which reacted to form a color with merocyanine dye *(4)*; creatinine was hydrolyzed to creatine, which in a reaction cascade, led to production of hydrogen peroxide that was measured by triaryl imidazole leuco-dye *(4)*; slides for phosphorus used the formation of an ammonium-phosphomolybdate complex and its reaction with *p*-methylaminophenol sulfate *(5)*; and uric acid was determined by the uricase peroxidase method *(6)*. The specific urine calibration was used.

The linear regression between the multiple dilution and single dilution methods was checked for urea, creatinine, phosphorus, and uric acid. In the case of sodium and potassium, the slopes were different from 1.0, and the intercepts were different from 0 ($P < 0.001$). Corrections were carried out by introduction of slope and intercept values established with the flame photometry reference method (Eppendorf apparatus) for calibration of each new batch of slides. The linearity limits were modified when a single dilution was used, as shown in Table 1. These new limits were determined by successive dilutions of samples with high concentrations of analytes.

The imprecision was checked by comparison of multiple dilutions and single dilution with the Vitros 250 (laboratory 1) and Vitros 700 (laboratory 2), using control urine (Biotrol, A 02262). The within-day CVs of the two methods (10 determinations) in the two laboratories were ≤1%. The mean values (± SDs) for different control ranges, respectively, in laboratories 1 and 2 were as follows: Na (127.3 ± 1.49 mmol/L; 89.3 ± 0.72 mmol/L), K (72 ± 0.67 mmol/L; 31.5 ± 0.3 mmol/L), urea (279.5 ± 3.19 mmol/L; 166 ± 2.06 mmol/L), creatinine (9.28 ± 0.09 mmol/L; 3.67 ± 0.04 mmol/L), phosphorus (17.91 ± 0.23 mmol/L; 2.65 ± 0.018 mmol/L), and uric acid (2.18 ± 0.02 mmol/L; 0.60 ± 0.006 mmol/L).

The day-to-day CVs (30 determinations in laboratory 1 and 50 determinations in laboratory 2 of control urine) were <2% for all analytes. One determination was carried out each day during a 1-month period in laboratory 1 and a 2-month period in laboratory 2. The freeze-dried control urine was reconstituted each week and stored at 4 °C. Every day, a fresh dilution was made. Laboratories 1 and 2, respectively, obtained the following results with a different lot in each laboratory: Na (122 ± 2.47 mmol/L; 121.6 ± 2.49 mmol/L), K (70.88 ± 1.10 mmol/L; 60.1 ± 0.95 mmol/L), urea (280.1 ± 5.83 mmol/L; 220 ± 2.74 mmol/L), creatinine (9.45 ± 0.20 mmol/L; 7.84 ± 0.09 mmol/L), phosphorus (17.13 ± 0.30 mmol/L; 15.85 ± 0.20 mmol/L), and uric acid (2.17 ± 0.04 mmol/L; 1.94 ± 0.02 mmol/L).

The agreement between the single dilution system and the standard multiple dilution system was assessed by analyzing patient urines by the two methods with both automatic (Vitros 250) and manual (Vitros 700) dilutions. Differences from the mean were calculated *(7)*. Fig. 1 shows the differences between methods for each analyte. The observed differences were small compared with the reference ranges. It is therefore clear that a single dilution of urine with aqueous Tween 20 can be used for assaying sodium, potassium, urea, creatinine, phosphorus, and uric acid without large error. Although this improved method is not yet endorsed by the manufacturer, it makes urine analysis on the Vitros apparatus much faster with or without automatic dilutions.

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Optimized Procedure for DNA Isolation from Fresh and Cryopreserved Clotted Human Blood Useful in Clinical Molecular Testing, *Luis A. Salazar, Mario H. Hirata, Selma A. Cavalli, Marcos O. Machado, and Rosario D.C. Hirata*[*] (Faculty of Pharmaceutical Sciences, University of São Paulo, CEP 05508-900, São Paulo, SP, Brazil; * address correspondence to this author at: Dept. of Clinical and Toxicological Analysis, Faculty of Pharmaceutical Sciences, University of São Paulo, Av. Prof. Lineu Prestes, 580, CEP 05508–900, São Paulo, SP, Brazil; fax 011-813-2197, e-mail mdchirta@usp.br)

In the routine clinical laboratory, large amounts of uncoagulated blood are collected and the blood clot usually is discarded. In molecular biology, cells from EDTA-anticoagulated or acid-citrate-dextrose-anticoagulated peripheral blood are used as sources of DNA *(1–3)*. After leukocyte isolation, most procedures utilize enzymatic cell digestion, followed by extraction with hazardous organic solvents (phenol-chloroform) and precipitation with ethanol *(4, 5)*. To minimize the volume of blood collected for laboratory tests, several authors have developed methodologies to isolate DNA from blood clots *(4–8)*. However, the techniques may be difficult or impractical and may require slicing of the clot with scalpels or other sharp instrument, exposing laboratory personnel directly to contaminated blood *(4, 7)*. Other techniques are time-consuming, using many chaotropic reagents, enzymes, RNA-removal steps, or large volumes of samples and reagents not suitable in the clinical laboratory *(4–8)*.

We have optimized a nonenzymatic, nontoxic procedure for efficient DNA extraction from fresh and cryopreserved clotted blood.

Blood samples were obtained from 24 unrelated individuals who had given informed consent. We compared 10 paired samples of EDTA-anticoagulated blood and fresh blood clot and studied 14 samples of cryopreserved

clot that had been frozen for 2 years at −20 °C. Blood clots were homogenized with 9 g/L NaCl, using a Potter-MARCONI MA 099 system, for 30 s. The homogenizing Teflon probe was cleaned three times with 700 mL/L ethanol and 9 g/L NaCl between samples to avoid cross-contamination. One milliliter of each homogenized sample was centrifuged at 1200*g* for 5 min, and the supernatant was removed. Blood cells were lysed with 1 mL of Tris buffer 1 (10 mmol/L Tris-HCl, pH 8.0, 10 mmol/L KCl, 10 mmol/L $MgCl_2$, 2 mmol/L EDTA, pH 8.0, and 25 mL/L Triton X-100). After centrifugation, the pellet was washed twice with Tris buffer 1 and lysed with 220 μL of Tris buffer 2 (10 mmol/L Tris-HCl, pH 8.0, 10 mmol/L KCl, 10 mmol/L $MgCl_2$, 2 mmol/L EDTA, pH 8.0, 0.4 mol/L NaCl, and 10 g/L sodium dodecyl sulfate) and incubated for 15 min at 56 °C. Cellular proteins were removed by precipitation, after addition of 100 μL of 5 mol/L NaCl. DNA was isolated by ethanol precipitation and solubilized in Tris-EDTA buffer (10 mmol/L Tris-HCl, pH 8.0, and 1 mmol/L EDTA).

The DNA concentration was measured by spectrophotometry at 260 nm, and DNA purity was determined by the A_{260}/A_{280} ratio *(3)*. The integrity of the DNA samples was verified by agarose gel electrophoresis after ethidium bromide staining under ultraviolet light. The suitability of the DNA obtained from clotted or anticoagulated blood was evaluated by PCR amplification of the *Hin*cII polymorphic region (exon 12) of the human low density lipoprotein receptor (*LDLR*) gene *(9)*. Analysis of the washes between samples by DNA examination and PCR amplification did not show evidence of cross-contamination. Results were expressed as mean ± SD. Student's *t*-test and regression analysis were used to evaluate differences of yield and purity between DNA extracts. Differences were considered significant at $P < 0.05$.

Clotted and whole blood provided similar yield and purity (A_{260}/A_{280}) of DNA (Table 1). These data are similar to those obtained when proteinase K treatment and organic extraction were used *(4)* and higher than reported for other salting-out procedures *(1, 6)*.

The reduction of the reagent volumes and replacement of Nonidet P40 with Triton X-100 (as described previously for DNA isolation from whole blood *(10)*) enhanced the feasibility and precision of the DNA isolation from fresh blood clot. The within-run CV of the DNA extraction from clotted blood was similar to that for the anticoagulated blood. The purity of DNA extracted from clot was comparable with whole blood (Table 1). All procedures can be performed at room temperature, providing similar results when compared with 4 °C (data not shown). We also could handle multiple samples in less time than with other protocols *(4–8)*. These features make the procedure even more appropriate for routine laboratory work.

The lower DNA recovery from cryopreserved clots (Table 1) probably reflected the higher number of wash steps necessary to remove hemoglobin from cryopreserved specimens *(11)*. The DNA purity and concentration were similar to those reported by other authors *(5)*.

DNA concentrations recovered from blood clot and whole blood were correlated ($P < 0.05$, Fig. 1, top). A significant correlation ($r^2 = 0.86$, $P < 0.05$) was also found between leukocyte counts in blood and DNA concentrations (data not shown). Gel electrophoresis showed high molecular weight DNA in all samples (Fig. 1, bottom). Moreover, the *Hin*cII region of the *LDLR* gene could be easily amplified from both DNA preparations. These results show that DNA can be efficiently extracted from cryopreserved samples, even after prolonged storage of the specimens.

In conclusion, the protocol described here enables molecular biologists to obtain DNA from blood clots by use of stable and nonhazardous reagents. The method is simple, fast, and reliable for obtaining high quantities of DNA suited for clinical molecular testing.

Table 1. Concentration and A_{260}/A_{280} ratio of DNA extracted from cryopreserved and fresh clotted blood and EDTA-anticoagulated blood.

Samples	DNA concentration, mg DNA/L blood[a]	A_{260}/A_{280} ratio[a]
EDTA-anticoagulated blood (10)[b]	60.6 ± 12.6	1.96 ± 0.17
Fresh clotted blood (10)[b]	61.5 ± 11.9	1.87 ± 0.19
Cryopreserved clotted blood (14)[b]	17.5 ± 2.8[c]	1.76 ± 0.08

[a] Mean ± SD.
[b] Number of samples.
[c] $P < 0.05$.

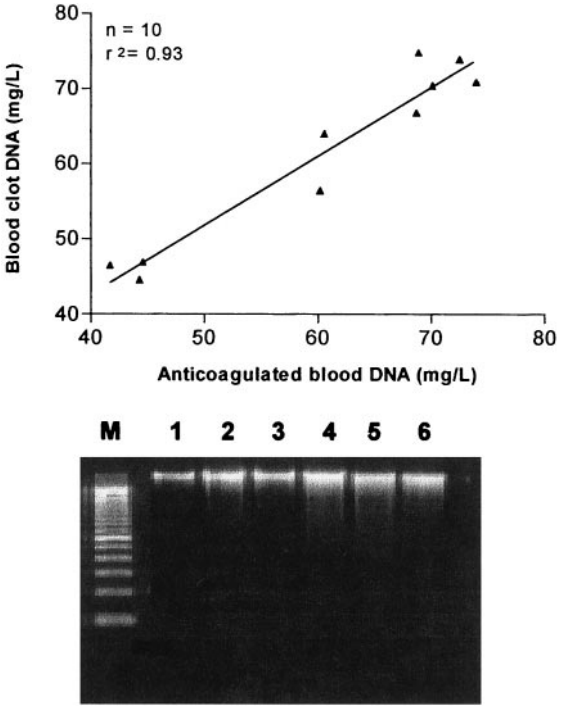

Fig. 1. Comparison of DNA from clotted blood and EDTA-anticoagulated or whole blood.

(*Top*) Correlation between concentrations of DNA extracted from fresh clotted blood (*y*) and EDTA-anticoagulated blood (*x*). (*Bottom*) Ethidium bromide-stained agarose gel electrophoresis of DNA extracted from clotted blood (*lanes 1–3*) and whole blood (*lanes 4–6*); *lane M*, DNA marker (1 Kb).

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Molecular Detection of Circulating Prostate Cells in Cancer II: Comparison of Prostate Epithelial Cells Isolation Procedures, *Pierre Berteau,[1] Frédéric Dumas,[1] Jean-Luc Gala,[2] Pascal Eschwège,[3] Bernard Lacour,[1] Marianne Philippe,[2] and Sylvain Loric[1,3]** ([1] INSERM U90 and Clinical Biochemistry Laboratory, CHU Necker, 149 rue de Sèvres, 75015 Paris, France; [2] Molecular Chemistry Laboratory, Saint-Luc Clinical University, Clos Chapelle aux Champs 30, Brussels, Belgium; [3] Urology Unit and Experimental Surgery Laboratory, CHU Bicêtre, 78 rue du Général Leclerc, 94270 Le Kremlin Bicêtre, France; * address correspondence to this author at: Laboratoire de Biochimie A, Hôpital Necker, 149 rue de Sèvres, 75015 Paris, France; fax 33-1-44495120)

The sensitive and specific detection of circulating tumor cells holds great promise for more accurate staging of cancer patients. Several reverse transcription-PCR (RT-PCR) procedures based on tissue-specific mRNA expression are now able to detect one cell derived from a given tissue among $>10^6$ peripheral nucleated blood cells [PNBCs; for a review, see *(1)*]. However, even for a single marker, highly discrepant results have been observed among the available clinical studies; e.g., the frequencies of positive prostate-specific antigen (PSA) RT-PCR results range from 25% *(2)* to 80% *(3)* in patients with metastatic prostate cancer (CaP), blurring the clinical relevance of these assays. Standardization and quality control in molecular diagnosis are crucial to the solution of this issue. We have previously studied factors potentially affecting RT-PCR results *(4)*, and this current work focuses on the approaches for harvesting prostate cells among nucleated blood cells.

Since the first 1992 clinical report describing the RT-PCR detection of circulating prostatic cells in CaP, the majority of reported assays have used gradient separations to recover nucleated cells from the peripheral blood *(1)*. We *(5)* and others *(6)* rather choose overnight hypoosmotic red blood cell lysis as an easier and more cost-effective protocol. Because the approach used to harvest PNBCs may account for discrepancies in clinical results, we have compared a panel of nucleated blood cell separation methods.

Fresh blood from four healthy blood donors (total volume, 400 mL) was sampled in 40 × 10-mL EDTA-treated tubes (Becton-Dickinson). The LNCaP cell line, derived from a metastatic prostate carcinoma, was cultured as described *(7)*. After trypsinization (2.5 mL/L trypsin), 10^5 confluent LNCaP cells were added into each of the 40 tubes, which were gently mixed. Six tubes were processed immediately by overnight hypoosmotic red blood cell lysis, as described *(5, 6)*: Two volumes of ammonium chloride (9 g/L) were mixed with 10 mL of blood in the first three tubes *(5)*, whereas 1.5 volumes of diethylpyrocarbonate (DEPC)-treated water was added for 5 min in the other three tubes *(6)*. After centrifugation and removal of the supernatant, 1 mL of guanidinium thiocyanate was added to each tube, and RNA was extracted as described *(8)*. Four commercial two-layer density gradients were utilized: LymphoPrep™ (Nycomed, d = 1.077 kg/L), Ficoll-Paque™ (Pharmacia Biotech, d = 1.077 kg/L), PolymorphPrep™ (Life Technologies, d = 1.113 kg/L), and NycoPrep™ (Life Technologies, d = 1.068 kg/L). Two additional density gradients (d = 1.095 kg/L and d = 1.050 kg/L) were prepared by diluting Percoll (Pharmacia Biotech) in 1.5 mol/L NaCl. The amount of Percoll added to reach an isoosmotic solution was determined according to the manufacturer's instructions. Samples of 10 mL of blood mixed with LNCaP cells were laid on top of each of 10-mL gradient. This was followed by centrifugation at 450*g* for 20 min at 4 °C.

The efficiency of nucleated cells separation was assessed by four different methods.

In method a, the banding of [^{3}H]-thymidine-incubated LNCaP cells, aggregate-free LNCaP cells were suspended in 1 mL of phosphate-buffered saline and incubated with [^{3}H]-thymidine (0.76 TBq/mmol [6-^{3}H]-thymidine, Amersham) for 12 h under sterile conditions and continuous agitation. The total activity of the cell suspension reached 3.0 kBq. Labeled cells were then added to 10 mL of peripheral blood samples and processed in density gradient medium as described above. After centrifugation of the gradient tubes at 450*g* for 20 min, 500-μL fractions of the gradient were poured into scintillation vials containing 5 mL of lysis buffer (75 mmol/L NaCl, 25 mmol/L Na_2EDTA, pH 8.0). [^{3}H]-thymidine β activity was mea-