both MN and NMN are stable for at least 1 month when frozen at $-20^\circ C$, also in the absence of a reducing agent.

On the basis of the present data, we give the following recommendations for handling, storage, and shipment: Blood should be kept at 4°C and must be centrifuged within 6 h. Even in the absence of a reducing agent, plasma can be kept at 4°C for 3 days without appreciable degradation and shipped if this does not take more than the same time span. Storage or shipment of longer duration must be at $-20^\circ C$ or lower.

References

Use of Magnetic Beads for Plasma Cell-free DNA Extraction: Toward Automation of Plasma DNA Analysis for Molecular Diagnostics, Christine Stemmer,1,2 Michèle Beau-Faller,1,2 Ewan Pencrach,1,2 Eric Guerin,1,2 Anne Schneider,1,2 Didier Jaquin,2 Elizabeth Quoix,3 Marie-Pierre Gaub,1 and Pierre Oudet1 (1 Service de Biochimie et Biologie Moléculaire, Hôpital de Hautepierre Hôpitaux Universitaires de Strasbourg, 67000 Strasbourg, France; 2 Service de Chirurgie Urologique and 3 Service de Pneumologie Lyautéy, Hôpitaux Universitaires de Strasbourg, 67000 Strasbourg, France; address correspondence to this author at: Service de Biochimie et Biologie Moléculaire, Hôpital de Hautepierre, Hôpitaux Universitaires de Strasbourg, 67098 Strasbourg cedex, France; fax 33-388127539, e-mail christine.stemmer@wanadoo.fr)

Urine, breast milk, plasma, and serum have been shown to contain cell-free DNA (1–7). For plasma DNA detection, several recent studies addressed the need for careful evaluation and standardization of preanalytical processes (8–12). Key problems appear, such as possible contamination of plasma by white blood cells; the generally low and variable amount of circulating DNA, making extraction/quantification difficult and time-consuming; poor DNA quality; and the presence of PCR inhibitors. In any case, automation of DNA extraction, which is a prerequisite for introduction of these diagnostic approaches in clinical laboratories, is difficult to achieve because of the volumes of plasma necessary to get sufficient DNA.

In this study (summarized in Fig. 1), we propose a new semiautomated, time-saving process for extraction of plasma cell-free DNA that provides high yields and is suitable for PCR amplification.

Two 5-mL blood samples from each of 23 patients being monitored for lung ($n = 19$) or colon cancer ($n = 4$) and 20 healthy controls were collected in EDTA-containing tubes. Informed consent was obtained for each. Blood was centrifuged at 800g for 10 min, and the collected plasma was transferred to a 15-mL BD Falcon™ polypropylene tube for an additional centrifugation step of 10 min at 1500g to remove any remaining leukocytes and platelets. An equal volume of 2× concentrated proteolytic buffer (2×: 20 mmol/L Tris-HCl, 50 mmol/L EDTA, 200 mmol/L NaCl, 10 g/L sodium dodecyl sulfate, and 400 mg/L proteinase K) was added to the plasma, mixed, and incubated 1 h at 37°C. After digestion, plasma samples were concentrated by centrifugation at room temperature (to avoid sodium dodecyl sulfate precipitation) for 20 min at 2600g in Amicon Ultra-15 filtration devices (Millipore). The concentrated plasma samples were stored at $-20^\circ C$ until DNA extraction (Fig. 1, step 1).

DNA was extracted with use of KingFisher silicate magnetic beads and a KingFisher ML robotic magnetic particle processor (ThermoLifeSciences) according to the manufacturer’s protocol. DNA was quantified by fluorometry (Fluoroskan; ThermoLifeSciences) with Pi-cogreen reagent (Molecular Probes) as recommended by the manufacturer. For plasma DNA extraction, slight modifications were introduced in the protocol: up to 300 µL of concentrated plasma was mixed with 950 µL of lysis buffer together with 80 µL of magnetic beads, and DNA was eluted in Tris-EDTA for 20 min. For comparison purposes, plasma DNA was extracted with use of the QIAamp DNA Midi reagent set (Qiagen), according to the

![Fig. 1. Experimental design.](image)

Step 1, plasma treatment; step 2, DNA extraction; step 3, analysis of plasma DNA quality by PCR.
“blood and body fluid” protocol. The following pipetting steps were performed by the Multiprobe II workstation (Perkin-Elmer): pipetting of KingFisher reagents, transfer of plasma samples into lysis buffer, transfer of extracted DNA to storage tubes, DNA dilutions for quantification, and distribution of Picogreen in 96-well plates. Special care was taken for the complete homogenization of the samples by up-and-down pipetting of the plasma with lysis buffer by the Multiprobe II robot. The manual steps were reduced to the transfer of tube-strip trays to the particle processor and the Picogreen plate to the fluorometer (Fig. 1, step 2).

To evaluate the quality of the plasma DNA, we subjected samples to microsatellite amplification using fluorescent primers, sequencing [both analyzed on an ABI Prism 3100 (Applied Biosystems); see the detailed method in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem. org/content/vol49/issue11/], and real-time PCR on the LightCycler (Roche; Fig. 1, step 3).

We digested 43 plasma samples from cancer patients (n = 23) and healthy controls (n = 20) with proteinase K and concentrated them in Amicon Ultra filtration devices. Depending on the initial volume of plasma, the concentration factor varied from 4 to 10. For all cases, 2 mL of plasma could be reduced to 150–250 μL (n = 15). When 4 mL of plasma was used (n = 27), the final concentrated volume was ≤500 μL for 15 cases and between 600 μL and 1 mL for 12 samples. No concentration could be achieved without proteinase K incubation, suggesting that the high proteinase K concentration (final concentration, 200 mg/L) allows efficient digestion of proteins and avoids clogging of the Amicon filter. No DNA was detected in the filtrates from the Amicon devices (data not shown).

To analyze DNA extraction yields after plasma concentration, we divided plasma samples from 16 cancer patients into three 1-mL aliquots: one aliquot was subjected to Qiagen extraction without concentration, one was concentrated and extracted on Qiagen columns, and one was concentrated and extracted with KingFisher magnetic beads. As shown in Table 1, extraction of concentrated plasma samples by the KingFisher method gave higher yields than the classic Qiagen protocol (P = 0.009). We observed no significant difference (P = 0.421) between the Qiagen method for concentrated samples and the KingFisher method. Overall these results suggest that the high yields obtained with the KingFisher method may be attributable to both the digestion/concentration step and use of magnetic beads.

To evaluate the background concentrations of plasma DNA obtained with magnetic beads, we extracted plasma DNA from 20 healthy controls. The amount of DNA extracted was 3–22 ng/mL of plasma; this range is in agreement with several studies (13–15). For the plasmas collected from cancer patients, we obtained 13–127 ng/mL. No higher values were observed, probably because these patients were not at initial diagnosis but under treatment (15). Special attention was paid to the already noticed importance of the centrifugation steps before extraction. We designed a two-step centrifugation protocol: low-speed centrifugation (800g) for whole blood, followed by higher speed centrifugation (1500g) for plasma to avoided lysis of leukocytes at the higher speed (11) and insufficient cell clearance at the lower speed. The lower values that we obtained may be attributable to efficient elimination of contaminating leukocytes by centrifugation at 800g and 1500g.

Finally, to assess the extraction capacity of magnetic beads, we prepared leukocytes from an unique buffy coat and added different volumes of these cells to 1 mL of a control plasma. These mixtures were then digested, concentrated, and extracted according to the KingFisher protocol. As a control, we extracted the same volumes of leukocytes kept in phosphate-buffered saline. Extraction yields were estimated by use of Picogreen. In both groups we found similar quantities of DNA (Table 2 in the online Data Supplement), showing that the whole process does not affect the recovery of the added material and allows purification of high amounts of DNA (up to 2 μg).

To limit human intervention, the magnetic bead extraction and quantification steps were automated. The complete process for extraction and quantification of 30 plasma samples was performed within 2.5 h. The absence of contamination was checked by the absence of additional detectable peaks after microsatellite amplification. To confirm the absence of contamination with a more sensitive approach, we set up a PCR on the LightCycler using a chromosome Y marker (Fig. 2A in the online Data Supplement), showing that the whole process does not affect the recovery of the added material and allows purification of high amounts of DNA (up to 2 μg).

### Table 1. Comparison of DNA yields obtained with three pretreatment/extraction methods. 

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Qiagen with no sample concentration</th>
<th>Qiagen with sample concentration</th>
<th>KingFisher</th>
</tr>
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<tbody>
<tr>
<td>1</td>
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<td>21</td>
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</tr>
<tr>
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<tr>
<td>3</td>
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</tr>
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<tr>
<td>6</td>
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<td>74</td>
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<td>16</td>
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<td>24</td>
<td>20</td>
</tr>
<tr>
<td>Range</td>
<td>0–76</td>
<td>9–128</td>
<td>13–127</td>
</tr>
</tbody>
</table>

Reference: P = 0.126; Reference²: P = 0.009

² Statistical significance for comparison among the three groups was calculated by Friedman ANOVA (P < 0.001).

² Statistical significance was calculated by bilateral Dunett ANOVA with:

² Qiagen/no concentration group as reference; ² KingFisher group as reference.
We amplified 20 female DNA samples extracted with the automated process in experiments also including male samples. We detected no amplification of male DNA at 45 PCR cycles based on the chromosome Y marker in these female samples (Fig. 2B in the online Data Supplement), whereas they all amplified in a control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR (data not shown). Systematic quality controls such as cultured cells or control leukocytes (buffy coat) for extraction control and a known DNA for Picogreen quantification were used. As an example, for these controls, for 12 automated extractions we obtained mean (SD) DNA concentrations of 1.4 (0.29) ng/µL after extraction of 20,000 cultured cells, 14 (3.8) ng/µL for 20 µL of buffy coat, and 96 (16) ng/µL for normal leukocyte DNA as quantified by Picogreen from a stock solution of 100 ng/µL, as determined by spectrophotometry. The controls were added once per automated process.

The quality of plasma DNA obtained with our protocol was first assessed by amplification of eight microsatellite markers (PCR products length, 100–250 bp in eight different PCR reactions) and comparison with DNA extracted from leukocytes. For comparison of PCR profiles, all DNA was quantified with Picogreen and normalized for PCR to 0.125 ng/µL (input of 0.5 ng per PCR reaction). Fig. 3A in the online Data Supplement summarizes the very close profiles routinely obtained with similar peak heights and peak shapes. We next sequenced the K-ras gene in plasma DNA from eight patients and compared the sequences with a control DNA extracted from normal leukocytes. We obtained high-quality sequences with 1 ng of plasma DNA as exemplified in Fig. 3B in the online Data Supplement. The mean raw signal of the sequences we obtained was 250 for plasma samples and in our protocol controls (data not shown).

Finally, we subjected six plasma DNA samples and one DNA control, all extracted with magnetic beads and quantified by Picogreen, to SYBR Green I PCR. A 110-bp GAPDH product was used to compare threshold cycles (Ct) for these samples based on a DNA input of 3 ng/PCR. We obtained a Ct value of 20 for the control DNA, whereas plasma DNA from different patients gave Ct values of 22–25. The most likely hypothesis for these differences is partial physiologic degradation of cell-free circulating DNA (16), meaning that not all extracted/quantified DNA can be amplified with the same efficiency. This phenomenon cannot be visualized in PCR at the plateau phase, similar to sequencing or microsatellite PCR. Moreover, the degradation may vary from patient to patient. Finally, these higher Ct values could also be attributable to chromosomal deletions in the genomic region bearing the GAPDH gene.

The protocol and the results described above show that concentrations of plasma samples allow miniaturization and automation of DNA extraction. The use of magnetic beads allows “fishing-out” of high-quality DNA with yields comparable to and even better than those obtained with commonly described techniques. The described process could also be used for urinary DNA extraction (data not shown). Despite additional steps (proteolytic digestion and concentration), the whole protocol is easily scalable and more reliable than manual handling of columns. Moreover, the use of a pipetting workstation and systematic use of controls considerably decreased error rates, and precision and reproducibility were much higher. Combined with automated PCR preparation and analysis, the process we describe here may be a first step toward rationalization of plasma DNA detection in a routine clinical setting.

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References

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