Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. 75 (8) 1053–1061 (2010)
JSCS–4030
UDC 547.963.32+547.455.5+57.088:
612.12:575.113
Original scientific paper

Comparison of phenol-based and alternative RNA isolation methods for gene expression analyses

KSENIJA V. JAKOVLJEVIĆ*, MILENA R. SPASIĆ, EMINA J. MALIŠIĆ, JELENA D. DOBRIČIĆ, ANA M. KRIVOKUĆA and RADMILA N. JANKOVIĆ

Department of Experimental Oncology, Institute of Oncology and Radiology of Serbia, Pasterova 14, 11000 Belgrade

(Received 23 December 2009, revised 5 February 2010)

Abstract: The widespread use of gene expression analyses has been limited by the lack of critical evaluations of the methods used to extract nucleic acids from human tissues. For evaluating gene expression patterns in whole blood or leukocytes, the method of RNA isolation needs to be considered as a critical variable in the design of the experiments. Quantitative real-time PCR (qPCR) is widely used for the quantification of gene expression in today's clinical practice. Blood samples as a preferred RNA source for qPCR should be carefully handled and prepared in order not to inhibit gene expression analyses. The present study was designed to compare the frequently employed guanidine thiocyanate–phenol–chloroform-based method (TRI Reagent®) with two alternative RNA isolation methods (6100 PrepStation and QIAamp®) from whole blood or leukocytes for the purpose of gene expression analysis in chronic myeloid leukemia (CML) patients. Based on the results of this study, for the best combination of yield and RNA extraction purity, taking into account the necessary amount of the clinical sample and performance time, the protocol using phenol-based TRI Reagent® for RNA extraction from leukocytes is suggested as the most suitable protocol for this specific gene expression analysis.

Keywords: RNA isolation; blood; leukocytes; TRI Reagent®; PCR.

INTRODUCTION

The development of molecular medicine, particularly in the last decade, undoubtedly put new molecular diagnostic tests into the focus. Most of these tests employ some kind of gene expression analysis. The widespread use of gene expression analyses has been limited by the lack of critical evaluation of the methods used to extract nucleic acids from human tissues. Quantitative real-time PCR (qPCR) is widely used for the quantification of gene expression in today's

* Corresponding author. E-mail: ksenija.jakovljevic@ncrc.ac.rs
doi: 10.2298/JSC091223084J

clinical practice.[1] Blood is an easy to obtain tissue and reflects the relevant information about the body, which makes it the preferred source of RNA for diagnostic tests. Blood samples for qPCR should be carefully handled and prepared so as not to inhibit gene expression analysis.[2] The absence of widely accepted protocols for blood sampling and further RNA extraction among laboratories is evident. Each laboratory has to establish the optimal procedure for the specific clinical application.

For evaluating gene expression patterns in whole blood or leukocytes, the method of RNA isolation needs to be considered as a critical variable in the design of the experiments.[3] Low quality and quantity of RNA often make all downstream applications impossible to conduct. Inadequate sampling, shipping and handling could easily cause degradation of RNA.[4] It is crucial to decrease the sampling time to a minimum and preferably store samples in RNA later.[5] Clinical samples of limited quantity are specially challenging, since unsuccessful RNA isolation means that the opportunity to analyze that particular sample is irretrievably lost.

Phenol-based methods are most commonly used for RNA isolation. When dealing with small clinical samples, a single extraction reagent (such as phenol--based TRI Reagent®) is crucial in order to obtain sufficient material for subsequent analyses.[6] Due to the high activity of RNAses in tissues, it is necessary to include a strong chaotropic (biologically disruptive) agent into the isolation reagent mixture. Guanidinium salts, together with phenol and chloroform (added to improve the deproteinization efficacy of phenol) denature and precipitate proteins without altering the solubility of RNA.[7]

In our laboratory, monitoring of the minimal residual disease in patients diagnosed with chronic myeloid leukemia (CML) is performed by detection of the bcr-abl fusion-gene (specific for chromosomal translocation t (9:22)) by qPCR.[8] The present study was designed to compare the mentioned guanidine thiocyanate–phenol–chloroform-based method with two alternative methods for RNA isolation from whole blood or leukocytes in order to establish the most adequate one for this specific downstream qPCR. To validate the quality of isolation process, both the yield and the purity of RNA were assessed and also the quality control of cDNA synthesis was performed by PCR amplification of reference genes. As a result of this evaluation, subsequent qPCR analysis was successfully conducted (the results are not included in this manuscript).

EXPERIMENTAL

Patient and control samples

The current study included five control (healthy) subjects and fourteen patients with CML. The patients were under medical treatment for CML and had been under constant observation for detection of minimal residual disease in our laboratory for several months. From each control and patient, 20 mL EDTA blood were drawn by venipuncture and further pro-

cessed within a few hours. According to the RNA isolation protocols from whole blood, specified amounts of blood, just from control samples, were transferred immediately into separate tubes and processed the same day. The rest of the blood was used for leukocyte isolation by centrifugation, according to the established procedure used in our laboratory. The isolated cells were counted and pellets were stored at –70 °C until RNA isolation.

RNA isolation

Three methods for extraction of total cellular RNA from blood and leukocytes were evaluated. The isolations were performed following the manufacturers' instructions with minor modifications.

Whole blood RNA isolation

Total RNA samples were isolated from whole blood only from the controls using TRI Reagent® BD kit (Sigma) (the TRI Reagent®), Applied Biosystems Total RNA Isolation Chemistry kit for Abi Prism™ 6100 Nucleic Acid PrepStation (the 6100 PrepStation) and QIAamp® RNA Blood Mini kit (Qiagen) (the QIAamp®). Briefly, 200 µL of the blood samples were lysed in TRI Reagent® BD supplemented with 5 M acetic acid, and the lysate was separated into aqueous and organic phases *via* chloroform addition and centrifugation. The RNA sample was then precipitated from the aqueous phase by 2-propanol and solubilized with 40 µL RNAase-free water. For the standard RNA isolation protocol on the 6100 PrepStation, 200 µL of the blood samples were lysed and purified using Applied Biosystems Total RNA isolation chemistry. For the QIAamp® method, the protocol for human whole blood was realized without any modification using 1 mL of blood as the starting material. The extracted RNA was eluted in 40 µL RNAase-free water.

Leukocyte RNA isolation

Total RNA samples were isolated from leukocytes from four controls (the fifth had to be discarded) and from all CML patients using the same methods, performing the protocols for whole blood according to the manufacturers' recommendations with minor modifications. The only difference in the phenol-based method was the first step, in which the cells (5×10^6 cells from controls; 10^7 cells from patients) were lysed with TRI Reagent® (Sigma) (TRI Reagent®). Aliquots of 2.1×10^6 and 1.3×10^6 cells from controls and patients, respectively, were taken for the standard RNA isolation protocol on the 6100 PrepStation. Finally, aliquots of 5×10^6 cell pellets from controls and patients were dissolved in 1 mL of normal saline solution and further processed according to the QIAamp® protocol for whole blood. This additional step was performed in order to selectively lyse the remaining erythrocytes in the pellets.

RNA quantization and visualization

The total RNA of each sample was quantified using a spectrophotometer (Eppendorf BioPhotometer) by the ultraviolet light absorbance at 260 nm. The ratio A_{260}/A_{280} was used to assess the purity of the isolated RNA. The RNA concentration was calculated in µg μL^{-1}. To analyze the RNA banding pattern, gel electrophoresis (Pharmacia Biotech) in 2 % agarose gels with ethidium bromide was performed. The RNA samples (6 µL) mixed with xylene cyanol color (3 µL; 0.25 % xylene cyanol in 30 % glycerol in water) and 0.5-µL portion (1.0 µg μL^{-1}) of molecular weight marker 1 kb DNA Ladder (Invitrogen) were run in 0.5xTBE buffer for 50 min at 25 mA. The RNA bands were visualized on a UV transilluminator (Hoefer) and photographed using a Nikon D70s camera.

cDNA Synthesis and PCR

cDNA synthesis (RT-PCR) from total RNA and following PCR reactions with Abl and p53 primers were performed only for the control (n = 4) and patient (n = 14) samples isolated from leukocytes by the TRI Reagent® method.

To perform RT-PCR with random primers, 2 μg of total RNA were used as template for MultiScribe™ Reverse Transcriptase (50 U μL^{-1}) in a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer's instructions. The reaction mixtures (20 μL) were incubated in a Mastercycler gradient (Eppendorf). cDNAs were analyzed by 2 % agarose gel electrophoresis and visualized on a UV transilluminator.

The PCR step was performed in a volume of 25 μL, including 2 μl 100 ng μL^{-1} of cDNA, 12.5 μL of AmpliTaq Gold PCR Master Mix (Applied Biosystems), 1 μL 10 pmol μL^{-1} each of the two Abl primers (Applied Biosystems) and 0.8 μL 10 pmol μL^{-1} each of the two p53 primers (Applied Biosystems). A tube with water instead of cDNA was used as the negative control (NTC – non-template control) in both PCR reactions. The sequence of the forward PCR primer for Abl was TGGAGATAACACTCTAAGCAT, whereas the sequence of the reverse PCR primer for Abl was GATGTAGTTGCTTGGGACCCA. The sequences of the forward and reverse PCR primers for p53 were ACTGGCCTCATCTTGGGCCT and TGTGCAGGGTGGCAAGTGGC, respectively. PCR for Abl was performed in a Mastercycler gradient for 30 s denaturation at 95 °C followed by 35 cycles consisting of 30 s at 94 °C, 1 min at 65 °C, 1 min at 72 °C and a hold at 16 °C. For the p53 PCR, the reaction mixtures were heated at 95 °C for 5 min and then subjected to 35 cycles at 95 °C for 1 min and at 60 °C for 1 min, followed by a hold at 4 °C in the same thermal cycler. A 6-μL portion of PCR products and 3-μL portion of ready-to-use molecular weight marker O'Gene Ruler 100 bp DNA Ladder (Fermentas) were analyzed by 40 min agarose electrophoresis. The PCR product bands were visualized on a UV transilluminator and photographed. A sample was considered positive for Abl when it generated a PCR product of the expected size of 123 bp in the Abl PCR reaction, whereas the generated product of 171 bp in p53 PCR reaction indicated a sample positive for p53.

RESULTS AND DISCUSSION

The RNA concentrations and A_{260}/A_{280} ratios of each control and patient sample obtained using the TRI Reagent®, 6100 PrepStation and QIAamp® method are given in Table I. In all tested samples, the highest concentration was achieved with the TRI Reagent® (between 0.888 and 6.338 μg μL^{-1}), while with QIAamp®, it was much lower (between 0 and 0.197 μg μL^{-1}). The 6100 PrepStation system showed a poor performance (between 0 and only 0.012 μg μL^{-1}). The average RNA concentrations for each method are shown in Fig. 1. There is a clear difference in mean RNA concentrations between the TRI Reagent® method and the other two methods (those obtained using the TRI Reagent® were noticeably higher). The results also show the difference between concentrations of the RNA samples isolated from blood and those from leukocytes (the blood RNA concentrations were lower).

One of possible explanations for the highest yield of RNA obtained with TRI Reagent® could be the amount of the starting material, as well as the different biochemical mechanism of cell lysis. In the cases of QIAamp® and the 6100

PrepStation, we were limited at the beginning by the maximum amount of starting material, recommended by manufacturer.

TABLE I. Concentrations and purity of the RNA samples

Material	Samples	c / μg μL^{-1} (A_{260}/A_{280} ratio)		
		RNA isolation methods		
		TRI Reagent®	6100 PrepStation	QIAamp®
		Blood		
Controls (n = 5)	C1	1.293 (1.43)	0.004 (NA)[a]	0.076 (1.38)
	C2	2.176 (1.75)	0.004 (NA)	0.163 (1.27)
	C3	2.711 (1.53)	0.000 (NA)	0.061 (1.92)
	C4	2.422 (1.55)	0.000 (NA)	0.062 (1.74)
	C5	2.007 (1.52)	0.000 (NA)	0.082 (1.71)
		Leukocytes		
Controls (n = 4)	C1	2.096 (1.50)	0.000 (NA)	0.012 (NA)
	C2	3.211 (1.57)	0.012 (NA)	0.197 (1.38)
	C3	3.611 (1.59)	0.000 (NA)	0.092 (1.54)
	C5	3.397 (1.54)	0.000 (NA)	0.072 (1.59)
Patients (n = 14)	1	4.100 (1.48)	0.008 (0.29)	0.016 (NA)
	2	2.014 (1.57)	0.000 (NA)	0.000 (NA)
	3	4.189 (1.55)	0.000 (NA)	0.000 (NA)
	4	3.333 (1.49)	0.000 (NA)	0.000 (NA)
	5	3.801 (1.47)	0.004 (NA)	0.008 (NA)
	6	6.338 (1.57)	0.000 (NA)	0.050 (1.80)
	7	3.458 (1.47)	0.000 (NA)	0.008 (NA)
	8	3.538 (1.46)	0.000 (NA)	0.036 (NA)
	9	0.888 (1.37)	0.000 (NA)	0.032 (NA)
	10	1.818 (1.43)	0.000 (NA)	0.000 (NA)
	11	3.039 (1.50)	0.000 (NA)	0.000 (NA)
	12	2.263 (1.41)	0.000 (NA)	0.024 (3.03)
	13	4.574 (1.50)	0.000 (NA)	0.016 (NA)
	14	3.517 (1.51)	0.000 (NA)	0.000 (NA)

[a]Not available, A_{280} = 0

The A_{260}/A_{280} ratios (Table I) had values lower than 2.0 (the expected value for a pure RNA sample) for most of the samples (just one sample had an A_{260}/A_{280} ratio 3.0). The QIAamp® method resulted in the highest values, ranging from 1.3 to 3.0, but many of the samples even had no available value (NA) because A_{280} was zero. For the TRI Reagent® method, these values ranged from 1.4 to 1.8 and practically all values for 6100 PrepStation, except one (0.3), were NA. The highest purity of RNA obtained by the QIAamp® method was expected due to high selective binding properties of the silica-based membrane, but the yield of RNA, regardless of its purity, was not sufficient for further cDNA synthesis and expression analysis by qPCR. During the isolation procedure with TRI Reagent®, one ethanol washing of the RNA pellet was omitted in an effort to ma-

ximize the yield, which may be the reason of the lower purity RNA. The 6100 PrepStation employs selective precipitation of RNA and its physical capture on a membrane, giving the possibility of isolating very pure RNA but, except in a few cases, measurable values of the RNA concentration were not obtained.

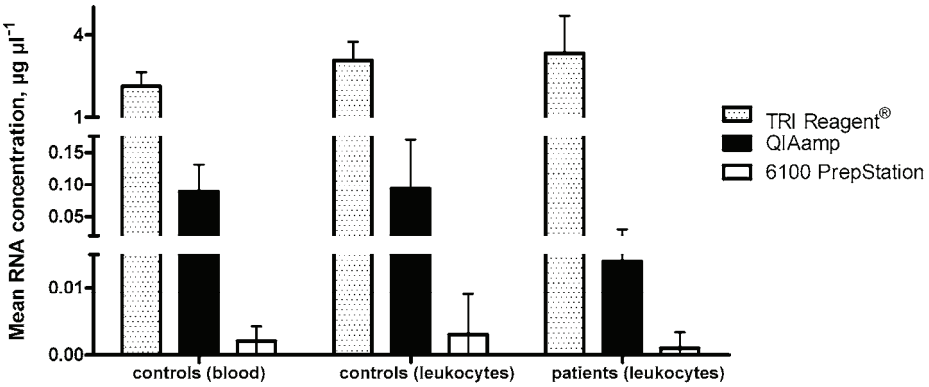

Fig. 1. Obtained RNA concentrations, the resulting bars shown are the average, and the standard deviation, from values obtained for each extraction method.

In order to verify the integrity, all RNA samples for each of the extraction methods were analyzed using 2 % agarose gel electrophoresis. High quality RNA was indicated by visible bands on the agarose gels only for certain methods. Agarose gel electrophoresis of isolated control RNA samples is shown in Fig. 2.

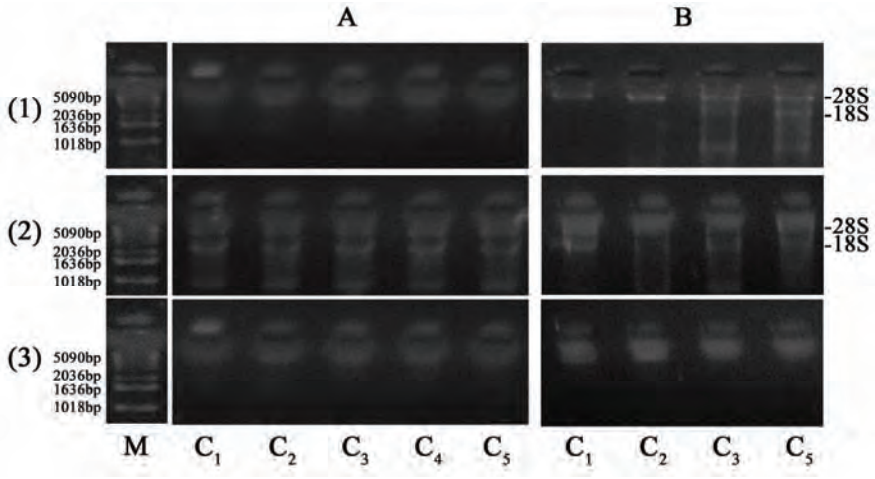

Fig. 2. Agarose gel electrophoresis of isolated control RNA samples. A) Whole blood; B) leukocytes. 1) TRI Reagent®, 2) QIAamp® and 3) 6100 PrepStation. C1–C5: control subjects, M: molecular weight marker.

From whole blood control samples, RNA bands are visible only for the QIAamp® method, while from leukocytes control samples, they are also visible for the QIAamp® and TRI Reagent® methods. Clearly, the RNA obtained from leukocytes showed more defined and visible bands, indicating a low level of degradation. Therefore, it was decided to use leukocytes as the RNA source. The RNA band patterns for patient samples isolated from leukocytes by the TRI Reagent® and QIAamp® methods are shown in Fig. 3 (the agarose gel for the 6100 Prep-Station method is not shown because there were no visible RNA bands). The RNA bands obtained by QIAamp® are more defined than those obtained by TRI Reagent®, due the higher purity of the former, but the problem of the amount remains. Since the next step of the analysis is RT-PCR, which requires in this specific case 2 μg of RNA (in order to obtain the minimal amount of cDNA for qPCR), it is evident that the QIAamp® method would not provide enough RNA from all samples. Although some control and patient samples may have sufficient RNA concentrations when obtained by the QIAamp® method, it is crucial that the method of choice always provides the necessary amounts of RNA. On the other hand, the TRI Reagent® method consistently provided sufficient amounts of RNA for all the analyzed samples and was therefore chosen as the most adequate protocol for this specific purpose.

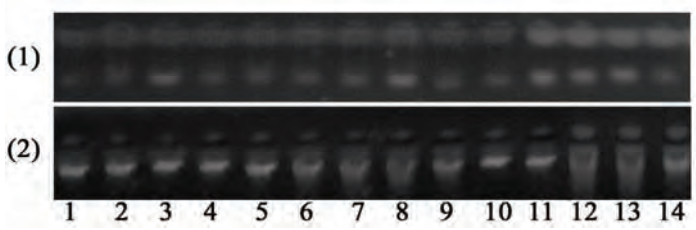

Fig. 3. Agarose gel electrophoresis of patients' RNA samples isolated from leukocytes. 1) TRI Reagent® and 2) QIAamp®. 1–14: Patients.

Following electrophoresis, RNA obtained with TRI Reagent® from leukocytes from each control and patient subject were assayed in RT-PCR, as described in the Experimental (results not shown). The synthesized cDNAs were further amplified with Abl and p53 primers in separate PCR reactions according to the protocol described in the Experimental. Agarose gels with PCR product bands and DNA molecular weight marker are represented in Fig. 4. PCR products of 123 bp in the Abl PCR reaction and of 171 bp in the p53 PCR reaction were obtained in practically all tested samples. The fact that visible bands of PCR products for both genes were obtained indicates that there are no inhibitors in template RNA preparations and that obtained cDNA was of satisfactory quality for further analysis. The subsequent gene expression analysis by qPCR for the detection of the bcr–abl fusion transcript was successfully performed on all patient

cDNA samples (the results are not shown). This diagnostic procedure is a part of everyday clinical routine in our laboratory.

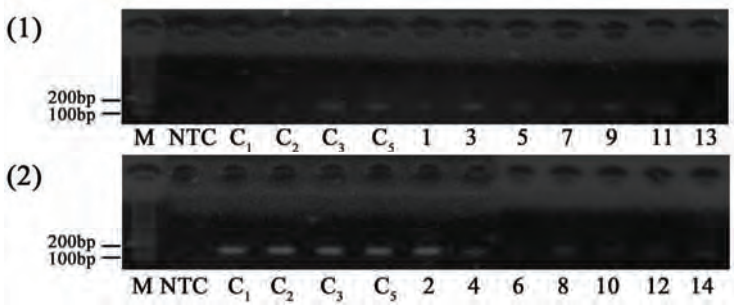

Fig. 4. Agarose gel electrophoresis of PCR products; 1) Abl gene primers and 2) p53 gene primers. M: Molecular weight marker; NTC: non-template control. C1, C2, C3, C5: Control subjects; 1–14: patients.

CONCLUSIONS

In light of the obtained results for the best combination of yield and RNA extraction purity, taking into account the required amount of the clinical sample and the performance time, the protocol using the phenol-based TRI Reagent® for RNA extraction from leukocytes is suggested as the most suitable protocol for this specific gene expression analysis.

Acknowledgements. The authors wish to thank Filip Stojanović and Gordana Kukić for their excellent technical assistance.

ИЗВОД

ПОРЕЂЕЊЕ ФЕНОЛСКЕ СА АЛТЕРНАТИВНИМ МЕТОДАМА ИЗОЛАЦИЈЕ РНК У СВРХУ АНАЛИЗЕ ГЕНСКЕ ЕКСПРЕСИЈЕ

КСЕНИЈА В. ЈАКОВЉЕВИЋ, МИЛЕНА Р. СПАСИЋ, ЕМИНА Ј. МАЛИШИЋ, ЈЕЛЕНА Д. ДОБРИЧИЋ, АНА М. КРИВОКУЋА и РАДМИЛА Н. ЈАНКОВИЋ

Одељење за експерименталну онкологију, Институт за онкологију и радиологију Србије, Пастерова 14, 11000 Београд

Недостатак критичне процене ефикасности метода за изолацију нуклеинских киселина из ткива ограничава шире коришћење анализа генске експресије. Изолација РНК представља критичну променљиву при одређивању генске експресије из узорака крви или леукоцита. Квантитативни „real-time“ PCR (qPCR) се у савременој клиничкој пракси често користи за квантификацију генске експресије. Узорци крви, као најчешћи извор РНК, морају бити пажљиво припремљени да не би дошло до инхибиције анализе генске експресије у току qPCR-а. Ова студија је спроведена у циљу упоређивања често коришћене гванидин-тиоцијанат–фенол–хлороформске методе изолације (TRI Reagent®) са друге две алтернативне методе за изолацију РНК (6100 PrepStation и QIAamp®) из пуне крви или леукоцита у сврху анализе генске експресије код пацијената са хроничном мијелоидном леукемијом. Имајући у виду количину клиничког узорка и време потребно за анализу, фенолска метода (TRI Reagent®) је

дала најбољу комбинацију приноса РНК и њене чистоће, те се стога ова метода предлаже за изолацију РНК из леукоцита у сврху ове специфичне клиничке анализе.

(Примљено 23. децембра 2009, ревидирано 5. фебруара 2010)

REFERENCES

1. S. A. Bustin, V. Benes, T. Nolan, M. W. Pfaffl, *J. Mol. Endocrinol.* **34** (2005) 597
2. L. Rainen, U. Oelmueller, S. Jurgensen, R. Wyrich, C. Ballas, J. Schram, C. Herdman, D. Bankaitis-Davis, N. Nicholls, D. Trollinger, V. Tryon, *Clin. Chem.* **48** (2002) 1883
3. R. J. Feezor, H. V. Baker, M. Mindrinos, D. Hayden, C. L. Tannahill, B. H. Brownstein, A. Fay, S. MacMillan, J. Laramie, W. Xiao, L. L. Moldawer, J. Perren Cobb, K. Laudanski, C. L. Miller-Graziano, R. V. Maier, D. Schoenfeld, R. W. Davis, R. G. Tompkins, *Physiol. Genomics* **19** (2004) 247
4. C. A. Perez-Novo, C. Claeys, F. Speleman, P. V. Cauwenberge, C. Bachert, J. Vandesompele, *Biotechniques* **39** (2005) 52
5. S. Fleige, M. Pfaffl, *Mol. Aspects Med.* **27** (2006) 126
6. A. Hummon, S. Lim, M. Difilippantonio, T. Ried, *BioTechniques* **42** (2007) 467
7. S. Mitra, *Sample Preparation Techniques in Analytical Chemistry*, Wiley, Hoboken, NJ, 2003, p. 306
8. T. Hughes, M. Deininger, A. Hochhaus, S. Branford, J. Radich, J. Kaeda, M. Baccarani, J. Cortes, N. C. P. Cross, B. J. Druker, J. Gabert, D. Grimwade, R. Hehlmann, S. Kamel-Reid, J. H. Lipton, J. Longtine, G. Martinelli, G. Saglio, S. Soverini, W. Stock, J. M. Goldman, *Blood* **108** (2006) 28.