAACATGGGGAGT	5' Phosphorylation
Dumbbell template DNA with 11 nt toehold	DB11	CTTTGTCCCCCCCCCACAAAGACTCCCCA*TAACAT CAGTG*CAACAACCTGGGGAGT	5' Phosphorylation
Dumbbell template DNA with 12 nt toehold	DB12	CTTTGTCCCCCCCCCACAAAGACTCCCC*ATAACAT CAGTG*CAACAACCCGGGGAGT	5' Phosphorylation
Dumbbell template DNA with 13 nt toehold	DB13	CTTTGTCCCCCCCCCACAAAGACTCCC*CATAACAT CAGTG*CAACAACCCCGGGAGT	5' Phosphorylation

Complementary sequences of DNA target to InDNA and ProDNA are marked in purple and orange, respectively. One invading nucleotide of InDNA is indicated in bold. Nucleotide mismatches compared with the sequences of DNA target are marked in red. Stem and toehold sequences in each DB are indicated in underline and italic, respectively.

Table S2. Summary of oligonucleotide sequences for the detection of RNA targets.

Name		Sequence (5’ to 3’)	Modification
RNA target for *ERBB2*	*ERBB2* target	CCAGCCUCGUUGGAAGAGGAACAGCACUGGGGAG UCUUUGUGGAUUCUGA GGCCCUGCCC	-
Reverse transcription primer for *ERBB2* target	*ERBB2* RT primer	GCAGGGCCTCAGAAT	-
Invading DNA for *ERBB2* target	InDNA (*ERBB2*)	GCCTCGTTGGAAGAGGAACAGCACC	
Probe DNA for *ERBB2* target	ProDNA (*ERBB2*)	CACTGATGTTATGGGGAGTCTTTGT	
Dumbbell template DNA for *ERBB2* target	DB template (*ERBB2*)	CTTTGTCCCCCCCCCACAAAGACTCCCCATAACATCA GTGCAACAACATGGGGAGT	5’ Phosphorylation
RNA target for *PPP1R1B*	*PPP1R1B* target	ACUCUAUCCUCACCCUGUUUUGUGCUCUUCCCCU CGCCUGCUAGGGCUGC GGCUUCUGAC	-
Reverse transcription primer for *PPP1R1B* target	*PPP1R1B* RT primer	CAGAAGCCGCAGCCC	-
Invading DNA for *PPP1R1B* target	InDNA (*PPP1R1B*)	CTATCCTCACCCTGTTTTGTGCTCC	
Probe DNA for *PPP1R1B* target	ProDNA (*PPP1R1B*)	CACTGATGTTATTCCCCTCGCCTGC	
Dumbbell template DNA for *PPP1R1B* target	DB template (*PPP1R1B*)	GCCTGCCCCCCCCCCGCAGGCGAGGGG AATAACATCAGTGCAACAACATTCCCCTC	5’ Phosphorylation

Table S3. qRT-PCR primer sequences used in this study.

Gene name	Accession number	Forward/reverse primer (5’ to 3’)
ERBB2	NM_001382782.1	CTCGTTGGAAGAGGAACAGC CTGAATGGGTCGCTTTTGTT
PPP1R1B	NM_032192.4	ATCCTCACCCTGTTTTGTGC CGGTTTTCTGGATTTTCCAAT
GAPDH	NM_001289745.3	GCTCTCTGCTCCTCCTGTTC TGACTCCGACCTTCACCTTC
β-Actin	NM_001101.2	TGCGTTACACCCTTTCTTGA CACCTTCACCGTTCCAGTTT

Table S4. Properties of the specified DB template structure obtained using NUPACK.

DB	Free energy[a] (kcal/mol)	Probability[b]	Ensemble defect[c] (nt)	Normalized ensemble defect[d] (%)	Nucleotide (nt)
DB9	-10.47	0.129	4.9	8.8	56
DB10	-9.39	0.122	4.7	8.4	56
DB11	-8.31	0.118	4.9	8.7	56
DB12	-7.16	0.126	4.8	8.6	56
DB13	-4.82	0.079	7.5	13.4	56

[a] Free energy of the specified secondary structure

[b] The probability for the formation of this structure

[c,d] The average number and percentage of nucleotides that are incorrectly paired at equilibrium relative to the specified secondary structure. 0 or 0% is best, and N or 100% is worst for a strand with N bases.

Table S5. Equilibrium concentrations and free energies of the DB template complexed with ProDNA, FlapDNA, and cProDNA in the diluted solution obtained using NUPACK. The concentration of each DNA strand was set as 200 nM.

Complex	**DB9/ProDNA**	**DB10/ProDNA**	**DB11/ProDNA**	**DB12/ProDNA**	**DB13/ProDNA**
Equilibrium concentration (nM)					
DB/ProDNA	190	190	200	200	200
ProDNA	15	8.7	4.7	2.8	1.1
DB	15	8.7	4.7	2.8	1.1
Free energy (kcal/mol)					
DB/ProDNA	-22.33	-22.41	-22.49	-22.45	-22.46
Complex	**DB9/FlapDNA**	**DB10/FlapDNA**	**DB11/FlapDNA**	**DB12/FlapDNA**	**DB13/FlapDNA**
Equilibrium concentration (nM)					
DB/FlapDNA	0.25	0.64	2.2	5.2	27
FlapDNA	200	200	200	190	170
DB	200	200	200	190	170
Free energy (kcal/mol)					
DB/FlapDNA	-14.03	-14.02	-14.10	-13.99	-13.99
Complex	**DB9/cProDNA**	**DB10/cProDNA**	**DB11/cProDNA**	**DB12/cProDNA**	**DB13/cProDNA**
Equilibrium concentration (nM)					
DB/cProDNA	0	0	0	0	0.63
cProDNA	200	200	200	200	200
DB	200	200	200	200	200
Free energy (kcal/mol)					
DB/cProDNA	-	-	-	-	-9.02
Complex	**DB9/ProDNA /FlapDNA /cProDNA**	**DB10/ProDNA /FlapDNA /cProDNA**	**DB11/ProDNA /FlapDNA /cProDNA**	**DB12/ProDNA /FlapDNA /cProDNA**	**DB13/ProDNA /FlapDNA /cProDNA**
Equilibrium concentration (nM)					
DB/ProDNA	190	190	200	200	200
ProDNA	15	8.7	4.8	2.9	1.2
FlapDNA	200	200	200	200	200
cProDNA	200	200	200	200	200
DB	14	8.7	4.7	2.8	1.0
Free energy (kcal/mol)					
DB/ProDNA	-22.33	-22.41	-22.49	-22.45	-22.46

Table S6. Summary of various methods using RCA for the detection of long DNA or RNA targets.

Method	Amplification mode	Detection signal	Temperature for RCA	Total assay time (total assay steps)	Detection limit (target gene)	Ref
iFEN-tRCA	Linear	Fluorescence	50 °C	1 h (iFEN and tRCA)	~10 pM (Two mRNA targets)	This work
RCA using a constrained DNA ring/hairpin	Linear	Fluorescence	37 °C	~3 h (RCA, enzyme deactivation, and incubation with signal probes)	0.48 pM, 0.56 pM (Two mRNA targets)	1
RNase H-assisted RNA-primed RCA	Linear	Fluorescence	37 °C	~2.5 h (Hybridization with a target, ligation, and RCA)	10^7 copies (Single mRNA target)	2
Target-catalyzed hairpin structure-mediated padlock cyclization-based RCA	Linear	Fluorescence	30 °C	~4.5 h (Ligation, enzyme deactivation, RCA, enzyme deactivation, hybridization with molecular beacons)	0.1 pM (Single DNA target)	3
RCA-FRET assay	Linear	Fluorescence	37 °C	~5 h (hybridization between a padlock and target, ligation, RCA, and probe hybridization)	0.19 pM, 0.06 pM (Two dsDNA targets)	4
Hybridization of hairpin probe pairs to a DNA nanowire generated by RCA	Linear	Fluorescence	37 °C	~7 h (RCA and enzyme deactivation, nanowire purification, hybridization with hairpins)	10.9 pM (Single mRNA target)	5
Graphene oxide-assisted RCA	Linear	Fluorescence	30 °C	16-18 h (overnight) (Hybridization between padlock and target, ligation, enzyme deactivation, exonuclease digestion, RCA, *etc.*)	1.4 pM (Single DNA target)	6
Split primer ligation-triggered 8-17 DNAzyme-assisted cascade RCA	Exponential	Electrochemical	37 °C	1.75 h (Ligation, padlock hybridization, enzyme deactivation, RCA, *etc.*)	60 pM (Two mRNA targets)	7

References:

1. W. Zhou, D. Li, R. Yuan and Y. Xiang, *Anal. Chem.*, 2019, **91**, 3628-3635.
2. H. Takahashi, M. Ohkawachi, K. Horio, T. Kobori, T. Aki, Y. Matsumura, Y. Nakashimada and Y. Okamura, *Sci. Rep.*, 2018, **8**, 1-5.
3. H. Song, Z. Yang, M. Jiang, G. Zhang, Y. Gao, Z. Shen, Z-S. Wu and O. Lou, *Talanta*, 2019, **204**, 29-35.
4. M. Dekaliuk, X. Qiu, F. Troalen, P. Busson and N. Hildebrandt, *ACS Sens.*, 2019, **4**, 2786-2793.
5. K. Ren, Y. Xu, Y. Liu, M. Yang and H. Ju, *ACS Nano*, 2019, **12**, 263-271.
6. J. Wen, W. Li, J. Li, B. Tao, Y. Xu, H. Li, A. Lu and S. Sun, *Sens. Actuator B-Chem.*, 2016, **227**, 655-659.
7. S. Dai, Y. Zhou, P. Dai, G. Cheng, P. He and Y. Fang, *Electroanalysis*, 2019, **32**, 554-560.