Electronic Supplementary Information

Specific Detection of RNA Mutation at Single-Base Resolution by Coupling Isothermal Exponential Amplification Reaction (EXPAR) with Chimeric DNA Probe-Aided Precise RNA Disconnection at the Mutation Site

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1. Experimental Section

Reagents and materials. Vent (exo-) DNA polymerase, and Nt.BstNBI nicking endonuclease were obtained from New England Biolabs (USA). Ribonuclease H, 5× Hybrid RNA Degeneration Buffer, dNTPs, and Recombinant RNase Inhibitor were purchased from TaKaRa (Dalian, China). SYBR Green I (20×) was supplied by Xiamen Bio-Vision (Xiamen, China). All the nucleic acid sequences (Table S1) used in this work were synthesized by TaKaRa (Dalian, China) and purified by HPLC. The real-time fluorescence measurement was conducted on a StepOne Real-Time PCR System (Applied Biosystems, USA).

In this study, a 41 nt-long tumor-associated mutant *BRAF* mRNA fragment with the V600E site mutation (mutRNA), in which an uracil ribonucleotide (U) in the wtRNA (wild-type sequence) is mutated to adenine ribonucleotide (A) (Table S1), is employed as a model target.

Table S1. Sequence of target mRNA fragments, DNA Probes and Template[a]

Nucleic acids	Sequence (5′-3′)
Mutant *BRAF* mRNA fragment (mutRNA)	rArUrArGrGrUrGrArUrUrUrUrGrGrUrCrUrArGrCrUrArC rArGrArGrArArArUrCrUrCrGrrArUrGrGrArG
Wild-type *BRAF* mRNA fragment (wtRNA)	rArUrArGrGrUrGrArUrUrUrUrGrGrUrCrUArGrCrUrArC rArGrUrGrArArArUrCrUrCrGrArUrGrGrArG
Chimeric DNA Probe (cmDNA)	AGA***TTTC***TCTGT
Normal DNA Probe (nDNA)	AGATTTCTCTGT
EXPAR template	AACTATCGACAACTTCCTCAGACTCAAACTATCGAC AACTTCTCTGTAGCTAGACCAAAATCACCTAT

[a]In mutRNA, the base rA highlighted in red color are the mutant site. The italicized and bold characters in the cmDNA indicate 2'-O-methyl-modified nucleotides. The underlined sequence (Z) in mutRNA is complementary with the Z' sequence (doubly underlined) in the EXPAR template.

Standard procedures of the RNase H-EXPAR assay for the detection of mutRNA. Typically, 1 μL series dilutions of *BRAF* mRNA fragment (mutRNA, or wtRNA) were added into a 4 μL mixture A

containing 500 nM cmDNA, 6 U RNase H, 1× hybrid RNA degeneration buffer and RNase-free water. Cleavage reaction was conducted by incubating the mixture at 42 °C for 2h, and then the RNase H was deactivated by heating at 70 °C.

Afterward, 1 μL digestion products was mixed with 4 μL mixture B solution consisting of 0.5× NEBuffer 3.1, 10 nM EXPAR template, 200 μM dNTPs, 0.4 U Recombinant RNase Inhibitor, 1× ThermoPol buffer, 0.4× SYBR Green I, 3 U nicking endonuclease, 0.1 U Vent (exo-) DNA polymerase and RNase-free water. The final mixture solution was immediately subjected to EXPAR under constant temperature of 55 °C on a StepOne Real-Time PCR System. Other conditions for the EXPAR are adopted from our previous work.[S1]

Extraction of total RNA from HeLa cells. HeLa cells were obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in the DMEM medium mixed with 10% (v/v) fetal calf serum, 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin at 37 °C with appropriate humidified atmosphere (5% CO_2 and 95% air). The HeLa cells were collected in the exponential phase of growth, and the total RNA was extracted with Trizol Reagent (Invitrogen, Beijing, China) following the manufacturer's instructions. The amount of extracted total RNAs were quantified with a Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, WA, USA).

2. Effect of temperature on the RNase H-based cleavage reaction with cmDNA

It is well recognized that in double-strand nucleic acid hybrids, one base mismatch may lead to the decrease of Tm compared with the fully complementary sequences. Therefore, in this RNase H-EXPAR strategy, an optimal RNase H reaction temperature should be low enough to form stable cmDNA/mutRNA heteroduplex structure while also should be higher than the Tm between wtRNA and cmDNA to ensure that no cmDNA/wtRNA duplexes can form. As can be seen from Fig. S1a and S1b, the Tm values of cmDNA/wtRNA, and cmDNA/mutRNA duplexes are determined to be 27 °C, and 45 °C, respectively. Accordingly, the effect of the RNase H digestion temperature on the specificity of the proposed assay was investigated by simultaneous detection of the blank (without mRNA), 1 fM and 100 fM mutRNA as well as 100 fM wtRNA under different temperatures from 26 °C to 45 °C. The point of inflection (POI) values, namely, the reaction time with the maximum slope of the fluorescent

curves, were recorded for quantitative analysis of target RNA.

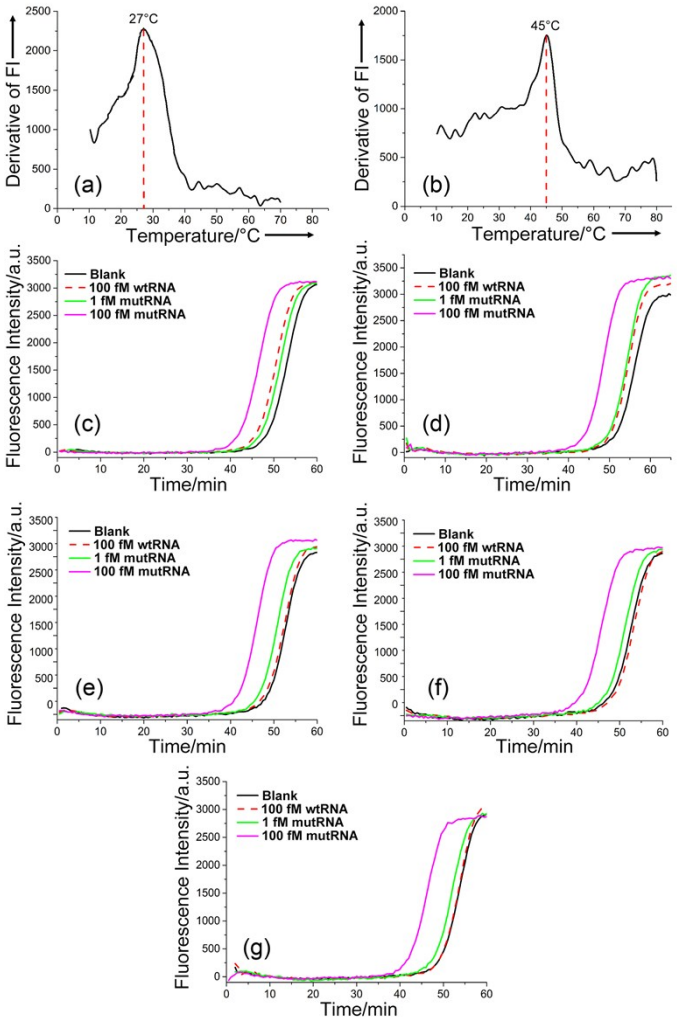

Fig. S1 Evaluation of the Tm values between (a) cmDNA/wtRNA and (b) cmDNA/mutRNA duplexes. (c-g) Effect of the cleavage temperature on the RNase H-EXPAR assay for mutRNA analysis. The real-time fluorescence curves produced by the mutRNA at 100 fM (pink line), 1 fM (green line), blank control (black line), and 100 fM wtRNA (red dashed line) are recorded simultaneously at each temperature. The digestion temperature is (c) 26 °C, (d) 33 °C, (e) 37 °C, (f) 42 °C, (g) 45 °C, respectively.

As shown in Fig. S1c-S1d, under the digestion temperature of 26~33 °C, the fluorescence curve aroused by 100 fM mutRNA can be clearly discriminated from that of 100 fM wtRNA, but the nonspecific signal of wtRNA will still exist. When the digestion temperature increases to 37 °C (Fig. S1e), the difference of the POI values produced by the 100 fM mutRNA and wtRNA curves will be obviously increased. More importantly, the curve aroused by 100 fM wtRNA become overlapped with

the blank control, suggesting the wtRNA will no longer interfere with the detection of mutRNA. It is reasonable that at relatively higher digestion temperatures, the cmRNA can also stably hybridize with mutRNA while the cmDNA/wtRNA heteroduplex structure will not be formed due to their much lower Tm. As shown in Fig. S1e~S1g, due to the large Tm difference between the cmDNA/wtRNA and cmDNA/mutRNA, the cmDNA-aided RNase H-EXPAR method can maintain similar high specificity when the digestion temperature is controlled in a wide range of 37 °C~45 °C. Meanwhile, the fluorescence responses aroused by 1 fM mutRNA can be all clearly discerned from the blank control under this temperature range. Therefore, 42 °C is employed as the cleavage temperature in this proposed RNase H-EXPAR assay.

3. Effect of temperature on the RNase H-based cleavage reaction with nDNA

Since the melting temperatures (Tm) of cmDNA and nDNA are different when hybridized with the target mRNA, the Tm values of nDNA/wtRNA and nDNA/mutRNA duplexes are also determined, which is ~25.5 °C and 38.5 °C (Fig. S2a-b), respectively. In order to obtain the best analytical results in the comparative experiments, the effect of temperature on the RNase H-based digestion with nDNA was also investigated. The blank (without mRNA), 1 fM, 100fM mutRNA and 100 fM wtRNA were simultaneously detected with the RNase H-EXPAR assay by using nDNA with the digestion temperature varying from 26 to 42 °C.

As depicted in Fig. S2c, with the digestion temperature of 26 °C, the fluorescence curve produced by 100 fM wtRNA cannot be clearly discriminated from that aroused by the same concentration of mutRNA. This indicates that at low temperature, both mutRNA and wtRNA can hybrid with the nDNA, both of which will be digested at random site by RNase H to trigger EXPAR.

When the reaction temperature increases to 33 °C or 38.5 °C (Fig. S2d, e), although the differences of POI values produced by the 100 fM mutRNA and wtRNA become more distinct, the nonspecific false-positive responses of wtRNA still remain obvious. Such results suggest that even though the reaction temperature is higher than the Tm of nDNA/ wtRNA duplexes, the wtRNA molecules may randomly and transiently pair with the nDNA, which can be also digested by RNase H at random sites. The nonspecific hydrolysis may lead to inevitable high nonspecific interference for the quantification

of mutRNA.

When the digestion temperature is further elevated to 42 °C, as displayed in Fig. S2f, the response of 1 fM mutRNA cannot be discriminated from the blank control. Meanwhile, the difference of the POI values between the 100 fM mutRNA and the blank control become much smaller. At such a high temperature, both the mutRNA and wtRNA could not form stable RNA/nDNA structures so that the RNase H digestion cannot proceed normally. Therefore, taking into consideration of both sensitivity and specificity, when nDNA is employed for the mutRNA analysis, 38.5 °C is used as the cleavage temperature throughout this work.

In addition, the comparison of the results shown in Fig. S1 (cmDNA) and Fig. S2 (nDNA) further suggests that compared with traditional nDNA, the introduction of 2'-O-methylated nucleotides in the cmDNA not only significantly enhances the Tm value of DNA/mutRNA duplexes, but also greatly enlarges the Tm difference between DNA/mutRNA and DNA/wtRNA, further highlighting the advantage of cmDNA in the RNase H-EXPAR strategy.

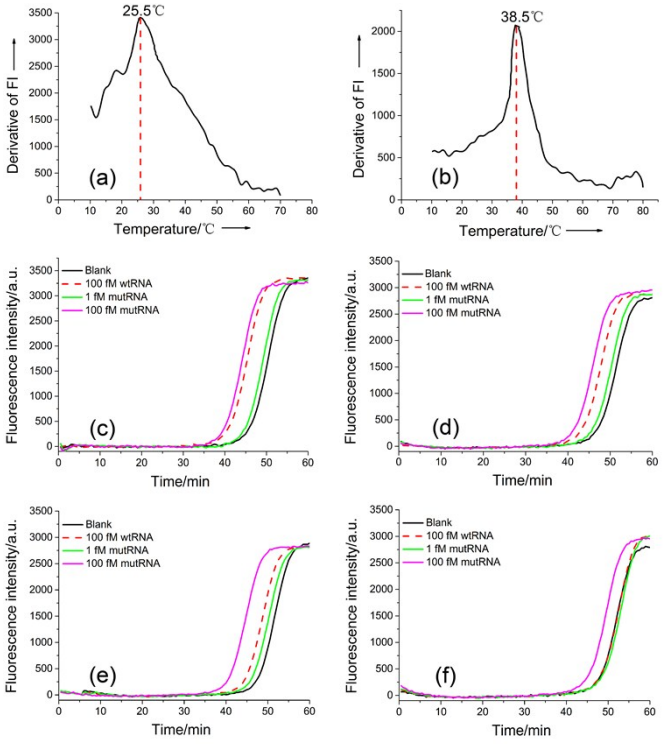

Fig. S2 Evaluation of the Tm values between (a) nDNA/wtRNA and (b) nDNA/mutRNA duplexes. (c-f) Effect of the cleavage temperature on the RNase H-EXPAR assay for the detection of mutRNA by using nDNA probe. The real-

time fluorescence curves produced by the mutRNA at 100 fM (pink line), 1 fM (green line), blank control (black line), and 100 fM wtRNA (red dashed line) are recorded simultaneously at each temperature. The digestion temperature is (c) 26 °C, (d) 33 °C, (e) 38.5 °C, (f) 42 °C, respectively. Other experimental conditions are the same as described in the Experimental Section except the substitution of cmDNA with nDNA.

4. Effect of the amount of RNase H on the detection of mutRNA

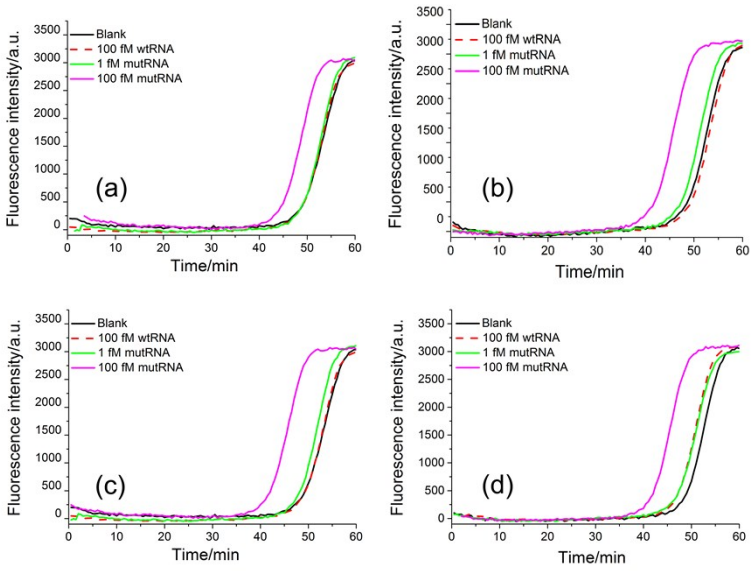

Fig. S3 Effect of the amount of RNase H on the detection of mutRNA. The real-time fluorescence curves were produced by 100 fM, 1 fM mutRNA, 100 fM wtRNA and blank (without target mRNA) respectively in each image. The amount of RNase H was (a) 3 U, (b) 6 U, (c) 9 U, and (d) 12 U. Other experimental conditions were the same as described in the Experimental Section.

The amount of RNase H is also a crucial factor for achieving high sensitivity and selectivity for mutRNA analysis. To test the effect of the amount of RNase H on the detection of mutRNA, the blank (without mRNA), 1 fM, 100 fM mutRNA and 100 fM wtRNA were simultaneously detected with the RNase H-EXPAR assay by using different amounts of RNase H. As shown in Fig. S3a, with 3 U RNase H, the difference of POI values produced by 100 fM mutRNA and the blank control is relatively small, indicating that 3 U RNase H may be not enough to completely disconnect mutRNA. One can see from Fig. S3b~c that if 6~9 U RNase H is employed, 1 fM mutRNA can be clearly detectable, and the difference of POI values produced by 100 fM mutRNA and the blank control reaches the maximum.

Meanwhile, the POI values produced by 100 fM wtRNA and the blank control are almost the same, indicating the RNase H-EXPAR assay has the best sensitivity and specificity with such RNase H dosages. However, it is obvious that too much RNase H (12 U, Fig. S3d) may inevitably lead to undesired nonspecific cleavage of wtRNA. Thus, taking into consideration of high sensitivity, assay cost and high specificity, 6 U RNase H is selected as the optimum amount for the RNase H-EXPAR assay.

5. Effect of the amount of cmDNA probe on the detection of mutRNA

According to the design principle, the performance of the RNase H-EXPAR assay also relies heavily on the amount of cmDNA, so the effect of cmDNA dosage on mutRNA detection was also investigated. As shown in Fig. S4, when cmDNA with a low concentration of 100 nM is employed, the fluorescence curve aroused by 1 fM mutRNA is overlapped with the blank control, suggesting the sensitivity of the assay is not satisfactory. When the amount of cmDNA increases to 500 nM, the response aroused by 1 fM mutRNA can be clearly discriminated from the blank control and meanwhile, the signal produced by 100 fM wtRNA overlaps with the blank control, suggesting that the RNase H-EXPAR assay exhibits both high sensitivity and specificity. Nevertheless, if the amount of cmDNA is further elevated to 1 μM, the 100 fM wtRNA will produce observable nonspecific interference signal. Thus, taking into consideration of both high specificity and sensitivity, 500 nM cmDNA is selected as the optimum amount for the RNase H-EXPAR assay.

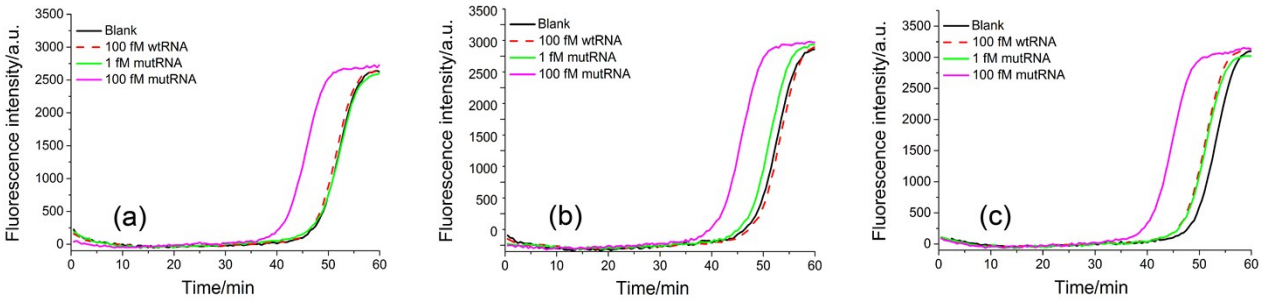

Fig. S4 The influence of the concentration of cmDNA. In each image, the real-time fluorescence curves produced by 100 fM mutRNA, 1 fM mutRNA, 100 fM wtRNA and blank control are displayed, respectively. The concentration of cmDNA is (a) 100 nM, (b) 500 nM, and (c) 1 μM, respectively.

6. Sample preparation for sequencing and the sequencing result of the *BRAF* mRNA in HeLa total RNA sample

Firstly, the total RNA was extracted from HeLa cells by using Trizol Reagent (Invitrogen, Beijing, China). The HeLa total RNA sample was amplified with RT-PCR before sequencing. Typically, the reverse transcription of total RNA sample (3 μg) was performed in the mixture of 50 nM reverse primer (5′-ATTATCTGGTCCCTGTTGTTGATGT-3′), 40 U ProtoScrip Ⅱ reverse transcriptase, 0.25 mM dNTPs and reaction buffer with a total volume of 10 μL at 50 °C for 30 min. Then the products of reverse transcription were amplified through PCR amplification. The PCR amplification was carried out in a thermal cycler with the mixture of PCR reaction buffer (50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3, 10 mM Tri-HCl), 1 U Taq Hot Strat DNA polymerase, 500 nM forward primer (5′ -TTACACGCCAAGTCAATCATCC-3′) and reverse primer, 0.25 mM dNTPs, and the products of reverse transcription in a total volume of 20 μL. The PCR system was first heated for 5 min at 94 ºC, followed by 30 cycles of 94 ºC 20 s and 60 ºC 1 min. Afterwards, the PCR product was finally sent to Sangon Biotech. (Shanghai, China) for sequencing.

The sequencing result is shown in Fig. S5, which demonstrates that the detected site is completely U in the total RNA extracted from HeLa cells. So, the target site of the *BRAF* mRNA is purely wild-type in HeLa cells.

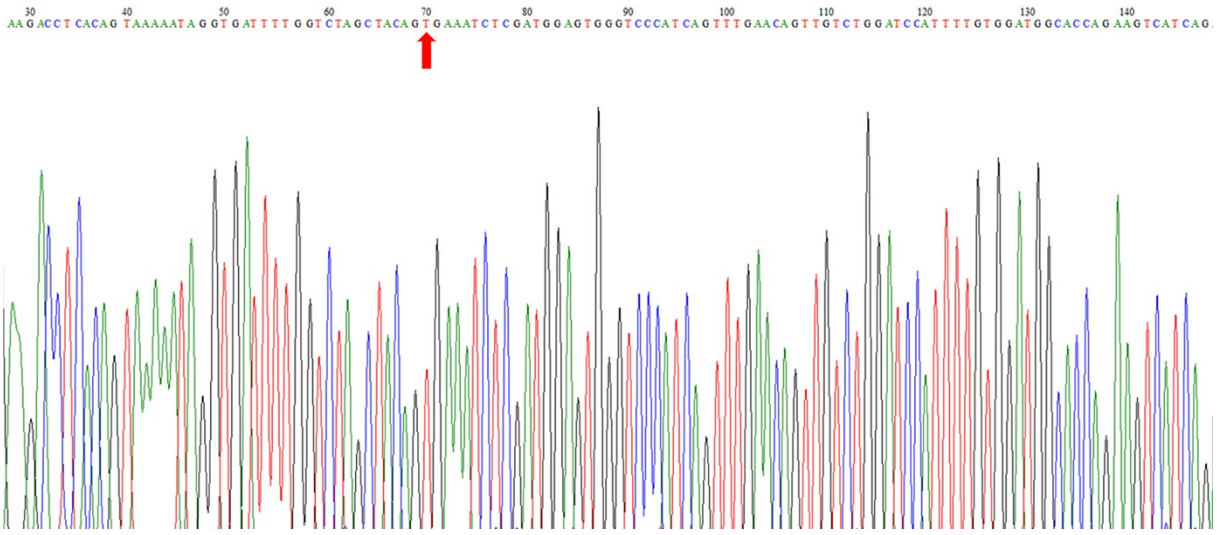

Fig. S5 The sequencing result of the *BRAF* mRNA in HeLa cells. The detected mutation site in this study was marked with red arrows.

7. Comparison of the RNase H-EXPAR assay with a PNA clamp-based RT-PCR method for the detection of the 299-nt mutRNA spiked in HeLa extracts

To more factually simulate the native mutRNA in HeLa cells, we obtained a 299-nt long *BRAF* mRNA fragment with the V600E mutation. The 299-nt mutRNA (5′-rUrUrArCrArCrGrCrCrArArGrUrCrArArUrCrArUrCrCrArCrArGrArGrArCrCrUrCrArArGrArGrUrArArArUrArArUrArUrArUrUrUrCrUrUrCrArUrGrArArGrArCrCrUrCrArCrArGrUrArArArArArUrArGrGrUrGrArUrUrUrUrGrGrUrCrUrArGrCrUrArCrArGrArGrArArArUrCrUrCrGrArUrGrGrArGrGrUrGrGrGrUrCrCrCrArUrCrArGrUrUrUrGrArArCrArGrUrUrGrUrCrUrGrGrArUrCrCrArUrUrUrUrGrUrGrGrArArArUrGrGrArUrGrGrCrArCrGrArArArGrUrCrArUrCrArGrArArUrGrCrArArGrArUrArArArArArUrCrCrArUrArCrArGrCrUrUrUrCrArGrUrCrArGrArArUrGrUrArUrArUrGrCrArUrUrUrGrGrArArUrUrGrUrArUrUrCrUrGrGrArCrUrGrGrArCrArGrUrUAArCrCrUrUrArUrUrCrArArArCrArUrCrArArCrArArCrArGrGrGrArCrCrArGrArUrArArU-3′) is provided by Takara Biotechnology (Dalian, China) by means of plasmid construction and T7 transcriptase-assisted in vitro transcription. In this study, 100 zmol, 200 zmol and 500 zmol of the 299-nt mutRNA is respectively spiked into the HeLa total RNA. The spiked samples are employed as simulated heterogeneous samples, which are detected by the RNase H-EXPAR assay and a peptide nucleic acid (PNA) clamp-based reverse transcription-PCR (RT-PCR) method, respectively.

The PNA clamp-based RT-PCR protocol is adopted from a previous literature with some modifications.[S2] A 16-nt PNA sequence is completely complementary to the wtRNA. In contrast, a A/A mismatch exists in the PNA/mutRNA duplex, which has an approximately 10~20 ºC lower Tm than that of the PNA/wtRNA. As such, the wtRNA will be firmly clamped by the PNA so that the subsequent PCR can be completely suppressed. However, the PNA cannot clamp the mutRNA, and the RT-PCR amplification can be performed normally on the mutRNA. The detailed experimental procedures are described as follows:

Reverse transcription. The reverse transcription was carried out in the 10 μL mixture including 5 nM the reverse primer (5′-ACAACTGTTCAAACTGATGGGAC-3′), 0.5 mM dNTPs, 1× RT buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, pH 8.3), 40 U ProtoScrip Ⅱ reverse transcriptase, series dilutions of synthetic 299-nt mutRNA or spiked HeLa total RNA samples were mixed, incubated at 50 °C for 30 min, and then inactivated at 80 °C for 10 min.

Real-time quantitative PCR. 1 μL of the reverse transcription products was transferred to the PCR reaction mixture with a final volume of 10 μL. The PCR reaction mixture contained 400 nM forward primer (5′-AATCATCCACAGAGACCTCAAGA -3′) and 400 nM reverse primer, PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin), 0.5 mM dNTPs, 0.5 U Taq Hot Start DNA Polymerase, 200 nM TaqMan probe (5′- 6FAM-CGAGATTTCTCTGTAGCTAG-MGBNFQ-3′) and 500 nM PNA (N′- GAGATTTCACTGTAGC-C′). The PCR reaction was carried out with a StepOne Real-Time PCR System (Applied Biosystems, USA) by hot start at 94°C for 5 min, followed by 50 cycles of 94°C for 20 s, 67°C for 20 s and 60°C for 1min.

As can be seen from Table S2, the quantitative determination results obtained by the proposed RNase H-EXPAR strategy are in good accordance with the values determined by the PNA clamp-based RT-PCR method.

Table S2. Comparison of the RNase H-EXPAR assay with the PNA clamp-based RT-PCR method for the detection of 299-nt mutRNA spiked in HeLa extracts

Sample	Detected Results/Recovery (%) by RNase H-EXPAR	Detected Results/Recovery (%) by PNA-clamp RT-PCR
3 ng HeLa total RNA spiked with 100 zmol mutRNA (299-nt)	97.3 zmol (97.3%)	101.5 zmol (101.5%)
3 ng HeLa total RNA spiked with 200 zmol mutRNA (299-nt)	211.1 zmol (105.6%)	195.8 zmol (97.9%)
3 ng HeLa total RNA spiked with 500 zmol mutRNA (299-nt)	477.5 zmol (95.5%)	495.8 zmol (99.2%)

Note: Each experiment performed in triplicates.

References

(S1) Y. Sun, Y. Sun, W. Tian, C. Liu, K. Gao and Z. Li, *Chem. Sci.*, 2018, **9**, 1344-1351.

(S2) Y. Sun, H. Tian, C. Liu, D. Yang and Z. Li, *ACS Sens.,* 2018, **3**, 1795-1801.