Klin. Biochem. Metab., 14 (35), 2006, No. 1, p. 21–24.

Comparison of various methods used for extraction of cell-free genomic DNA from human plasma

Beránek M., Vlčková J., Hypiusová V., Živný P., Palička V.

Institute of Clinical Biochemistry and Diagnostics, Charles University in Prague, Faculty of Medicine in Hradec Králové and University Hospital Hradec Králové

SUMMARY

Objective: For many years it has been known that free DNA of various origin is released into peripheral blood. An extremely short halftime of plasma DNA and many pre-analytical variables may affect the number of free DNA molecules in plasma specimens. The goal of our study was to compare six nucleic acids extraction protocols to define the optimal pre-analytical approach for further molecular analyses based on plasma DNA.

Material and Methods: Extraction of nucleic acids (NA) from 900 μl of the aliquoted pool were performed by phenol/chloroform DNA extraction method after SDS and proteinase K lysis without or with addition of a polyacryl DNA carrier before lysis, salting-out protein precipitation, DNA extraction with Qiagen spin microcolumns without or with the carrier addition, and a guanidinium isothiocyanate-based RNA extraction method. Extracts were characterized spectrophotometrically, electrophoretically, and according to the PCR amplificability.

Results: Spectrophotometric analysis shows that the guanidinium and phenol/chloroform extraction methods provide the highest yields of nucleic acids. The mean yield values from 900 μl of plasma were 2.4 μg and 1.4 μg, respectively. The lowest yield was reached using commercial microcolumns without (0.4 ± 0.1 μg) or with the DNA carrier (0.2 ± 0.1 μg). However, fluorescence intensities of the extracts obtained by the phenol/chloroform procedure (with addition of the carrier) and both the microcolumn-based methods manifested very similar values. Examining by crossing-point analysis, microcolumn methods revealed the lowest number of cycles necessary to reach the exponential part of amplification. No fluorescence was detected in the guanidinium RNA extracts.

Conclusion: Our data report that microcolumn extraction techniques are very suitable for preparation of plasma DNA specimens. Addition of the polyacryl DNA carrier does not seem to significantly influence the extraction yield.

Key words: DNA; genomic DNA, free-cell DNA, extraction, nucleic acids, plasma, PCR, pre-analytical phase, spectrophotometry, electrophoresis.

For many years it has been known that free DNA of various origin is released into peripheral blood. Experimental studies reported that the excess of DNA circulating in plasma was positively associated with presence of fetuses, tumors, or viruses in the body [1–3]. A significant increase of plasma DNA was also found in trauma patients early after injury or in transplanted subjects when the graft was being rejected [4, 5]. The relationship between free-cell DNA and pathological processes has revealed a possible way how to define new biological markers for non-invasive cancer or prenatal diagnostics [6, 7].

Real time PCR-based methods are able to detect and quantify extremely small amounts of nucleic acids. Recently published papers show that fetal plasma DNA is even amplifiable at concentrations of 1–5 genome-equivalents/ml of blood [8]. However, an extremely short halftime of plasma DNA [9] and many pre-analytical variables (type of blood anticoagulant, transport conditions, delayed plasma separation, centrifugation force, freezing-thawing, storage, etc.) may affect the number of free DNA molecules in plasma specimens. Also, additional plasma modifications (filtration or fractionation on gradients) and adaptations of extraction protocols significantly affect the quantity and variation of plasma DNA [10].

The goal of our study was to compare six nucleic acids extraction protocols to define the optimal pre-analytical approach for further molecular analyses based on plasma DNA.

Material and Methods

A blood pool was made from venous EDTA blood specimens of ten healthy individuals. Plasma was separated by centrifugation at 1600 g for 10 min within 3 h after collection, pooled, divided into thirty 1-ml aliquots, and stored at –70 °C prior to analysis. Cell lysis, protein denaturation, and isolation of nucleic acids (NA) from 900 μl of the aliquoted pool were performed by six different extraction procedures:

a) phenol/chloroform DNA extraction method after SDS and proteinase K lysis [11];
b) phenol/chloroform DNA extraction with addition of a polyacryl DNA carrier (Molecular Research Center, Cincinnati, Ohio, USA) before lysis;
c) salting-out protein precipitation method with 6M sodium chloride [12];
d) DNA extraction with commercial spin microcolumns (QIAamp Blood Mini Kit, Qiagen, Hilden, Germany) [13];
e) DNA extraction by the microcolumns with the carrier addition [14];
f) guanidinium isothiocyanate-based RNA extraction method [15].

The final volume of NA samples after the extraction process was 75 μl. Each extraction was performed simultaneously in pentaplicate.

Subsequently, the extracts were characterized spectrophotometrically. Optical densities at 260 nm and 280 nm were used to estimate the purity and yield of

nucleic acids. The integrity of NA was further determined electrophoretically on 1% agarose gel. The extracts were examined using the Factor V Leiden Kit (Roche Diagnostics, Mannheim, Germany) according to recommendations of the manufacturer. The PCR amplificability was evaluated via crossing-point (CP) analysis and determination of fluorescence of PCR products on 2% agarose gel.

Statistical analysis was based on calculations of means, standard deviations (SD), and coefficients of variations (CV, in percentages). The extraction methods were compared using the Student pair t test. Significance was established at $P < 0.05$.

Results

Spectrophotometric analysis shows that the guanidinium and phenol/chloroform extraction methods provide the highest yields of nucleic acids from 900 μl of plasma. As demonstrated in Table 1, the mean yield values were 2.4 μg and 1.4 μg, respectively. The lowest yield of nucleic acids was reached using commercial microcolumns without (0.4 ± 0.1 μg) or with the DNA carrier (0.2 ± 0.1 μg). The differences were found statistically significant. In the case of the phenol/chloroform extraction, addition of the carrier increased neither the yield (1.4 ± 0.2 vs. 1.1 ± 0.3 μg) nor the purity (1.4 ± 0.2 vs. 1.7 ± 0.2 μg) of NA extracts. The quanidinium method provided nucleic acids of excellent purity (1.9 ± 0.1).

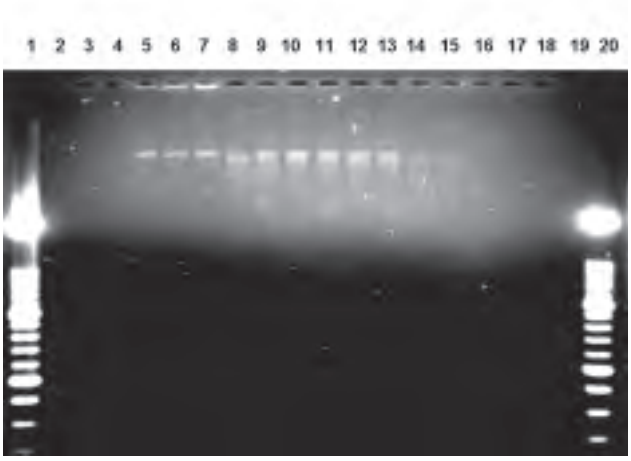

Fig. 1. Electrophoretical pattern of nucleic acids extracted from plasma
Lines 2–4 phenol-chloroform isolation; 5–7 phenol-chloroform + DNA carrier; 8–10 microcolumns; 11–13 microcolumns + DNA carrier; 14–16 protein salting-out procedure; 17–19 guanidinium (RNA) extraction; 1 and 20 DNA Marker MXIII (Roche).

Despite the spectrophotometric differences, fluorescence intensities of the NA extracts obtained by the phenol/chloroform procedure (with addition of the carrier) and both the microcolumn-based methods manifested very similar values (Fig. 1). The conformation of DNA differed according to the used isolation procedure (compare lines 5–7 with lines 8–10 or 11–13 in Fig. 1). No clear bands reflecting the presence of DNA in the protein precipitation NA extracts or guanidinium extracts were observed.

Crossing-point (CP) Leiden Factor V analysis determining the PCR cycle of the first significant increase of the amplified sequence was in consonance with the data from electrophoresis. Microcolumn methods (with or without addition of the carrier) revealed the lowest number of cycles necessary to reach the exponential part of amplification (CP medians 32.16 and 32.14). Successful amplification was also observed in the DNA extracts received from the carrier-enriched phenol/chloroform method (CP median 33.27), protein precipitation (34.59), and standard phenol/chloroform extraction (37.52). No fluorescence was detected in the guanidinium RNA extracts. Complete amplification data are provided in Table 2.

End-point electrophoretic analysis shows that fluorescence intensities of all the Leiden Factor V PCR products are very comparable after 45 cycles of amplification (see Fig. 2).

Table 1. Spectrophotometric characteristics of nucleic acids (NA) obtained from 900 ul of plasma

Extraction method	NA concentration (μg/ml)	NA yield (μg)	Purity (A260/A280)
Phenol/chloroform	18.3 ± 2.11***	1.4 ± 0.2***	1.4 ± 0.2
Phenol/chloroform + carrier	14.1 ± 3.8*	1.1 ± 0.3**	1.7 ± 0.2
Microcolumns	**4.8 ± 0.7**	**0.4 ± 0.1**	**1.7 ± 0.5**
Microcolumns + carrier	2.1 ± 0.9***	0.2 ± 0.1*	0.9 ± 0.6
Protein precipitation	11.4 ± 6.2	0.8 ± 0.5	1.1 ± 0.3
Guanidinium RNA extraction	32.6 ± 3.3***	2.4 ± 0.3***	1.9 ± 0.1

Extractions were performed in pentaplicate. Data presented as mean ± standard deviation.
*P < 0.01, **P < 0.005, ***P < 0.001 referred to the microcolumn extraction

Table 2. Amplificability of extracted nucleic acids

Extraction method	Crossing-point (CP) (FV Leiden)	CV (%)
Phenol/chloroform	37.52 ± 0.36***	0.96
Phenol/chloroform + carrier	33.27 ± 0.23*	0.69
Microcolumns	**32.16 ± 0.08**	0.24
Microcolumns + carrier	32.14 ± 0.08	0.25
Protein precipitation	34.59 ± 0.25**	0.72
Guanidinium RNA extraction	no amplification	

Real-time PCR was performed in triplicate. CP data presented as mean ± standard deviation. CV = coefficient of variation
*P < 0.01, **P < 0.005, ***P < 0.001 referred to the microcolumn extraction

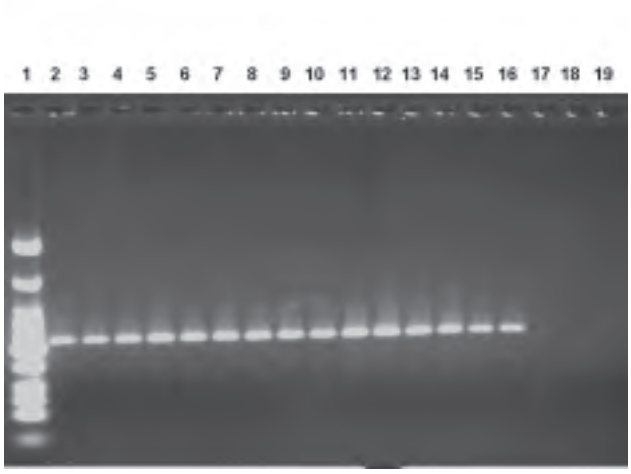

Fig. 2. PCR products (222 bp) of the factor V gene amplified by real-time PCR

Lines 2–4 phenol-chloroform isolation; 5–7 phenol-chloroform + DNA carrier; 8–10 microcolumns; 11–13 microcolumns + DNA carrier; 14–16 protein salting-out; 17–19 guanidinium RNA extraction, line 1 Low Molecular Weight DNA Ladder, New England Biolabs, Hitchin, UK.

Discussion

Cell-free DNA circulating in human plasma or serum seems to be promising biological material for performing molecular biology analyses. In our work we preferred plasma to serum because an additional release of DNA from hematopoietic cells during the clotting process can influence the content of DNA in serum [16]. Plasma was separated from blood cells within 3 h after collection. We took into account published recommendations that blood samples should be handled within 6 h after collection and repeated freezing and thawing of plasma samples lead to extensive DNA fragmentation [17]. For separation we used 1600 g centrifugation for 10 min. Spinning at 400–3000 g does not significantly change total plasma DNA levels [18].

Using β-actin sequences and quantitative PCR Herrera et al. found that the mean plasma DNA concentration in healthy subjects is 10.6 μg/l (range 7.0–14.0 μg/l) [6]. In our work the amount of circulating nucleic acids in plasma extracts was determined via spectrophotometric analysis. Our concentrations of nucleic acids ranged from 2.1 to 32.6 μg/ml. We explain this discrepancy by spectrophotometric interference of RNA molecules and DNA fragments appearing in large numbers in plasma.

The guanidinium isothiocyanate-based procedure was wittingly performed in the conditions for preferential isolation of fragmented RNA (without ice cooling of processed samples, addition of RNAse inhibitors, using re-sterilized plastic tubes and tips, buffers containing DEPC water, etc.). This protocol reduced probability of RNA interference in amplification reactions. Our results suggest the high UV absorbance of guanidinium extracts reflects much better the large content of RNA than the presence of integral DNA molecules. On the basis of these findings spectrophotometric analysis should not be performed for determination of DNA content in plasma extracts. Electrophoretical characteristics and PCR amplificability describe DNA amounts in plasma samples more exactly.

In this study we tested six extraction protocols used in our laboratory. A lot of other analytical approaches have been published. Both, guanidinium isothiocyanate and guanidinium hydrochloride procedures also serve to standard extraction of DNA molecules if experimental conditions are properly modified [19, 20]. We did not include those methods into our study because the guanidinium salts are present in two buffers of used Qiagen commercial kits [13]. Boiling DNA extractions were omitted in principle because of low DNA purity (1.1–1.4; our unpublished results).

In conclusion, our data report that microcolumn extraction techniques are very suitable for preparation of plasma DNA specimens. Performing forty-five cycles of amplification we achieved the plato-phase of the real-time PCR curve in all examined DNA extracts. That is the reason why the fluorescence of PCR products was very comparable. Evaluating the amplificability by the conventional end-point analysis we approved the phenol/chloroform extraction and salting-out method to be also acceptable laboratory approaches. Addition of the polyacryl DNA carrier does not seem to significantly influence the extraction yield.

The pre-analytical phase is a very important premise for successful amplification of DNA from any clinical sample. Both plasma separation and DNA extraction should be performed by standardized and validated procedures. Taking into account the results of our experiments and contemporary laboratory trends, we prefer plasma DNA extraction with spin microcolumns – the procedure providing genomic DNA of high integrity, purity, and amplificability.

References

1. **Lo, Y. M. D., Corbetta, N., Chamberlain, P. F. et al.** Presence of fetal DNA in maternal plasma and serum. *Lancet*, 1997, 350, p. 485–487.
2. **Chen, X. Q., Stroun, M., Magnenat, J. L. et al.** Microsatellite alterations in plasma DNA of small cell lung cancer patients. *Nat. Med.*, 1996, 2, p. 1033–1035.
3. **Johnson, P. J., Lo, Y. M. D.** Plasma nucleic acids in the diagnosis and management of malignant disease. *Clin. Chem.*, 2002, 48, p. 1186–1193.
4. **Lo, Y. M. D., Rainer, T. H., Chan, L. Y., Hjelm, N. M., Cocks, R. A**. Plasma DNA as a prognostic marker in trauma patients. *Clin. Chem.*, 2000, 46, p. 319–323.
5. **Lo, Y. M. D., Tein, M. S., Pang, C. C., Yeung, C. K., Tong, K. L., Hjelm, N. M**. Presence of donor-specific DNA in plasma of kidney and liver-transplant recipients. *Lancet*, 1998, 351, p. 1329–1330.
6. **Herrera, L. J., Raja, S., Gooding, W. E. et al.** Quantitative analysis of circulating plasma DNA as a tumor marker in thoracic malignancies. *Clin. Chem.*, 2005, 51, p. 113–118.
7. **Farina, A., LeShane, E. S., Lambert–Messerlian, G. M. et al**. Evaluation of cell-free fetal DNA as a second–trimester

maternal serum marker of Down syndrome pregnancy. *Clin. Chem.*, 2003, 49, p. 239–242.
8. **Birch, L., English, C. A., O' Donoghue, K., Barigye, O., Fisk, N. M., Keer, J. T.** Accurate and robust quantification of circulating fetal and total DNA in maternal plasma from 5 to 41 weeks of gestation. *Clin. Chem.*, 2005, 51, p. 312–320.
9. **Lo, Y. M. D., Zhang, J., Leung, T. N., Lau, T. K., Chang, A. M., Hjelm, N. M.** Rapid clearance of fetal DNA from maternal plasma. *Am. J. Hum. Genet.*, 1999, 64, p. 218–224.
10. **Chiu, R. W. K., Poon, L. L. M., Lau, T. K., Leung, T. N., Wong, E. M. C., Lo, Y. M. D.** Effects of blood-processing protocols on fetal and total DNA quantification in maternal plasma. *Clin. Chem.*, 2001, 47, p. 1607–1613.
11. **Sidransky, D., Tokino, T., Hamilton, S. R. et al.** Identification of ras oncogene mutations in the stool of patients with curable colorectal tumours. *Science*, 1992, 256, p. 102–105.
12. **Miller, S. A., Dykes, D. D., Polesky, H. F.** A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.*, 1988, 16, p. 1215.
13. QIAamp DNA Mini Kit and QIAamp DNA Blood Mini Kit Handbook, Qiagen, version 2001, p. 1–66.
14. **Ryan, B. M., Lefort, F., McManus R. et al.** A prospective study of circulating mutant KRAS2 in the serum of patients with colorectal neoplasia: strong prognostic indicator in postoperative follow up. *Gut*, 2003, 52, p. 101–108.
15. **Chomczynski, P., Sacchi, N.** Single-step method of RNA isolation by acid guanidinium-phenol-chloroform extraction. *Anal. Biochem.*, 1987, 162, p. 156–159.
16. **Lui, Y. Y. N., Chik, K. W., Chiu, R. W. K., Ho, C. Y., Lam, C. W. K., Lo, Y. M. D.** Predominant hematopoietic origin of cell-free DNA in plasma and serum after sex-mismatched bone marrrow transplantation. *Clin. Chem.*, 2002, 48, p. 421–427.
17. **Chan, K. C. A., Yeung, S. W., Lui, W. B., Rainer, T. H., Lo, Y. M. D.** Effects of preanalytical factors on the molecular size of cell-free DNA in blood. *Clin. Chem.*, 2005, 51, p. 781–784.
18. **Lui, Y. Y. N., Chik, K. W., Lo, Y. M. D.** Does centrifugation cause the ex vivo release of DNA from blood cells? *Clin. Chem.*, 2002, 48, p. 2074–2076.
19. **Ciulla, T. A., Sklar, R. M., Hauser, S. L.** A simple method for DNA purification from peripheral blood. *Anal. Biochem.*, 1988, 174, p. 485–488.
20. **Průša, R., Vošmik, M.** Extrakce DNA metodou s guanidinhydrochloridem a uchovávání vzorků v DNA bance. *Klin. Bioch. Metab.*, 1996, 4, p. 233–236.

The study was supported by the grant IGA MZ ČR NR7884–3.

Do redakce došlo 29. 8. 2005.

Adresa pro korespondenci:
PharmDr. Martin Beránek, Ph.D.
Ústav klinické biochemie a diagnostiky LF UK a FN
Sokolská 581
500 05 Hradec Králové
e-mail: beranek@lfhk.cuni.cz

Tematický plán kurzů Katedry klinické biochemie IPVZ pro období září – prosinec 2006 (část 3)

211010 Kurz – Aktuality v klinické biochemii pro zdravotní laboranty

Určeno pro zdravotní laboranty a pracovníky laboratorního komplementu.
Předběžný program: Vnitřní prostředí organismu. Regulace homeostázy vody a iontů. Aktuální otázky klinicko-biochemického vyšetření ledvin.
Vedoucí kurzu: doc. MUDr. A. Jabor, CSc.
Místo konání: Praha 4, Budějovická 15
Termín konání: 25. 10. 2006
Kurzovné: 500,– Kč

211011 Kurz – Příprava klinických laboratoří k akreditaci

Určeno pro pracovníky laboratoří klinické biochemie, hematologie, imunologie a mikrobiologie.
Předběžný program: Aktuální informace o procesu přípravy klinických laboratoří na akreditace podle ISO 15189 a norem souvisejících, vytváření příručky jakosti, směrnic, standardních operačních postupů, laboratorní příručky a ostatních dokumentů, procvičování tvorby dokumentů na příkladě systému SLP. Změny, které je nutné realizovat v laboratoři. Etapy přípravy a vlastní akreditace. Zkušenosti z praxe. Praktická cvičení v počítačové učebně.
Vedoucí kurzu: ing. M. Zámečník
Místo konání: Praha 4, Budějovická 15
Termín konání: 21.–22. 11. 2006
Kurzovné: 1000,– Kč

211012 Kurz – Validace metod v klinické laboratoři

Určeno pro pracovníky laboratorního komplementu.
Předběžný program: Pojem validace a verifikace. Doporučení o validaci – národní a mezinárodní zdroje. Statistické nástroje pro validaci metody. Provedení validace v jednotlivých skupinách laboratorních vyšetření. Výběrový kurz v rámci specializační přípravy lékařů a biochemiků-analytiků.
Vedoucí kurzu: ing. L. Šprongl
Místo konání: Praha 4, Budějovická 15
Termín konání: 23. 11. 2006
Kurzovné: 600,– Kč

(pokračování na s. 32)