Gene expression profiling is increasingly important in human health research and applications (1). Unfortunately, the human tissue samples required for this process, particularly those from healthy individuals, are not safely and easily available. Therefore, the use of peripheral blood mononuclear cells (PBMCs) as surrogate material for high-throughput analysis of gene expression is currently being explored. These cells are involved in a large variety of immune-related diseases, including infection and cancer. Moreover, in recent studies, characteristic sets of transcriptional changes in PBMCs were associated with physiologic or pathologic states (2–4). Thus, a PBMC transcriptome may be used as an individual’s health sensor, a concept referred to as the sentinel principle (5).

In large multicenter studies, the reliable and reproducible detection of transcript concentrations from PBMCs requires standardization of blood sampling and an efficient method of conservation. Indeed, many preanalytical factors during collection, processing, and storage of blood specimens may affect RNA and its subsequent use as a biomarker (6). Although numerous technical and clinical aspects of blood sampling have been addressed (7–9), comprehensive data on the long-term storage and stability of RNA from PBMCs are needed. Here we report experiments performed over 15 months to test the preanalytical conditions involved in blood collection procedures and PBMC storage. Blood samples were collected from healthy donors into EDTA and sodium heparin tubes. RNA extractions were performed on isolated PBMCs stored at −80 °C up to 15 months in 4 different lysis buffers. Using spectrometry and real-time PCR, we compared the concentration, purity, integrity, and stability of the total RNA. We propose a quality-assured and controlled protocol of PBMC banking for further mRNA expression analysis.

After informed consent was obtained from 12 unrelated adult volunteers, whole blood (10 mL) was collected by standardized venipuncture in EDTA (EDTA-blood; n = 12) and heparin (heparin-blood; n = 12) tubes (Vacutainer™, Becton Dickinson) and processed for PBMC preparation. Briefly, blood samples were homogenized with 10 mL of Hanks Balanced Salt Solution, Modified (Sigma-Aldrich). PBMCs were prepared by Ficoll density-gradient centrifugation (Ficoll-Paque™ PLUS; Amersham) at 300g for 30 min at 20 °C. The ring of high-density PBMCs was isolated and washed twice in 50 mL of Hanks buffer. After cell survival was determined with the trypan-blue exclusion test, the PBMC concentration was normalized to 10⁶ cells/mL in Hanks buffer. PBMC populations were evaluated by microscopic observation after May–Grunwald–Giemsa staining. After centrifugation for 6 min at 700g (4 °C), 200 μL of cell lysis buffer was immediately added to the PBMC pellet. Four different commercially available buffers were tested (n = 24 for each buffer, including 12 EDTA-blood and 12 heparin-blood samples): RNA InstaPure [Eurogentec (E)], lysis/binding buffer from the MagNA Pure LC RNA Isolation Kit I [Roche Diagnostics (R)], buffer RTL from the QiAamp® RNA Blood Mini Kit [Qiagen (Q)], and RNA lysis buffer from the StrataPrep® Total RNA Miniprep Kit [Stratagene (S)]. The PBMC lysates were used to extract total RNA immediately (t₀) or after storage at −80 °C for 3, 5, 10, and 15 months (t₃, t₅, t₁₀, t₁₅, respectively).

For total RNA extraction, we used an automated process with the MagNA Pure LC instrument (Roche Diagnostics) and the MagNa Pure LC RNA Isolation Kit I according to the manufacturer’s instructions, including DNase treatment. Purified RNA was eluted at 70 °C with 100 μL of low-salt buffer. Total RNA quality and quantity were assessed in 2 ways. In the first method, we estimated the RNA concentration by ultraviolet absorbance at 260 nm (1 absorbance unit at 260 nm = 40 ng/μL RNA) and the RNA purity by measuring the ratio of absorbance at 260 nm and 280 nm (1.8 < A₂₆₀/A₂₈₀ < 2.1 for pure RNA). Total RNA was run on 1% agarose gels to check size and integrity. In the second method, RNA from each sample was assessed for integrity by a 2-step reverse transcription–PCR assay for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; NM_002046). cDNA synthesis was performed at 37 °C for 60 min with 80 ng of extracted RNA, 0.25 μg of oligo(dT)₁₅ primer, and 200 U of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega). After each reverse transcription, cDNA was amplified by a standard real-time protocol in the LightCycler instrument (Roche Diagnostics). The 20-μL PCR reaction for the GAPDH gene included 1× LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics), 4 mM MgCl₂, 0.2 μM each of 5’à-CAGGATTTGGTCGTAT-TGGG-3’ (forward primer) and 5’à-CGGTCTCGGAAGAT-GGTGAT-3’ (reverse primer; Sigma-Aldrich), and 2 μL of 1:100-diluted template cDNA. Cycling settings were an initial denaturation step at 95 °C for 8 min and 45 cycles of 95 °C for 6 s, 60 °C for 5 s, and 72 °C for 15 s, followed by melting curve analysis to evaluate the specificity of PCR products. To ensure amplification of the appropriately sized fragment, PCR products were analyzed by electrophoresis in 3% agarose gels. PCR controls included a separate reaction in which either cDNA template or the reverse transcriptase was eliminated. None of these controls showed detectable amplification product (data not shown). GAPDH mRNA detection was estimated by use of the crossing point (CP), defined by the LightCycler software (Ver. 3.5) as the cycle at which each reaction reaches the logarithmic portion of the PCR curve. PCR determinations in each sample were performed in triplicate. To improve our results, we analyzed the ADM (NM_001124), and IL-8 (NM_000584) genes, which are known to quickly change expression in response to technical influences such as temperature. The PCR conditions for these reactions are shown in Table 1.

Technical Briefs

Collection and Storage of Human Blood Cells for mRNA Expression Profiling: A 15-Month Stability Study, Jean-Brice Marteau, Steve Mohr, Michele Pfister, and Sophie Visvikis-Siest* (Institut National de la Santé et de la Recherche Médicale, U525 Equipe 4, Faculté de Pharmacie, Nancy, France; * address correspondence to this author at: INSERM U525 Equipe 4, 30 rue Lionnois, 54000 Nancy, France; fax 33-3-83321322, e-mail Sophie.Visvikis-Siest@nancy.insERM.fr)
of the Data Supplement that accompanies the online version of this Technical Brief at http://wwwclinchem.org/content/vol51/issue7/.

Statistical analysis was performed with StatView software (Ver. 5; SAS Institute Inc.). Differences in mean RNA concentration or CP value according to either anticoagulant (EDTA, heparin) or lysis buffer (E, R, Q, and S) were estimated by paired Student t-test. Variation of RNA stability according to different storage times was calculated by the Friedman test. Values are reported as the mean (SE). A P value ≤0.05 was considered statistically significant.

As determined by trypan-blue counting, PBMC viability was 97.5 (1.3)%. According to results obtained with May–Grunwald–Giemsa staining, PBMC populations usually contained >97% mononuclear cells. To compare the purity and yield of total RNA obtained from the whole-blood collection under the conditions being investigated, we performed spectrometric analysis on the RNA samples extracted at $t_0$. Regardless of the cell lysis buffer used, we observed no difference in the $A_{260}/A_{280}$ ratio between anticoagulants [1.95 (0.12) and 1.94 (0.11) for EDTA and heparin, respectively; $P = 0.24$], suggesting that RNA purity was equivalent for all samples. In contrast, the yield of total RNA at $t_0$ was higher in EDTA-blood than in heparin-blood: 23.9 (15.2) vs 19.1 (10.5) ng/μL ($P < 0.0001$; Fig. 1A). The CP value of the GAPDH gene determined by real-time PCR indicated that RNA integrity was better in EDTA-blood than in heparin-blood (Fig. 1A in the online Data Supplement). The measured mean CP was 38.0 (2.0) cycles in EDTA-blood and 40.4 (2.3) cycles in heparin-blood ($P < 0.0001$; $n = 24$). Melting curve analysis confirmed the presence of a single GAPDH-specific amplicon for all samples (data not shown). These results indicate that the use of EDTA as anticoagulant in blood collection ensures the best total RNA yield and quality. This conclusion was further supported by other findings involving blood-based RNA assays (10, 11).

To further improve the yield of total RNA, we tested 4 different cell lysis buffers combined with automated MagNA Pure LC extraction. Typical results obtained for 12 donors at $t_0$ are shown in Fig. 1B and in Fig. 1B of the online Data Supplement. RNA concentrations were significantly higher with use of the E buffer than with the other buffers ($P < 0.01$; Fig. 1B). The RNA yield obtained in buffers Q and S did not differ significantly from the yield obtained in buffer R. Neither EDTA nor heparin affected this result (Fig. 1B). In addition, RNA integrity was best in EDTA-blood with buffer E (Fig. 1B of the online Data Supplement). Results were similar for the ADM and IL-8 genes (panels A and B, respectively, of Fig. 2 of the online Data Supplement). We therefore propose that buffer E rather than buffer R be used to constitute PBMC banks for the purpose of RNA expression analysis.

Fig. 1. Impact of preanalytical factors on PBMC RNA concentrations.
Impact of anticoagulants (EDTA and heparin; $n = 48$ for both anticoagulants; A), cell lysis buffers (Eurogentec, Roche, Qiagen, and Stratagene; $n = 12$ for all groups; B), and storage time (3, 5, 10, and 15 months; $n = 8$ for all time points) in Eurogentec buffer (EDTA-blood; C) on RNA concentration. The RNA concentrations in all panels are in ng/μL. The results are shown as box-plots with medians (lines inside boxes), 25th and 75th percentiles (limits of boxes), and the 10th and 90th percentiles (whiskers). The ● in panel A indicate outliers. NS, not significant.
Finally, to determine whether buffer E would preserve the RNA of PBMCs over time, we analyzed total RNA obtained from 8 individuals at t₀, t₁₀, t₁₀₀, and t₁₅₀ of PBMC storage time with reference to their t₀ values. For all samples examined, RNA purity, determined by the A₂₆₀/A₂₈₀ ratio, was 1.8–2.1, indicating that highly pure RNA could be obtained with storage in buffer E. In addition, for all 3 genes, we observed no significant variations in either the amount (Fig. 1C; Friedman test, P = 0.0812) or the integrity (Fig. 1C of the online Data Supplement; Friedman test, P = 0.3682) of RNA at t₀, t₁₀, t₁₀₀, and t₁₅₀ compared with t₀. Thus, buffer E provides advantageous conditions for PBMC storage at −80 °C. In addition, it is noteworthy that buffer E is also suitable for manual extraction (see the manufacturer’s instructions), which does not necessarily require specific and costly automation.

The kinetics of gene expression and RNA stability in blood samples processed ex vivo (preanalytical variables) are currently under intense investigation (6, 12, 13). To guarantee optimal conditions, samples reach our laboratory for processing after a transportation time of <2 h at 4 °C. Indeed, gene expression was shown to change 2 h after blood collection (13), probably because of significant stress responses (12) or possible contact activation of cells in the collection tubes (9). These findings underscore the extreme sensitivity of blood cells to ex vivo handling. One study has suggested that EGTA is preferable to EDTA as an anticoagulant to prevent the irreversible loss of antigen-specific lymphoproliferative responses in instances in which unclotted blood may be stored for long periods (14). Methods such as the PAXgene tube system or addition of acidic phenol and guanidine isothiocyanate have been proposed to stabilize blood cell gene expression after prolonged incubation of blood ex vivo, but new methods and protocols are needed (8, 12).

Our studies demonstrate that PBMC RNA is stable over time (up to 15 months) when blood samples are collected into EDTA tubes and when PBMCs are stored at −80 °C in buffer E. We provide here a simple assay for rapid, efficient, and standardized banking of PBMCs, a key step in large gene expression studies. To our knowledge, this is the first comprehensive report on RNA stability over 15 months. Moreover, preliminary observations suggest that our protocol provides successful results in gene expression assays such as quantitative reverse transcription-PCR or microarrays (data not shown). Finally, our recommendations may be helpful for clinicians and researchers involved in experimentation with PBMC transmitters in molecular medicine and epidemiologic surveillance, an emerging field of noninvasive health applications.

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References


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Denaturing HPLC Coupled with Multiplex PCR for Rapid Detection of Large Deletions in Duchenne Muscular Dystrophy Carriers, Chia-Cheng Hung,1,2 Yi-Ning Su,2 Chia-Yun Lin,1 Chih-Chao Yang,3 Wang-Tso Lee,4 Shu-Chin Chien,2 Win-Li Lin,1 and Chien-Nan Lee5*

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Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) affect 1 in 3500 newborn male infants (1). DMD and BMD are both inherited in an