Journal of Thrombosis and Haemostasis, **1**: 987–991

ORIGINAL ARTICLE

Logistics and quality control for DNA sampling in large multicenter studies

R. J. NEDERHAND,*† S. DROOG,* C. KLUFT,* M. L. SIMOONS,† AND M. P. M. DE MAAT* on behalf of the investigators of the EUROPA trial

*Gaubius Laboratory, TNO Prevention and Health, Leiden, the Netherlands; and †Department of Cardiology, Erasmus University, Rotterdam, the Netherlands

Summary. To study associations between genetic variation and disease, large bio-banks need to be created in multicenter studies. Therefore, we studied the effects of storage time and temperature on DNA quality and quantity in a simulation experiment with storage up to 28 days frozen, at 4 °C and at room temperature. In the simulation experiment, the conditions did not influence the amount or quality of DNA to an unsatisfactory level. However, the amount of extracted DNA was decreased in frozen samples and in samples that were stored for >7 days at room temperature. In a sample of patients from 24 countries of the EUROPA trial obtained by mail with transport times up to 1 month DNA yield and quality were adequate. From these results we conclude that transport of non-frozen blood by ordinary mail is usable and practical for DNA isolation for polymerase chain reaction in clinical and epidemiological studies.

Keywords: clinical trial, DNA isolation, quality control.

Introduction

Recent advances in molecular technology and molecular genetics are increasing our knowledge of the DNA sequence and its variants. With this immense amount of data on human genetic variation that is available now, the comprehension of these data becomes a challenging task. One field of interest is the dissection of the genetic architecture of complex diseases, and for that purpose the analysis of genetic variation can be considered as the most fundamental approach. Another area of interest is pharmaco-genetics, the prediction of the response of individuals to treatment. For these reasons, many clinical and epidemiological studies now include the analysis of the role of genetic variation with regard to the risk of disease or the response to treatment.

Correspondence: Moniek P. M. de Maat, TNO Prevention and Health, Gaubius Laboratory, Department of Vascular and Connective Tissue Research, PO Box 2215, 2301 CE Leiden, the Netherlands.
Tel.: +31 0 71 518 1502; fax: +31 0 71 518 1904; e-mail: mpm.demaat@pg.tno.nl

Received 10 June 2002, accepted 6 January 2003

In order to study associations between genetic polymorphisms and the risk of disease with sufficient statistical power, large DNA bio-banks need to be created. A DNA bio-bank can be defined as a collection of stable genomic DNA, which enables investigators to perform genetic studies for a long time. DNA can be extracted from almost all body tissues [1–3] and body fluids [4]. Blood is usually the biological material of choice because it is often available in clinical and epidemiological studies and well-established protocols for DNA extraction with a good yield are available [5].

Some guidelines on the optimal storage of blood for DNA extraction have been reported [6]. For maximum DNA yield and quality, these guidelines advise storage and shipment at 4 °C for a maximum of 8 days after blood collection, while freezing should only be considered for long-term storage. In addition, by preference freezing of the leukocyte pellet should be performed instead of whole blood freezing, and storage at room temperature should be considered only when DNA is extracted within 24 h after blood collection [6]. Only for small, single-center studies can it be expected that these guidelines can be fulfilled. For most large studies it is not possible to fulfill these ideal circumstances, because multiple centers participate in these studies, many of which do not have the experience or facilities necessary for optimal DNA extraction. This usually means that blood samples need to be transported to a central facility. In addition, batch-processing and central analysis of samples is an important cost-reducing procedure. The use of a central facility thus implies that shipment and storage of blood will take place over a prolonged period of time and under various conditions.

Furthermore, many clinical and epidemiological studies include participants from many countries, and in this situation the period between blood collection and DNA extraction is often more than 1 week. Hence, it follows that these samples will be affected to some degree by the storage conditions and the time between blood collection and extraction of the DNA.

To document the effects of conditions occurring during collecting, transport and storage of blood on DNA quality and quantity, we studied several quality and quantity aspects of DNA that was extracted from blood that was stored at room temperature (RT), 4 °C, and −20 °C, for different prolonged periods of time with a maximum of 1 month. Based on these results a logistic procedure and quality program were set up for the creation of a large DNA bio-bank and the effect of DNA

transport and storage conditions on DNA quality and quantity was evaluated in a large multicenter clinical study.

Materials and methods

Simulation experiments

Blood samples and storage conditions Blood (45 mL) was collected from eight healthy volunteers in K3-EDTA tubes. Blood was pooled per individual and divided into aliquots of 2 mL. Aliquots were placed at RT, 4 °C, or were frozen at −20 °C and stored for 0, 3, 7, 14, 21, or 28 days. In addition, severe mechanical transport circumstances were mimicked by harshly treating whole blood for 24 h at RT on a Heidolph head-over (15 rounds min^{-1}), vortex (500 rotations min^{-1}), and by transporting blood samples by ordinary mail.

The degree of hemolysis was determined by observing the color of the plasma after sedimentation of the erythrocytes. The size of the leukocyte pellet was determined by observing the leukocyte pellet after the centrifugation step, which follows the last lysis step. The amount of hemolysis and the size of the pellet were registered semiquantitatively. All observations and analyses were carried out by one trained technician to reduce interindividual interpretation errors and deviations in the DNA extraction procedure. All procedures and observations were recorded according to ISO-9001 standards.

DNA was extracted according to the salting-out procedure published by Miller, with brief modifications to adapt the protocol to the small volume of blood [5]. Briefly, erythrocytes were lyzed by mixing 2 mL blood with 10 mL erythrocyte cell lysis buffer (3 M NH_4Cl, 240 mM $KHCO_3$, 20 mM EDTA) followed by a 20-min incubation on ice and centrifugation ($3000 \times g$, 10 min) until the leukocyte pellet was off-white. The pellet was dissolved in 1 mL nucleus buffer (75 mM NaCl, 25 mM EDTA, pH 7.4) containing 0.75% SDS and 0.25% pronase (Roche Diagnostics, Almere, the Netherlands) for digestion until the leukocyte pellet was completely dissolved. If the leukocyte pellet was not completely dissolved after 48 h extra pronase was added. Proteins were precipitated by mixing with 336 μL 8 M ammonium acetate. After centrifugation ($4000 \times g$, 15 min), the mixture was transferred to one volume of isopropanol to precipitate the DNA. DNA was washed with 70% ethanol and rehydrated in 75 μL TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.4) and stored at 4 °C.

Variables for DNA quality

The amount of DNA was calculated from the optical density (OD) at $\lambda = 260$ nm using the molar extinction coefficient $\epsilon = 20$ mL mg^{-1} cm^{-1}. Contamination with proteins or RNA was determined by calculating the ratio of the OD at $\lambda = 260$ nm and $\lambda = 280$ nm. Ideally, this ratio should be between 1.7 and 2.0 [7]. The isopropanol fraction was centrifuged ($13\,000 \times g$, 10 min), the pellet dissolved in TE buffer and analyzed by agarose gel electrophoresis to examine DNA loss and DNA degradation during the isolation procedure. The presence of DNase was determined by visualizing the DNA on agarose gel after an overnight incubation at 37 °C. When DNase is absent, a clear high molecular band is observed, while a smear indicates the presence of DNase. In order to test the suitability of the DNA for polymerase chain reaction (PCR) a small 200 base and a large 1.2-kb fragment were amplified using routine protocols [8,9].

Multicenter study

A logistic procedure and a quality program for the creation of a DNA bio-bank were established within the EUROPA trial (EUropean trial on the Reduction Of cardiac events with Perinodopril in stable coronary Artery disease), a large multicenter study including more than 12 000 patients from 527 centers in 22 European countries [10]. In a trial with the size and complexity of the EUROPA study it is essential that the collection procedure is very simple. Therefore, each sample was sent to a central laboratory in a protective box by ordinary mail. Upon arrival at the central laboratory, the erythrocytes were lyzed and the leukocyte pellet was stored at −20 °C. The effect of transport time on the DNA quality and quantity in the EUROPA trial was tested in a selection of 61 blood samples representing a wide range of transport times (0–28 days) and countries. Plasma color, size of the leukocyte pellet, OD_{260}/OD_{280} ratio, DNase test, and PCR amplification were determined as described above.

Results

Simulation experiment

Storage at room temperature. The lysis of the erythrocytes increased with time and after 14 days the erythrocytes of blood samples, that were stored at RT, were severely lyzed. This affected the color of the leukocyte pellet, which gradually changed from white to brown. Therefore, for the samples that were stored for 1 week, the lysis step of the erythrocytes had to be repeated twice. In addition, extra pronase had to be added to all samples stored for ≥7 days and extra pronase needed to be added to complete the full lysis of the leukocyte pellet. The incubation time had to be extended to 1 week to complete the lysis of all blood samples stored for 21 days or longer. In all samples except for one, DNA precipitates were present. These DNA precipitates became visibly smaller after 14 days. The amount of DNA that could be isolated on day 0 was 43.1 μg mL^{-1} blood (SD 11.2). The amount of DNA that could be isolated from blood samples stored for a prolonged period decreased in time (Fig. 1). After 28 days (17.8 μg mL^{-1} blood; SD 10.9) less a half the DNA amount of day 0 was isolated. On agarose gel it could be seen that all DNA was of high molecular weight (Fig. 2). No DNA could be isolated from one blood sample after 28 days and one sample ($t = 28$) showed minimal DNase activity. The mean DNA/protein absorbance ratio ($OD_{260/280}$) for blood samples stored for ≥3 days was 1.7 (SD 0.1) (Fig. 3). It was possible to amplify the small and the large PCR products from all DNA samples.

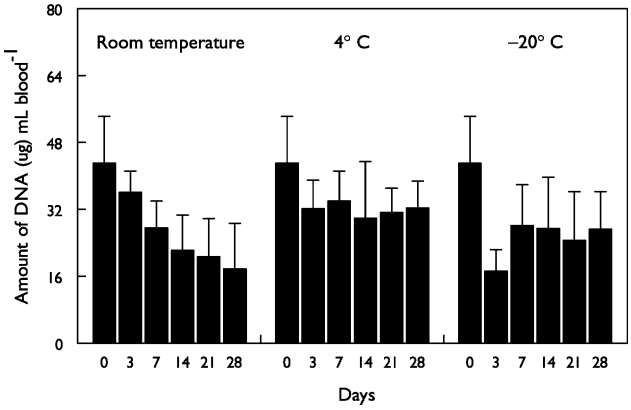

Fig. 1. Mean amount (SD) of DNA isolated from blood samples stored at room temperature, 4 °C, and −20°C, at different time points.

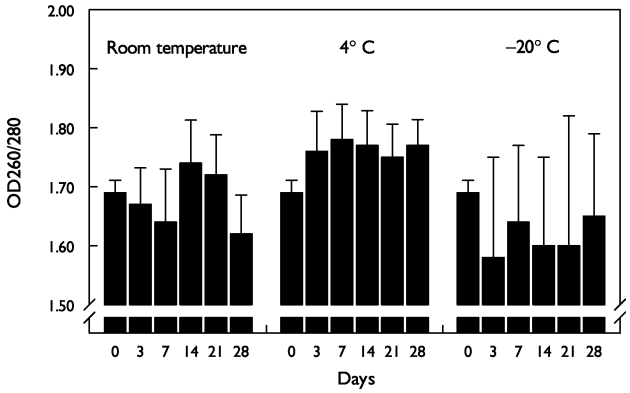

Fig. 3. Mean protein/DNA ratio (SD) as a measure for the DNA quality from blood samples stored at room temperature, 4 °C, and −20°C, at different time points.

Storage at 4 °C. As expected, blood stored at 4 °C showed less hemolysis. For blood samples stored for ≥21 days, the leukocyte pellets had to be washed with erythrocyte buffer twice, and extra pronase and an extended incubation time were necessary to dissolve the leukocyte pellet completely. In contrast to blood samples stored at RT, the amount of DNA extracted was stable at all time points (24.4 μg mL^{-1} blood) (see Fig. 1). In addition, the mean DNA/protein absorbance ratio ($OD_{260/280}$) from samples stored for ≥3 days was 1.8 (SD 0.1, Fig. 3). From all DNA samples it was possible to amplify the short and the long fragments by PCR and no DNase activity was observed in any of the samples.

Storage at −20 °C. DNA could be extracted from all samples that were frozen and stored at −20 °C. However, the quality of this DNA, as reflected by the $OD_{260/280}$ ratio, was very variable among the samples (see large SD in Fig. 3). The amount of DNA that could be isolated was comparable to the amount in blood samples that were stored at RT for 14–21 days (24.6 μg mL^{-1} blood (SD 10.3); see Fig. 1). On all samples both PCR reactions could be performed and no DNase activity was observed.

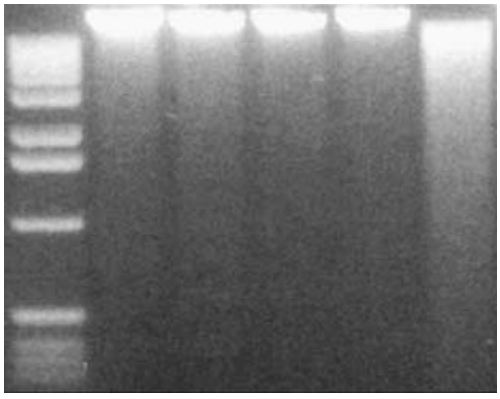

Fig. 2. Representative result from a DNase test showing genomic DNA obtained from blood samples stored for 28 days at room temperature. The first lane contains a 1kb base pair ladder, subsequently 4 samples with a high molecular band, and a positive control (DNase activity present) are shown.

Mechanical transport conditions. Samples that underwent severe mechanical transport conditions where the blood was harshly treated for 24 h on a Heidolph head-over (15 rounds min^{-1}), vortex (500 rotations min^{-1}), and where blood samples were transported by ordinary mail resulted in severe hemolysis of the blood samples comparable to samples that were frozen. In addition, the DNA concentration (18.3 μg mL^{-1} blood (SD 9.7) and highly variable protein/DNA ratio were comparable to blood samples stored at −20 °C.

Logistic data from the EUROPA trial

A total of 11 241 blood samples from 22 countries have been received in the EUROPA trial. The average time between blood

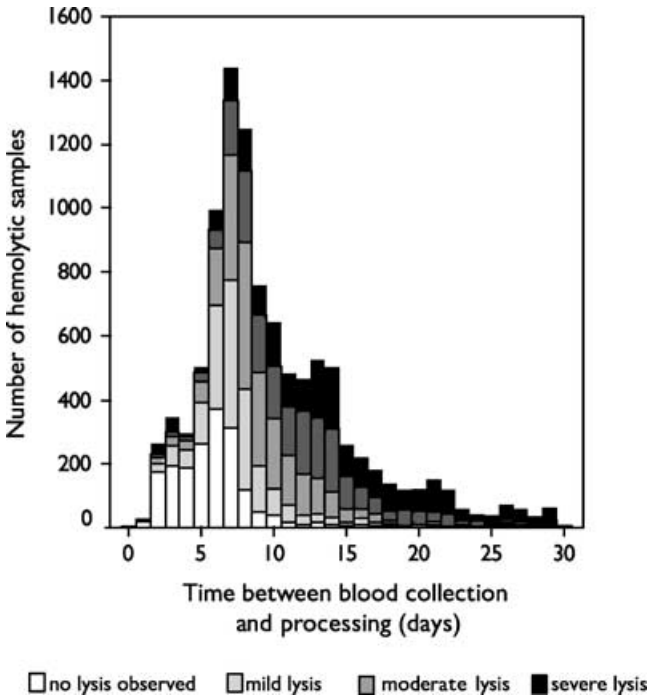

Fig. 4. Number of severely haemolysed blood samples that were received in the EUROPA trial in time.

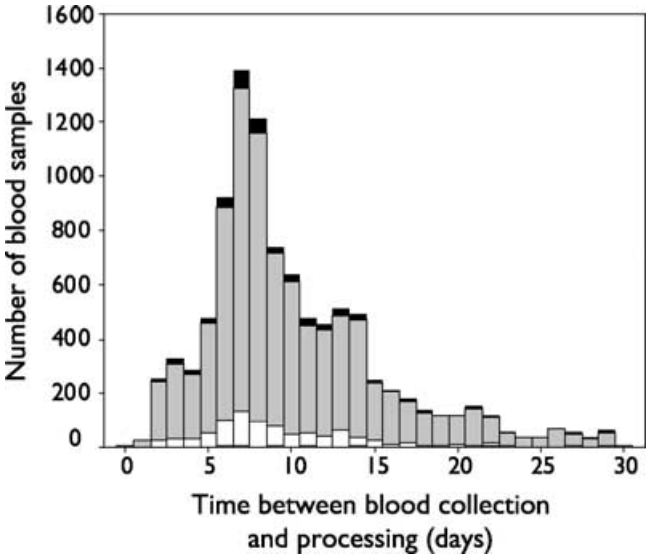

Fig. 5. Leukocyte pellet size after the erythrocyte lysis step in blood samples that were received from the EUROPA trial in time. □ = small pellet; ⊠ = medium sized pellet; ■ = large pellet.

collection and processing was 10.1 (SD 8.4) days (see Fig. 4). The period of transport covered 2 years including the hot summer of 1999. As expected, many of the blood samples were partially lyzed (62%) (Fig. 4). In 93% of the samples the leukocyte pellet size was not visibly decreased. In addition, the amount of isolated DNA was independent of the pellet size. In a selection of 61 blood samples from all countries, no association was found between the leukocyte pellet size and the amount of DNA that could be isolated, and also no association between leukocyte pellet size and the $OD_{260/280}$ ratio. We observed no effect of the region of origin (with varying environmental temperatures) and the amount of DNA that could be isolated. In addition, in none of the DNA samples was DNase activity visible on agarose gel. All DNA samples showed up as high-molecular DNA, with no degradation products visible. We were able to perform a 1.2-kb DNA amplification using PCR, suggesting that all DNA obtained could be used for genetic testing.

Discussion

High expectations have been placed on the analysis of genetic variants and their role in disease. Therefore, numerous clinical studies, which include this type of analysis, require standardization and definition of laboratory organization. Recent publications have tried to fill this gap, but only for the laboratory assays, not for the logistics before the DNA isolation procedure and DNA storage [11,12].

Our simulation experiments show that DNA can be extracted with sufficient yield (approx. 5 μg mL^{-1} blood) and quality from blood samples that are stored at room temperature, 4 °C, or are frozen at −20 °C for a maximum of 1 month.

The extracted DNA may be amplified by PCR and is usable for genotyping analysis. These results suggest that the storage and transport conditions for blood do not need to be very strict. The only two requirements are measures to remove hemoglobin contamination in the leukocyte pellets by reinforcing washing, and that pronase treatment is also reinforced.

We defined a procedure for the multicenter trial without freezing the samples, but simply transporting the blood at environmental temperature. We then used the DNA isolation procedure of Miller, which is routinely used by many laboratories. However, we expect that other isolation methods will give comparable results. Since there is always a great difference between well-controlled laboratory conditions and real-life conditions, we also determined the relationship between DNA quality and quantity in this large multicenter trial: the EUROPA trial, which is conducted over 527 centers in 22 countries.

We saw comparable results for the samples studied for the simulation study and the EUROPA trial. In the trial, blood samples were transported by ordinary mail under varying temperatures over a period of 2 years, including the warm summer of 1999, and under all transport conditions. We were able to extract sufficient quantities of DNA (17.8 μg (SD 10.9) mL^{-1} blood) from blood samples that were processed for up to 28 days after collection and stored at room temperature using the adapted procedure. We even observed that the severe lysis of the erythrocytes in many of the old samples still resulted in DNA of a quality that allowed for PCR amplification of both large and small DNA fragments, and could thus be considered suitable for many genetic analyses.

Prolonged storage causes lysis of the erythrocytes, which can be visualized by observing the color of the plasma. Hemoglobin is an inhibitor of the Taq polymerase enzyme in the PCR reaction and therefore much effort must be made to reduce the amount of hemoglobin in the leukocyte pellet [13,14]. In our experiments the leukocyte pellets often needed to be washed twice to reduce the amount of cell remnants in the pellet. Although erythrocytes lyze more easily than leukocytes, when there is severe hemolysis, it is expected that some of the leukocytes have lyzed as well, which will result in loss of DNA during the leukocyte harvesting step in DNA extraction. In the end, this will result in a reduced DNA yield. Indeed, in the freezing or thawing step rapid hemolysis occurred and less DNA could be extracted, independently of the time the sample was stored at −20 °C. This observation is in agreement with other studies [6,15,16]. In addition, one study showed that an increased protein contamination rate was found to affect the efficiency of a PCR reaction and the reliability of the restriction analysis pattern, but we did not experience that in our study [17]. To reduce these lysis-related problems our advice is not to freeze whole blood but to perform the erythrocyte-lysis step as soon as possible after blood collection, and if required to store the leukocyte pellet for later DNA extraction. This procedure always gives better yields when blood is stored at 4 °C and for the first week when blood is kept at ambient temperature.

Hemolysis can be considered a marker of cell disruption. This implies not only that due to this disruption DNA is lost, but that the contents of the cells may contaminate the DNA as well. DNA-degrading proteins and hemoglobin both affect the amount and quality of the DNA extracted. Therefore, most studies advise against storage as unprocessed whole blood. In our

study this advice cannot be supported when storage is at 4 °C or for the first week at room temperature. On the contrary, a larger reduction in yield was observed in blood samples that were stored in a frozen condition. This observation is in agreement with other studies [6,15–18]. Few studies advise against the freezing of whole blood, but this should be supported because lysis of the erythrocytes will occur and contribute to DNA degradation. In this case, storage in processed form, after the first lysis of the erythrocytes, might be an alternative.

In many studies that include the analysis of genetic variation, there is also a wish to analyze plasma levels. From many of the blood samples that are processed using the procedure we suggest in this paper, the quality of the collected plasma will be insufficient for protein analysis.

In conclusion, storage and shipment of whole blood samples at 4 °C yields optimal DNA quantity and quality. However, storage of whole blood at room temperature provides a simple and usable alternative for large multicenter studies. Freezing of whole blood samples should only be considered in the case of prolonged storage (several months).

Acknowledgements

The authors thank Institut de Recherches Internationales SERVIER (IRIS) for an unconditional grant that allowed us to perform this study, and Sükran Demir and René Westhof for their help with blood sample handling and DNA isolation in the EUROPA trial.

References

1 Hanson CA, Kersey JH. A modified method of DNA extraction from peripheral blood and bone marrow specimens. *Am J Hematol* 1988; **28**: 176–80.
2 Sepp R, Szabo I, Uda H, Sakamoto H. Rapid techniques for DNA extraction from routinely processed archival tissue for use in PCR. *J Clin Pathol* 1994; **47**: 318–23.
3 Thomson DM, Brown NN, Clague AE. Routine use of hair root or buccal swab specimens for PCR analysis: advantages over using blood. *Clin Chim Acta* 1992; **207**: 169–74.
4 Mage RG, Harindranath N, Hole NJ, Newman B, Perez R, Alexander CB, Young-Cooper GO. Genetic analyses of restriction fragment length polymorphisms using high molecular weight DNA from sperm or lymphocytes embedded in agarose. *Gene Anal Tech* 1988; **5**: 94–6.
5 Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucl Acids Res* 1988; **16**: 1215.
6 Gomma AH, Fox KM. The EUROPA trial: design, baseline demography and the status of the substudies. *Cardiovasc Drugs Ther* 2001; **15**: 169–79.
7 Farkas DH, Kaul KL, Wiedbrauk DL, Kiechle FL. Specimen collection and storage for diagnostic molecular pathology investigation. *Arch Pathol Lab Med* 1996; **120**: 591–6.
8 Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning, A Laboratory Manual.* Cold Spring Harbor: Cold Spring Harbor Press, 1989.
9 Cascorbi I, Drakoulis N, Brockmoller J, Maurer A, Sperling K, Roots I. Arylamine N-acetyltransferase (NAT2) mutations and their allelic linkage in unrelated Caucasian individuals: correlation with phenotypic activity. *Am J Hum Genet* 1995; **57**: 581–92.
10 Edberg JC, Wainstein E, Wu J, Csernok E, Sneller MC, Hoffman GS, Keystone EC, Gross WL, Kimberly RP. Analysis of FcgammaRII gene polymorphisms in Wegener's granulomatosis. *Exp Clin Immunogenet* 1997; **14**: 183–95.
11 Bladbjerg EM, Gram J, Jespersen J, de Maat MPM. Internal quality control of PCR-based genotyping methods in research studies and patient diagnostics. *Thromb Haemost* 2002; **87**: 812–6.
12 Neumaier M, Braun A, Wagener C. Fundamentals of quality assessment of molecular amplification methods in clinical diagnostics. International Federation of Clinical Chemistry Scientific Division Committee on Molecular Biology Techniques. *Clin Chem* 1998; **44**: 12–26.
13 Busch MP, Wilber JC, Johnson P, Tobler L, Evans CS. Impact of specimen handling and storage on detection of hepatitis C virus RNA. *Transfusion* 1992; **32**: 420–5.
14 Panaccio M, Lew A. PCR based diagnosis in the presence of 8% (v/v) blood. *Nucl Acids Res* 1991; **19**: 1151.
15 Madisen L, Hoar DI, Holroyd CD, Crisp M, Hodes ME. DNA banking: the effects of storage of blood and isolated DNA on the integrity of DNA. *Am J Med Genet* 1987; **27**: 379–90.
16 Lahiri DK, Schnabel B. DNA isolation by a rapid method from human blood samples: effects of MgCl2, EDTA, storage time, and temperature on DNA yield and quality. *Biochem Genet* 1993; **31**: 321–8.
17 Cushwa WT, Medrano JF. Effects of blood storage time and temperature on DNA yield and quality. *Biotechniques* 1993; **14**: 204–7.
18 Visvikis S, Schlenck A, Maurice M. DNA extraction and stability for epidemiological studies. *Clin Chem Lab Med* 1998; **38**: 551–5.