Electronic Supplementary Information

Sensitive detection of fusion transcripts with padlock probe-based continuous cascade amplification (P-CCA)

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1. Table S1. The sequences of the synthetic RNA target, DNA probes and primers used in the P-CCA assay

name	sequence (5′-3′)
PML-RARα: 6-3	rCrArGrUrGrGrCrGrCrCrGrGrGrGrArGrGrCrArG*rCrCrArUrUrGrAr GrArCrCrCrArGrArGrCrArGrC
PML-RARα: 3-3	rUrUrGrCrArUrCrArCrCrCrArGrGrGrGrArArArG*rCrCrArUrUrGrAr GrArCrCrCrArGrArGrCrArGrC
Padlock probe (for PML-RARα: 6-3)	CTGCCTCCCCGTTTTCGACACGACACGATTTTGGAACTCTGCT CGACGGATTAAAATAATACAGTCTGCCCACAACCTTTTTGGG TCTCAATGG
Padlock probe (for PML-RARα: 3-3)	CTTTCCCCTGGGTTTTTCGACACGACACGATTTTGGAACTCTG CTCGACGGATTAAAATAATACAGTCTGCCCACAACCTTTTTGG GTCTCAATGG
FP	CGACACGACACGAAAAAAATCCGTCGAGCAGAGTTCC
BP	ATCGTCGTGACTGTTTTCCCTAACCCTAACCCTAACCC
RSLP	ATCGTCGTGACTGTTTTCCCTAACCCTAACCCTAACCCTTTTC AGTCACGACGATTTTTTAATACAGTCTGCCCACAACC
single-base mismatched PML-RARα: 6-3	rCrArGrUrGrGrCrGrCrCrGrGrGrGrArGrGrCrArA*rCrCrArUrUrGrAr GrArCrCrCrArGrArGrCrArGrC
double-base mismatched PML-RARα: 6-3	rCrArGrUrGrGrCrGrCrCrGrGrGrGrArGrGrArArA*rCrCrArUrUrGrAr GrArCrCrCrArGrArGrCrArGrC
BCR-ABL: e1a2	rGrArGrGrGrCrGrCrCrUrUrCrCrArUrGrGrArGrArCrGrCrArGr*ArAr GrCrCrCrUrUrCrArGrCrGrGrCrCrArGrUrArGrCrArUrCrU
Reverse primer for real-time RT-PCR	GCTGCTCTGGGTCTCAAT

Forward primer for real-time RT-PCR	CAGTGGCGCCGGG

Note: The letter "r" indicated ribonucleotides. The asterisk (*) indicates the fusion junction. In the padlock probe, the sequences binding with PML-RARα fusion transcripts are underlined, complementary with the shadow regions in PML-RARα: 6-3 or PML-RARα: 3-3. The part that hybridizes with FP is marked in purple, the part that same as RSLP is marked in orange, the mismatched bases are marked in red.

2. Optimization of the concentration of padlock probe

The padlock probe binds to the target and is subsequently cyclized to form the RCA template, which plays a crucial role in the entire P-CCA method. Therefore, we firstly study the impact of padlock probe usage on the analytical performance.

As illustrated in Fig. S1A-B, when the concentration of the padlock probe is increased from 50 pM to 200 pM, the specific ligation efficiency gradually increases, meanwhile, the 10 fM PML-RARα: 6-3 can be discriminated from blank. However, when the probe concentration is further increased to 1 nM and 2 nM (Fig. S1C-D), the POI value of blank sample decreases, indicating the concentration of padlock probe is excessive to lead to the nonspecific ligation and cascade amplification. Therefore, we chose 200 pM of padlock probe for the follow-up reaction.

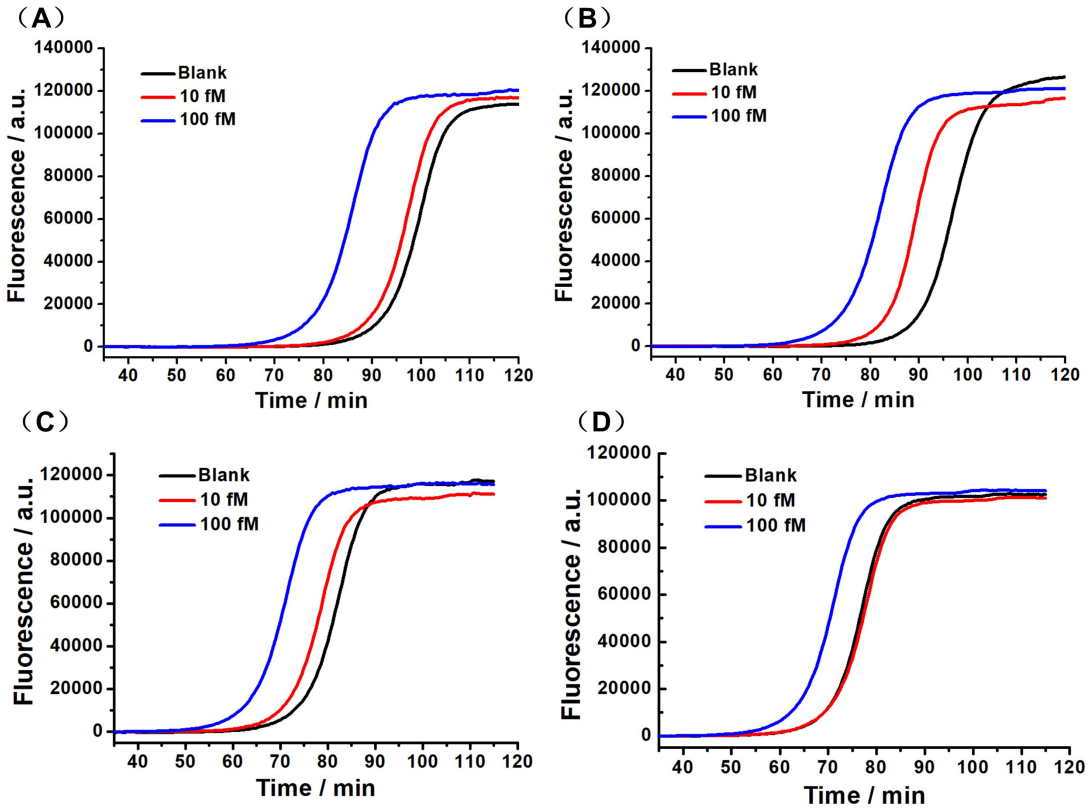

Fig. S1 Influence of the concentration of the padlock probe on the P-CCA assay. The real-time fluorescence curves are produced from 0 (Blank), 10 fM and 100 fM PML-RARα: 6-3. The padlock probes are used as 50 pM (A), 200 pM (B), 1 nM (C) and 2 nM (D), respectively.

3. Optimization of the amount of SplintR ligase

The SplintR ligase is the catalyst of the ligation reaction, so we investigate the analytical performance of P-CCA assay by using 1.25 U to 7.5 U SplintR ligase. When the amount of SplintR ligase is used from 1.25 U to 5.0 U (Fig. S2A-C), the POI value of the 10 fM and 100 fM PML-RARα: 6-3 gradually decrease, indicating the specific ligation efficiency is elevated. Further increasing the amount of SplintR ligase to 7.5 U (Fig. S2D), the POI value of blank reduces further and the difference of the 10 fM PML-RARα: 6-3 with blank was smaller, indicating the SplintR ligase is excessive to result in nonspecific ligation. Therefore, we chose 5.0 U SplintR ligase to circulate the padlock probe.

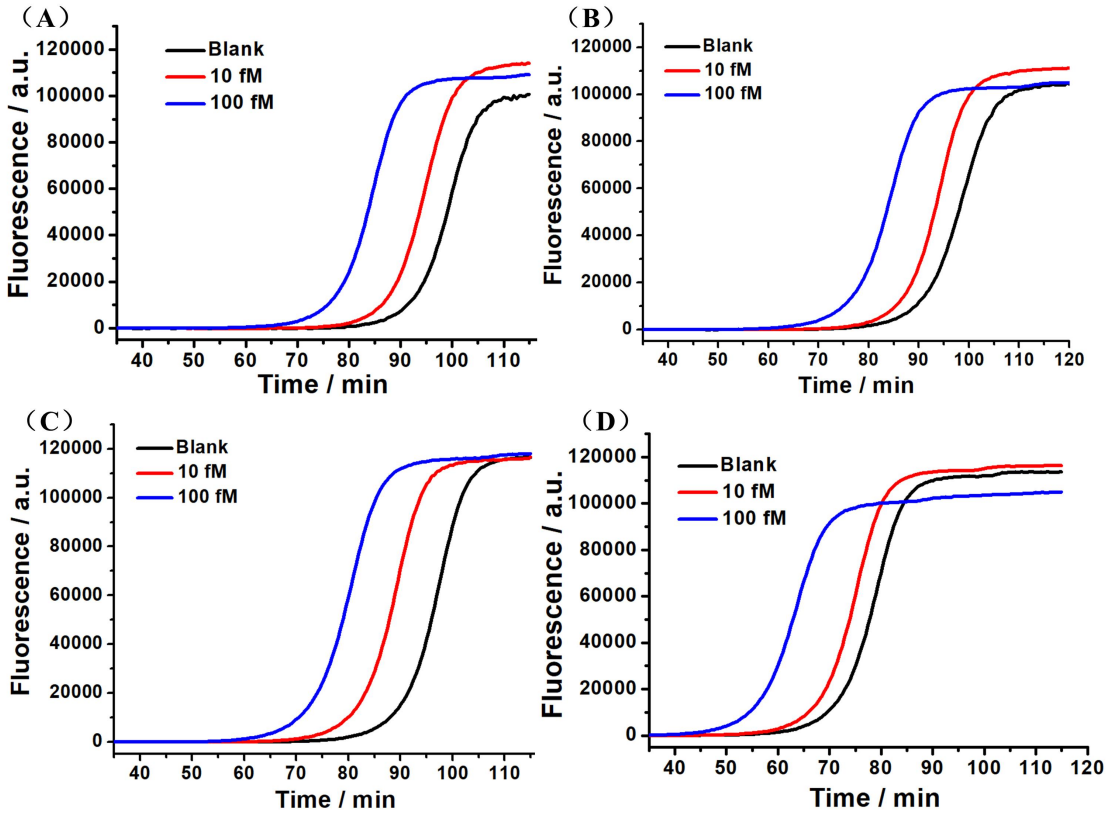

Fig. S2 Effect of the amount of SplintR ligase on the P-CCA assay. The fluorescence curves are respectively produced from 0 (Blank), 10 fM, 100 fM PML-RARα: 6-3 in the presence of different SplintR ligase dosages of 1.25 U (A), 2.5 U (B), 5.0 U (C) and 7.5 U (D).

4. Optimization of the concentration of RSLP

During the amplification process, RSLP is used as a reverse primer to bind with RCA product and subsequently forms a double stem-loop DNA structure to initiate LAMP. As an essential primer for P-CCA assay, we evaluate the effect of its concentration on analytical performance. As shown in Fig. S3A-B, with the concentration of RSLP increased from 10 nM to 50 nM, the amplification efficiency of P-CCA gradually increases, and 10 fM target can be clearly detected. When the concentration of RSLP further increases to 100 nM and 200 nM (Fig. S3C and D), the POI of blank reduce, verifying that the RSLP is excessive to cause the non-specific amplification. Therefore, we chose 50 pM RSLP for amplification.

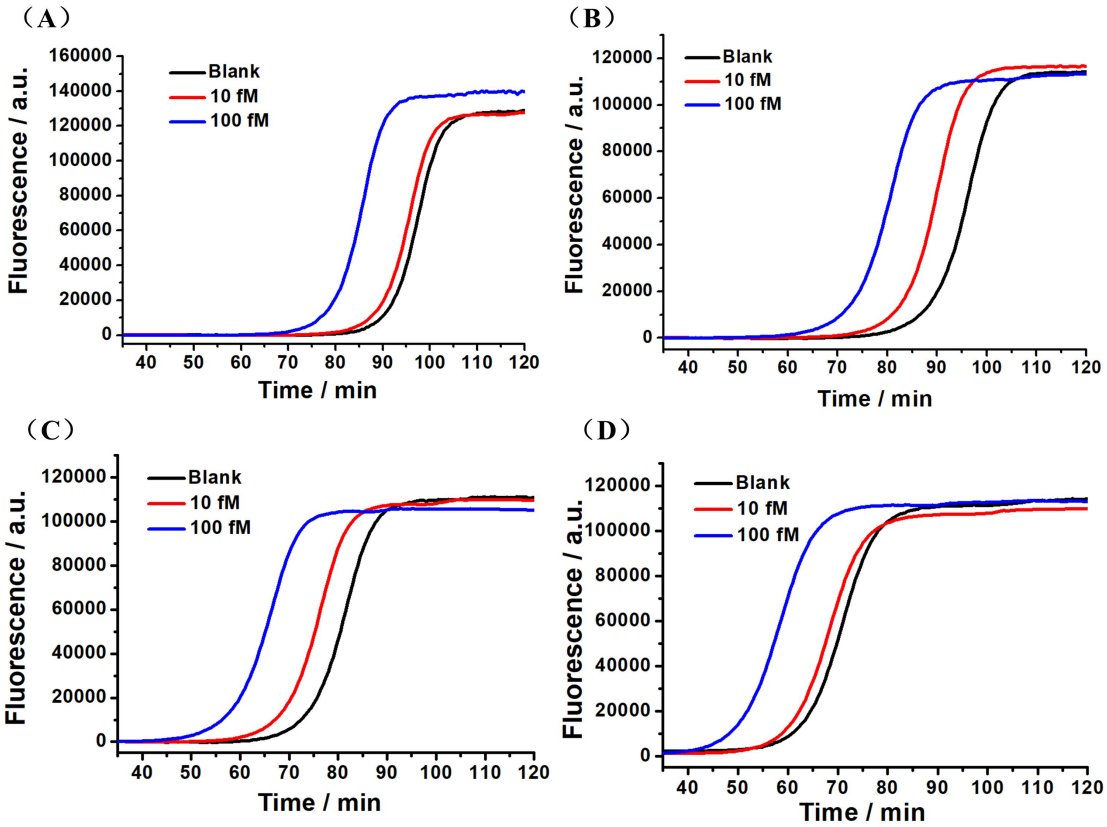

Fig. S3 Influence of the concentration of the RSLP on the P-CCA assay. The real-time fluorescence curves were respectively produced from 0 (Blank), 10 fM, 100 fM PML-RARα: 6-3 by using 10 nM (A), 50 nM (B), 100 nM (C) and 200 nM (D) of RSLP.

5. Optimization of the amount of Bst 2.0 Warmstart DNA polymerase

Bst 2.0 Warmstart DNA polymerase is used to catalyze the CCA reaction, so we investigate the effect of different polymerase amounts from 0.4 U to 3.2 U on analytical performance. As shown in Fig. S4A, when the amount of DNA polymerase is as low as 0.4 U, no obvious amplification can be detected even if the reaction time reached 120 min, indicating that the efficiency of LAMP is extremely low. When the amount of the DNA polymerase increases to 0.8 U, the amplification efficiency greatly improves and well-defined fluorescence curves are obtained. However, as the amount of DNA polymerase continues to increase to 1.6 U and 3.2 U (Fig. S4C and D), the POI of blank decrease, indicating the polymerase is excessive to lead the nonspecific amplification. Therefore, we chose 0.8 U Bst 2.0 Warmstart DNA polymerase for the P-CCA assay.

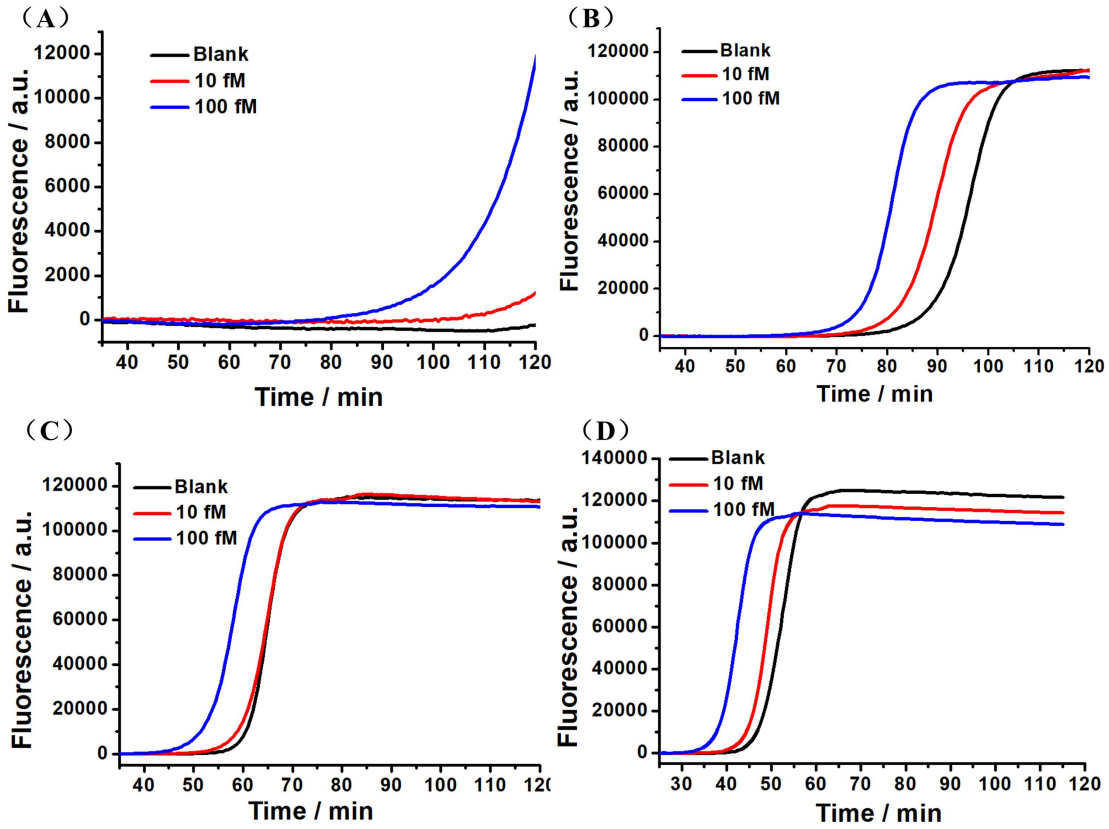

Fig. S4 Effect of the amount of Bst 2.0 Warmstart DNA polymerase in the proposed P-CCA assay. The real-time fluorescence curves are produced from PML-RARα: 6-3 by using 0.4 U (A), 0.8 U (B), 1.6 U (C) and 3.2 U (D) Bst 2.0 Warmstart DNA polymerase, respectively.

6. Table S2. The sequences of RT-PCR primers used in Fig. 4 in the main text

Primer name	sequence (5′-3′)
Reverse transcription primer	CTGCAGGACCTCAGCT
PCR-FP	GCTGGGCACTATCTCTT
PCR-RP	CTGCAGGACCTCAGCT

* The RT-PCR primers were synthesized by Sangon Biotech (Shanghai, China).

7. The PML-RARα: 6-3 by only RCA reaction

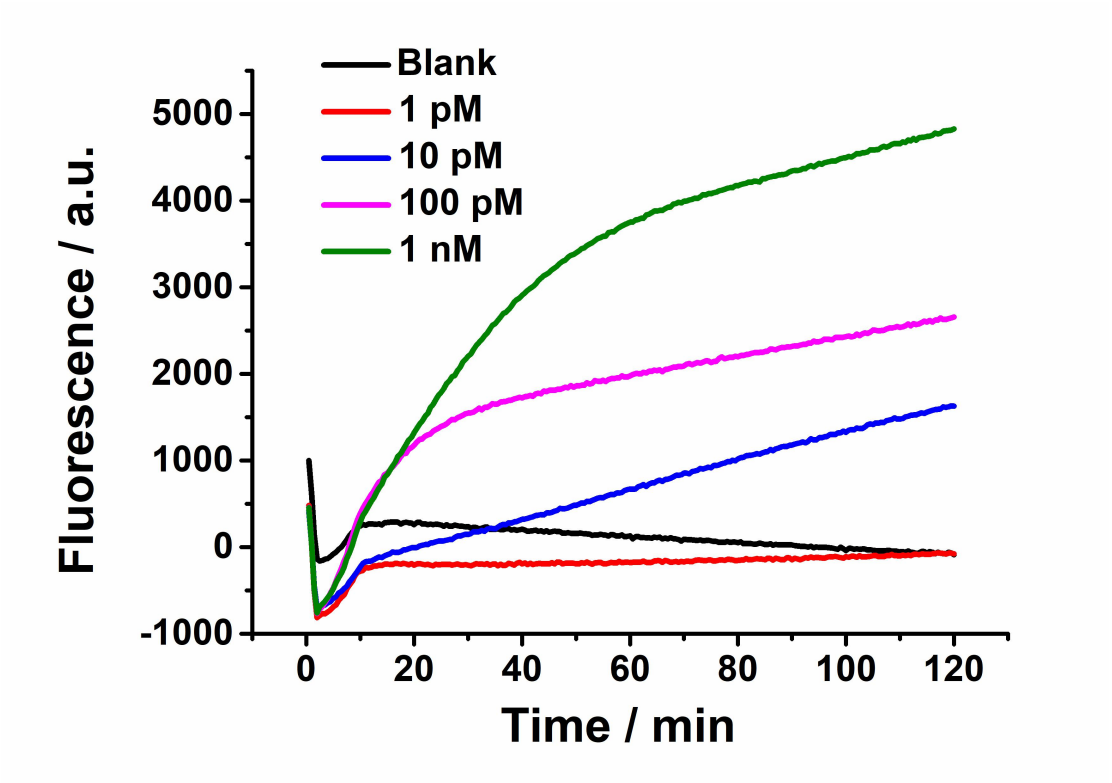

Fig. S5 The real-time fluorescence curves are produced from different concentrations of PML-RARα: 6-3 by padlock-based RCA reaction only.

8. The single-base, double-base mismatched PML-RARα: 6-3 and other sequence detection by P-CCA

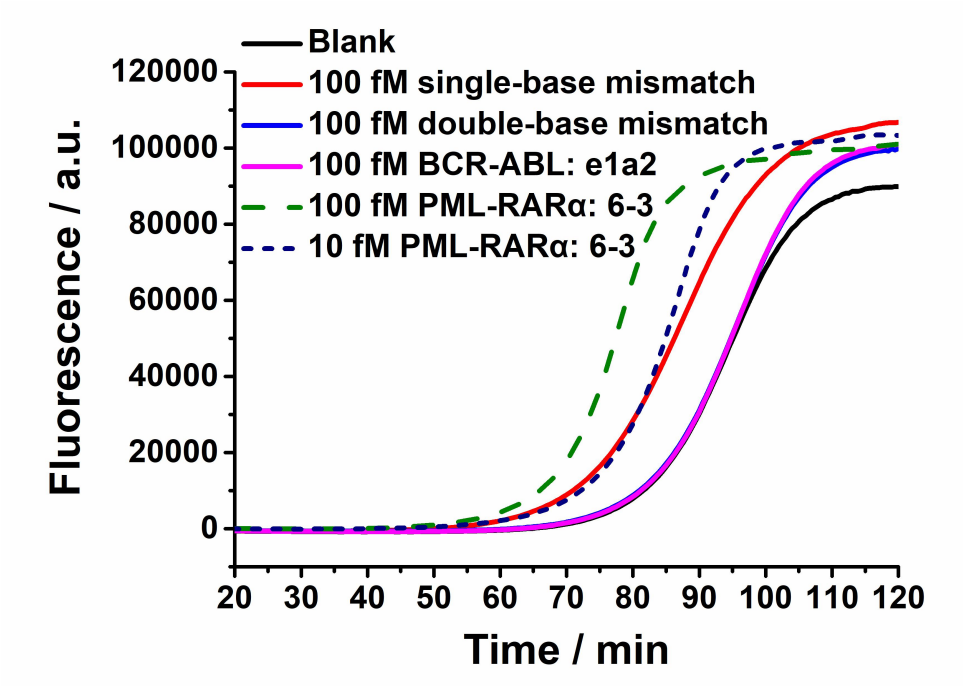

Fig. S6 The real-time fluorescence curves are produced from fully matched PML-RARα: 6-3, single-base and double-base mismatched PML-RARα: 6-3, and BCR-ABL: e1a2 by P-CCA with the PML-RARα: 6-3 specific probe.

9. The sequencing results of RT-PCR products of total RNA from NB4 cells

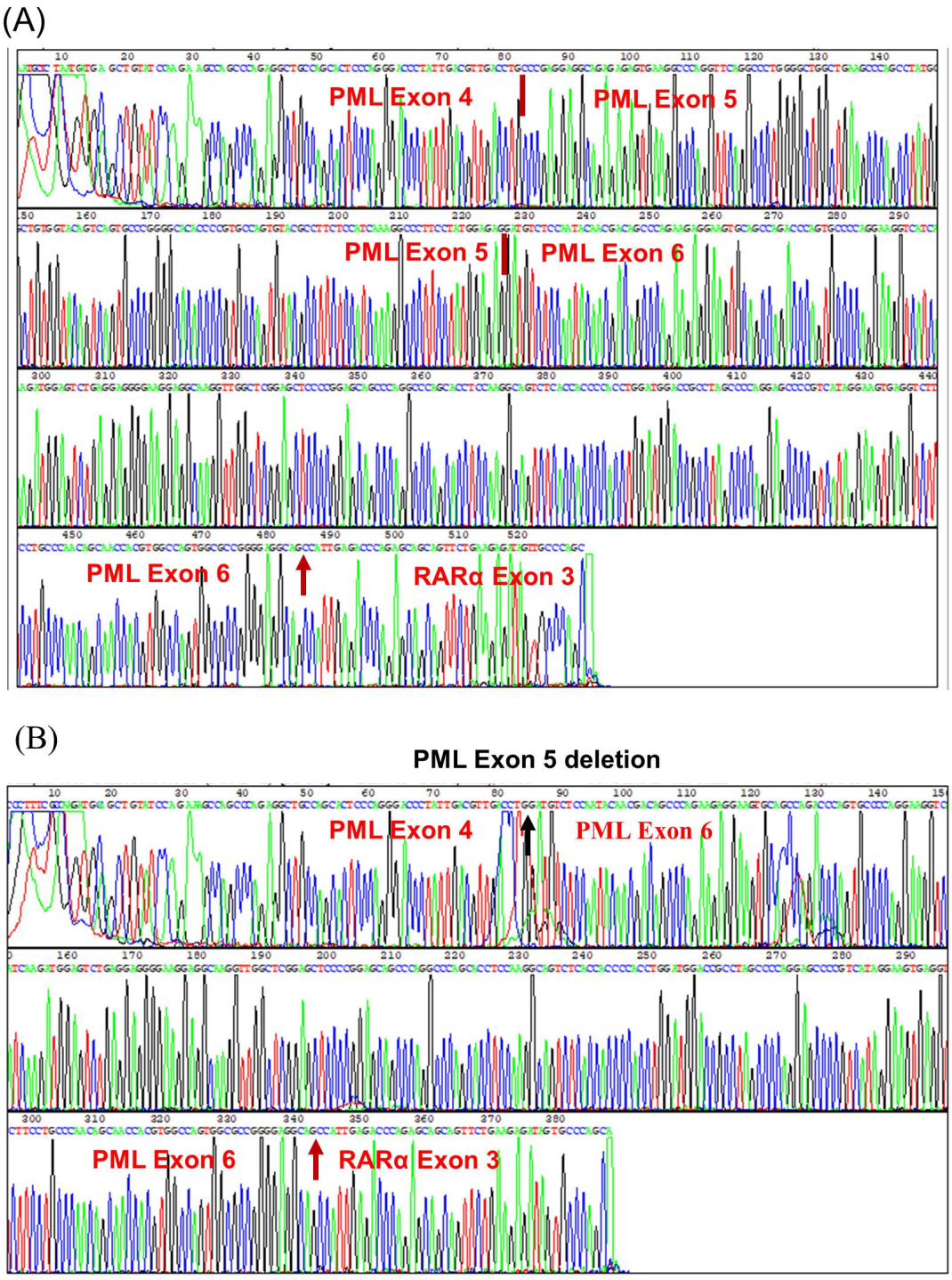

Fig. S7 (A) The sequencing result of RT-PCR product of the band of 555 bp in Fig. 4B. (B) The sequencing result of RT-PCR product of the band of 411 bp in Fig. 4B. The red arrow indicates the fusion site of PML and RARα gene. The black arrow indicates the deletion of PML Exon 5.

10. Table S3 The recovery test of PML-RARα: 6-3 from 100 ng total RNA extracted from NB4 cells by the P-CCA method

sample	copy number of PML-RARα: 6-3 in sample 1	copy number of PML-RARα: 6-3 in sample 2	copy number of PML-RARα: 6-3 in sample 3
100 ng total RNA from NB4	12645	11401	14518
100 ng total RNA from NB4 +60221 copy number of PML-RARα: 6-3	66309	64059	71049
Recovery	89.1%	87.5%	93.9%
Average recovery	90.2% (RSD = 3.7%)		

11. The real-time RT-PCR assay for PML-RARα:6-3

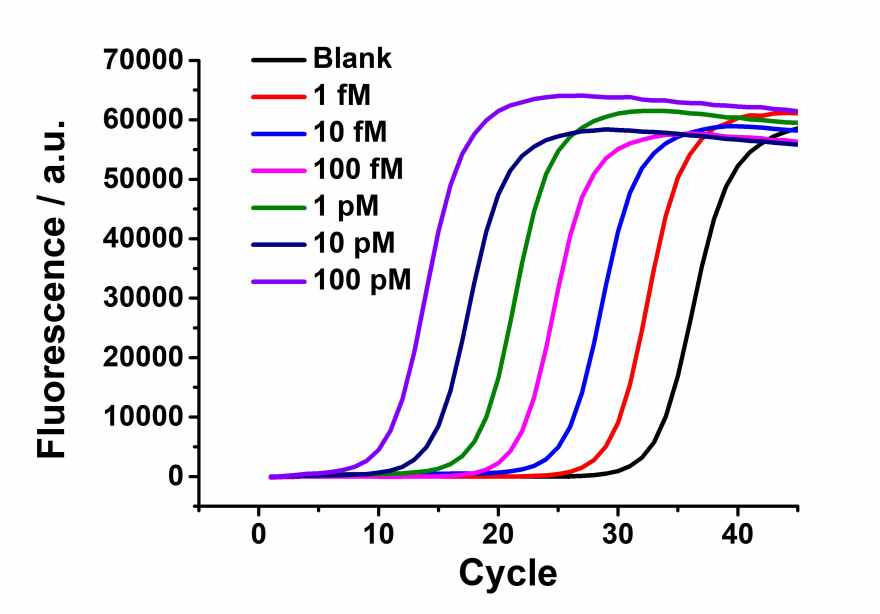

Fig. S8 The real-time fluorescent curves of RT-PCR for assaying different concentrations of PML-RARα: 6-3. The concentrations of PML-RARα: 6-3 is blank, 1 fM, 10 fM, 100 fM, 1 pM, 10 pM and 100 pM, respectively.

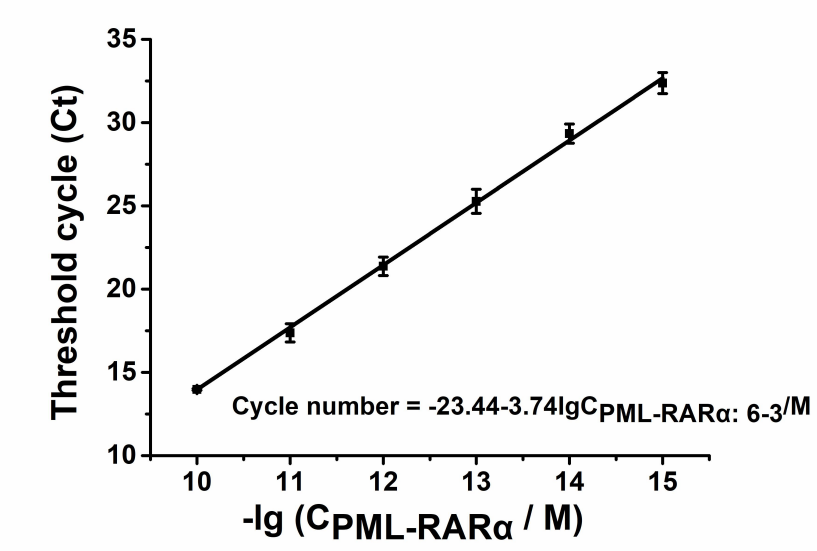

Fig. S9 The relationship of the Ct value of the fluorescent curves in Fig. S8 with the negative logarithm of the target concentrations.

Table S4 The comparison of the P-CCA and RT-PCR method for quantification the PML-RARα: 6-3 in 100 ng total RNA from NB4 cells.

Method	Sample 1	Sample 2	Sample 3	average
P-CCA	2.10 fM	1.89 fM	2.41 fM	2.13 fM (RSD=12.2%)
RT-PCR	2.44 fM	2.35 fM	2.91 fM	2.57 fM (RSD=11.7%)

12. Table S5 Comparison of different methods for the detection of gene fusion

Method	target	Reverse transcription	LOD	linear range	Reference
P-CCA	PML-RARα	No	1 fM	1 fM-100 pM	*This work*
RT-PCR	PML-RARα	Yes	10 copies/reaction	10^1-10^6copies/reaction	*PLoS One,* 2015, 10(3): e0122530.
FISH	BCR-ABL EWSR1-FLl1	No	Single-molecule		*PLoS One,* 2014, 9(3): e93488.
LAMP	BCR-ABL	No	100 aM	100 aM-100 pM	*Anal. Chem.,* 2019, 91(19): 12428-12434.
LCR	BCR-ABL	No	1 fM	1 fM-100 pM	*Analyst,* 2020, 145(11): 3977-3982.