Electronic Supplementary Information

Specific recognition and sensitive quantification of mRNA splice variants *via* one-pot ligation-depended loop-mediated isothermal amplification

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1. The sequences of the nucleic acids used in this assay.

Table S1. The sequences of the nucleic acids used in this assay.

Name	Sequence (5′-3′ direction)
α-deletion	CCGCCUGAGCUGUACUUUGUCAAGGACAGGCUCACGGAG GUCAUCGCC
β-deletion	GUCCGCAAGGCCUUCAAGAGCCACGUCCUACGUCCAGUG CCAGGGGAU
γ-deletion	CACCCACGCGAAAACCUUCCUCAGCUAUGCCCGGACCUCC AUCAGAGC
$P1_\alpha$	/PO$_4$/CTTGACAAAGTACAGCTCAGTTTATCGTCGTGACTGTT TGTAATAGGACAGAGCCCCGCACTTTCAGTCACGACGAT
$P2_\alpha$	CGACAGCAGAGGATTTGTTGTGTGGATATCTGAGCGGATTT TCCTCTGCTGTCGTTTATGACCTCCGTGAGCCTGTC
$P1_\beta$	/PO$_4$/GTGGCTCTTGAAGGCCTTGCTTTATCGTCGTGACTGTT TGTAATAGGACAGAGCCCCGCACTTTCAGTCACGACGAT
$P2_\beta$	CGACAGCAGAGGATTTGTTGTGTGGATATCTGAGCGGATTT TCCTCTGCTGTCGTTTCCTGGCACTGGACGTAGGAC
$P1_\gamma$	/PO$_4$/CTGAGGAAGGTTTTCGCGTGTTTATCGTCGTGACTGTT TGTAATAGGACAGAGCCCCGCACTTTCAGTCACGACGAT
$P2_\gamma$	CGACAGCAGAGGATTTGTTGTGTGGATATCTGAGCGGATTT TCCTCTGCTGTCGTTTTGATGGAGGTCCGGGCATAG
U-FIP	ATCGTCGTGACTGAAAGTGCGGGGCTCTGTCCTATTAC
U-BIP	CGACAGCAGAGGATTTGTTGTGTGGATATCTGAGCGGA

Notes: /PO$_4$/ indicates the phosphate group.

2. The experimental procedures of two-step ligation-LAMP-based mRNA splicing variants assay

The ligation reaction mixture consisted of 1 μL mRNA splice variants or 1 μL total RNA samples, 1 μL 1 nM stem-loop DNA probe 1 (P1), 1 μL 1 nM stem-loop DNA probe 2 (P2), 1.0 μL 10 × SplintR ligase reaction buffer, 0.1 μL 25 U/μL SplintR ligase, 5.9 μL RNase-free water. Before testing, the RNA samples, P1 and P2 probes were annealed at 65 ℃ for 5 minutes and incubated at 35 ℃ for 5 minutes.

The ligation reaction mixture was incubated at 35 °C for 20 minutes to perform the ligation reaction.

2 μL ligation product was added to 8 μL LAMP amplification solution which included 0.5 μL 8 U/μL *Bst* DNA polymerase, 1.0 μL 10× ThermoPol reaction buffer, 1.0 μL 2.5 mM dNTPs, 0.4 μL 20 μM U-FIP, 0.4 μL 10 μM UBIP, 0.2 μL 20× SYBR Green I, and 4.5 μL RNase-free water. The LAMP amplification reaction was performed in StepOne real-time quantitative PCR system under 65 °C, and the real-time quantitative fluorescence signals were monitored once per minute.

3. Optimization of the experimental conditions and procedures.

3.1 Concentration of stem-loop DNA probe.

By using two-step ligation-LAMP, the experimental conditions of the ligation reaction, including the concentration of stem-loop DNA probes, the dosage of SplintR ligase, and the reaction time and temperature of ligation have been investigated.

Firstly, we investigated the influence of probes (P1 and P2) concentration on the assay by detecting 10 fM, 100 fM, and 1 pM synthetic α-deletion splicing variant RNA with different probe concentrations. As results shown in Fig. S1, when the probe concentration decreased from 1 nM to 10 pM, the reaction speed gradually reduced, especially when the probe concentration dropped to 10 pM, and the ligation reaction efficiency was too low. However, too high probe concentrations may aggravate non-specific ligation, such as 1 nM probe concentration. Considering the ligation specificity and efficiency, the probe concentration of 100 pM was selected as the optimal condition for this ligation-LAMP assay.
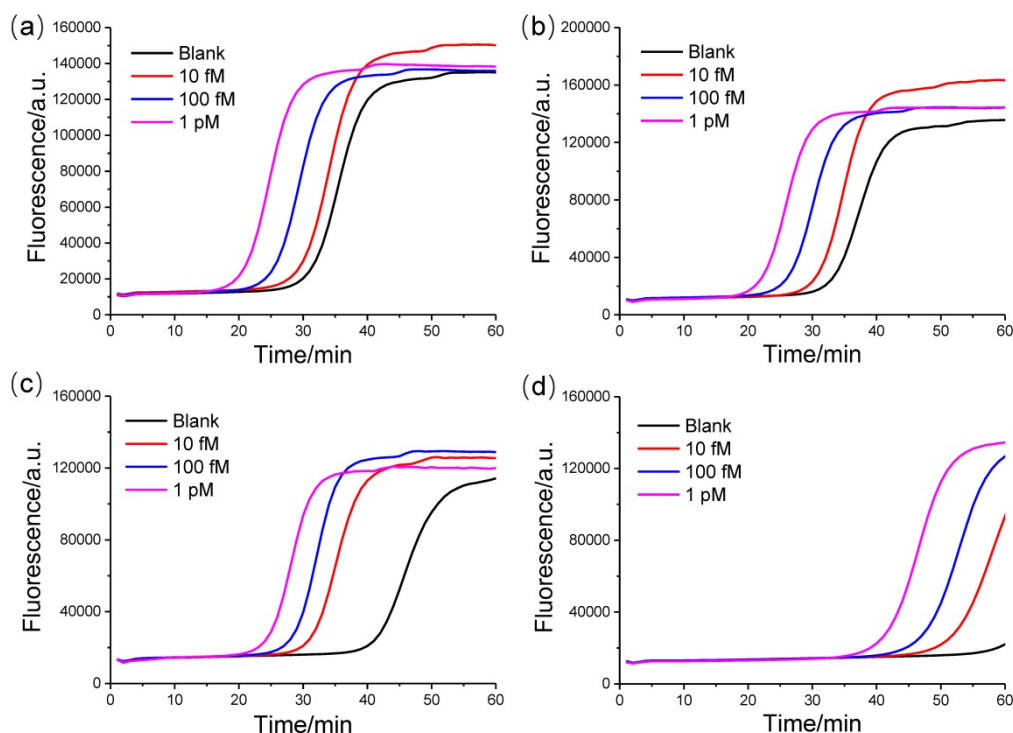
Fig. S1. Effect of the probes (P1 and P2) concentration on the ligation-LAMP assay. The probes concentration was 1 nM (a), 500 pM (b), 100 pM (c), and 10 pM (d), respectively. The blank control was treated with the same procures but without any splice variant.

3.2 Amount of SplintR ligase.

To investigate the effect of the amount of SplintR ligase on the ligation-LAMP-based assay, 10 fM, 100 fM, and 1 pM synthetic α-deletion splicing variant RNA were tested with different amounts of SplintR ligase. As exhibited in Fig. S2, by increasing the amount of SplintR ligase from 1.25U to 5 U, the reaction efficiency was gradually accelerated. However, when the dosage of SplintR ligase increased to 5 U, it was observed that the fluorescence curve of the blank showed a growing trend, indicating that the increased dosage of SplintR ligase may aggravate the non-specific ligation. In contrast, there was no significant difference between the SplintR ligase dosage of 2.5 U and 3.75 U. Finally, 2.5 U was chosen for further studies.
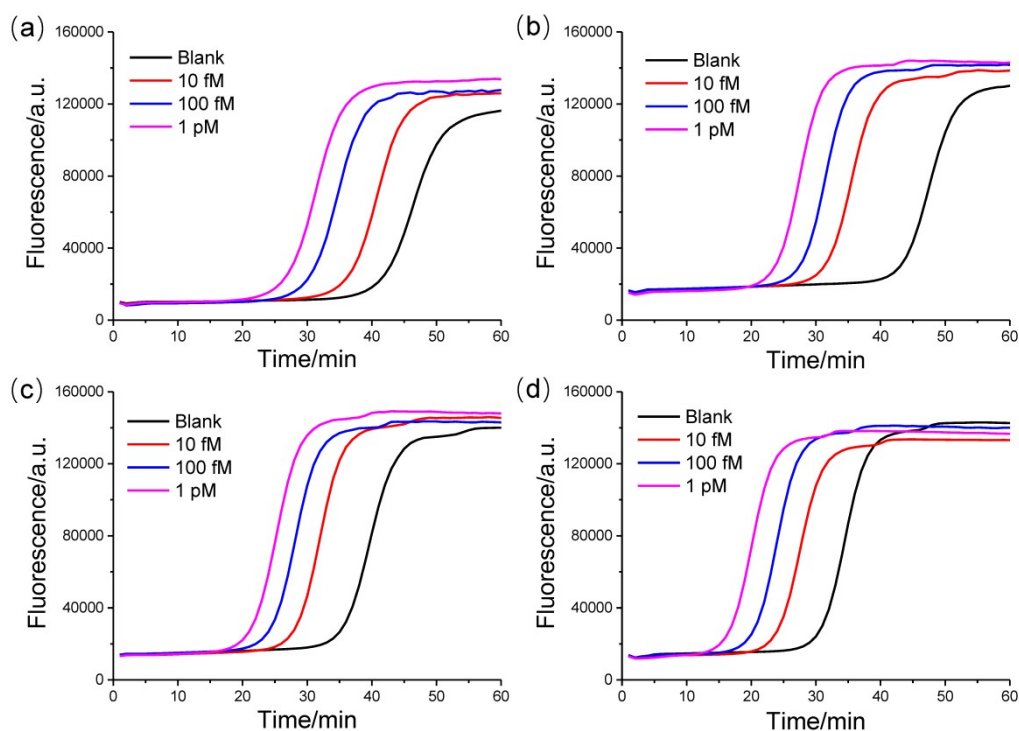
Fig. S2. Effect of the SplintR ligase dosage on the ligation-LAMP. The SplintR ligase dosage was 1.25 U (a), 2.5 U (b), 3.75 U (c), and 5 U (d), respectively. The blank control was treated with the same procures but without any splice variant.

3.3 Ligation reaction time.

Next, we investigated the influence of ligation time on the ligation-LAMP-based assay. The ligation times, including 15 min, 20 min, 25 min, and 30 min, were respectively used to detect 10 fM, 100 fM, and 1 pM synthetic α-deletion splicing variant RNA. As illustrated in Fig. S3, when the ligation time was extended from 15 min to 30 min, the reaction speed gradually increased, but the longer reaction time of ligation may increase the risk of template-independent ligation. Considering the reaction speed and ligation specificity, 20 min ligation time was chosen as the best condition.
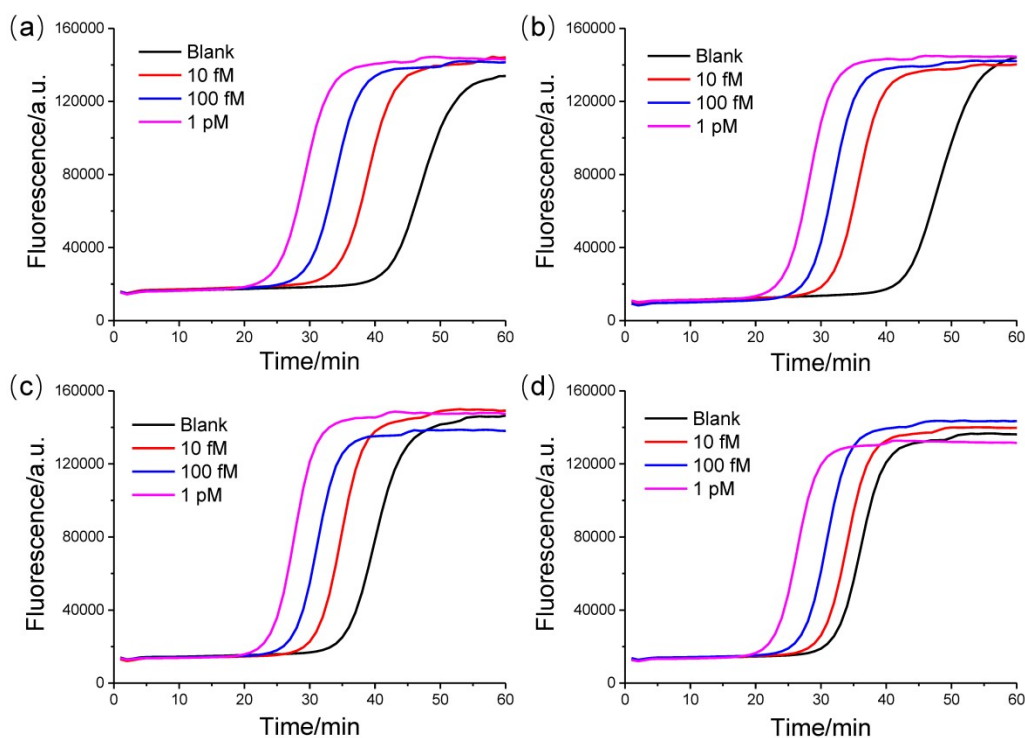
Fig. S3 Effect of the ligation reaction time on the ligation-LAMP assay. The probe concentration was 15 min (a), 20 min (b), 25 min (c), and 30 min (d), respectively. The blank control was treated with the same procures but without any splice variant.

3.4 Temperature of the ligation reaction.

The temperature of the ligation reaction largely affects the activity of SplintR ligase. To investigate the effect of ligation temperature on splice variant detection, the ligation-LAMP was performed by using the following temperatures, 16 ℃, 25 ℃, 35 ℃, and 40 ℃. As shown in Fig. S4, with increasing the ligation reaction temperature from 16 ℃ to 40 ℃, there was no significant difference in the reaction efficiency. but when the ligation temperature was 25 ℃, the nonspecific ligation of the probes (P1 and P2) was least significant. Finally, 35 ℃ was selected as the reaction temperature in this work.
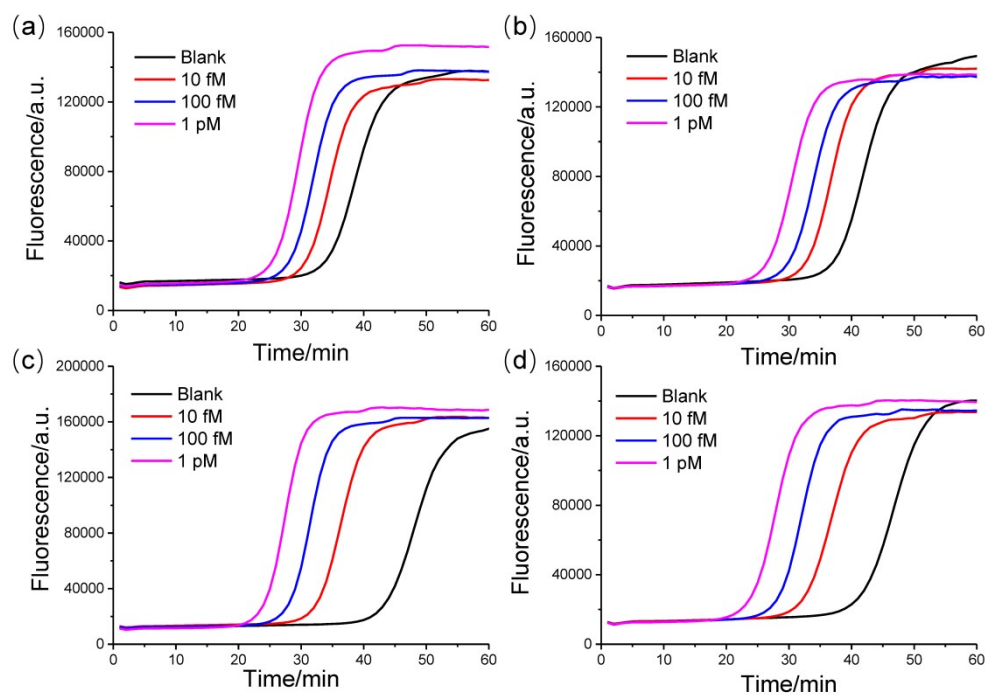
Fig. S4. Effect of the ligation reaction temperature on the ligation-LAMP assay. The ligation temperature was 16 ℃ (a), 25 ℃ (b), 35 ℃ (c), and 40 ℃ (d), respectively. The blank control was treated with the same procures but without any splice variant.

4. Analytical performance comparison of two-step and one-step ligation-LAMP-based assay.
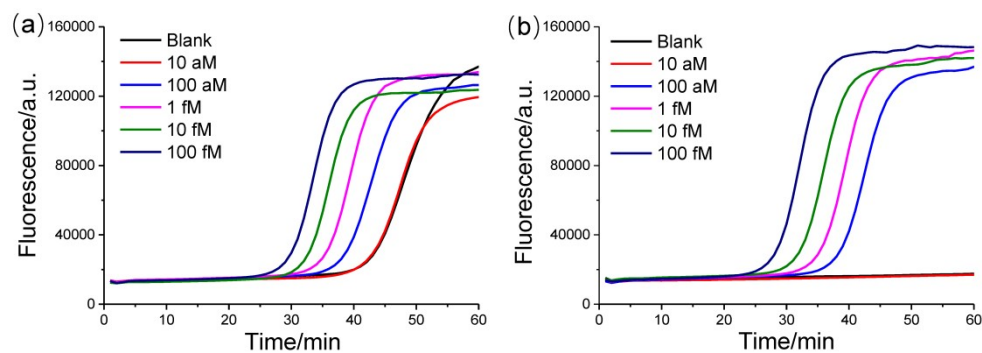
Fig. S5 Analytical performance comparison of two-step (a) and one-pot (b) ligation-LAMP-based assay. (a) Real-time fluorescence curves produced by blank 0 (without any splice variant)，10 aM, 100 aM, 1 fM, 10 fM, and 100 fM synthetic α-deletion splicing variant by two-step ligation-LAMP assay. (b) Real-time fluorescence curves produced by blank，10 aM, 100 aM, 1 fM, 10 fM, and 100 fM synthetic α-deletion splicing variant by one-pot ligation-LAMP assay.
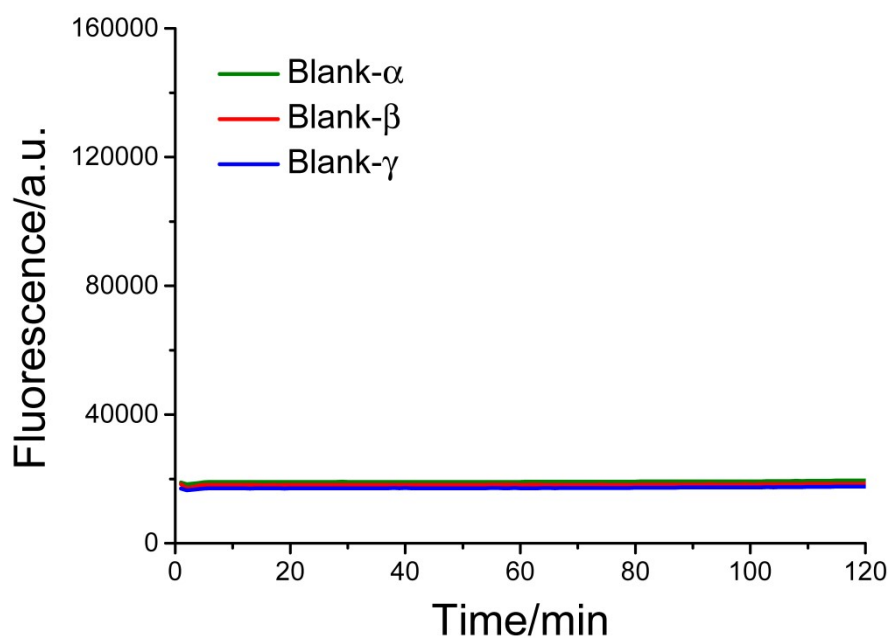
5. No specific amplification evaluation.

Fig. S6. No specific amplification evaluation of the one-pot ligation-LAMP assay. The real-time fluorescence curves were produced by blank control (without any splice variant) with $P1_\alpha$ and $P2_\alpha$ (Blank-α), $P1_\beta$ and $P2_\beta$ (Blank-β), and $P1_\gamma$ and $P2_\gamma$ (Blank-γ) within 120 min.

6. Comparison of the performance of one-step ligation-LAMP with other methods for mRNA splice variants analysis.

Table S2. Comparison of the performance of one-step ligation-LAMP with other methods for mRNA splice variants analysis.

Method	Number of enzymes	Experimental processes	Preparation process and time of sensors	Analysis time	Dynamic range	Sensitivity	Ref
Plasmonic ISH	No need	Multiple operations	Complex, >24 h	~ 1 h	N/A	1 copy	[1]
SPR biosensor	1	Multiple operations	No need	~ 2 h	0 pM−50 nM	387 pM	[2]
RETF probe assay	No need	Multiple operations	Complex, >24 h	~ 0.5 h	N/A	50 nM	[3]
SpliceRCA	2	Multiple operations	No need (in viro); Complex, ~ 17 h (in situ)	3 h (in viro); ~ 0.5 h (in situ)	10 pM-100 nM (in viro)	10 pM (in viro); single cell (in situ)	[4]
EJNC probe assay	No need	Multiple operations	Complex, > 24 h	1 h	N/A	Single cell	[5]
RNA-primed RCA	5	Multiple operations	Complex, > 24 h	1 h	N/A	Single cell	[6]

RCA	3	Multiple operations	No need	~ 3.3 h	100 fM−20 pM.	29 fM	[7]
Ligation-PCR	2	Multiple operations	No need	~ 1.5 h	100 aM-100 pM	100 aM	[8, 9]
RT-PCR	2	Multiple operations	No need	~ 2 h	100 aM-100 pM	100 aM	[10-12]
RT-LAMP	3	Multiple operations	No need	~ 3 h	100 aM-100 pM	100 aM	[13]
One-pot ligation-LAMP	2	All in one-pot	No need	1.5 h	100 aM-100 pM	100 aM	This work

Notes:

ISH: fluorescence in situ hybridization.

SPR: surface plasmon resonance.

RETF: reduction-triggered fluorescence.

SpliceRCA: splice-junction anchored padlock-probe-mediated rolling circle amplification.

EJNC: exon‐exon junction.

RT-PCR: reverse transcription-polymerase chain reaction.

N/A: Not available.

7. Quantification results of the hTERT splicing variants in total RNA and spiked RNA samples.

Table S3. Quantification results of the hTERT splicing variants in total RNA and spiked RNA samples.

Cell lines	Splice variants	Sample (n=3)	Average (zmol)	Recovery (%)
HeLa	α-deletion	10 ng total RNA	6.8	90.3
		10 ng total RNA+ 30 zmol	33.9	
	β-deletion	10 ng total RNA	3.8	103.0
		10 ng total RNA + 20 zmol	24.4	
	γ-deletion	10 ng total RNA	0.7	103.3
		10 ng total RNA + 3 zmol	3.8	
A549	α-deletion	10 ng total RNA	4.9	89.3
		10 ng total RNA + 30 zmol	31.7	
	β-deletion	10 ng total RNA	1.2	106.0
		10 ng total RNA + 5 zmol	6.5	
	γ-deletion	10 ng total RNA	0	91.0
		10 ng total RNA + 10 zmol	9.1	
K562	α-deletion	10 ng total RNA	1.8	109.0
		10 ng total RNA + 10 zmol	12.7	
	β-deletion	10 ng total RNA	0.7	106.0
		10 ng total RNA + 3 zmol	3.9	
	γ-deletion	10 ng total RNA	0.4	105.0
		10 ng total RNA + 2 zmol	2.5	
MRC-5	α-deletion	10 ng total RNA	2.2	107.0
		10 ng total RNA + 10 zmol	12.9	
	β-deletion	10 ng total RNA	1.1	96.0
		10 ng total RNA + 5 zmol	5.9	
	γ-deletion	10 ng total RNA	0.6	90.0
		10 ng total RNA + 3 zmol	3.3	

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