Electronic Supplementary Information

Ultrasensitive quantification of multiplexed mRNA variants via splice-junction anchored DNA probes and SplintR ligase-initiated PCR

Yuting Jia,[a] Jun Han,[b] Hui Wang,[a] Weixiang Hong,[a] Honghong Wang,*[a] Mai Zhang[a] and Zhengping Li*[a]

[a]*Beijing Key Laboratory for Bioengineering and Sensing Technology; School of Chemistry and Biology Engineering, University of Science and Technology Beijing, Beijing, 100083, P. R. China.*

[b]*National Textile and Leather Product Quality Supervision Testing Center, Beijing 100025, China.*

Email: wanghh@ustb.edu.cn; lzpbd@ustb.edu.cn

Content List

15. Detection of the mRNA splice variants from the total RNA samples

1. Materials and apparatus

SplintR ligase (25 U/μL), SplintR ligase Reaction Buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT, pH 7.5 @ 25 °C) were purchased from New England Biolabs (USA). Taq™ Hot Start Version (5 U/μL), 10×PCR Buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, pH 8.9 @ 25 °C), RNase-free water, RNase inhibitor (40 U/μL) and dNTP Mixture (2.5 mM) were purchased from TaKaRa Biotechnology Co. Ltd. (Dalian, China). SYBR Green I (20 ng/μL stock solution in DMSO) was purchased from Xiamen Bio-Vision Biotechnology (Xiamen, China). All of the oligonucleotides including synthetic mRNA splice variants of the *hTERT*, DNA probes, and PCR primers were purified by using HPLC and purchased from TaKaRa Biotechnology Co. Ltd. (Dalian, China). TaqMan probes were purified by using HPLC and purchased from Thermo Fisher Scientific. The HeLa, K562, A549, MRC-5 cell lines were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). All the reagents were of analytical grade and were used as received without further purification. The sequences of the oligonucleotides were given in **Table S1**.

2. Table S1. The sequences of the oligonucleotides used in this work

Name	Sequences (5′-3′ direction)
α-deletion	CCGCCUGAGCUGUACUUUGUCAAGGACAGGCUCACGGAGGUCAUCGCC
β-deletion	GUCCGCAAGGCCUUCAAGAGCCACGUCCUACGUCCAGUGCCAGGGGAU
γ-deletion	CACCCACGCGAAAACCUUCCUCAGCUAUGCCCGGACCUCCAUCAGAGC
$P1_{\alpha}$	CCATCTCATCCCTGCGTGTCATGACCTCCGTGAGCCTGTC(OH)
$P2_{\alpha}$	(PO_4)CTTGACAAAGTACAGCTCAGGCCCATAGAGAGGAAAGCGG
$P1_{\beta}$	CCATCTCATCCCTGCGTGTCCCTGGCACTGGACGTAGGAC(OH)
$P2_{\beta}$	(PO_4)GTGGCTCTTGAAGGCCTTGCACTGCCCATAGAGAGGAAAGCGG
$P1_{\gamma}$	CCATCTCATCCCTGCGTGTCTGATGGAGGTCCGGGCATAG(OH)
$P2_{\gamma}$	(PO_4)CTGAGGAAGGTTTTCGCGTGCCGACTGCCCATAGAGAGGAAAGCGG
UFP	CCGCTTTCCTCTCTATGGGC
URP	CCATCTCATCCCTGCGTGTC
TaqMan probe for α-deletion	FAM-TGAGCCTGTCCTTGAC-MGBNFQ
TaqMan probe for β-deletion	VIC-ACGTAGGACGTGGCTC-MGBNFQ
TaqMan probe for γ-deletion	NED-CATAGCTGAGGA AGGT-MGBNFQ

3. Cell culture and total RNA extraction

Cervical carcinoma cell (HeLa), embryonic lung fibroblast cell (MRC-5) were cultured in MEM Medium supplemented with 10% (v/v) fetal bovine serum (HyClone), 100 U/mL penicillin, 100 μg/mL streptomycin, 1% essential and 1% non-essential amino acids (Invitrogen). Leukemia cell (K562) was cultured in IMDM Medium supplemented with 10% (v/v) fetal bovine serum (HyClone), 100 U/mL penicillin, 100 μg/mL streptomycin, 1% essential and 1% non-essential amino acids. Lung adenocarcinoma epithelial cell A549 was cultured in F-12K Medium supplemented with 10% (v/v) fetal bovine serum (HyClone), 100 U/mL penicillin, 100 μg/mL streptomycin, 1% essential and 1% non-essential amino acids. The cells were maintained at 37 °C under a humidified atmosphere containing 5% CO_2. When the bottom of the culture bottle was filled with cells, the Medium was removed from the culture bottle, and the cells were washed three times with PBS (10 mM sodium phosphate buffer, 0.1 M NaCl, pH 7.4 @25 °C). Then, isolation procedures of total RNA from cells were operated according to the instructions of the TRIZOL® Reagent (Invitrogen). Finally, the concentrations of total RNA extracts were quantified using the NanoDrop One (Thermo Fisher Scientific, USA).

4. Standard procedures for the detection of mRNA splice variant

(1) Ligation reaction of splice-junction anchored DNA probes using SplintR ligase:

1.0 μL mRNA splice variant solution or 1.0 μL total RNA extracts were added to the mixture containing 1.0 μL 10 nM P1 and 1.0 μL 10 nM P2 of corresponding the target mRNA splice variants, 0.2 μL 40 U/μL RNase inhibitor, 0.6 μL SplintR ligase Reaction Buffer and 2.2 μL RNase-free water with the final volume of 6.0 μL. The mixture was heated at 65 °C for 5 min and 35 °C for 5 min. Then 0.4 μL 10× SplintR ligase Reaction Buffer, 0.2 μL 25 U/μL SplintR ligases and 3.4 μL RNase-free water were added to the mixture, and the reaction mixture was incubated at 35 °C for 20 min to complete the ligation reaction. A 2720 Thermal Cycler (Applied Biosystems, USA) was used to control the reaction temperature.

(2) PCR amplification reaction using SYBR Green I:

2.0 μL ligation product was added to 8.0 μL PCR mixture containing 1.0 μL 10× PCR Buffer, 1.0 μL 2.5 mM dNTPs, 0.2 μL 10 μM UFP, 0.2 μL 10 μM URP, 0.2 μL 5 U/μL Taq™ Hot Start Version, 0.2 μL 20 ng/μL SYBR Green I, and 5.2 μL RNase-free water with final volume of 10.0 μL. PCR reaction was carried out with the Quantstudio™ 3 Real-Time PCR System to monitor real-time fluorescence signal by using the hot start of 94 °C for 2 min, followed by 50 cycles of 94 °C for 20 s, 65 °C for 30 s, and 72 °C for 20 s.

(3) PCR amplification reaction using TaqMan probes for detection of the single-plex mRNA splice variants:

2.0 μL ligation product was added to 8.0 μL PCR reaction mixture containing 1.0 μL 10×PCR Buffer, 1.0 μL 2.5

mM dNTPs, 1.0 μL 10 μM UFP, 1.0 μL 10 μM URP, 0.2 μL 5 U/μL Taq™ Hot Start Version, 0.05 μL 20 ng/μL TaqMan Probe and 3.75 μL RNase-free water with final volume 10.0 μL. PCR reaction was carried out with Quantstudio™ 3 Real-Time PCR system to monitor real-time fluorescence signal by using the hot start of 94 °C for 2 min, followed by 50 cycles of 94 °C for 20 s, 65 °C for 30 s, and 72 °C for 20 s.

(4) PCR amplification reaction using TaqMan probes for detection of multiple mRNA splice variants:

2.0 μL ligation product was added to 8.0 μL PCR reaction mixture containing 1.0 μL 10×PCR Buffer, 1.0 μL 2.5 mM dNTPs, 1.0 μL 10 μM UFP, 1.0 μL 10 μM URP, 0.2 μL 5 U/μL Taq™ Hot Start Version, 0.05 μL 20 ng/μL TaqMan Probe of α-deletion, 0.05 μL 20 ng/μL TaqMan Probe of β-deletion, 0.05 μL 20 ng/μL TaqMan Probe of γ-deletion, and 3.65 μL RNase-free water with final volume 10.0 μL. PCR reaction was carried out with Quantstudio™ 3 Real-Time PCR system to monitor real-time fluorescence signal by using the hot start of 94 °C for 2 min, followed by 50 cycles of 94 °C for 20 s, 65 °C for 30 s, and 72 °C for 20 s.

5. Optimization of the dosage of SplintR ligase for mRNA splice variant assay

Ligase dosage can affect the efficiency of the ligation reaction. Therefore, the dosage of SplintR ligase was optimized by detecting Blank and β-deletion at 1 fM, 10 fM, 100 fM, respectively with the proposed assay. 0.25 U, 0.5 U, and 1.0 U SplintR ligase dosage were investigated. When the amount of ligase is 0.25 U, it can be seen from Fig. S1A that the C_T values show smaller difference between Blank and 1 fM β-deletion compared with that shown in Fig. S1B. This is because the low amount of ligase leads to the decrease of the ligation efficiency of the splice-junction anchored probes. When the amount of ligase is 0.5 U, the ligation efficiency increased and the difference of C_T value between Blank and 1 fM target mRNA reached the maximum. When the amount of ligase is 1 U, the C_T values of Blank becomes smaller, resulting the difference of the C_T values between Blank and 1 fM target mRNA the also become smaller. This may be due to the high dosage of ligase causing the non-specific ligation of DNA probes, which in turn leads to non-specific amplification. Taking into consideration of detection sensitivity and interference of non-specific amplification, 0.5 U was selected as the optimum amount of SplintR ligase for the mRNA splice variant assay.

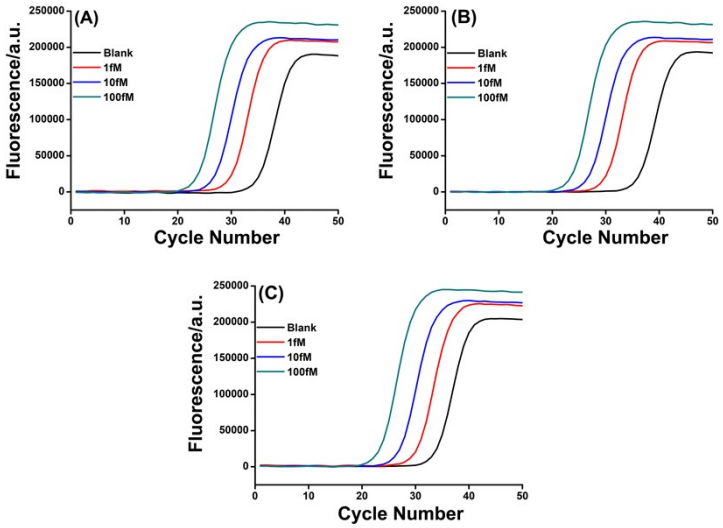

Fig. S1 The effect of the dosage of SplintR ligase on the mRNA splice variant assay. The dosage of SplintR ligase are 0.25 U (A), 0.5 U (B) and 1.0 U (C).

6. Optimization of the temperature of the ligation reaction

The temperature of the ligation reaction is closely associated with the sensitivity of mRNA splice variant detection. The influence of the temperature of the ligation reaction was investigated by detecting the Blank, 1 fM, 10 fM, and 100 fM β-deletion with the proposed assay at different ligation temperatures. When the ligation temperature is 16 °C, it can be seen from Fig. S2A that the fluorescence signal of Blank appears early. This is because more probe-dimers are formed at a lower temperature, which leads to serious non-specific amplification reactions and speeds up the non-specific amplification reaction of Blank. When the temperature is raised to 35 °C, the non-specific amplification caused by probe-dimer is inhibited, which increases the difference of C_T value between the Blank and 1 fM target mRNA. However, when the reaction temperature is 37 °C, the C_T values of the target mRNA are delayed, which is because the high temperature can reduce the hybridization efficiency between the target mRNA and splice-junction anchored DNA probes. As a result, the amount of the ligated DNA probes that can be used as the PCR templates are reduced and the reaction speed is slowed down. Therefore, taking into consideration of both detection sensitivity and interference of non-specific amplification, 35 °C was selected as the optimized temperature of the ligation reaction in this work.

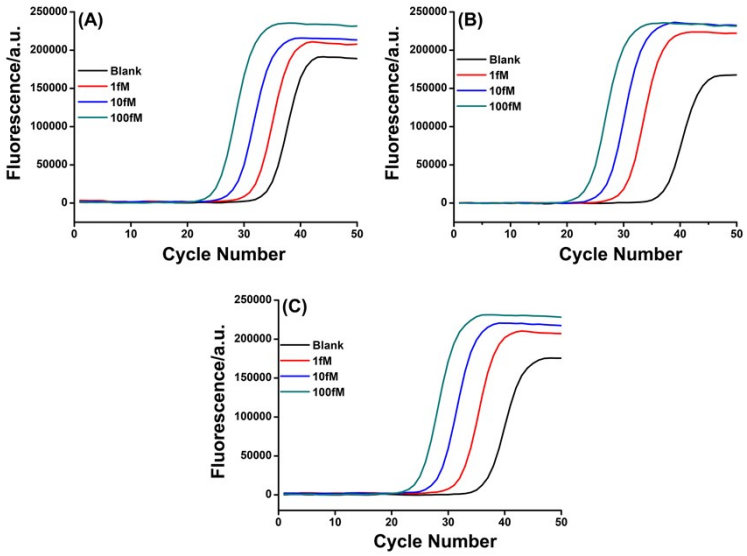

Fig. S2 The effect of the temperature of ligation reaction on the mRNA splice variant assay. The temperature of the ligation reaction is 16 °C (A), 35 °C (B) and 37 °C (C).

7. Optimization of the dosage of splice-junction anchored probes

To investigate the influence of the dosage of splice-junction anchored probes, 500 pM, 1 nM, 2 nM and 10 nM $P1_{\beta}$ and $P2_{\beta}$ were investigated by detecting the Blank, 1 fM, 10 fM and 100 fM β-deletion with the proposed assay. As depicted in Fig. S3 (A-D), with increasing the $P1_{\beta}$ and $P2_{\beta}$ concentration from 500 pM to 10 nM, the C_T values of the β-deletion and Blank was all gradually decreased. Firstly, the amount of probe-dimers will increase with increasing the amount of the probes, resulting in more non-specific amplification, which can decrease the C_T. values of the Blank. Secondly, the amount of splice-junction anchored probes will affect the hybridization efficiency of the probes and the target mRNAs and consequently affect the ligation efficiency. So increasing the probe amounts can result in more ligation products and high speed of PCR leading to the decreased C_T values. One can see from Fig. S3, integration of the effects in the two aspects, the differences of the C_T values between 1 fM and Blank reached their maximum when 1 nM $P1_{\beta}$ and $P2_{\beta}$ were used. Therefore, taking into consideration of both detection sensitivity and interference of non-specific amplification, 1 nM $P1_{\beta}$ and $P2_{\beta}$ were employed for the mRNA splice variant assay.

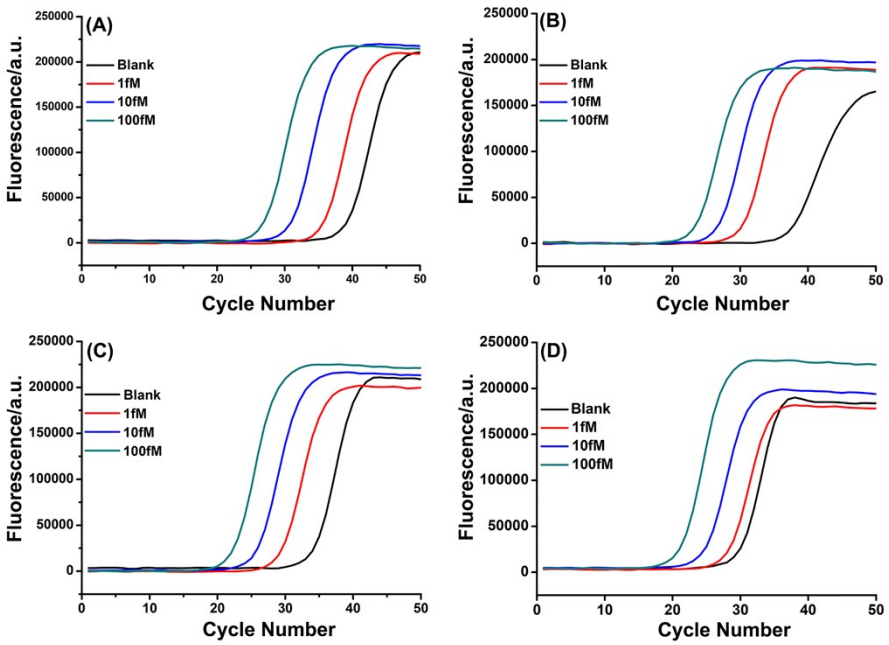

Fig. S3 The effect of the dosage of splice-junction anchored probes on the mRNA splice variant assay. The dosage of splice-junction anchored probes are 500 pM (A), 1 nM (B), 2 nM (C) and 10 nM (D).

8. Optimization of the dosage of TaqMan probe

To investigate the influence of the dosage of TaqMan probe, 50 nM, 100 nM, and 200 nM TaqMan probe were investigated by detecting the Blank, 10 aM, 100 aM, and 1 fM γ-deletion with the proposed assay. As depicted in Fig. S4 (A-C), with increasing TaqMan probe concentration from 50 nM to 200 nM, the fluorescence signals of the 10 aM γ-deletion were gradually enhanced. The fluorescence signal of 10 aM γ-deletion cannot be observed in Fig. S4A, indicating that the 50 nM TaqMan probe is too low to detect the specific PCR products when γ-deletion concentration was extremely low. When 100 nM TaqMan probe was used, the Blank fluorescence signal kept very small level and 10 aM γ-deletion could produce enough detectable fluorescence signal. However, when the concentration of the TaqMan probe increased to 200 nM, the fluorescence signal of the Blank was observed and increased with the PCR cycles, which may be because the TaqMan probe can partially hybridize with non-specific amplification products, resulting in the generation of background signal. Therefore, taking into consideration of both detection sensitivity and interference of non-specific amplification, 100 nM TaqMan probe was employed for the mRNA splice variant assay.

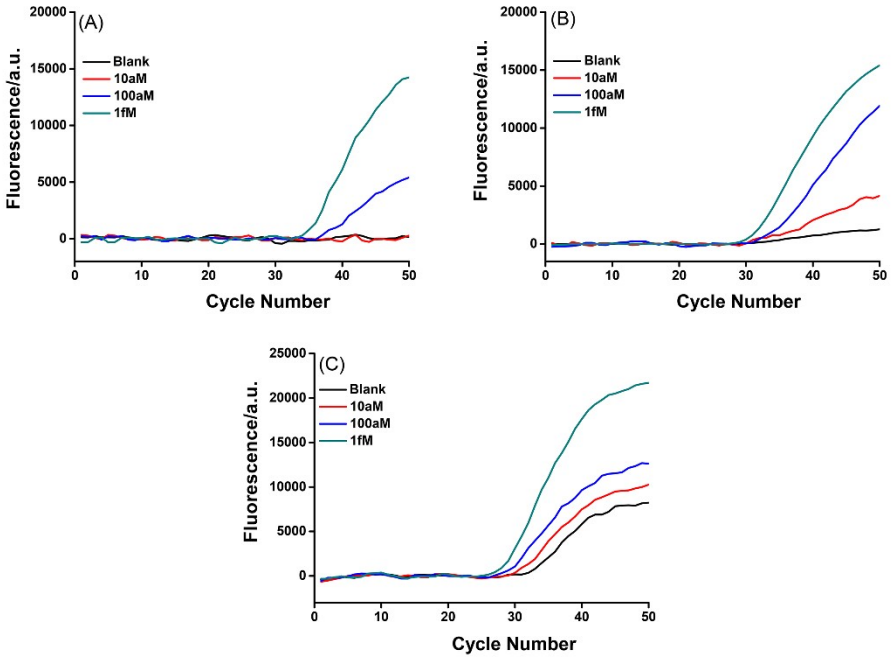

Fig. S4. The effect of the dosage of the TaqMan probe on the mRNA splice variant assay. The dosage of TaqMan probe is 50 nM (A), 100 nM (B), 200 nM (C), respectively.

9. The standard curves for detection of single-plex mRNA splice variant using TaqMan probe

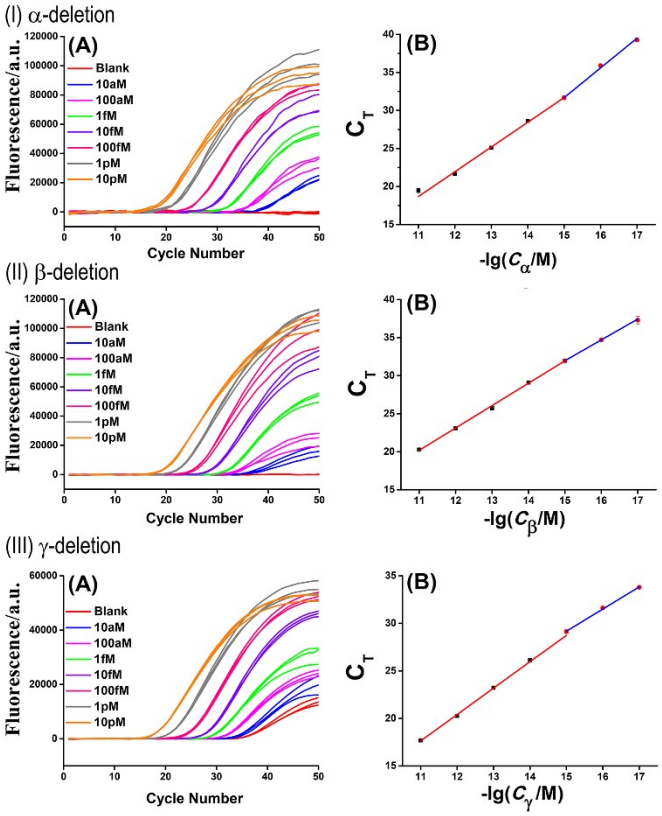

Fig. S5 (A) Real-time fluorescence curves aroused by various mRNA splice variants with different concentrations by using TaqMan probe. The concentrations of the mRNA splice variants (from left to right) were successively 10 pM, 1 pM, 100 fM, 10 fM, 1 fM, 100 aM, 10 aM and 0 (Blank). The concentration of TaqMan probe was 100 nM.

(B) The linear relationship between C_T values of the fluorescence curves and lg of the mRNA concentrations. Error bars indicate the standard deviation of three replicative experiments.

10. Table S2. The correlation equations and corresponding correlation coefficients for single-plex detection of mRNA splice variants

Splice variant	The correlation equation and corresponding correlation coefficient (10 pM-1 fM)		The correlation equation and corresponding correlation coefficient (1 fM-10 aM)	
α-deletion	C_T=-17.11-3.25lgCα (M)	R^2=0.9956	C_T=-26.92-3.92lgCα (M)	R^2=0.9940
β-deletion	C_T=-12.18-2.94lgCβ (M)	R^2=0.9992	C_T=-9.09-2.74lgCβ (M)	R^2=0.9994
γ-deletion	C_T=-12.90-2.78lgCγ (M)	R^2=0.9970	C_T=-5.84-2.33lgCγ (M)	R^2=0.9972

11. Table S3. The correlation equations and corresponding correlation coefficients for multiplexed detection of mRNA splice variants

Splice variant	The correlation equation and corresponding correlation coefficient (10 pM-1 fM)		The correlation equation and corresponding correlation coefficient (1 fM-10 aM)	
α-deletion	C_T=-17.92-3.34lgCα (M)	R^2=0.9995	C_T=-27.05-3.94lgCα (M)	R^2=0.9999
β-deletion	C_T=-12.76-3.02lgCβ (M)	R^2=0.9990	C_T=-9.70-2.77lgCβ (M)	R^2=0.9958
γ-deletion	C_T=-12.10-2.78lgCγ (M)	R^2=0.9914	C_T=-5.52-2.32lgCγ (M)	R^2=0.9913

12. Specificity evaluation for the detection of mRNA splice variants using SYBR Green I

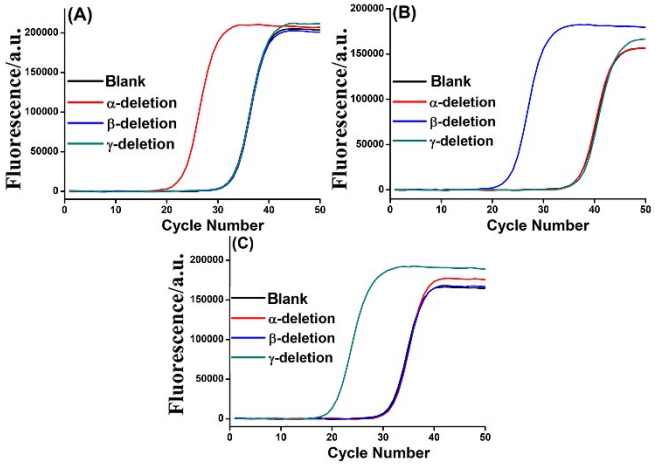

Fig. S6 Real-time fluorescence curves aroused by mRNA splice variant by using (A) α-deletion-specific DNA probes, (B) β-deletion-specific DNA probes, and (C) γ-deletion-specific DNA probes, respectively. The concentration of each mRNA splice variant was 100 fM. The Blank control was detected with the same procedures but without the mRNA splice variants.

13. Specificity evaluation for the detection of mRNA splice variants using TaqMan probe

To further evaluate the specificity for the detection of the mRNA splice variant using TaqMan probe, α-deletion

with different concentrations were detected by using SplintR ligase to ligate the α-deletion-specific DNA probes, $P1_{\alpha}$ and $P2_{\alpha}$. While the β-deletion and γ-deletion were employed as the interference variants and were simultaneously detected by using $P1_{\alpha}$ and $P2_{\alpha}$. As demonstrated in Fig. S6, similar to the results shown in Fig. 2, only α-deletion can produce the positive real-time fluorescence signal. In addition, the fluorescence curves produced by β-deletion and γ-deletion overlap completely with the Blank control. These results clearly show that the proposed method shows high specificity at both high and low concentrations of target mRNA.

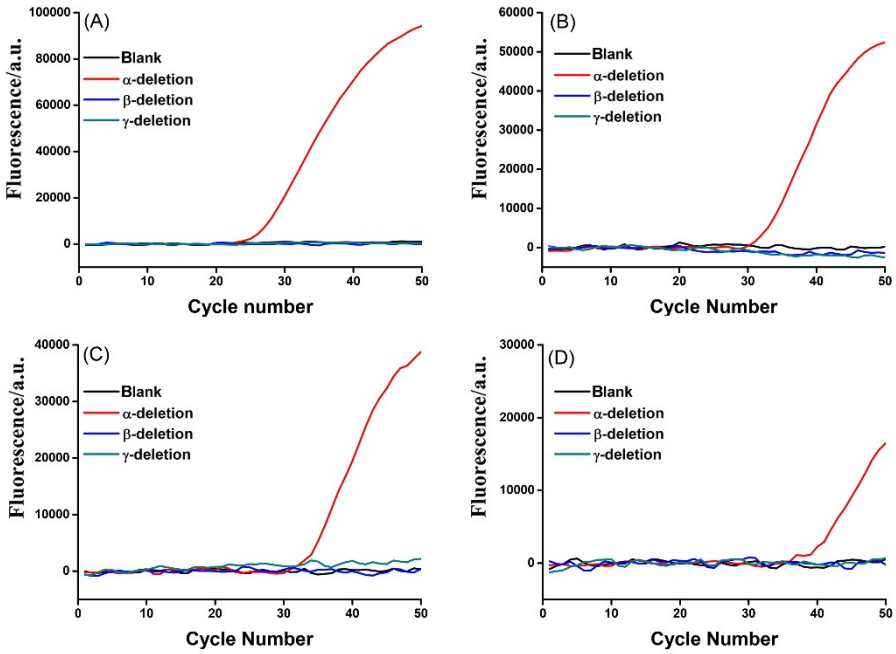

Fig. S7 Real-time fluorescence curves produced by different mRNA splice variants by using α-deletion-specific DNA probes. The concentration of each mRNA splice variant was 100 fM (A), 1 fM (B), 100 aM (C), and 10 aM (D), respectively. The Blank control was detected with the same procedures but without the mRNA splice variants.

14. Discussion of the background signal and non-specific amplification

Although the sensitivity of the proposed method is quite high, the non-specific amplification can be observed from the fluorescence signal of the Blank as shown in Fig. 1A. The non-specific amplification is a key problem in nucleic acid amplification-based detection methods because it is an important issue that limits the sensitivity of such methods. There are three main possibilities for the source of background signal (Blank) in this work, including the non-specific amplification of the PCR primer-dimer, the non-specific ligation of the splice-junction anchored DNA probes, and the probe-dimer. The negative test control (NTC) is a control experiment and in which no mRNA splice variants and the splice-junction anchored DNA probes were added to the PCR reaction system. From Fig. 1, one can see that the NTC is a constant straight line, indicating that no amplification reaction has occurred, so the non-specific signals should not be produced by the PCR primer-dimers.

Generally, the mRNA template-independent ligation (non-specific ligation) may exist in the ligation reaction. The Fig. 3 shows the results by using TaqMan probe to detect the mRNA splice variants. The TaqMan probes can specifically hybridize to the target-specific sequences of PCR products and will be cleaved with the 5'-3' exonuclease activity of DNA polymerase to generate a fluorescence signal. If the non-specific ligation exists in the ligation reaction, it will result in the non-specific amplification with the same PCR products, which can produce the background fluorescence signals. However, the fluorescence signal of the background in Fig. 3 can basically be ignored, which confirms that our experimental scheme does not have non-specific ligation of the splice-junction anchored DNA probes. This may be because the SplintR ligase we used is highly dependent on the ligation reaction using mRNA splice variants as the templates.

Since the probe has a long sequence including the partial complementary sequence of mRNA splice variant and the primer sequence of PCR. The two probes are easy to partially hybridize with each other to produce probe-dimers. Fig. S7 shows the possible structure of the probe-dimers which is estimated through the IDT website (https://sg.idtdna.com/calc/analyzer). Although the probes only partially bybridize with each other, the 3'-terminus extension catalyzed with DNA polymerase can also produce the non-specific amplification in the PCR processes. In this work, we first used SYBR Green I as a fluorescent dye for the real-time monitoring of the PCR products. SYBR Green I has a high affinity for all double-stranded DNA, and thus, cannot distinguish the non-specific products generated by the probe-dimer from the specific products of PCR. The TaqMan probe has good specificity for PCR product detection, which can effectively eliminate the fluorescence signal of Blank as shown in Fig. 3. So the Blank has a high background when SYBR Green I is used, and the source of this high background is mainly the non-specific amplification caused by the probe-dimers.

(A) probe-dimer of P1α and P2α

```
Delta G: -9.76 kcal/mole  Base Pairs: 5
5' CCATCTCATCCCTGCGTGTCATGACCTCCGTGAGCCTGTC
                     :   :   :::  :: |||||
3'                GGCGAAAGGAGAGATACCCGGACTCGACATGAAACAGTTC
```

(B) probe-dimer of P1β and P2β

```
Delta G: -8.16 kcal/mole  Base Pairs: 4
5' CCATCTCATCCCTGCGTGTCCCTGGCACTGGACGTAGGAC
         :  :  : :         |||| :: : :     :
3'       GGCGAAAGGAGAGATACCCGTCACGTTCCGGAAGTTCTCGGTG
```

(C) probe-dimer of P1γ and P2γ

```
Delta G: -11.23 kcal/mole  Base Pairs: 5
5' CCATCTCATCCCTGCGTGTCTGATGGAGGTCCGGGCATAG
                      ::        :  |||||
3'                 GGCGAAAGGAGAGATACCCGTCAGCCGTGCGCTTTTGGAAGGAGTC
```

Fig. S8 Schematic illustration of possible probe-dimer structures.

15. Detection of the mRNA splice variants from the total RNA samples

To evaluate the practicality, the proposed assay has been applied to detect hTERT splicing events in 10 ng total RNA samples derived from different cell lines, including cervical carcinoma cell (HeLa), leukemia cell (K562), lung adenocarcinoma cell (A549), and normal cell (MRC-5). As shown in Fig. S8, the expression levels of α-deletion, β-deletion and γ-deletion from different cell lines were different. The quantification results presented in Fig. S8 reveal that the α-deletion transcript and β-deletion transcript have the highest expression level in HeLa, a significantly lower expression level in A549, MRC-5 and K562. The expression level of the γ-deletion transcript was not much different in HeLa, K562, A549 and MRC-5. Thus, our mRNA splice variant assay by splice-junction anchored DNA probes and SplintR ligase can be applied successfully to detect mRNA splice variants in total RNA samples from various cell lines.

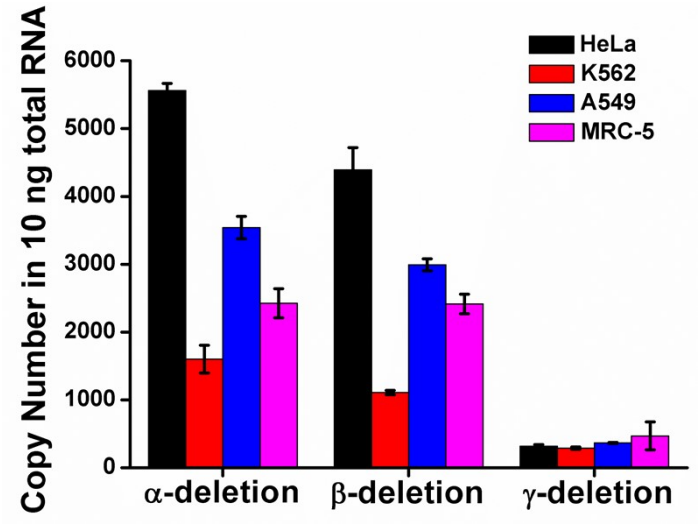

Fig. S9 Simultaneously quantification of hTERT splicing events in 10 ng total RNA samples by using TaqMan probe (100 nM). The total RNA samples were extracted from the HeLa, K562, A549, and MRC-5. Error bars indicate the standard deviation of three replicative experiments.