Supplementary Information for

An ultrasensitive label-free RNase H assay based on *in vitro* transcription of fluorogenic light-up aptamer

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Table S1 Oligonucleotide sequences used in this work.

Name	Sequence (5' → 3')
$HP_{(20)}$[a], [b], [c]	rCrCrCrUrArUrArGrUrGrArGrUrCrGrUrArUrUrA T TTT TTT TTA ATA CGA CTC ACT ATA GGG
$HP_{(15)}$[a], [b], [c]	CCC rUrArUrArGrUrGrArGrUrCrGrUrArU TAT TTT TTT TTA ATA CGA CTC ACT ATA GGG
$HP_{(11)}$[a], [b], [c]	CCC TA rUrArGrUrGrArGrUrCrGrU AT TAT TTT TTT TTA ATA CGA CTC ACT ATA GGG
$HP_{(8)}$[a], [b], [c]	CCC TAT rArGrUrGrArGrUrC G TAT TAT TTT TTT TTA ATA CGA CTC ACT ATA GGG
$HP_{(6)}$[a], [b], [c]	CCC TAT A rGrUrGrArGrU CG TAT TAT TTT TTT TTA ATA CGA CTC ACT ATA GGG
$HP_{(4)}$[a], [b], [c]	CCC TAT AG rUrGrArG TCG TAT TAT TTT TTT TTA ATA CGA CTC ACT ATA GGG
DNA HP[b], [c]	CCC TAT AGT GAG TCG TAT TAT TTT TTT TTA ATA CGA CTC ACT ATA GGG
TP[b], [d]	GGA GCC CAC ACT CTA CTC GAC AGA TAC GAA TAT CTG GAC CCG ACC GTC TCC CCT ATA GTG AGT CGT ATT A

[a] The red letters with the prepositive 'r' in HP indicate the RNA sequence whose length is denoted in the bracket.

[b] The blue letters represent T7 promoter sequences.

[c] Underlined sequences represent the stem region of HP and DNA HP.

[d] The green letters represent the antisense of broccoli aptamer.

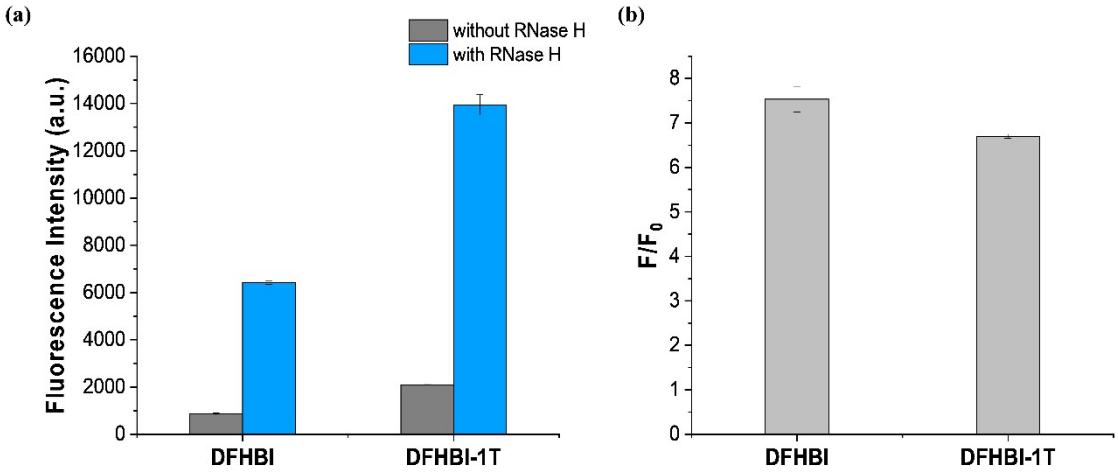

Fig. S1 Comparison of DFHBI and DFHBI-1T for the RNase H activity assay. (a) Fluorescence intensities at 508 nm from the samples containing DFHBI and DFHBI-1T. (b) The signal-to-background ratio (F/F_0, where F and F_0 are the fluorescence intensities at 508 nm from the samples with and without RNase H, respectively.) was obtained from the samples containing DFHBI and DFHBI-1T. The final concentrations of HP, TP, rNTP mix, fluorogenic dyes, T7 RNA polymerase, and RNase H were 200 nM, 200 nM, 3.3 mM, 100 μM, 1.67 U μL^{-1}, and 1 U mL^{-1}, respectively. The error bars were calculated from three independent experiments.

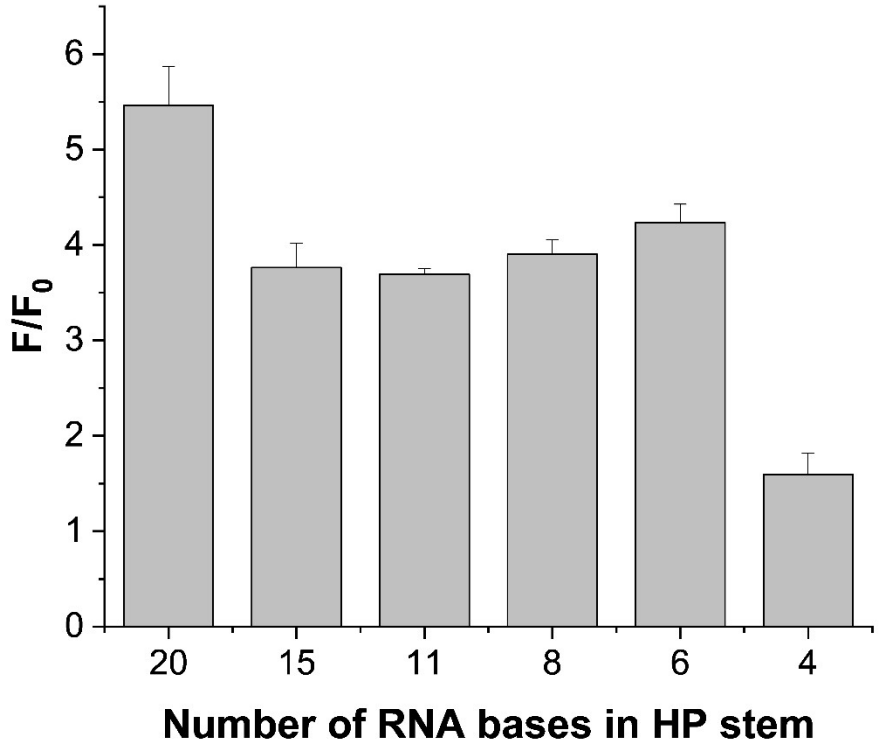

Fig. S2 Optimization of the number of RNA bases in HP stem. The signal-to-background ratio (F/F_0, where F and F_0 are the fluorescence intensities at 508 nm from the samples with and without RNase H, respectively.) was obtained by comparing the fluorescence intensity of the sample with or without RNase H. The final concentrations of HP, TP, rNTP mix, DFHBI, T7 RNA polymerase, and RNase H were 200 nM, 200 nM, 3.3 mM, 100 μM, 1.67 U μL^{-1}, and 1 U mL^{-1}, respectively. The error bars were calculated from three independent experiments.

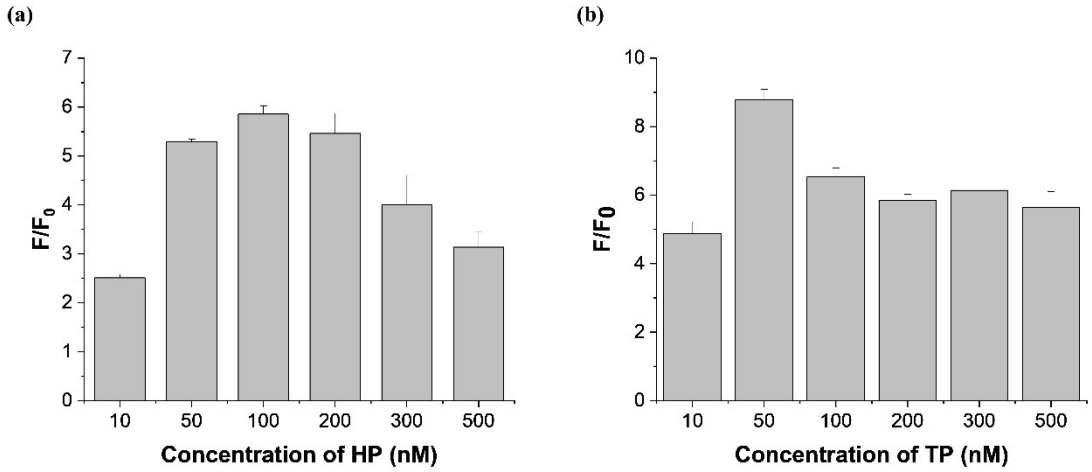

Fig. S3 Optimization of probe concentrations. The signal-to-background ratio (F/F_0, where F and F_0 are the fluorescence intensities at 508 nm from the samples with and without RNase H, respectively.) was obtained by comparing the fluorescence intensity of the sample with or without RNase H. (a) The signal-to-background ratios (F/F_0) for HP at varying concentrations. The final concentration of TP was 200 nM. (b) The signal-to-background ratios (F/F_0) for TP at varying concentrations. The final concentration of HP was 100 nM. Through all the experiments, the final concentrations of rNTP mix, DFHBI, T7 RNA polymerase, and RNase H were 3.3 mM, 100 μM, 1.67 U μL^{-1}, and 1 U mL^{-1}, respectively. The reaction times for RNase H and T7 *in vitro* transcription were 30 min and 60 min, respectively. The error bars were calculated from three independent experiments.

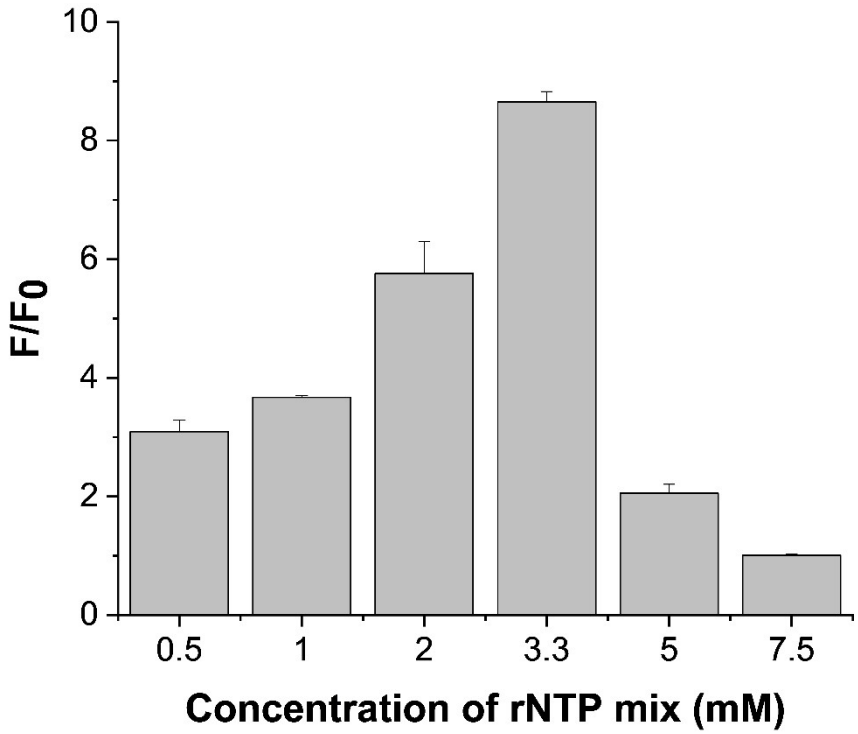

Fig. S4 Optimization of rNTP mix concentration. The signal-to-background ratio (F/F_0, where F and F_0 are the fluorescence intensities at 508 nm from the samples with and without RNase H, respectively.) was obtained by comparing the fluorescence intensity of the sample with or without RNase H. The final concentrations of HP, TP, DFHBI, T7 RNA polymerase, and RNase H were 100 nM, 50 nM, 100 μM, 1.67 U μL^{-1}, and 1 U mL^{-1}, respectively. The reaction times for RNase H and T7 *in vitro* transcription were 30 min and 60 min, respectively. The error bars were calculated from three independent experiments.

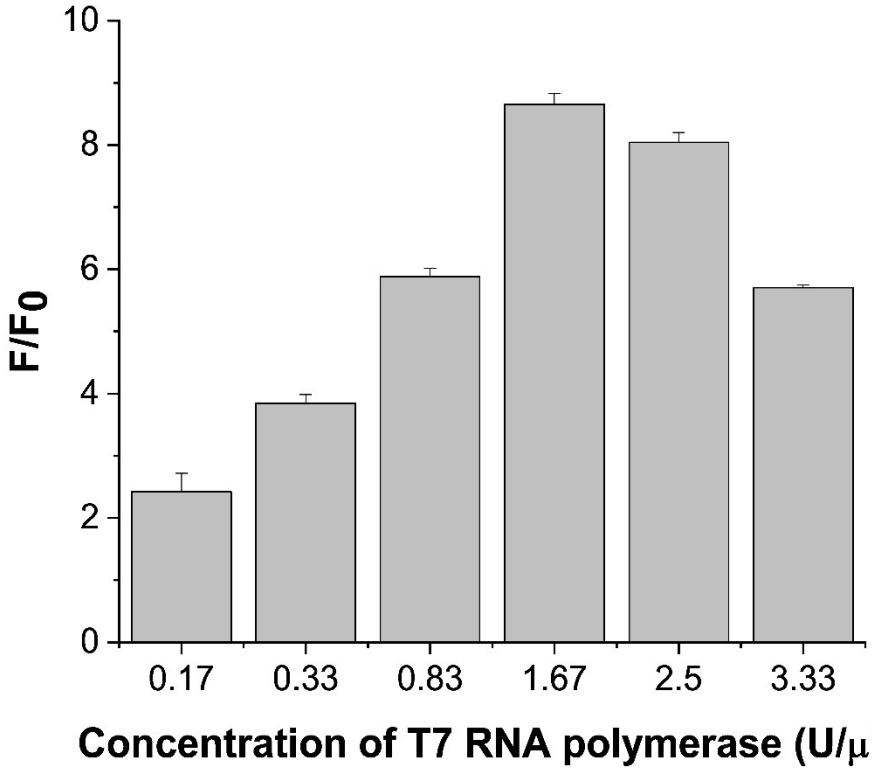

Fig. S5 Optimization of T7 RNA polymerase concentration. The signal-to-background ratio (F/F_0, where F and F_0 are the fluorescence intensities at 508 nm from the samples with and without RNase H, respectively.) was obtained by comparing the fluorescence intensity of the sample with or without RNase H. The final concentrations of HP, TP, rNTP mix, DFHBI, and RNase H were 100 nM, 50 nM, 3.3 mM, 100 μM, and 1 U mL^{-1}, respectively. The reaction times for RNase H and T7 *in vitro* transcription were 30 min and 60 min, respectively. The error bars were calculated from three independent experiments.

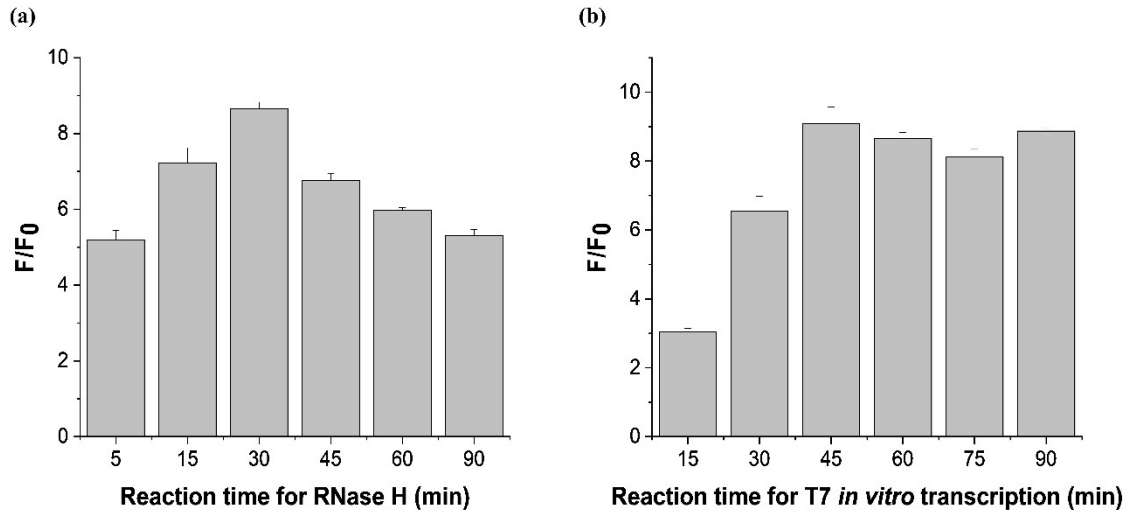

Fig. S6 Optimization of reaction times. The signal-to-background ratio (F/F_0, where F and F_0 are the fluorescence intensities at 508 nm from the samples with and without RNase H, respectively.) was obtained by comparing the fluorescence intensity of the sample with or without RNase H. (a) The signal-to-background ratios (F/F_0) of varying reaction times for RNase H. The reaction time for T7 *in vitro* transcription was 60 min. (b) The signal-to-background ratios (F/F_0) of varying reaction times for T7 *in vitro* transcription. The reaction time for RNase H was 30 min. Through all the experiments, the final concentrations of HP, TP, rNTP mix, DFHBI, T7 RNA polymerase, and RNase H were 100 nM, 50 nM, 3.3 mM, 100 μM, 1.67 U μL^{-1}, and 1 U mL^{-1}, respectively. The error bars were calculated from three independent experiments.

Table S2 Comparison of the developed strategy with alternative methods for RNase H assay.

Key components/Methods	**Limit of detection (U mL^{-1})**	**Limitations**	**Reference**
Molecular beacon	15	- Modification of target substrate with fluorophore and quencher - A single signaling event in response to target - Low sensitivity	S1
Pyrene-modified molecular beacon	5	- Modification of target substrate with pyrene molecules - A single signaling event in response to target - Low sensitivity	S2
Target-triggered release of DNAzyme	0.01	- Modification of detection probe with fluorophore and quencher - Low sensitivity	S3
Target-triggered rolling circle amplification (RCA)	0.019	- Modification of target substrate with amino groups - Low sensitivity	S4
Target-activated DNA polymerase	0.016	- Modification of detection probe with fluorophore and quencher - Low sensitivity	S5
Tb^{3+}-induced G-quadruplex	2	- A single signaling event in response to target - Low sensitivity	S6
DNAzyme-powered on-particle DNA walker	0.00847	- Synthesis of nanomaterials	S7
In vitro transcription of light-up aptamers	0.000156	-	This work

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