

## Development of Reverse Transcription Loop-Mediated Isothermal Amplification for Simple and Rapid Detection of *Promyelocytic Leukemia–Retinoic Acid Receptor α* mRNA

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### ABSTRACT

**Background** Acute promyelocytic leukemia (APL) is a disease characterized by expression of *promyelocytic leukemia–retinoic acid receptor α* (*PML-RARα*) chimeric mRNA. Although APL is curable, early death due to hemorrhage is a major problem. Here, we report the development of a simple and rapid diagnostic method for APL based on reverse transcription loop-mediated isothermal amplification (RT-LAMP).

**Methods** An RT-LAMP primer set was designed to detect three types of *PML-RARα* mRNA in a single reaction. Serial dilutions of plasmid DNA containing bcr1, bcr2, or bcr3 *PML-RARα* sequences and RNA extracted from bone marrow aspirates of 6 patients with APL were used to compare the results of RT-LAMP and nested PCR assays.

**Results** Plasmid DNA was amplified by RT-LAMP, for which the reaction time was > 4 h shorter and the lower detection limit was higher than for nested RT-PCR. Six of 7 samples tested positive by both methods.

**Conclusion** We developed an RT-LAMP assay for simple and rapid *PML-RARα* mRNA detection that may be clinically useful for point-of-care testing and APL diagnosis.

**Key words** acute promyelocytic leukemia; polymerase chain reaction; *promyelocytic leukemia–retinoic acid receptor α*; reverse transcription loop-mediated isothermal amplification; t(15;17) chromosome translocation

Loop-mediated isothermal amplification (LAMP) is an established nucleic acid amplification method that requires a set of 4 specially designed, highly specific inner and outer primers.<sup>1</sup> Additional loop primers can further accelerate the reaction.<sup>2</sup> The LAMP product is detected by turbidity arising from magnesium pyrophosphate formed during the reaction.<sup>3</sup> This method is very rapid and directly amplifies the target region from an RNA sample, and has been used to detect various pathogens, including bacteria and viruses.<sup>4</sup> In addition, LAMP has

now been developed as commercial kits, some of which have been adopted as officially recommended methods for routine pathogen examination and surveillance in Japan.<sup>5</sup> However, there have been few applications of LAMP to the field of hematological malignancy, although one notable exception is the detection of *Wilms' tumor-1* gene mRNA.<sup>6</sup>

Acute promyelocytic leukemia (APL) is a distinct subtype of acute myeloid leukemia (AML) accounting for approximately 10% of cases. APL is cytogenetically characterized by balanced reciprocal translocation between chromosomes 15 and 17, which results in the fusion of the *promyelocytic leukemia* (*PML*) and *retinoic acid receptor α* (*RARα*) genes.<sup>7</sup> The *PML-RARα* chimeric gene is detected in nearly all APL patients and is used as a marker for clinical diagnosis. In addition, the mRNA is detected by reverse transcription polymerase chain reaction (RT-PCR) and/or real time RT-PCR for minimal residual disease monitoring of APL.<sup>8–10</sup> Three different types of *PML-RARα* mRNA exist owing to different breakpoints in the *PML* gene, although these do not result in different treatment outcomes: the long-, variable-, and short-form types are derived from breakpoints at bcr1 (intron 5), bcr2 (exon 6), and bcr3 (intron 3), respectively, and occur at relative frequencies of 55%, 5%, and 40%, respectively.<sup>11</sup>

APL is the most curable form of AML because of an established molecular targeted therapy combining all-trans retinoic acid (ATRA) and arsenic trioxide.<sup>12</sup> Before starting the treatment, it is necessary to verify that the patient has the *PML-RARα* fusion gene.<sup>13</sup> However, early death—mostly due to severe hemorrhage—remains

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Abbreviations: AML, acute myeloid leukemia; APL, Acute promyelocytic leukemia; ATRA, all-trans retinoic acid; LAMP, loop-mediated isothermal amplification; *PML*, *promyelocytic leukemia*; *RARα*, *retinoic acid receptor α*; RIN, RNA integrity number; RT-LAMP, reverse transcription loop-mediated isothermal amplification; RT-PCR, reverse transcription polymerase chain reaction

the main cause of APL treatment failure according to recent population-based studies carried out in developed countries.<sup>14</sup> Some reports recommend early and timely initiation of ATRA and provision of aggressive supportive care at the first suspicion of the disease, without awaiting molecular confirmation, in order to reduce hemorrhagic deaths in APL.<sup>14</sup> An improved *PML-RAR $\alpha$*  mRNA detection test is also needed.

We report here the development of a simple reverse transcription loop-mediated isothermal amplification (RT-LAMP) method for rapid *PML-RAR $\alpha$*  mRNA detection. The advantages of this method with respect to RT-PCR are also discussed.

## MATERIALS AND METHODS

### Samples

Three different plasmids—containing *bcr1*, *bcr2*, and *bcr3* *PML-RAR $\alpha$*  mRNA sequences—were purchased from Eurofins Genomics (Louisville, KY) for use as positive controls. Vector plasmid was pTAKN-2 and chimeric sequences were made of sequences derived from the Genbank sequence database (Accession numbers are M73778, NM\_000964, and AB067754). IVS-0035 RNA (Invivoscribe Technologies, San Diego, CA) was used as a negative control. Clinical samples used in this study were archived specimens from APL patients diagnosed by detection of APL cells and *PML-RAR $\alpha$*  mRNA by nested RT-PCR at our hospital. RNA was extracted from patient bone marrow aspirates using the RNeasy Plus Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions; these were used for diagnosis, and then frozen at  $-80^{\circ}\text{C}$  until the study. RNA concentrations were measured and 260/280 nm absorbance ratios were determined with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) prior to RT-LAMP and nested RT-PCR

**Table 1. Primers for detection of *PML-RAR $\alpha$*  mRNA by RT-LAMP**

Primers	Sequence (5' → 3')
<i>bcr1,2</i> FIP	TCCCTCCTCAGACTCCATCTTGAAGAGGAAGTGCAGCCAGA
<i>bcr1,2</i> F3	CTCCAATACAACGACAGCC
<i>bcr3</i> FIP	TTGAACTCGTCGAAGCCATCGGCTGGACATGCACGGTT
<i>bcr3</i> F3	AAGTGCTACGCCCTCGGA
BIP	TGAGACCCAGAGCAGCAGTTGTAGATGCCGGGTAGA
B3	TGACAGACAAGCAAGGC
<i>bcr1,2</i> LF	GATGACCTTCTGGGGCA
<i>bcr3</i> LF	ACGGCAGCTTGACAGCT
LB	TGAAGAGATAGTCCCAGCCCT

*PML-RAR $\alpha$* , promyelocytic leukemia–retinoic acid receptor  $\alpha$ ; RT-LAMP, reverse transcription loop-mediated isothermal amplification.

assays. The study protocol was approved by the Tottori University Hospital ethics committee (number 2326).

### Primer design

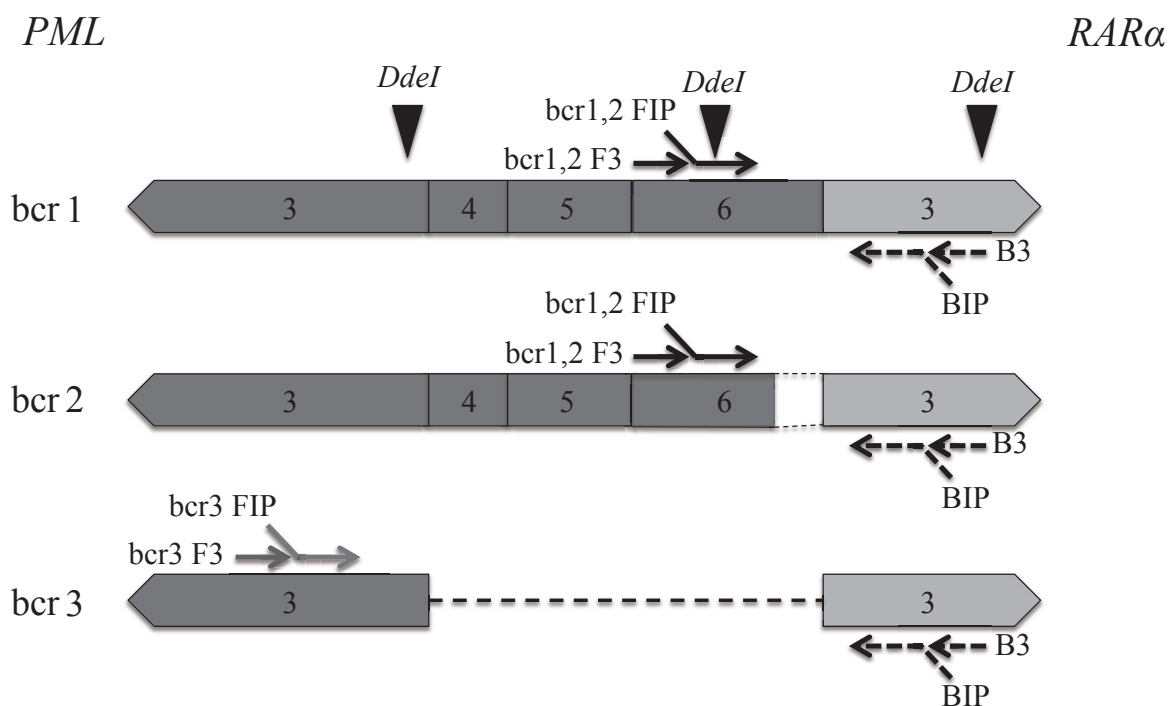
Primer sets for detection of *PML-RAR $\alpha$*  mRNA by RT-LAMP (Table 1) were designed using PrimerExplorer ver. 4 software (Eiken Chemical, Tokyo, Japan). The primer sets were modified to detect the three types of *PML-RAR $\alpha$*  mRNA in a single tube—i.e., *bcr1* and *bcr2* were amplified with the same primer sets and an additional set consisting of FIP, F3, and LF were included in one reaction to amplify *bcr3* (Fig 1).

### RT-LAMP

The RT-LAMP assay was carried out using the Loopamp RNA Amplification kit (Eiken Chemical, Tokyo, Japan) under the following conditions: 1  $\mu\text{L}$  sample (RNA or DNA) was mixed with 20 pmol each of FIP and BIP primers, 10 pmol each of LF and LB primers, 2.5 pmol each of F3 and B3 primers, 1  $\mu\text{L}$  Enzyme Mix, and 12.5  $\mu\text{L}$  of 2  $\times$  Reaction Mix, with distilled water added to obtain a final volume of 25  $\mu\text{L}$ . For real-time turbidity monitoring, the reaction mixture was incubated at  $68^{\circ}\text{C}$  for 60 min in an RT-160C Loopamp Realtime Turbiditymeter (Eiken Chemical). The specificity of LAMP was confirmed by restriction enzyme digestion using *DdeI* (Takara Bio, Otsu, Japan) under the following conditions: 5  $\mu\text{L}$  LAMP product was mixed with 2  $\mu\text{L}$  of 10  $\times$  K buffer (Takara Bio), 1  $\mu\text{L}$  *DdeI*, with distilled water added to obtain a final volume of 20  $\mu\text{L}$ , followed by incubation at  $37^{\circ}\text{C}$  for 120 min. *DdeI* was inactivated at  $70^{\circ}\text{C}$  for 15 min and the digestion products (10- $\mu\text{L}$  aliquots) were mixed with loading buffer (Nippon Gene, Toyama, Japan) and separated by 1.5% agarose gel electrophoresis (100 V, 35 min) with 0.005% ethidium bromide using a Mupid mini gel electrophoresis system (Advance, Tokyo, Japan), with 5  $\mu\text{L}$  Gene Ladder 100 (Nippon Gene) used as a molecular size marker.

### Nested RT-PCR

The RT reaction was carried out using 1  $\mu\text{L}$  RNA sample and the ThermoScript RT-PCR system (Thermo Fisher Scientific) according to the manufacturers' protocol. Three types of *PML-RAR $\alpha$*  mRNA were detected using two forward primer sets designed according to published sequences.<sup>10</sup> Specifically, *bcr1* and *bcr2* types were amplified with the same primer set. The PCR reaction mixture was prepared using AmpliTaq Gold with 10  $\times$  PCR Gold Buffer and  $\text{MgCl}_2$  (Thermo Fisher Scientific) and the GeneAmp dNTP mix (Thermo Fisher Scientific) under the following conditions: 1  $\mu\text{L}$  plasmid DNA, 2  $\mu\text{L}$  cDNA, or 1  $\mu\text{L}$  first PCR product was mixed with



**Fig. 1.** Schematic representation of primer design for detection of *PML-RARα* mRNA by RT-LAMP and cutting sites of *DdeI* restriction enzyme assay. Exon numbers of the *PML* gene (dark) and the *RARα* gene (light) are shown. *PML*, promyelocytic leukemia; *RARα*, retinoic acid receptor  $\alpha$ ; RT-LAMP, reverse transcription loop-mediated isothermal amplification.

2.5  $\mu$ L 10  $\times$  PCR Gold buffer, 2.25 nmol  $MgCl_2$  solution, 500 pmol dNTP mix, 0.2  $\mu$ L AmpliTaq Gold DNA polymerase, and 0.25 pmol each forward and reverse primers, with distilled water added to obtain a final volume of 25  $\mu$ L. The forward primers were as follows: 5'-AGTCAGTGCCCCGGGCACAC-3' for bcr1 and bcr2 and 5'-AGCTGCTGGAGGCTGTGGACG-3' for bcr3 in the first PCR; and 5'-AGTGTACGCCTTCTCATCAAAG-3' for bcr1 and bcr2 and 5'-TGTGCTGCAGCGACTCCGCA-3' for bcr3 in the second PCR. The reverse primers were as follows: 5'-AGGGCTGGGCACTATCTCTTC-3' for bcr1, bcr2, and bcr3 in the first PCR; and 5'-CAGAACTGCTGCTCTGGGTCTCAAT-3' for bcr1, bcr2, and bcr3 in the second PCR. Reaction conditions on the MultiGene Mini Personal Thermal Cycler (Labnet International, Edison, NJ) were as follows: 94  $^{\circ}C$  for 5 min; 35 cycles at 94  $^{\circ}C$  for 30 s, 62  $^{\circ}C$  for 1 min, and 72  $^{\circ}C$  for 1 min; and 72  $^{\circ}C$  for 7 min. The nested PCR products (10- $\mu$ L aliquots) were mixed with loading buffer and separated along with 5  $\mu$ L of molecular size marker by 1.5% agarose gel electrophoresis (100 V, 35 min) with 0.005% ethidium bromide.

#### RNA integrity number (RIN) analysis

The quality of clinical RNA samples (expect for S-4,

for which the amount was insufficient) was evaluated by RIN as previously described<sup>15</sup> on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

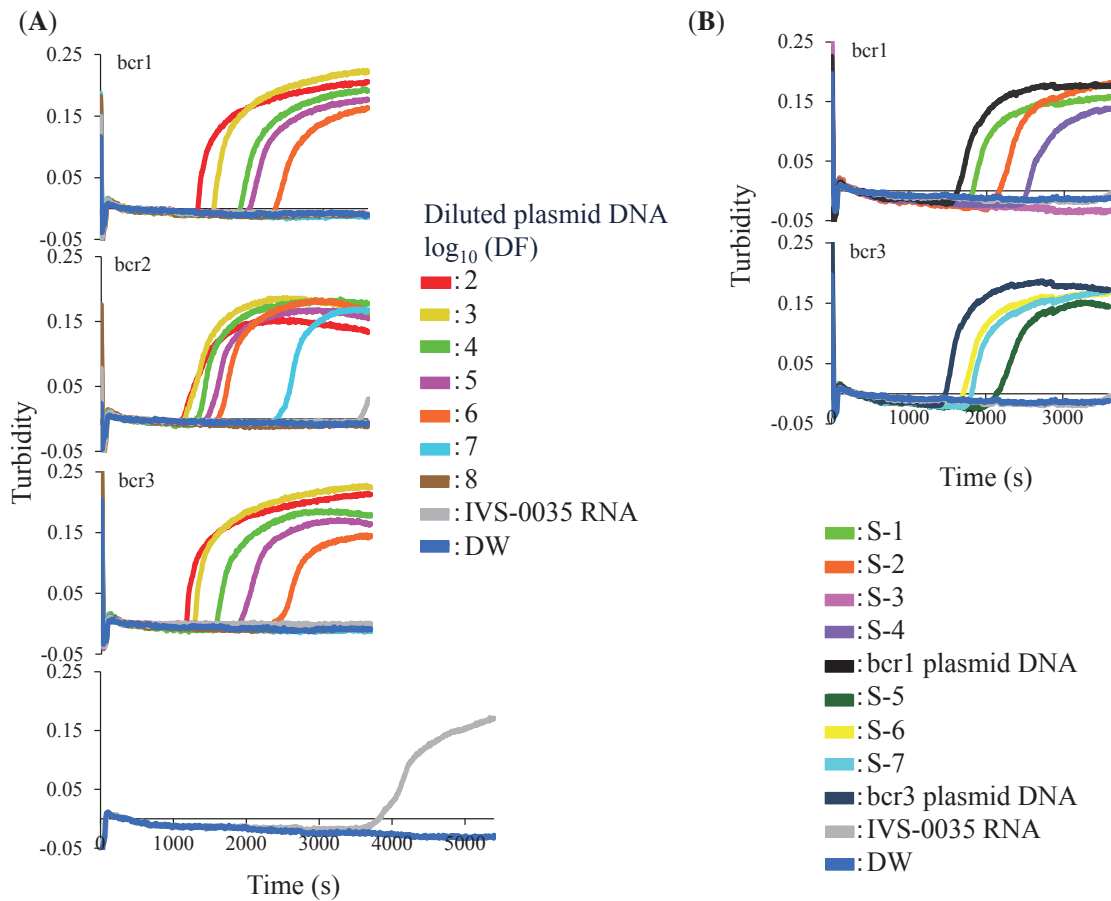
## RESULTS

#### Specificity of amplification by RT-LAMP

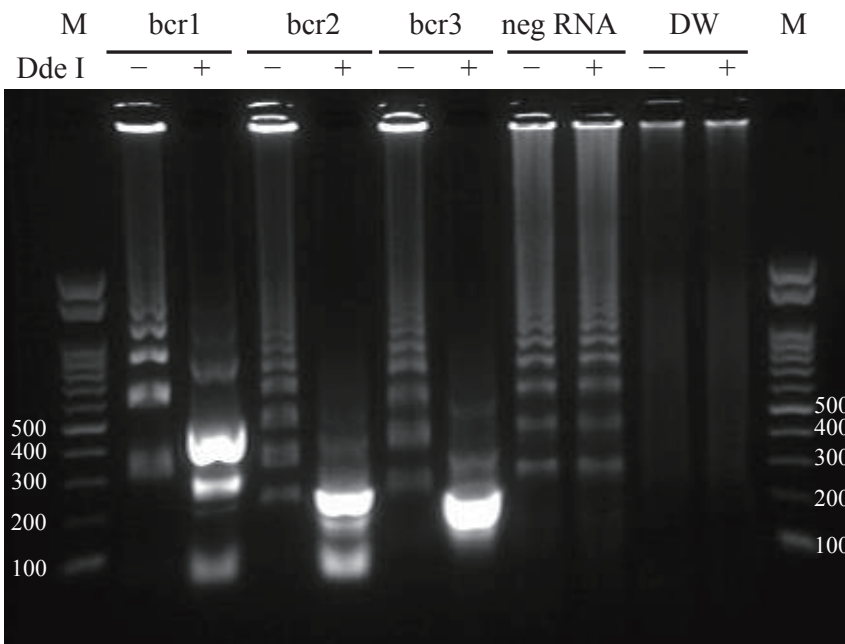
We performed the RT-LAMP assay with two concentrations of plasmid DNA followed by *DdeI* restriction enzyme analysis. All plasmid DNAs were detected by RT-LAMP in 60 min (data not shown, Fig. 2A) and yielded the expected products after digestion with *DdeI* (Fig. 3), which differed for each breakpoint. Non-specific amplification products obtained after 60 min from *PML-RARα*-negative RNA were not digestible by *DdeI* (Fig. 3).

#### Sensitivity of the RT-LAMP assay

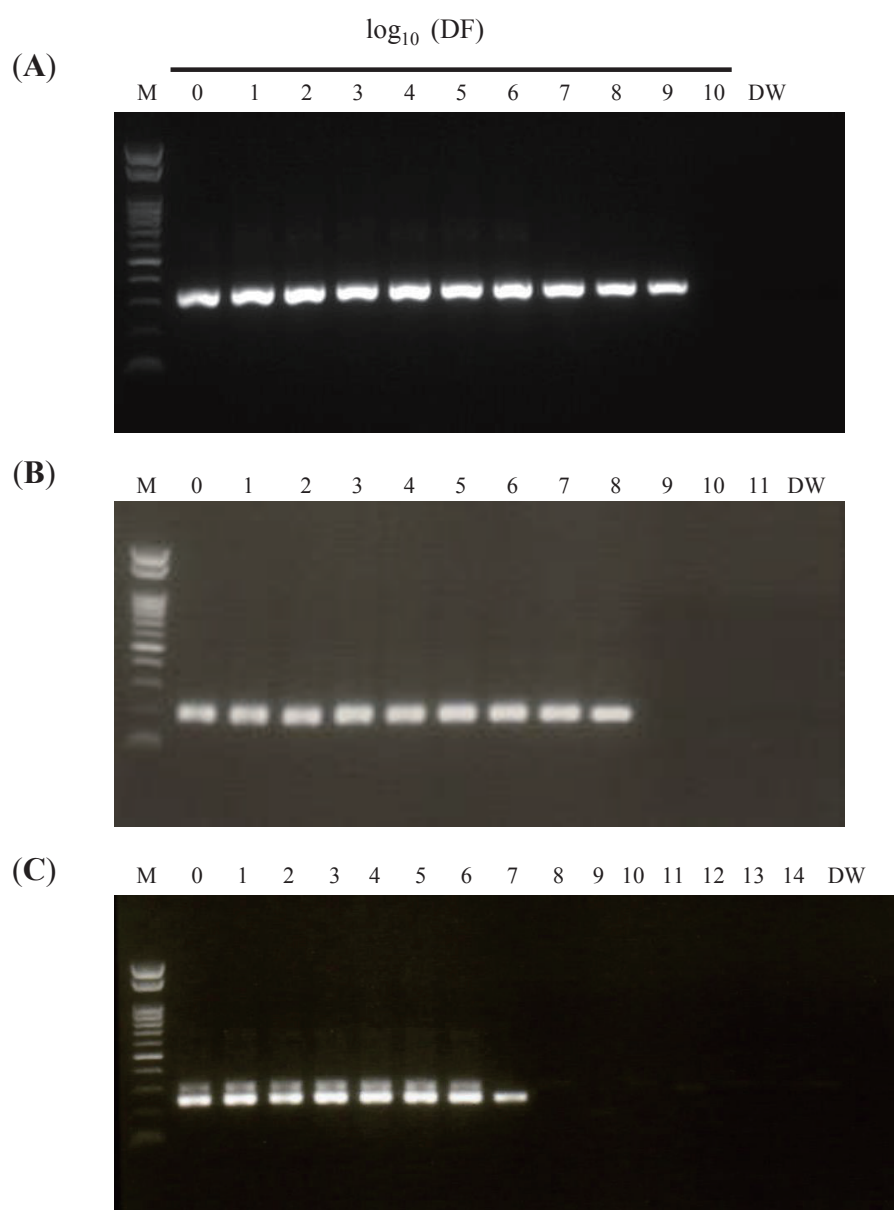
The lower detection limit of the RT-LAMP assay was determined using 10-fold serial dilutions of bcr1, bcr2, and bcr3 plasmid DNA; the result was compared to that obtained by nested RT-PCR. RT-LAMP was able to detect 880, 78, and 800 copies/tube of bcr1, bcr2, and bcr3 plasmid, respectively (Fig. 2A), as compared to 0.88, 7.8, and 80 copies/tube, respectively, by nested RT-PCR (Figs. 4A–C). The specificity of all RT-LAMP products was confirmed by *DdeI* restriction enzyme digestion.



**Fig. 2.** Detection of *PML-RAR $\alpha$*  in plasmid DNA and clinical RNA samples by RT-LAMP. (A) Ten-fold serial dilutions of bcr1, bcr2, and bcr3 plasmid DNAs were used as template; the starting amounts were  $8.8, 7.8,$  and  $8.0 \times 10^8$  copies/tube, respectively. DF of the indicated plasmid DNA is shown as a logarithmic value. (B) Clinical samples from APL patients (S-1 through -7) were analyzed by RT-LAMP. IVS-0035 RNA and distilled water were used as negative controls. A 100-fold dilution of corresponding plasmid DNAs served as a positive control. The amplification reaction was carried out at 68 °C for indicated times. APL, acute promyelocytic leukemia; DF, dilution factor; *PML-RAR $\alpha$* , *promyelocytic leukemia-retinoic acid receptor  $\alpha$* ; RT-LAMP, reverse transcription loop-mediated isothermal amplification.



**Fig. 3.** *DdeI* restriction enzyme digestion of RT-LAMP products. Products of bcr1, bcr2, and bcr3 plasmid DNA amplification were incubated with (+) or without (-) *DdeI* at 37 °C for 120 min, followed by deactivation at 70 °C for 15 min and separation by 1.5% agarose gel electrophoresis (100 V, 35 min). The amounts of bcr1, bcr2 and bcr3 plasmid DNAs were  $8.8, 7.8,$  and  $8.0 \times 10^6$  copies/tube, respectively. The expected sizes of digested RT-LAMP products are the followings: 468 bp, about 234 bp (stem and loop structure) and 83 bp for bcr1; 232 bp, about 116 bp (stem and loop structure) and 83 bp for bcr2; and 139 bp and 121 bp (merged in Figure) for bcr3. DW, distilled water (negative control); M, 100-bp ladder size marker; neg RNA, IVS-0035 RNA (negative control); RT-LAMP, reverse transcription loop-mediated isothermal amplification.

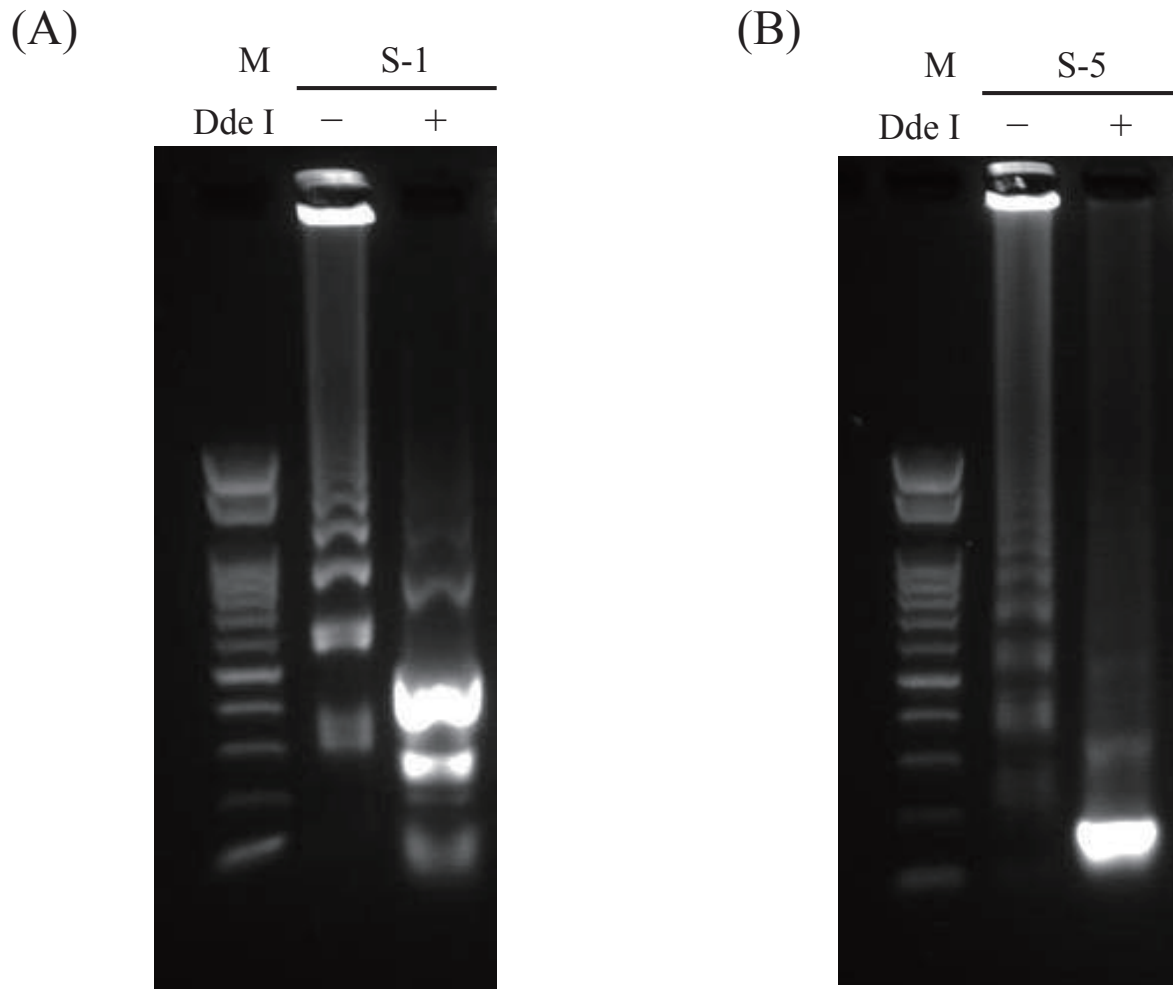


**Fig. 4.** Nested RT-PCR amplification using 10-fold serial dilutions of plasmid DNA. Plasmid DNAs containing (A) *bcr1*, (B) *bcr2*, and (C) *bcr3* sequences (starting amounts:  $8.8 \times 10^8$ ,  $7.8 \times 10^8$ , and  $8.0 \times 10^8$  copies/tube, respectively) were diluted by the indicated DF and amplified by nested RT-PCR. Amplification products were separated by 1.5% agarose gel electrophoresis (100 V, 35 min). DF, dilution factor; DW, distilled water; M, 100-bp ladder size marker; RT-PCR, reverse transcription polymerase chain reaction.

**Table 2. Comparison between RT-LAMP and nested RT-PCR using clinical samples**

Sample number	Breakpoint type	RNA concentration (ng/ $\mu$ L)	Absorbance ratio (260/280 nm)	RIN value	RT-LAMP	Nested RT-PCR
S-1	<i>bcr1</i>	250.8	2.04	9.60	+	+
S-2	<i>bcr1</i>	535.0	2.04	10	+	+
S-3	<i>bcr1</i>	361.9	1.96	NM	-	-
S-4	<i>bcr1</i>	1141.0	2.06	NA	+	+
S-5	<i>bcr3</i>	389.8	2.06	9.50	+	+
S-6	<i>bcr3</i>	309.2	2.05	9.90	+	+
S-7	<i>bcr3</i>	59.9	2.04	8.50	+	+

NA, not available; NM, not measured due to pattern deformation; RIN, RNA integrity number; RT-LAMP, reverse transcription loop-mediated isothermal amplification; RT-PCR, reverse transcription polymerase chain reaction; Samples 5 and 6 were derived from the same patient: S-5, at the initial visit and S-6, at the time of relapse.



**Fig. 5.** Confirmation of *bcr1* and *bcr3* of clinical samples after RT-LAMP assay. The RT-LAMP products of clinical sample RNAs of S-1 (**A**) and S-5 (**B**) were incubated with (+) or without (-) *DdeI* at 37 °C for 120 min, followed by deactivation at 70 °C for 15 min and separation by 1.5% agarose gel electrophoresis (100 V, 35 min). M, 100-bp ladder size marker; RT-LAMP, reverse transcription loop-mediated isothermal amplification.

#### RT-LAMP of clinical samples

Clinical samples were analyzed by RT-LAMP and nested RT-PCR; these included four *bcr1*- and three *bcr3*-type *PML-RAR $\alpha$*  RNAs. The plasmid DNAs were used as positive controls, and IVS-0035 RNA and distilled water were used as negative controls. Three *bcr1*- and three *bcr3*-type RNAs were detected by RT-LAMP (Fig. 2B); the products were confirmed by *DdeI* restriction enzyme digestion (Fig. 5), and the results were the same as those obtained by nested RT-PCR (Table 2). One sample yielded no product with both assays; this RNA had been stored for the longest period (about 9 years) and was likely degraded, as indicated by RIN analysis of RNA quality.<sup>15, 16</sup>

#### DISCUSSION

Each type of *PML-RAR $\alpha$*  sequence was specifically amplified by RT-LAMP; moreover, each *PML-RAR $\alpha$*  breakpoints as well as non-specific amplification could be distinguished by digesting the products with *DdeI*. The sensitivity of the RT-LAMP assay was inferior to that of nested RT-PCR by 10 fold for *bcr2* and *bcr3* and by 1000 fold for *bcr1*. Some RT-LAMP assays have higher sensitivity than nested RT-PCR,<sup>17-20</sup> while others have lower sensitivity.<sup>21, 22</sup> This depends on the primers used, which determine the amplification efficiency and has a significant effect on the sensitivity of gene amplification. In this study, we selected 5 primer sets from more than 1000 candidates listed by PrimerExplorer ver. 4. It is virtually impossible to test all candidates, although a primer set with better amplification efficiency

may exist.

RT-LAMP and nested RT-PCR yielded similar results in the analysis of clinical samples. This suggests that RT-LAMP has clinical utility as a diagnostic test, especially at initial presentation when a sufficient amount of *PML-RAR $\alpha$*  mRNA is available.

The nested RT-PCR assay requires about 6 h from RNA extraction to target mRNA detection. In contrast, *PML-RAR $\alpha$*  mRNA was amplified in 30 min by RT-LAMP, and is therefore more useful for early diagnosis and determination of an appropriate intervention. In addition, the amount of sample that is lost with RT-LAMP is minimal because the extracted RNA is used directly in the amplification reaction even in one tube. This simplifies the procedure, thereby reducing the labor and cost of laboratory testing.

Spinelli et al. recently reported another assay using RT-LAMP to detect *PML-RAR $\alpha$*  chimeric gene.<sup>23</sup> Using fluorescence, the assay appeared to be more rapid and sensitive than our assay. However, the detection of fluorescence requires a dedicated device and their assay needs 2 reaction tubes to detect 3 *PML-RAR $\alpha$*  mRNAs. In addition, primer sequences were not described. Therefore, our assay has advantages of simple procedure and low cost, and could readily be applied in developing countries. Such simplicity can lead to the development of an automated system that can be used for point-of-care testing and diagnosis of APL.

In conclusion, we developed an RT-LAMP assay for detection of *PML-RAR $\alpha$*  mRNA in clinical samples from APL patients. This assay is more simple and rapid than nested RT-PCR, and can contribute to early diagnosis of APL.

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*The authors declare no conflict of interest.*

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