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Comparison of a Selection of Rapid Automated DNA and RNA Extraction Technologies for Detection of Somatic or Constitutional Gene Abnormalities in Cancer Diagnosis

FRANÇOIS LASSAILLY,[1,2,5] MARIE-JOËLLE MOZZICONACCI,[3,5] TETSURO NOGUCHI,[4,5]
DIDIER BECHLIAN,[1,5] JOSÉ ADELAIDE,[3,5] FRÉDÉRIC FINA,[2,6]
PIERRE-MARIE MARTIN,[2,6,7] HAGAY SOBOL,[4,5,7] LUC XERRI,[3,5,7] DANIEL BIRNBAUM,[3,5]
and CHRISTIAN CHABANNON[1,2,5]

ABSTRACT

Molecular analyses of large numbers of patient samples are increasingly used for diagnostic applications as well as for understanding cancer biology. Their accuracy depends on the quality and quantity of nucleic acids extracted from human cells or tissues. To optimize these preanalytical steps, we evaluated several automated technologies for nucleic acid purification from clinical samples. Three automated platforms were compared. DNA was extracted from peripheral blood leukocytes from five normal individuals, and its quality was assessed by D-HPLC and sequencing after PCR. Clinical samples from acute leukemia patients were used for automated RNA extractions; results were compared to our standard manual technique. RNA qualification was done using capillary gel electrophoresis and analysis of the Abelson gene transcript by real-time PCR. One robot produced higher total output for both DNA and RNA. While the quality of DNAs obtained from the three workstations allowed implementation of their analysis for detection of germinal mutations, important differences were observed in the quality of RNAs. One robot isolated RNA with similar quality and quantity to the manual technique, but the resulting products displayed low concentrations. A robust technique had therefore to be evaluated and validated to allow the implementation of this workstation within the daily diagnostic practices. This study is of interest at a time when hospital-based laboratories are bringing molecular signatures to the clinic.

INTRODUCTION

PREANALYTICAL PROCESSING of biological samples is an important step to ensure subsequent reliable analysis for both diagnostic and research applications. This includes collection of patient samples, isolation of subcellular species, and secured identification, storage, and distribution. Biobanking today emerges as a new and complex activity, with a strong in-

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[1]Biothèque, Tumorothèque, Centre de Ressources Biologiques en Oncologie, Institut Paoli-Calmettes, Marseille, France.
[2]Réseau des Centres de Ressources Biologiques et Tumorothèques de l'agglomération Marseillaise, France.
[3]Département de Biopathologie, Institut Paoli-Calmettes, Marseille, France.
[4]Département d'Oncogénétique Moléculaire, Institut Paoli-Calmettes, Marseille, France.
[5]Centre de Recherche en Cancérologie de Marseille, UMR599, Marseille, France.
[6]Laboratoire de Transfert d'Oncologie Biologique et Tumorothèque de l'Assistance Publique—Hôpitaux de Marseille, Faculté de Médecine de Marseille, Marseille, France.
[7]Université de la Méditerranée, Marseille, France.

centive to define its own quality standards and needs.[1–3] In the field of oncology, several initiatives are being developed at regional, national, and international levels to achieve the homogenization of practices and the constitution of large-scale catalogs.[3–8]

Identification of numerous molecular abnormalities associated with cancers resulted in their introduction as predictive, diagnostic, or prognostic markers in the clinical practice of oncology, and the increasing use of high throughput molecular biology techniques in hospital laboratories. National and international initiatives aimed at normalization and standardization of analytical steps, including analysis of DNA or RNA by real-time PCR,[9–14] D-HPLC,[15–17] and sequencing.[18] In contrast, standardization of nucleic acid extraction procedures—preanalytical steps—have received little attention, except for paraffin embedded tissues.[19–33]

We compared the performance of several commercially available automates to extract DNA and RNA from various cell populations. Only silica-based technologies were included in these evaluations, because they minimize storage and handling of chemical compounds. Silica can be immobilized as a filter within columns, or can be coated on magnetic microparticles.

Two types of devices are available for nucleic acid extractions using silica technologies: "closed systems" and "open platforms." The first ones are characterized by software and consumables that cannot be modified by the operator. Their use is simple, because of preestablished protocols. Based on magnetic particle technologies, they offer a small/median throughput (1–6 to 1–48 samples). Conversely, open platforms are liquid handling customizable systems, suitable to automate a large variety of processes, including nucleic acid isolations. Potentially any type of purification kit for DNA and/or RNA can be adapted on such systems. Consequently, the training time for personnel is longer. Throughput is higher than for closed systems (96- or 384-well plates).

We initially identified four devices that could be used for two diagnostic applications that are routinely done at our institution: one "closed/ dedicated system" (Biorobot EZ-1; Qiagen, Chatsworth, CA), two open platforms (Biorobot 8000; Qiagen and Freedom-Evo75, Tecan, Lyon, France) equipped with silica columns kits and a "semiopened system" (KingFisher mL-Thermo/Biosprint15; Qiagen) running either dedicated or customized kits. This latter device was rapidly abandoned, because it requires too much input from the operator. The three other devices were tested in validated and standardized conditions in compliance with the French Haute Autorité de Santé (HAS, formerly ANAES, public agency that defines good practices for healthcare in France[34]) reference frame for genetic testing and the recommendations of the Europe Against Cancer (EAC)[11,13] and the European Leukemia Network (LeukemiaNet) programs.[35] Finally, since automated systems produced low concentration RNAs, a device allowing for their subsequent reconcentration was evaluated and validated for routine diagnosis processes.

MATERIALS AND METHODS

Study design

Simultaneous nucleic acid isolation from cell samples using several techniques in parallel turned out to be impossible due to equipment availability. Thus, two small collections of leukemic cell samples and apheresis samples from normal volunteers were prepared. The same samples were used for DNA or RNA extraction with different techniques on different days.

Clinical samples

Apheresis products from five immobilized normal adult volunteers were obtained from the Establishment Français du Sang, after informed consent. Total peripheral blood leukocytes were isolated and stored for subsequent DNA extractions, following the laboratory standard operating procedures used for detection of hereditary mutations associated with cancer predisposition. Total leukocytes were recovered after erythrocyte lysis. Briefly, fresh samples were diluted in hypotonic solution (10 mM NaCl; 5 mM $MgCl_2$; 10 mM Tris pH 7.4 in

distilled water) and centrifuged. This treatment was repeated until total elimination of erythrocytes. Dry pellets were stored in a −80°C mechanical freezer under permanent remote monitoring and temperature recording.

Peripheral blood and bone marrow mononuclear cells obtained from acute leukemia patients at diagnosis were identified from the collections stored at the Biological Resource Center at our institution, following patient information and consent. After Ficoll gradient separation and washing with phosphate-buffered saline (PBS), mononuclear cells were cryopreserved in 5% human albumin and 10% DMSO. Twenty million cell containing cryovials were stored in liquid nitrogen tanks under permanent remote monitoring and temperature recording. Eleven samples were selected from our database on the following criteria: (1) the subtype of acute leukemia, (2) the duration of cryopreservation, and (3) cell origin. Sample characteristics are summarized in Table 1.

Platforms and kits used for nucleic acid extractions

The King Fisher® mL (Thermo™ France, La Garenne-Colombes, France)/Biosprint 15 (Qiagen, Courtaboeuf, France) device was used with Biosprint 15 DNA Blood kit (Qiagen) and

TABLE 1. LEUKEMIC SAMPLES

Patient no.	FAB	Sample type	Years in liquid nitrogen
1	AML-M5	BM	2.1
2	AML-M5	BM	6.6
3	AML-M5	BM	1.6
4	AML-M5	PB	1.6
5	BAL[a]	BM	9.2
6	AML-M5	BM	1.8
7	AML-M1	PB	1.2
8	AML-M5	PB	5.7
9	AML-M2	PB	2.3
10	AML-M5	BM	6.5
11	B-ALL	PB	6.2

To standardize the comparative analysis of the different extraction procedures, the same 11 samples were used for the different tests. Samples were selected from 11 patients, including different subtypes of acute leukemia (French American British classification), different types of biological material (bone marrow [BM] or peripheral blood [PB]), and different duration of conversation (between 1 and 9 years).

[a]Biphenotypic acute leukemia.

mag® Mini RNA Isolation (Agowa, Berlin, Germany) kits for DNA and RNA isolation, respectively. The Biorobot® EZ-1 DNA Tissue kit (Qiagen) and Biorobot® EZ-1 RNA Tissue Mini Kit (Qiagen) were used for extractions of DNA and RNA, respectively, with the Biorobot® EZ-1 workstation (EZ-1) (Qiagen). NucleoSpin® 8 Tissue (Macherey-Nagel, Hoerdt, France), and NucleoSpin® 8 RNA (Macherey-Nagel) kits were adapted to the Freedom-evo® 75 (Tecan, Lyon, France) for DNA and RNA isolation, respectively (condition subsequently abbreviated as Freedom-MN). QIAamp® 96 DNA (Qiagen) and Rneasy 96 (Qiagen®) kits were used with the Biorobot® 8000 (BRB8000) workstation for extraction of DNA and RNA, respectively.

DNA extractions and analyses

Frozen cell pellets were exposed to cell lysis and proteinase K digestion immediately after recovery from the −80°C freezer. After purification, genomic DNA concentration was determined by conventional absorbance measurement at 260 nm. Protein contamination was measured by absorbance at 280 nm to determine the standard 260/280 nm ratio.

DNA quality was further assessed in the same conditions as routinely performed for determination of familial cancer predisposition, in accordance with the French HAS reference frame for genetic testing.[34]

PCR amplification of BRCA1, exons 6 and 8 were performed using 25 ng of genomic DNA, HotStarTaq DNA Polymerase (Qiagen) and the following primers sets: CTTATTTTAGTGTC-CTTAAAAG (exon 6, fw); TTTCATGGACAG-CACTTGAGT (exon 6, rev); TGTTAGCTGA-CTGATGATGATGCT (exon 8, fw); ATCCA-GCAATTATTATTAAATAC (exon 8, rev). PCR amplification was performed as follow: 95°C for 10 min, followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec, and 77°C for 30 sec.

D-HPLC analysis of BRCA1 exons 6 and exons 8: PCR products were heteroduplexed and analyzed using a Transgenomic WAVE DHPLC instrument (Transgenomic, Inc., Carpentaria, CA) with product-specific melting temperatures and gradient conditions for each exon.

Sequencing of BRCA1 exons 6 and exons 8 were done after PCR product purification us-

ing MinElut™ 96UF (Qiagen). Amplicons were sequenced with BigDye® Terminator Cycle Sequencing kits v.1.1 (ABI, Courtaboeuf, France). Sequencing reactions were then purified using G50® (GE-Healthcare Bio-Science, Piscataway, NJ) and loaded on a 3100 Genetic Analyzer® (ABI). Sequencing data were analyzed using Sequencing Analysis Software v.5.1 and SeqScape Software v.2.5.

RNA extractions and analyses

Preparation of cell samples and RNA extractions. For each tested technology, a vial containing 20×10^6 cryopreserved cells was used. After thawing at 37°C, cells were washed in RPMI supplemented with 10% fetal calf serum. Cell concentration and viability were determined using the Trypan blue exclusion method. Cells were then maintained at +4°C before chaotropic lysis using commercial solutions provided with the different kits. RNA extraction and purification were done following manufacturers' instructions.

Manual RNA extractions. Total RNA was isolated using guanidinium isothiocyanate solution and centrifugation on cesium chloride cushion (CsCl), as previously described.[36] RNA was dissolved in 10 μL RNAse free water.

Total RNA concentration was determined as previously described for DNA. Yields of RNA were defined as the ratio of total RNA output to the number of viable cells.

Concentration of RNA before analysis. RNAs obtained from automated procedures were concentrated by precipitation before analyses. Purified RNAs obtained in a volume of 100 μL were precipitated by addition of 15 μL NaCl 5 M, 2 μL glycogen 20 mg/mL (Boehringer Mannhein, Indianapolis, IN), and 300 μL 100% ethanol. After mixing, tubes were incubated overnight at -20°C and then centrifuged for 30 min at 13,000 rpm at +4°C. After washing with 300 μL 70% ethanol, RNA pellets were dried at room temperature and finally recovered in 10 μL RNase free water. RNA concentration was determined by 260 nm absorbance measurement. A heating vacuum device that allows the concentration of purified RNA was also evaluated (see below).

RNA integrity was monitored by automated capillary gel electrophoresis using the 2100 Bioanalyzer® workstation with the RNA 6000 Nano LabChip Kit® (Agilent Technologies, Palo Alto, CA) following the manufacturer's instructions. At that time, our system was not equipped with the software allowing for the production of the RNA Integrity Number (RIN) used to score the quality.[37–39]

RNA quality was assessed by real-time quantitative RT-PCR (RQ-PCR), following protocols routinely used at our institution with the TaqMan 7700® technology (Applied Biosystems, Bedord, MA) and in accordance with the EAC program recommendations.[11,13] For each sample, the Abelson transcript (*ABL*)—an internal control—was amplified as recommended by LeukemiaNet.[35] PCR amplification was realized in duplicate for each sample to calculate the median cycle threshold, or Ctm value (median number of amplification cycles necessary to reach detection threshold established by EAC standards).

RNA and DNA simultaneous coextractions

The possibility to simultaneously extract DNA and RNA from the same cell lysate was assessed with the Biorobot® EZ-1 and the Biorobot® EZ-1 RNA Universal Tissue Kit. A modification of the protocol used for the Nucleospin® 8 RNA kits Macherey-Nagel was also evaluated using the NucleoSpin® RNA/DNA Buffer Set with the Freedom evo (Tecan) workstation; this condition is referred to as Freedom-MN "+" in the text and figures, to signal modifications of the standard procedure.

Semiautomated vacuum concentration of RNAs

The Concentrator 5301 (Eppendorf, Waterbury, CT) was assessed for its ability to improve RNA concentration without deteriorating the quality for subsequent RQ-PCR analyses. Large quantities of highly concentrated RNAs were purified with the RNA Midi kit (Qiagen) from three normal leukocyte samples. For each sample, four daughter solutions each of 350 μL, were obtained by diluting the initial RNA sample with the appropriate amount of RNase free water to reach concentrations of 10, 35, 70, and 100 ng/μL, respectively. Actual concentrations were measured

by nano-spectrophotometry (Nanodrop, Nyxor Biotech, Paris, France), using 1 μL of each sample. Each new solution was distributed in three tubes of 100 μL each, which corresponds to a total quantity of 1, 3.5, 7, and 10 μg RNA per tube, respectively. The resulting 36 samples were placed in the rotor of the Concentrator 5301. One tube containing 100 μL of RNase free water was intercalated between two consecutive triplicates (total of 12 tubes containing only RNase free water). These H_2O tubes were further treated in the same conditions as test tubes (see below). Total evaporation of the liquid phase in all tubes (48 tubes) was obtained after heating the chamber to 45°C under vacuum conditions for 1 h 20 min. Tubes were then placed in wet ice and 12 μL of RNase free water were added in each tube, including those initially containing only RNase free water. RNA concentration was determined for each sample by nano-spectrophotometry measurement. Yield was defined as the ratio between RNA mass recovered after evaporation to the real initial RNA mass contained in each tube before evaporation, expressed as a percentage.

RNA quality was assessed before and after evaporation: the three tubes of a given triplicate were pooled to analyze RNA integrity by automated capillary gel electrophoresis and ABL transcript detection by RQ-PCR.

RQ-PCR analyses for crosscontamination assessment. The 12 tubes initially containing only 100 μL of RNase free water were carefully analyzed by standard RQ-PCR for the presence of RNA (*ABL* transcripts) reflecting an intra-run cross-contamination. For this purpose, 3 μL from each tube (out of the final 12 μL H_2O—see above) were used as samples.

Because the Concentrator 5301 had been used previously to concentrate human DNA samples, the presence of DNA traces within the 12 H_2O tubes would reflect an inter-run cross-contamination. To analyze this possibility, 3 μL from each tube (out of the final 12 μL H_2O— see above) were used as samples for genomic *ABL* detection by RQ-PCR.

Statistical analyses

Results obtained with the different techniques were compared using the paired Student *t*-test. Descriptive statistics and comparisons were computed with the Microsoft® Excel 97 SR-1 for PC computers.

RESULTS

DNA extractions from dry pellets of blood leukocytes and characterization of DNA quality

We first determined the maximum cell capacity for each platform. Considering both total output (in μg of DNA) and extraction yields (in μg of DNA per million of lysed cells), the maximal cell number for DNA extractions was 5×10^6 cells per silica column (for both BRB 8000 and Tecan-MN); exceeding this value lead to frequent collapsing of the matrix. For magnetic particle-based technologies, 5×10^6 and 10×10^6 cells could efficiently be processed with the KingFisher mL/Biosprint 15 and Biorobot EZ-1, respectively.

A greater number of cells usually caused difficulties in pipetting, or induced a dramatic decrease in the yield of purification. For subsequent experiments these maximum numbers of cells were used. Because handling conditions with the KingFisher mL/Biosprint 15 was time consuming, we did not further evaluate this technology for DNA extraction. Total peripheral blood leukocytes obtained from five normal volunteers were used to compare the three other devices. The Biorobot EZ-1 was the most efficient system, systematically providing better results than the two other automates. The BRB 8000 platform reproducibly exhibited lower results (Fig. 1A). The median DNA output obtained with BRB 8000, Tecan-MN and EZ-1 robots were 3.4 ± 0.9 μg, 8.8 ± 2.2 μg and 16.3 ± 2.4 μg, respectively (median yield of 0.68 ± 0.15, 1.76 ± 0.38, and 1.63 ± 0.21 μg DNA per 10^6 cells, respectively). The three systems produced very low protein contaminations (Fig. 1B). The high 260/280 ratio (>2) obtained with the BRB8000 is likely to be due to very low level protein contamination, but we could not completely exclude at this stage a minor DNA degradation.

To further characterize the quality of recovered DNAs, they were analyzed in standard conditions routinely used for genetic testing and predictive oncology. After targeted PCR
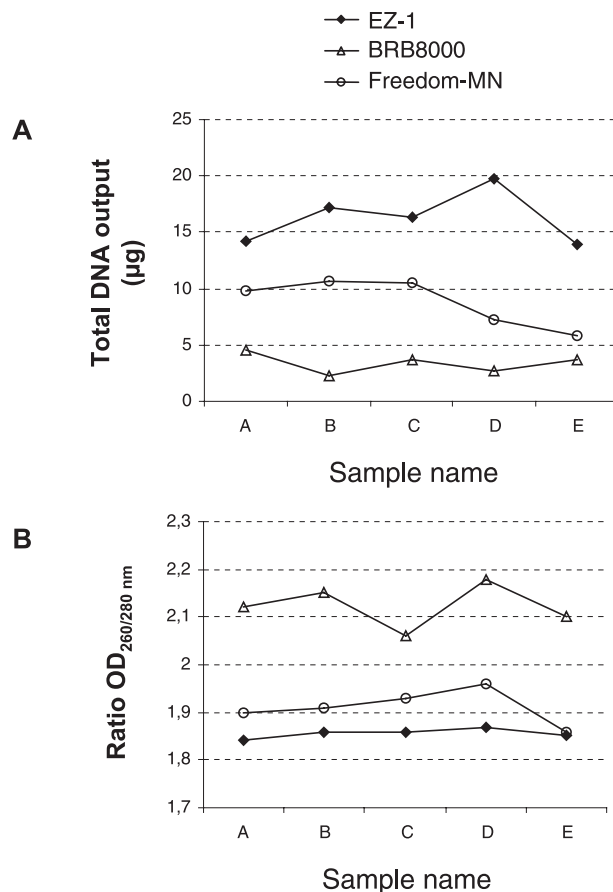
FIG. 1. DNA extraction results. DNA extractions were realized using frozen leukocyte samples obtained from five normal volunteers (**A–E**), following the manufacturer's indications. Cells (5×10^6 and 10×10^6) per sample were used for BRB8000 and Freedom-MN, and EZ-1, respectively. Absorbance measurements at 260 and 280 nm were used to determine DNA concentration in order to calculate the total DNA output (**A**) and to quantify the presence of residual protein contaminants, calculating the 260/280nm ratio (**B**).

amplification, the presence of heterozygous mutations is detected by denaturing HPLC (D-HPLC), and further characterized by sequencing. We analyzed exons 6 and 8 of the *BRCA1* gene, which is well known to be associated with hereditary breast and ovarian cancers.[15] All samples were amplified by PCR successfully, whatever the technology used for DNA extraction. Fifty analyses by D-HPLC and 100 sequencing reactions were validated in conditions used for genetic diagnostic; typical results are presented in Figure 2A and B. No DNA purification-related difference was observed. We conclude that DNA samples obtained with the three workstations have similar qualities, and can be used for diagnostic tests.

RNA extractions and quality evaluation

As for DNA extractions, preliminary tests were realized in order to determine the maximum number of cells to be used for each extraction procedure. Cells (2.5×10^6 and 5×10^6) per column were the highest quantity of primary cells to use with the BRB 80000 and Tecan-MN, respectively. The Agowa kit adapted to the King-Fisher mL allowed to extract RNA from a maximum of 2.5×10^6 cells. The Biorobot EZ-1 extraction procedure allowed to process up to 20×10^6 cells with satisfactory results. These maximum numbers of cells were not exceeded for subsequent experiments. As for DNA isolation, the KingFisher mL/Biosprint 15 technique was time consuming, and not evaluated further.

Mononuclear cells from 11 acute leukemic patients at diagnosis were used to test the reproducibility of each RNA extraction technology. Since it was not possible to realize all extractions in parallel on the same day with different devices, one vial of each of the 11 samples was thawed at different time points; to eliminate any bias, the quality of samples thawed on different days was checked and were comparable. Mean cell viabilities were $66 \pm 22\%$, $74 \pm 20\%$, $63 \pm 26\%$, $66 \pm 20\%$, $72 \pm 23\%$ for CsCl, EZ-1, Tecan-MN, Tecan-MN+, and BRB8000 runs, respectively ($p > 0.1$).

Results obtained with robots were compared to those obtained with the manual technique routinely used for the diagnosis of hematological malignancies: guanidinium isothiocyanate lysis and centrifugation on CsCl cushion.[36] The Biorobot EZ-1 allowed the recovery of two- to three-fold more RNA than other automated techniques (Fig. 3A). This result is directly related to the initial quantity of loaded biological material, since the yield of purification was comparable between the different extraction procedures: median extraction rates were 0.81 ± 0.29, 0.97 ± 0.85, 1.03 ± 1.06, 0.93 ± 0.67, and 0.91 ± 0.56 μg RNA/10^6 viable cells for CsCl, EZ-1, Freedom-MN, BRB 8000, and Freedom-MN+, respectively. The relatively high standard deviations observed can be explained by the variability of the sample quality (cf Table 1 and cell viability). The mean quantity of RNA recovered after Biorobot EZ-1 extraction was 43% higher than after manual CsCl extraction (7 samples out of 11), but this difference did not reach statistical dif-
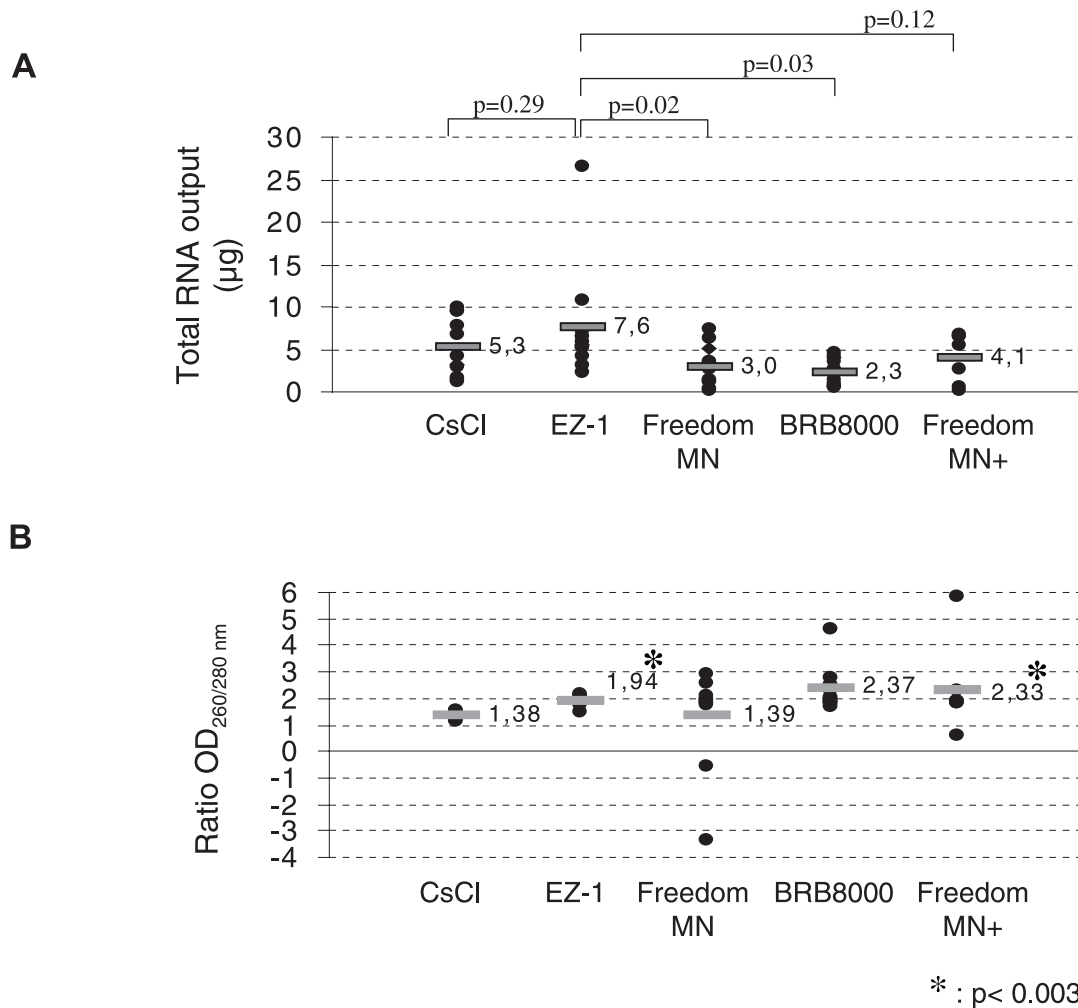
A

BRCA1 Exon 6   **BRCA1 Exon 8**

B

BRCA1 Exon 6   **BRCA1 Exon 8**

A

B

* : p< 0.003
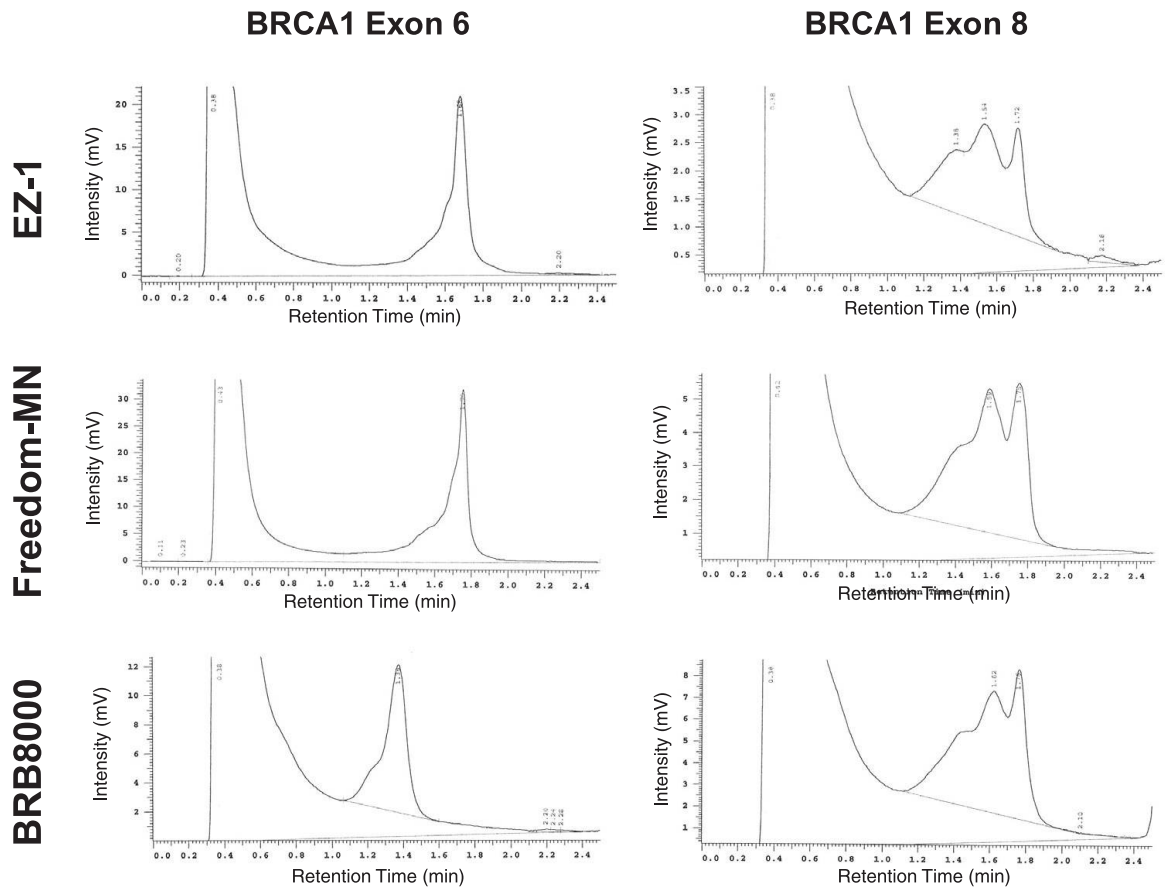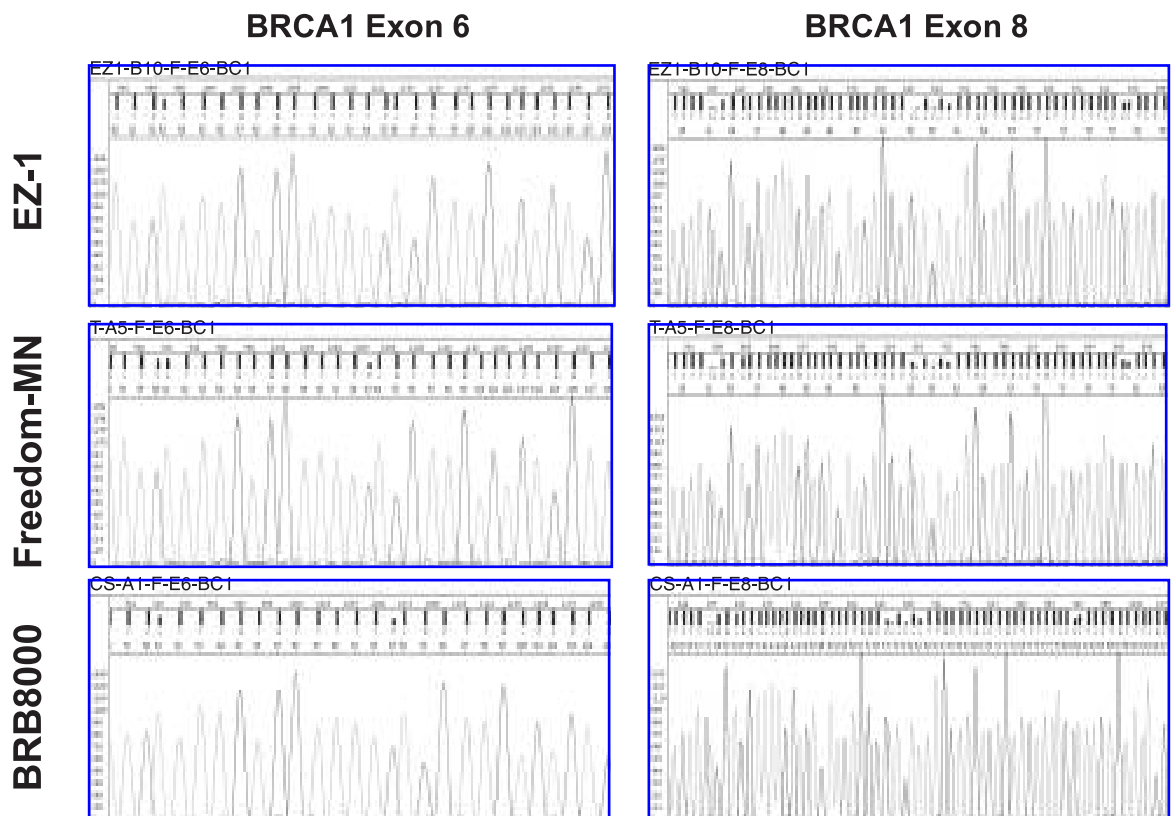
FIG. 3. RNA extraction results. For each of the 11 selected samples and each extraction procedure (CsCl, EZ-1, Freedom-MN, BRB8000, and Freedom-MN+), an aliquot of 20×10^6 cells, cryopreserved in DMSO and stored in liquid nitrogen, was thawed immediately before RNA extraction ($n = 11$). The simultaneous DNA and RNA coextraction procedure was tested, using 7 of the 11 samples (Freedom-MN+). Measurement of absorbance at 260 nm was used to calculate RNA concentration and the total RNA output (**A**). Yield of purification was calculated as the ratio of the quantity of RNA recovered to the number of viable cells (not shown). Protein contamination was evaluated by calculating the standard 260/280 nm ratio (**B**).

ference ($p = 0.29$). Spectrophotometry analysis of protein contamination indicated a significantly better quality for Biorobot EZ-1 extraction in comparison with the CsCl manual procedure (Fig. 3B). An analysis of handling time was realized for RNA extractions from the same 11 samples: the procedure with the Biorobot EZ-1 was ninefold faster than with the CsCl protocol (2 h vs. 18 h).

RNA integrity was assessed by automated capillary gel electrophoresis using the 2100 Bioanalyser platform (Fig. 4A). Both the manual RNA extraction and the Biorobot EZ-1 allowed recovery of high-quality RNA, as illustrated by migration profiles of both 18S and 28S RNA subpopulations. With these techniques, 9/11 RNA samples displayed high-quality RNA migration profiles. One particular sample repro-

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FIG. 2. Representative results obtained after D-HPLC and sequencing analysis. The sequence of *BRCA1* exons 6 and exon 8 were analyzed as routinely performed in our laboratory for cancer predisposition testing. For each DNA sample, both exons were separately amplified by PCR. PCR products were first analyzed by D-HPLC (**A**). Graphs presented here show typical profiles, as expected for each exon. PCR products were also purified for subsequent sequencing (**B**). Representative illustrations show that the signal/background ratio is very low.
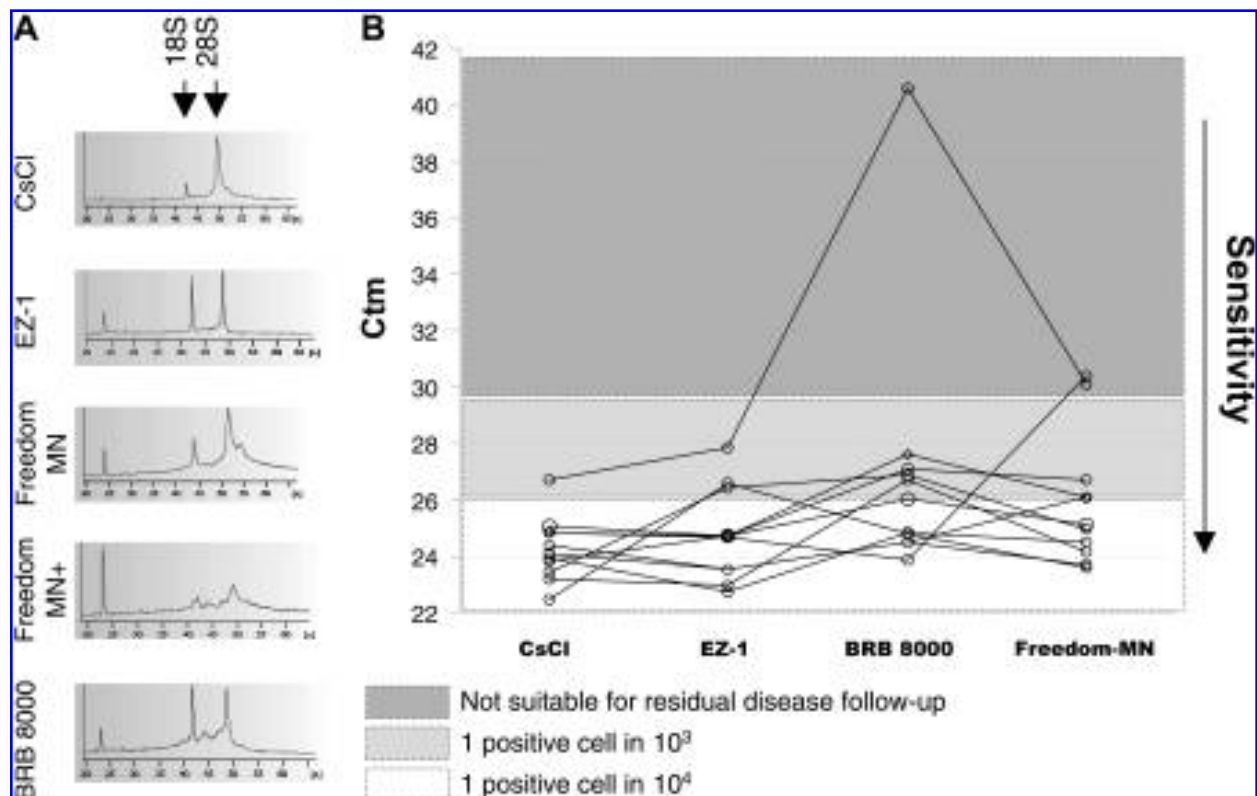
FIG. 4. RNA quality. After extraction, RNAs were precipitated and rehydrated in 10 μL RNase free water. RNA integrity was subsequently assessed by automated microelectrophoresis (**A**). The relative proportion of both 18S and 28S RNA subpopulations are represented by the surface under the curve. An ideal separation would result in a ratio of 2 when referring to the manufacturer's indications. Typical high-quality RNAs are presented for each extraction procedure. Freedom-MN+ corresponds to a modification of the Freedom-MN procedure allowing the sequential extraction of DNA and RNA from a single cell lysate, in a single run. Concentrated RNAs were used to detect by quantitative RQ-PCR, the specific transcripts for the Abelson gene (*ABL*) in the 11 RNA samples. For each sample, the Ctm values (median number of amplifications cycle necessary to reach detection threshold established by EAC standards) for *ABL* are represented. To improve readability, three zones are represented: the white zone represents results allowing the detection of 1 positive cell out of 10^4 cells, and the clear gray area a sensitivity of 1 out of 10^3. The darker gray zone represents sensitivities that are not compatible with minimal residual disease detection.

ducibly displayed evidence of RNA degradation, independently of the technique used for processing (data not shown). Some high-quality RNAs were also obtained with the Freedom-MN workstation and the BRB8000, but the proportion of such profiles was lower (4/11 and 5/11 samples, respectively).

Finally, quantification of the *ABL* transcript was done for each single RNA sample, with RQ-PCR and standard conditions.[11,13] The quality of RNAs obtained by both the CsCl manual technique and the Biorobot EZ-1 were compatible with high sensitivity detection of *ABL* transcripts (Fig. 4B); the difference in the number of amplification cycles was not statistically different between these two techniques

($p > 0.3$). However, more samples displayed a sensitivity of 10^{-4} when RNAs were obtained by CsCl extraction than when they were obtained with EZ-1 (10/11 and 8/11, respectively) (Fig. 4B). All samples obtained with these two techniques were validated for detection of minimal residual disease. cDNAs obtained by reverse transcription of RNAs purified using the Freedom-MN and the BRB8000 techniques were efficiently amplified, but needed additional amplification cycles to reach the threshold, as shown by the mean Ctm values: 24.2 ± 1.1, 24.8 ± 1.6, 25.9 ± 2.3, and 27.0 ± 4.7 for CsCl, EZ-1, Freedom-MN, and BRB8000, respectively ($p < 0.05$). RQ-PCR results—expressed as levels of sensitivity (Fig. 4B)—corroborated qual-

ity controls obtained with automated capillary gel electrophoresis for each single sample.

RNA and DNA simultaneous extraction

The manual CsCl extraction procedure presents a major advantage because it allows for the simultaneous recovery of both DNA and RNA from a same sample. With the Biorobot EZ-1, it is possible to treat samples with QIAzol reagent to separate RNA from DNA and proteins, and then to perform RNA and DNA cleanup using standard procedures. The first step of the extraction is not automated; thus, is time consuming and might be operator dependent. Preliminary evaluations of this approach were undertaken: using 5, 10, and 20×10^6 cells per extraction, we obtained 0.3 to 0.5 μg RNA/10^6 viable cells, and around 0.8 μg DNA/10^6 viable cells, with 260/280 ratios superior to 2 and 1.7, respectively, showing the feasibility of this approach.

The Macherey-Nagel Company has developed a sequential procedure with first elution of DNA, followed by RNA recovery from standard silica columns. We tested the possibility to automate this procedure with the freedom evo 75 workstation (Tecan-MN+). Using a panel of nine different leukemic cell samples (out of the 11 previously tested samples), we obtained a mean of 0.9 μg DNA/10^6 viable cells (0.7–1.4 μg) and 0.9 μg RNA/10^6 viable cells (0.2–1.5 μg) (Fig. 4B). 260/280 nm ratios were measured at 2.3 in both cases. RNA migration profiles obtained by automated capillary gel electrophoresis exhibited clear evidences of RNA degradation; thus, RQ-PCR analyses were not done.

Evaluation of a semiautomated device for reconcentration of RNA after extraction

The different automated techniques we have evaluated gave rise to low RNA concentrations because of the high volume of buffer needed to elute nucleic acids. Therefore, we had to find a reliable way to increase the concentration of our samples to fit with the criteria of our standard operating procedures. Different technologies are available for this purpose, including precipitation of the nucleic acids (high concentration of salts ± glycogen), filtration on silica columns, or evaporation in a heating vacuum chamber. The latest approach was particularly attractive because of a minimal intervention of the manipulator and the absence of a consumable to be used. Thus, we evaluated the Concentrator 5301 for its ability to improve RNA concentration without deteriorating the quality for subsequent transcript analyses by real-time RQ-PCR. As shown in Fig. 5A, the percentage of RNA recovery was systematically superior to 83% for the 36 samples (mean value of 89.7% ± 8%), even when the initial quantity of RNA was of 1 μg or 3.5 μg RNA (18 tubes in this study).

RNA quality was assessed before and after evaporation: the three tubes of a given triplicate were pooled to analyze RNA integrity by capillary gel electrophoresis and *ABL* transcript detection by RQ-PCR. Using three high-quality RNA samples, we observed that tubes initially containing 7 μg or 10 μg RNA conserved a migration profile very similar to the initial RNA sample after volume reduction (Fig. 5B). However, a tiny but reproducible degradation—materialized by the diminution of the 28S RNA peak—was observed for five out of six samples, which contained initially 1 μg or 3.5 μg.

After reverse transcription in EAC standard conditions, ABL transcripts were quantified by RQ-PCR before and after volume reduction (Fig. 5C). The low Ctm values observed for unmanipulated RNA solutions (Ctm < 26) confirmed their high quality. The volume reduction procedure did not alter the overall quality of RNA: only 1 sample (C—1 μg) displayed a Ctm value superior to 26. The mean Ctm value for the 18 samples treated with the Concentrator 5301 was increased by 0.99 cycles after volume reduction, when compared to unmanipulated RNAs; this difference is equivalent to a loss of sensitivity of 4%. As observed before, samples initially containing a small RNA quantity (1 μg) were more susceptible to degradation during volume reduction. Indeed, the mean Ctm value was increased by 1.8 cycles, in comparison with the mother RNA solution. We conclude that observed differences are acceptable and do not significantly change the reliability of the technique for diagnostic purposes.
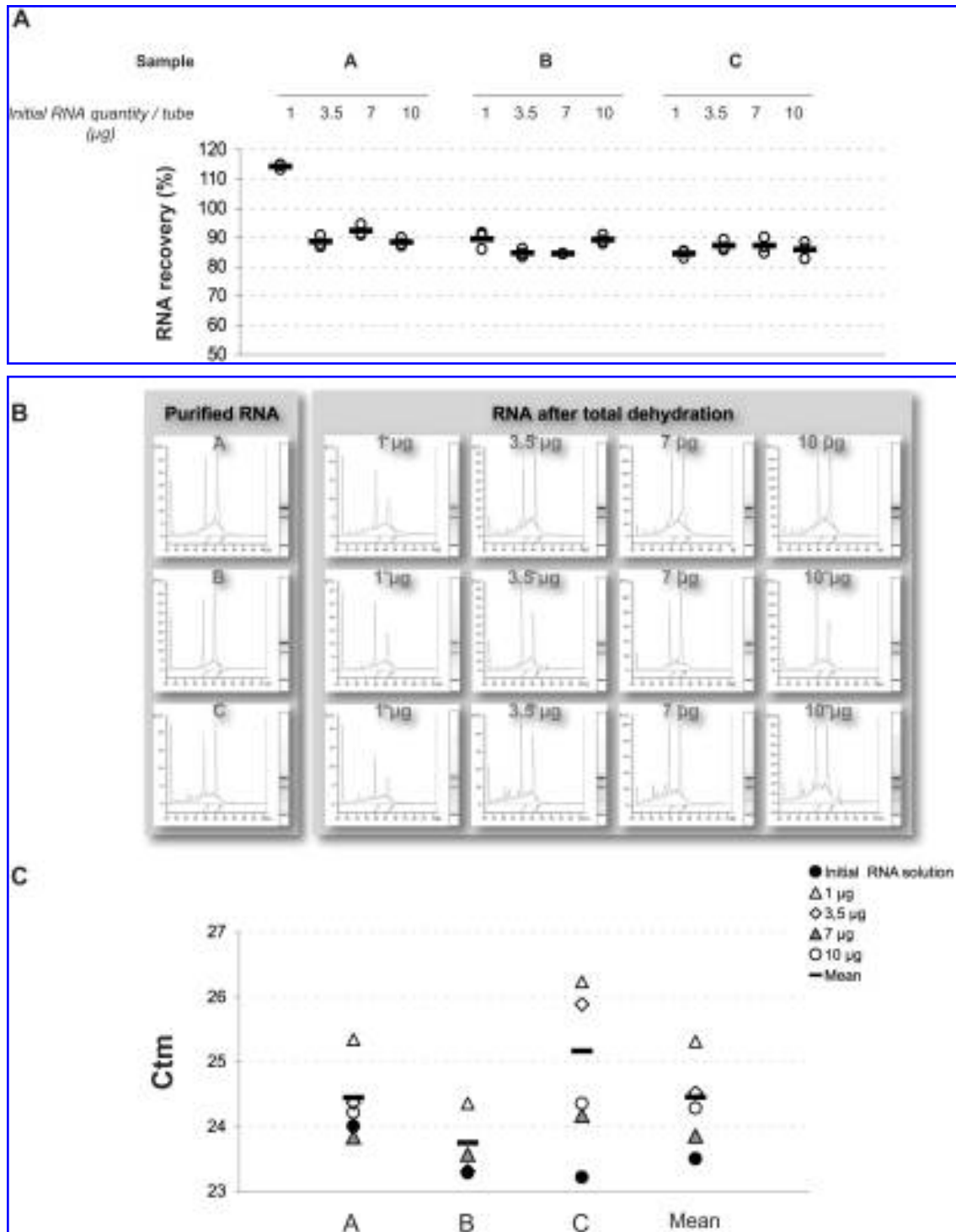
FIG. 5. Evaluation of the Concentrator 5301 for purified RNA reconcentration. (**A**) RNA recovery after a 10-fold concentration of varying quantities of RNA. Concentrated RNA obtained from leukocyte samples from three normal donors (**A**, **B**, and **C**) were diluted in order to obtain, for each sample, 1, 3.5, 7, and 10 μg RNA per tube, in triplicate, under a volume of 100 μL. After total evaporation of the aqueous phase by heating at 45°C under low pressure during 1 h 20 mn, RNAs were solubilized in 12 μL RNase free water. RNA concentrations were precisely determined before and after evaporation using UV nano-spectrophotometry in order to calculate the percentage of RNA recovery (yield). Each dot of the diagram represents the median yield of RNA recovery for a given triplicate, expressed as the percentage of final output over initial RNA quantity. (**B**) RNA integrity analysis by capillary gel-electrophoresis. After a 10-fold volume reduction, the three tubes of a given triplicate were pooled in a single tube. RNA integrity was analyzed the same day on the same chip to compare the migration profiles of the initial RNA suspension before (**A**, **B**, and **C**) and after (1, 3.5, 7, and 10 μg) volume reduction. (**C**) Abelson transcript detection before and after volume reduction. *ABL* transcripts were quantified before (black circles) and after (white or gray symbols) volume reduction by quantitative RQ-PCR. The mean numbers of PCR amplification are represented for each sample. The black bars represent the mean values for the four different samples for a given patient. The mean results were also calculated for each one of the three different samples (black lines) and for each experimental condition (before and after volume reduction).

*Analysis of intra- and
interrun crosscontamination*

RQ-PCR for *ABL* gene transcript quantification systematically led to Ctm values of 50 cycles (data not shown) for the 12 tubes containing only 100 μL of RNase free water. This result indicates that RQ-PCR failed to detect the presence of human RNA crosscontamination trace. We also assessed the possibility of interrun crosscontamination caused by DNA aerosols, using standard RQ-PCR amplification in the same tubes but omitting the reverse transcriptase treatment. Similarly, Ctm values were systematically of 50 cycles for the same 12 samples and detection of the *ABL* genomic sequence, demonstrating the absence of detectable traces of human DNA contaminants (data not shown).

DISCUSSION

We have compared three automated devices for their ability to extract DNA, RNA or both from human biological samples, in conditions that are designed for further diagnostic testing. DNA from blood leukocytes was analyzed in search of genomic mutations that predict an elevated risk of developing breast or ovarian cancers. RNA from leukemic blood or marrow cells was analyzed for *ABL* transcript quantification as an internal control commonly used to assess RNA quality in minimal residual disease detection. Using blood leukocytes from normal individuals, the BRB 8000, the Freedom evo75, and the Biorobot EZ-1 allowed efficient purification of DNAs with quality that was systematically compatible with implementation of diagnostic procedures. The CsCl manual technique and the Biorobot EZ-1 allowed for isolation of higher quantities of RNAs from human primary leukemic cells, with better RNA integrity and higher quality for RQ-PCR analyses. Further, the Biorobot EZ-1 presents an interesting advantage over the other tested automated approaches, allowing the treatment of up to 10 and 20×10^6 cells per extraction for DNA and RNA, respectively.

Potential advantages associated with the use of automated techniques for nucleic acid extraction include the reduction of operator workload, rapid and simultaneous analyses of high numbers of samples, as well as improvements in reliability and traceability. Conversely, a potential problem observed with automated silica-based technologies is the high volume (100 μL) and low concentrations (<100 ng/μL) under which purified RNA is recovered. This situation imposes an additional step and workload before RQ-PCR analyses, and this can potentially damage RNA quality or decrease the final quantity of available RNA. We tested different techniques, including NaCl + glycogen precipitation and the Concentrator 5301 (Eppendorf). Excellent recovery of high-quality RNA was observed with the Concentrator 5301, demonstrating the possibility to easily overcome this difficulty.

Substitution of manual techniques for automated techniques will favor standardization at all steps of biological analyzes, including the preanalytical steps. Available information regarding the standardization of nucleic acid extraction procedures from clinical samples in the field of cancer diagnosis is scarce in the literature. Recently, regulatory agencies including the U.S. Food and Drug Administration (FDA) have classified all the preanalytical reagents and technologies as "Class II medical devices."[40] This rule, effective September 26, 2005, covers RNA collection, stabilization, and purification for subsequent molecular diagnostic testing. In Europe, scientific societies are involved in the design and publication of "good laboratory practices" and recommendations (two examples are the EAC and the LeukemiaNet programs). The CE-IVD label ("CE" labeling for *in vitro* diagnosis devices) has been used for many years for viral testing, and is likely to be extended to other fields including cancer diagnosis. Standards will include quality controls for DNA and RNA, as well as predefined ranges of expected values. Capillary gel electrophoresis is one possibility for qualification of RNA samples before RQ-PCR analyses, because we observed a good correlation between the global RNA migration profile and our ability to detect the presence of a control gene in the sample. Improvements—including recently developed software[37–39]—will help interpret electrophoregrams and assess RNA integrity. Unfortunately, at the time we realized this study, our system was not equipped with

the version of the program that allows for the production of the RNA Integrity Number (RIN) cited above.

Finally, the validation of protocols allowing for the concurrent recovery of DNA, RNA, and proteins from a single sample will require further testing. Depending on the nature and rarity of a sample, this may be a critical issue for both diagnostic and research applications, enabling simultaneous analysis of both molecular species (combining a cDNA microarray and a CGH array, for example). This is particularly true for nonreplicable samples, which is the case for many samples of human origin, including tumors. It is likely that a single automated device will not fulfill all needs, and that a high throughput platform may operate several different devices to suit variable applications.

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Address reprint requests to:
Francois Lassailly
Hematopoietic Stem Cell Laboratory
London Research Institute—Cancer Research UK
44 Lincoln's Inn Fields
London, WC2A 3PX, UK

E-mail: Francois.Lassailly@cancer.org.uk