Clinical Usefulness of the PAXgene™ Bone Marrow RNA System for Stabilizing Total RNA

Mayu Takeda,^{1,5*} Tadao Funato,² Masaki Ikemoto,¹ Toru Nanmoku,³ Yoshimasa Urasaki,⁴ and Yoshinori Iwatani⁵

¹Department of Clinical Laboratory Science, Faculty of Health Care, Tenri Health Care University, Tenri, Japan

²Division of Healthcare Economics and Quality Management, Tohoku Fukushi University, Sendai, Japan

³Department of Clinical Laboratory, Tsukuba University Hospital, Tsukuba, Ibaraki, Japan

⁴Transfusion Center, University of Fukui Hospital, Fukui, Japan

⁵Department of Biomedical Informatics, Division of Health Sciences, Graduate School of Medicine, Osaka University, Suita, Japan

The collection of clinical samples, such as bone marrow (BM) and peripheral blood, is an important procedure for the extraction of the cellular RNA. It is essential to preserve the extracted RNA during and after the collection of clinical samples to ensure the accurate analysis of gene expression. To date, the PAXgene™ Blood RNA System has been proven useful for stabilizing RNA extracted from peripheral blood; however, a problem concerning the stability of the total RNA stored using the system has been identified. The PAXgene™ Bone Marrow RNA System (BM system) is a newly developed system, and its clinical usefulness as a stabilizer for the cellular RNA in BM and peripheral blood was inves-

tigated with respect to the quality of RNA extracted using this system. A quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was carried out using total RNA extracted with the BM system, which showed that total RNA was more stable in the BM system than in the conventional system, indicating that the BM system can be applied to RT-PCR. The BM system enabled us to detect Wilms' tumor suppressor gene (WT1) more effectively than the conventional system. In conclusion, the BM system is clinically valuable for extracting and stabilizing total RNA of high quality. *J. Clin. Lab. Anal.* 29:61–67, 2015. © 2014

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INTRODUCTION

In the gene analyses, cellular RNA is commonly used to analyze oncogenes. The first step is to safely retrieve the bone marrow (BM) from a patient with a disease such as leukemia. To diagnose leukemia discriminatively, minimal residual disease (MRD) is detected using RNA extracted from the BM of the patient (1). The clinical conditions of patients undergoing chemotherapy are also monitored by detecting MRD in the peripheral blood as an indicator using real-time quantitative polymerase chain reaction (q-PCR) and reverse transcriptase (RT)-PCR (2, 3). For the correct diagnosis of leukemia and subsequent follow-up of such patients throughout the whole course of the disease, it is essential to extract RNA of high quality from

both the BM and peripheral blood. The immune cells present in the peripheral blood, such as neutrophils and lymphocytes, are commonly used as a clinical sample for the gene transcript analysis. The accurate quantification of mRNA in the whole blood and BM is difficult due to the simultaneous degradation of gene transcripts

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*Correspondence to: Mayu Takeda, Department of Clinical Laboratory Science, Faculty of Health Care, Tenri Health Care University, 80-1 Bessho-cho, Tenri, Nara 632-0018, Japan. E-mail: takeda@tenriyozu-u.ac.jp

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and unintended gene induction caused by sample handling or the uncontrolled activation of coagulation (4, 5). Therefore, RNA of a lower quality may result in serious misunderstandings in subsequent interpretations due to a reduction in the accuracy of the gene transcript analysis. For the follow-up of a patient's condition during and postchemotherapy, it is clinically important to analyze RNA of the highest quality obtainable. To date, the PAXgeneTM Blood RNA System (the Blood system) has been proven to be useful for stabilizing RNA extracted from peripheral blood; however, it is difficult to use the Blood system for RNA extraction from BM due to the fact that BM has a different volume and cell count in comparison to peripheral blood. In clinical practice, BM is the most important sample type used for the diagnosis and follow-up monitoring of leukemia. The problem with BM is the viscosity of the sample. BM samples, instead of peripheral blood samples, were examined after using an RNA stabilization system in this study. During a gene expression analysis, the deterioration of extracted RNA during its storage should be limited as much as possible. The PAXgeneTM Bone Marrow RNA System (the BM system) was developed to guarantee the quality of samples during the collection, stabilization, storage and transport of human BM specimens (6, 7). The capacity and the ingredient ratio of the stabilizer in the BM system are different from those of the Blood system. Standardization of the BM system was performed simultaneously using real-time q-PCR, RT-PCR, and a microarray system. A system originally developed for peripheral blood has been used to extract RNA from the BM of patients with serious diseases; however, this system has not been adequate to obtain RNA from the samples without resulting in their degradation. A new system that enables the effective extraction of RNA has recently been developed. A conventional system, which contains RPMI-1640 culture medium, heparin sodium, and other proprietary constituents, has also been used in Japan.

In the present study, we compared the BM system with the conventional system from the viewpoints of the stability and integrity of RNA, and also investigated the sensitivity of both systems by assaying for the level of Wilms' tumor suppressor gene (WT1), for which the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as an internal control (8). Finally, the clinical usefulness and significance of the BM system was discussed.

MATERIALS AND METHODS

Materials

The human myeloid leukemia cell line, K562, was obtained from the Cancer Cell Repository of the Institute of

Development, Aging and Cancer. RPMI-1640 medium, 10% fetal calf serum, penicillin, and streptomycin were purchased from Gibco Laboratories, Grand Island, NY. The BM system was obtained from Qiagen GmbH, Hilden, Germany. Clinical samples ($n = 9$) of BM were collected from patients suspected to have leukemia, who provided informed consent for the use of their samples.

Methods

RNA extraction

K562 cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 IU of penicillin, and 100 mg/l streptomycin in 5% CO₂ at 37°C. Cells were immediately transferred to the BM system according to the manufacturer's instructions, and gently mixed for a short time at room temperature. The minimum incubation time required to achieve the highest yield of RNA in the BM system was 6 h. Total cellular RNA was extracted from 1 ml of the cell suspension (1×10^7 /ml) or from the BM samples of patients with suspected leukemia using the BM system according to the manufacturer's instructions. Similarly, the sample was transferred to the conventional system (containing RPMI-1640 culture medium, heparin sodium, and other proprietary constituents). The total cellular RNA was extracted using the RNeasy Mini kit (Qiagen). The extracted RNA was dissolved in 20 μ l of nuclease-free water and kept frozen at -80°C until use.

Concentration and purity of RNA samples

The absorbance of each sample containing RNA at 260 and 280 nm was determined using a spectrophotometer. The absorbance at 260 nm was converted to the concentration of total RNA. The contamination of the sample with protein was evaluated by calculating the ratio of absorbance at 260 nm to 280 nm (A_{260}/A_{280}) (9). Two samples were prepared for every method, and the data determined the mean values of duplicate experiments.

Integrity of the RNA samples

The RNA integrity was analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) according to the manufacturer's instructions. Using this instrument, which was equipped with a capillary electrophoretic device, 18S and 28S ribosomal RNA (rRNA) peaks were commonly seen in the electropherograms. Based on the peak area, the ratio of 28s to 18s rRNAs was used as an index of the RNA integrity. The RNA integrity number (RIN) is a new index that can be characterized by the Agilent 2100 Bioanalyzer equipped with a specific software program (10, 11), and

helps estimate the integrity of the total RNA present in clinical samples.

Quantitative real-time RT-PCR analysis

The analysis of the relative mRNA gene expression was performed using the TaqMan PCR system (PE Applied Biosystems, Carlsbad, CA, USA). The PCR reaction mixture was prepared using a TaqMan PCR Master Reagent Kit according to the manufacturer's instructions. For each reverse transcription reaction, cDNA was synthesized from the extracted total RNA using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). The assay conditions of the thermal cycling protocol were as follows: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of amplification (denaturation for 15 s at 95°C and extension for 1 min at 60°C). Finally, the data analysis was carried out using the ABI PRISM 7700 Sequence Detector equipped with the relevant software program.

The sequences of the synthetic oligonucleotide primers and probes used are shown below. For the WT1 gene, the sequences of the double dye-labeled probe (a-830T), forward primer (a-811F), and reverse primer (a-936R) were 5'- FAM-AGGGTTACAGCACGGTCACCTTCGA-TAMRA-3', CAGCCCGCTATTCGCAAT, and CATGGGAT- CCTCATGCTTGA, respectively. For the GAPDH gene, the sequences of the double dye-labeled probe, forward primer, and reverse primer were 5'-FAM-TCCGCGTGTTCAGTGCAACCC-3'-TMR, ATGACGTCTCCCGCTTCCT, and GGACGCGTG-GCGGATATAC, respectively, and TaqMan GAPDH Detection Reagents (PE Applied Biosystems) were used as a control. The analytical precision was indicated by the mean $\pm$ 2 standard deviations (mean $\pm$ 2SD). In all samples, a background signal in the absence of target mRNAs was determined simultaneously, and was subtracted from the signal obtained in the presence of target mRNAs.

Statistical analysis

The statistical analysis was performed using the parametric test for pairwise comparisons with controls. Significant differences between groups were identified using two-tailed Student's *t*-tests (for the differences between two means). Values of *P* < 0.05 were considered to be significant.

Fig. 1. The changes in the concentration of total RNA after extraction. Total RNA was extracted from K562 cells at room temperature and at 4°C using the BM and conventional systems. Co-RT (Δ) and Co-4°C ($\circ$) show the changes in the yield of RNA at room temperature and at 4°C, respectively, over the period of storage in the conventional system. BM-RT ($\blacktriangle$) and BM-4°C ($\bullet$) show the changes in the yield of RNA at room temperature and at 4°C, respectively, during the period of storage in the BM system. The total amount of RNA was indicated in nanogram per microliters. The results are the mean values for duplicate determinations of samples obtained from K562 cells.

RESULTS

Changes in the Concentration and Purity of Total RNA After Extraction

The extraction of intracellular RNA from K562 cells was successfully performed using both the BM and conventional systems, such that a high yield of total RNA was obtained as indicated at the 0 h time point (Fig. 1). The yield of total RNA in the former system was about half that of the latter one. Although the yield of extracted RNA markedly decreased in both systems 24 h after extraction, the yield of RNA in the BM system was higher than that of the conventional system (Fig. 1). From the viewpoint of stability, the level of RNA maintained in both systems was constantly kept at 4°C and room temperature during storage, and the concentration of total RNA in the BM system was almost double that in the conventional system under both the conditions. To confirm the purity of the cellular RNA, the A_{260}/A_{280} was determined at different time points using a spectrophotometer, and no significant changes in these ratios were observed in either system, indicating that the purity of RNA was maintained, without its deterioration during storage (Fig. 2).

Fig. 2. The changes in the purity of the total RNA after extraction. Total RNA was extracted from K562 cells at room temperature and 4°C using the BM and conventional systems. The purity of the RNA in each sample was indicated by the A_{260}/A_{280} ratio. Co-RT (Δ) and Co-4°C ($\circ$) show the changes in the purity of RNA at room temperature and at 4°C, respectively, over the period of storage in the conventional system. BM-RT ($\blacktriangle$) and BM-4°C ($\bullet$) show the changes in the purity of RNA at room temperature and at 4°C, respectively, over the period of storage in the BM system.

Changes in the Integrity of RNA During Storage

The integrity of RNA after extraction was time-dependently confirmed using the Agilent 2100 Bioanalyzer and was indicated by the RIN (Fig. 3). When RNA was kept at 4°C in the BM system, no change in RNA integrity was observed, although it slightly decreased at room temperature. In contrast, the integrity of RNA kept in the conventional system significantly decreased at both 4°C and room temperature during the storage period. These findings indicate that the RNA integrity was conserved for at least 3 days after extraction in the new system.

Electrophoresis was automatically carried out in the Bioanalyzer after the injection of RNA samples to visually verify the RNA integrity. Two prominent peaks of 18S and 28S rRNAs, which were extracted with the conventional and BM systems, were clearly observed at the time point 0 h after their extraction (Fig. 4, panels A2 and A3); however, when the total RNA was kept in the conventional system at room temperature, the two prominent peaks of rRNAs disappeared 72 h after their extraction, and many peaks of RNA fragments with a smaller size than those at the time point 0 h then appeared (Fig. 4, panel B2), thus indicating the degradation of RNA. In contrast, when the BM system was used, there was almost no degradation

Fig. 3. The changes in the integrity of RNA during storage. Total RNA was extracted from K562 cells at room temperature and at 4°C using the BM and conventional systems. Co-RT (Δ) and Co-4°C ($\circ$) show the changes in the RIN at room temperature and at 4°C, respectively, over the period of storage in the conventional system. BM-RT ($\blacktriangle$) and BM-4°C ($\bullet$) show the changes in the RIN at room temperature and at 4°C, respectively, over the period of storage in the BM system. The RIN is a new index of the RNA integrity that was obtained using the Agilent 2100 Bioanalyzer, which is characterized by a special software program for the analysis. To assess the overall RNA quality, the RIN includes an analysis of the 18S and 28S complete rRNA patterns on electrophoresis. The RIN method takes into account the complete microcapillary electrophoretic trace and assigns each sample a value between 1 (degraded) and 10 (high-quality RNA). For successful transcriptional analyses, RIN values exceeding a score of 7 or 8 are recommended.

of rRNAs over the period investigated (Fig. 4, panels A3 and B3). The integrity of most rRNAs was maintained for at least 3 days after isolation, or if there was any degradation of rRNAs, it was visually limited to a small amount of rRNAs at room temperature (Fig. 4, panel B3).

Gene Expression Analysis of Extracted RNA Samples

Amplification of the WT1 and GAPDH genes was performed using quantitative real-time RT-PCR. The normalization of the WT1 gene expression was also performed using clinical samples from nine patients with leukemia, in which the housekeeping gene, GAPDH, was used as a control. When the WT1 and GAPDH genes extracted using the BM and conventional systems were amplified, the WT1 gene was more sensitively, in all cases, detected in the former system ($2.8 \times 10^4 \pm 3.9 \times 10^4$ copy/ μ g RNA) than the latter ($4.0 \times 10^4 \pm 5.7 \times 10^4$ copy/ μ g RNA; $P < 0.05$). In addition, the BM system was more sensitive in each case (Fig. 5).

Fig. 4. Representative electropherograms of the total RNA after its electrophoresis. The electrophoresis of total RNA was automatically carried out in the Agilent Bioanalyzer after the injection of RNA samples extracted with the conventional and BM systems. In panels A1 and B1, Co and BM, which were indicated by horizontal bars, corresponded to the conventional and BM systems, respectively. The total RNA used in lanes 1, 3, 5, and 7 was extracted at room temperature, and that in lanes 2, 4, 6, and 8 was extracted at 4°C. Lane M in panels A1 and B1 show the markers for gene size. Panels A2 and A3 are electropherograms of lanes 1 and 3 in panel A1, respectively. Panels B2 and B3 are electropherograms of lanes 5 and 7 in panel B1, respectively.

DISCUSSION

We herein described the clinical usefulness of the newly developed BM system. An analysis of the gene expression of RNA derived from the blood and/or BM of patients is clinically helpful not only for obtaining an accurate diagnosis of various diseases, but also for the follow-up of

patients with serious diseases, such as malignant tumors and hereditary diseases. It is often difficult to extract the target mRNA from patient samples, because total RNA, including mRNA, easily decomposes after the extraction of cellular RNA from both peripheral blood and human BM. To overcome this difficulty, the development of a new system to extract total RNA, without any significant

Fig. 5. The results of the gene expression analysis of WT1 in the total RNA from patients with leukemia. Amplification of the WT1 genes from the total RNA of patients with leukemia ($n = 9$) was performed using quantitative real-time RT-PCR. The dotted and closed bars indicate values that were obtained using the BM and conventional (Co) systems, respectively. The standard deviation (mean $\pm$ 2SD) is indicated by bars.

degradation, from the BM of patients with diseases with different genetic backgrounds is strongly desired. To achieve this, a new matrix, the BM system, has recently been developed. We found that total RNA could be more safely recovered using the BM system than using the conventional system. To verify the clinical usefulness of the BM system, we extracted the cellular RNA from K562 cells and assayed the quality of RNA based on electropherograms, and found that the integrity of the cellular RNA in the BM system was maintained for at least 3 days at 4°C, but that of the RNA in the conventional system gradually declined, even at 4°C (Fig. 3). When RNA disintegrates, accurate quantification is difficult. Therefore, an evaluation of the quality (the degree of resolution) of the RNA using the RIN is important. In the examination using the cells, the concentration of RNA at 0 h obtained using the conventional system was higher than that of the BM system. This indicates that if the RNA will be used immediately after collection, it is desirable to extract it without adding a stabilizer. In addition, it is thought that a mixture of DNA with the RNA increases the A_{260} , so it is important to know whether a DNase was included in the extraction process. These observations suggest that there are many factors that affect the yield of total RNA, independent of the specific kits used. A further investigation to enhance the recovery of total RNA from clinical samples may be needed in the future.

The WT1 gene is an excellent biomarker that can be used to diagnose leukemia, and it is also clinically useful for monitoring the postdiagnostic conditions of patients with this disease. The WT1 gene in the BM of several patients who received medical treatment with anticancer

agents because of leukemia was assayed using both the BM and conventional systems. Among nine cases in our study, the WT1 gene was positive in five patients (Fig. 5, cases 1, 3, 4, 7 and 9) who received no medical treatment with anticancer agents, indicating that the WT1 gene was still present in the BM of these patients. Interestingly, this gene was almost negative in three patients (Fig. 5, cases 2, 6, and 8), likely because these patients were undergoing medical treatment with anticancer agents. This indicates that the assay for WT1 using the BM system can preserve and help distinguish oncogenes in the BM of patients with leukemia. Based on our findings, more sensitive detection of the WT1 gene may be possible using the BM system than using the conventional system. Such an advantage may lead to a better follow-up of patients receiving medical treatment with anticancer agents (12). Therefore, the BM system is clinically useful to sensitively detect the target mRNA, and to help identify MRD derived from leukemia.

In this context, our analysis may be noteworthy in that, despite the similar RNA extraction systems and technical procedures, the BM and conventional systems differ in the purity, integrity, and recovery of total RNA. This observation suggests the superiority of the BM system over the conventional one, and presents the possibility that we can improve the BM system to provide an even higher grade of mRNA by performing a more efficient extraction and recovery of total RNA, because there are various other factors affecting the stability of the RNA, especially that in the BM. In conclusion, the BM system was more effective than the conventional system for extracting and storing total RNA, without resulting in the deterioration of its purity and integrity, supporting future efforts to develop a new system for RNA extraction to detect oncogenes circulating in the blood and/or in the BM of patients with leukemia.

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ABBREVIATIONS

GAPDH	=	glyceraldehyde-3-phosphate dehydrogenase;
MRD	=	minimal residual disease;
PCR	=	polymerase chain reaction;
RT-PCR	=	reverse transcriptase polymerase chain reaction;
rRNA	=	ribosomal RNA;
WT1	=	Wilms' tumor suppressor gene

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