The impact of RNA stabilization on minimal residual disease assessment in chronic myeloid leukemia

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Background and Objectives. Accurate quantification of *BCR-ABL* mRNA is of critical importance for managing patients with chronic myeloid leukemia (CML) who are receiving imatinib therapy. RNA degradation thus constitutes a potential problem for laboratories quantifying minimal residual disease (MRD). Patients' samples that take a long time to be transported from the hospital to the analyzing laboratory may be subject to RNA degradation with a corresponding loss in sensitivity and possible generation of false negative results. Recently, RNA preservation systems have been developed in order to improve RNA stability. The aim of the present study was to investigate such a system.

Design and Methods. We evaluated the performance of the PAXgene Blood RNA Kit in follow-up CML peripheral blood samples and compared the results to those from unstabilized parallel Trizol extracted samples. The different sample processing methods were evaluated by real-time polymerase chain reaction (PCR) analysis.

Results. RNA isolated with the PAXgene system gave a superior yield per milliliter of blood than did the routine Trizol extraction method. However, although of comparable quality, the RNA did not PCR-amplify as efficiently as equal amounts of RNA from routinely processed samples. Therefore, RNA processed with the PAXgene system showed decreased sensitivity for MRD detection, resulting in false negative results. The sensitivity was comparable to that of samples processed routinely 20-30 hours after phlebotomy.

Interpretation and Conclusions. We conclude that routinely processed, i.e. unstabilized, peripheral blood that reaches the laboratory and is processed within 30 hours is preferable for MRD detection. Optimal results were achieved with fresh samples processed within 5 hours with the Trizol method. However, RNA stabilization may be useful if sample transit is expected to exceed 30 hours.

Key words: PAXgene, MRD, BCR-ABL, real-time PCR, CML.

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Assays based on the detection and quantification of specific RNA are widely used to monitor therapy response in patients with hematologic malignancies.[1] Since the advent of the real-time quantitative polymerase chain reaction (PCR), this has become the method of choice for assessment of minimal residual disease (MRD) by quantification of leukemia-specific fusion genes.[2,3] In particular, in chronic myeloid leukemia (CML), therapeutic decisions are often based on the kinetics of *BCR-ABL* mRNA and thus reliable quantification of *BCR-ABL* mRNA is of critical importance.[4] Specifically, since the introduction of imatinib as frontline therapy, molecular end-points are becoming increasingly important for determining the efficacy of therapy.[5] Numerous reports have been published over the last few years describing the feasibility and clinical application of MRD monitoring in CML with real-time PCR.[6-11] However, different methodologies complicate both the comparison of results between laboratories and the extrapolation of findings. Thus, it has been difficult to assess the impact of MRD monitoring on patient management and to establish universal molecular thresholds that indicate the need for further therapeutic intervention. An important step towards standardization was accomplished in a recently published report by a consortium of 25 different European academic laboratories,[3] who developed and extensively tested standardized assays for quantifying the recurrent fusion genes found in acute and chronic leukemias. Standardization efforts have been mainly limited to the PCR reactions and thus far little work has addressed standardization of pre-PCR

steps.[12] Although the quality of input material is of critical importance, few attempts have been made to establish the optimal methods for treating the samples before the actual RNA extraction. This is particularly important for patients' samples that are taken at relatively large distances from the laboratory performing the analysis, as is frequently the case in multicenter trials. Delayed processing may result in variable degradation of RNA, with consequent decreased sensitivity of MRD detection. RNA stabilization systems, such as the recently introduced PAXgene Blood RNA Kit®, have been designed to prevent RNA degradation during transportation of blood samples to the laboratories performing the analysis. RNA extracted from samples using the PAXgene methodology has been shown to be superior to unstabilized samples which have been stored for 72 hours.[13] However, no data are available on the performance of PAXgene-stabilized samples compared to unstabilized samples processed on the same or following day that the patient gave blood, which represent the majority of samples that are received for MRD monitoring in CML.

The aim of this study was to assess the performance of the PAXgene Blood RNA Kit in follow-up CML peripheral blood samples. We compared RNA obtained by the standard procedure of Trizol® RNA extraction of blood samples collected in EDTA tubes with the bedside RNA stabilization system, the PAXgene Blood RNA Kit®. The RNA yield and quality and the impact on MRD quantification in CML were determined.

Design and Methods

Patients and samples

During the period from May 2002 to May 2003, duplicate peripheral blood samples from 30 patients with chronic myeloid leukemia were systematically collected into conventional 14 mL EDTA Vacutainer® tubes (Becton Dickinson, Heidelberg, Germany) and into 5 mL PAXgene Vacutainer® tubes (Preanalytix, Hornbrechtikon, Switzerland). Twenty-two of the patients had undergone allogeneic bone marrow transplantation and the remaining eight patients were receiving imatinib treatment. The study was approved by the ethical committee at Uppsala University Hospital and all patients gave informed consent.

A total number of 187 peripheral blood samples were analyzed (Figure 1). Seventy-seven samples were collected in PAXgene Vacutainers®, stored for the indicated time and thereafter RNA was isolated using the PAXgene Blood RNA Kit® (Preanalytix, Hornbrechtikon, Switzerland). RNA was extracted

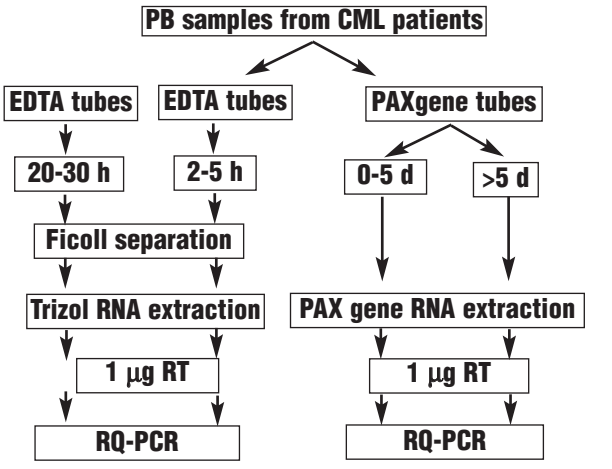

Figure 1. Schematic diagram of the experimental procedure to process peripheral blood follow-up samples from CML patients. PB: peripheral blood, RT: reverse transcription, RQ-PCR: real-time quantitative polymerase chain reaction.

from 35 samples within 5 days (median 2 days, range 0-5 days) after blood sampling, while the remaining 42 samples were stored for a longer period of time (median 11 days, range 6-16) at 4°C before the RNA was extracted. Parallel samples in EDTA tubes were processed 2-5 hours after blood extraction and subsequently frozen as cell lysates in Trizol® (Molecular Research Center Cincinnatti, OH, USA). Where indicated, additional samples in EDTA tubes were sent to the laboratory by mail and processed using Trizol 20-30 hours after sample withdrawal (n=33).

RNA extraction and c-DNA synthesis

Samples collected in EDTA tubes were density separated using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and mononuclear cells were lyzed in Trizol® and stored at -20°C until the RNA was extracted. Samples processed using the PAXgene system were stored at 4°C for the indicated time and thereafter processed according to the recommended protocol. Briefly, following red cell lysis, white cells were pelleted and lysed in buffer containing proteinase K. The RNA was subsequently purified on PAXgene spin columns and eluted in 80 µL of elution buffer. All RNA concentrations were determined spectrophotometrically. Whenever possible, 1 µg of RNA was reverse transcribed according to the guidelines published by Gabert *et al.*[3] In 18 of the samples tested with the PAXgene method, the RNA concentration was too low to include 1 µg of RNA in a 20 µL reverse transcription reaction and thus less RNA was used. Equivalent amounts of RNA were used from the parallel Trizol® extraction. RNA integrity was investigated for ten representative paired samples using the Bioanalyzer as described in the RNA 6000

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Nano Assay (Agilent Technologies, Santa Clara, CA, USA).

Real-time PCR

All PCR amplifications were performed on the ABI 7700 instrument (Applied Biosystems, Foster City, CA, USA). *BCR-ABL*, *ABL* and *GAPDH* were amplified and quantified as described elsewhere.[3,8] The results are expressed as cycle threshold (Ct) values, which represent the number of PCR cycles necessary to detect a signal above the threshold and are directly, inversely proportional to the amount of target present at the beginning of the reaction.[14] Where indicated the results are expressed as *BCR-ABL* copy number relative to 100 *ABL* or 10000 *GAPDH* molecules. Plasmid dilution series were used to calculate the copy number of the respective target as described previously.[3,8]

Statistical analysis

The statistical significance of the results was determined using a t-test for independent samples from Statistica 6 (Statsoft Tulsa, OK, USA).

Results

RNA yield and concentration

The RNA yield and concentration were investigated for the two different sample processing methods, i.e. with and without RNA stabilization. The median yield of RNA per milliliter of blood using the PAXgene method was 3.5 µg (n=34) from samples undergoing extraction within 5 days, and 2.1 µg from samples in which the extraction took place later (n=41) (Table 1). The recovery from the Trizol method was 1.5 µg/mL blood for samples processed within 2-5 hours (n=65) and 0.85 µg/mL blood for samples processed 20-30 hours post-extraction (n=30). Thus, the PAXgene system allows a higher recovery of RNA per milliliter of blood (p<0,001). It should be noted that with the PAXgene system, RNA is extracted from all leukocytes present in the sample, whereas Ficoll separation of mononuclear cells was performed prior to RNA extraction with Trizol.

The RNA concentration was compared within the PAXgene group and within the Trizol group as a function of time from blood collection to RNA extraction. The median RNA concentration for the PAXgene-processed samples stored for 0-5 days (n=34) was 109.5 ng/µL, and for samples stored longer than 5 days (n=41) was 66.4 ng/µL (Table 1). This suggests a lower yield after a longer storage time; however, the difference was not statistically significant. The median RNA concentration for the samples undergoing RNA extraction with the Trizol method processed the same or the following day, was 1515 ng/µL and 962 ng/µL, respectively (p=0.017).

Table 1. Median RNA concentration and yield per milliliter blood using either the PAXgene Blood RNA Kit or the standard EDTA/Trizol method.

RNA yield [µg/mL blood]			
PAXgene		*EDTA/Trizol*	
≤ 5 days	>5 days	2-5 h	20-30 h
3.5	2.1	1.51	0.85

RNA concentration [ng/µL]			
PAXgene		*EDTA/Trizol*	
≤ 5 days	>5 days	≤ 6h	20-30 h
109	66	1515	962

Analysis of RNA quality

The expression of two endogenous reference genes, *ABL* and *GAPDH* was quantified with real-time PCR in order to evaluate the amplification efficiency of the RNA samples processed by the two methods.[15,16] Equal amounts of RNA from the paired samples were reverse-transcribed and equal amounts of cDNA were used as a template in the following PCR reactions.

The median cycle threshold (Ct) value for *ABL* mRNA was 25.6 for RNA from the PAXgene method extracted 0-5 days after phlebotomy (n=28), and 26.9 for samples extracted 6-16 days after phlebotomy (n=22) (Figure 2A). RNA extracted with the routine method, processed within 5 hours (n=50), showed a median Ct of 23.4 while samples mailed to our laboratory and processed within 20-30 hours (n=32) showed a median Ct value of 25.2 (Figure 2A).

Equivalent results were observed for *GAPDH* mRNA (Figure 2B). Quantification of *GAPDH* for 30 PAXgene-processed samples undergoing extraction within 0-5 days showed a median Ct value of 19.1, whereas 38 samples extracted within 6-16 days showed a median Ct of 20.3. The median Ct for the Trizol-processed samples (n=68) was 17.8 (Figure 2B), and for samples processed within 20-30 hours, the median Ct value was 19.1. Thus, for both *ABL* and *GAPDH* mRNA quantification, the Ct was 1-2 cycles lower in the Trizol-processed samples compared to the PAXgene system, indicating more easily amplifiable RNA in the Trizol-processed samples despite equal RNA input. The Ct values of samples processed within 0-5 days with the PAXgene system were statistically significant from those of the RNA extracted within 2-5 hours (p<0.01), and those processed after 6-16 days with the PAXgene system (p< 0.001) (Figure 2A and B).

In addition, ten representative samples from which

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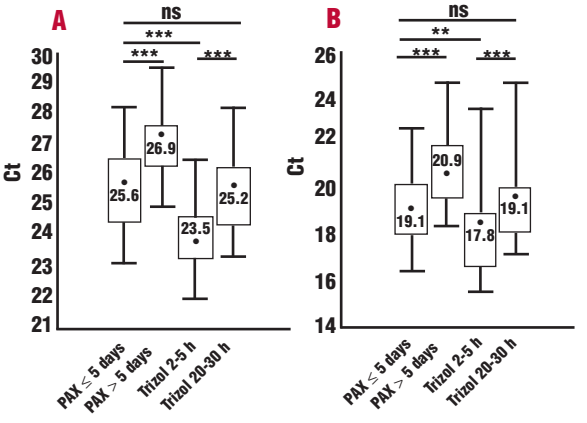

Figure 2. Comparison of the control gene expression for the different sample processing methods. The results are expressed as cycle threshold (Ct) values. A low Ct indicates more target molecules present at the beginning of the PCR reaction. A shows the median and 25th to 75th percentile for *ABL* expression. B shows the median and 25th to 75th percentile for *GAPDH* expression. Statistical analysis was performed with a t-test for independent samples. $^{**}p<0.01$, $^{***}p<0.001$, ns: not significant.

Table 2. Number of follow-up samples in which BCR-ABL mRNA was detectable.

	Trizol positive	*Trizol negative*
PAXgene (≤ 5 days) positive	12	1
PAXgene (≤ 5 days) negative	5	12

	Trizol positive	*Trizol negative*
PAXgene (> 5 days) positive	10	1
PAXgene (> 5 days) negative	12	15

The PAXgene method results were analyzed separately for samples processed within 5 days and 6 to 16 days after blood collection. Samples were considered positive when at least one out of two replicates had a Ct value <45.

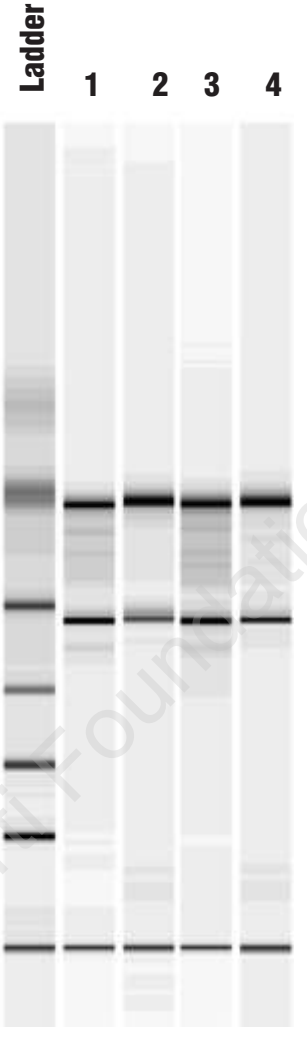

Figure 3. Representative RNA samples fractionated on the Bioanalyzer. Lanes 1 and 3 represent PAXgene-processed samples whereas 2 and 4 represent Trizol-processed samples.

RNA was extracted with both methods were run on the Bioanalyzer to examine the quality of the RNA, showing no difference in RNA quality obtained using the two methods (Figure 3).

Relative quantification of BCR-ABL

In order to determine the impact of the RNA stabilization/preparation method on the quantification of minimal residual disease, *BCR-ABL* fusion mRNA was quantified relative to the two reference genes, *ABL* and *GAPDH*. Of 77 follow-up samples the corresponding paired sample was not available in nine cases. Overall, of the 68 paired samples, 39 Trizol-processed samples (55%) were positive for *BCR-ABL* mRNA, whereas only 24 (35%) of the PAXgene-processed samples were positive. In total, 19 samples showed a discordant result between the two methods (Table 2). Seventeen samples that were positive only with the Trizol method, had a median Ct value of 39.6, corresponding to approximately one copy of *BCR-ABL* mRNA. Similarly, two samples were negative with the Trizol method but positive with the PAXgene method (Ct values 37 and 40). When samples processed using the PAXgene method between 0 and 5 days only were considered, 13 samples scored positive for *BCR-ABL* mRNA compared to 17 positive Trizol-processed samples (Table 2).

The *BCR-ABL* ratios for the paired samples processed with both methods showed a good correlation, irrespective of which reference gene was used; R^2 was 0.94 for the *BCR-ABL/ABL* ratio and 0.80 for the *BCR-ABL/GAPDH* ratio (Figure 4).

Discussion

Samples for MRD assessment are occasionally collected in clinics which are at long distances from the analyzing laboratory, entailing variable transit times

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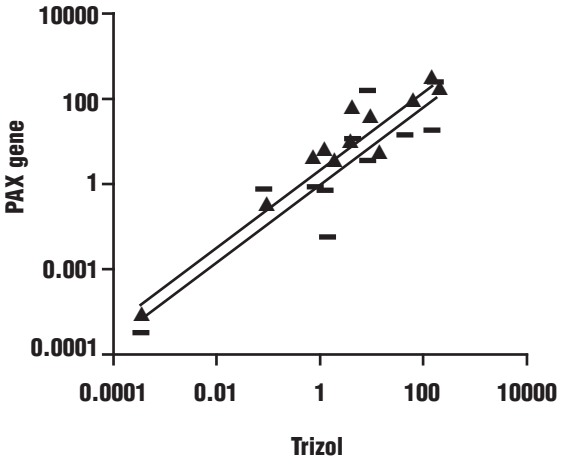

Figure 4. Correlation between the PAXgene and Trizol methods for paired samples. *BCR-ABL* expression relative to *ABL* (▲) and *GAPDH* (–) expression was plotted for the two methods.

prior to processing and partial RNA degradation as a consequence. The potential implications are decreased sensitivity for partially degraded samples and possible inaccurate MRD quantification if the target gene and internal reference gene are differentially regulated during the transport time. RNA stabilization systems have been developed to circumvent these problems, and a previous study demonstrated that samples stabilized with the PAXgene system were better for MRD quantification than were unstabilized samples stored for 72 hours before RNA extraction.[13] In the present report we evaluated the impact of the bedside RNA stabilization method, PAXgene, on the assessment of MRD in CML, and compared performance with fresh, unstabilized samples processed 2-5 hours and also 20-30 hours after sample collection. The yield of RNA per milliliter of blood from the PAXgene-processed samples was higher than that from the Trizol-processed samples. This is most likely explained by the fact that RNA is extracted from all the white blood cells in the PAXgene method, but only from mononuclear cells in the routine Ficoll/Trizol method. It should be noted that when using the PAXgene method, we found that storage of samples at 4°C for more than five days before RNA extraction negatively influences RNA yield and should be avoided.

With the PAXgene system, the large elution volume which results in a lower RNA concentration is a potential disadvantage, since for some of the samples it may not be possible to include RNA from enough cells in the reverse-transcription and subsequent PCR reactions to achieve the desired sensitivity. Sensitivity is particularly important for patients who achieve a complete cytogenetic response, since these represent the majority of patients who are treated with imatinib and who thereafter need to be monitored with real time-quantitative PCR[17] and also for patients who have undergone bone marrow transplantation.[18]

A potential source of inaccuracy during RNA quantification is differential RNA stability or transcription induction of individual RNA once the samples have been collected. Breit *et al.* have shown reproducible deregulated expression of large sets of genes commencing even during short-term storage of bone marrow samples.[19] The changes in expression of individual transcripts could be partially avoided by RNA stabilization agents, such as the PAXgene system.[16] However, we observed a good correlation in the *BCR-ABL* to control gene ratio between stabilized and unstabilized samples, which indicates that the RNA analyzed for MRD detection are not differentially regulated upon sample collection.

It is interesting to note that, despite the fact that granulocytes had largely been removed from the routinely processed samples by Ficoll density centrifugation prior to RNA extraction, the ratios of *BCR-ABL* to control genes showed a good correlation. This suggests that both mononuclear cells and total white cells express comparable levels of *BCR-ABL* mRNA, and that either red cell lysis or Ficoll density sedimentation can be performed prior to RNA extraction, giving comparable results and sensitivity for MRD assessment. It also suggests that there are no relative differences between the two methods in amplification efficiency for any of the transcripts analyzed.

The Ct values for RNA obtained using the PAXgene method were on average 1-2 Ct higher than those for the corresponding Trizol-extracted RNA for both the control genes and *BCR-ABL*, despite equivalent RNA input. This result indicates that the PCR amplification is more efficient with RNA extracted with the Trizol method. The median Ct values of RNA extracted with the PAXgene method were comparable to those of Trizol-extracted RNA from samples that had been mailed to our laboratory and processed within 20-30 hours of phlebotomy. No consistent difference in RNA integrity was seen when representative parallel samples from the two methods were run in the Bioanalyzer, which indicates that the observed differences in Ct values could be due to the presence of PCR inhibitors rather than to partial RNA degradation. The less robust amplification of the PAXgene-processed RNA resulted in slightly decreased sensitivity for MRD assessment. In all cases, the false negative samples did have a low *BCR-ABL* mRNA copy number, which leads to stochastic sampling effects[20] and can explain the false negativity. Indeed, the PAXgene method displayed a higher ratio of false negative samples, probably due to less efficient amplification.

We conclude that optimal results for MRD quan-

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tification are obtained with RNA which has been isolated using Trizol, from fresh, unstabilized blood samples which are processed within 5 hours after blood extraction. RNA stabilized with the PAXgene method and extracted 0-5 days after blood collection performed similarly to unstabilized samples which had been shipped to our laboratory and processed 20-30 hours after blood collection. RNA stabilization did not affect the *BCR-ABL* to control gene ratio, and we therefore consider this method suitable for MRD monitoring in CML patients if the sample transit time to the analyzing laboratory is expected to exceed 30 hours.

IT contributed to the acquisition, analysis and interpretation of data, and drafting the article; UO-S and BS contributed to the conception and design of the study, and revising the manuscript; CO contributed to the acquisition and analysis of data and revising the manuscript; A-MJ and UK contributed to the acquisition of data and revising the manuscript; GB contributed to the conception and design of the study, interpretation of data, and drafting the article.

IT created and is responsible for Tables 1 and 2, and Figures 2-4; GB created and is responsible for Figure 1.

All the authors approved the final version of the manuscript to be published and declare that they have no potential conflict of interest. *This work was supported by the Swedish Cancer Foundation, Swedish Children Cancer Foundation and Lions Cancer Foundation. We are grateful to Sarah Walsh for linguistic revision of this manuscript.*

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