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Journal of Chromatography A, 852 (1999) 305–312

JOURNAL OF CHROMATOGRAPHY A

Short communication

Introduction of the gene amplification technique to decrease the risk of hepatitis C virus transmission by plasma products

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Abstract

The viral safety of plasma-derived products with respect to hepatitis C virus (HCV) is assured by selection of donors, screening of individual donations for antibodies to HCV and the incorporation of effective viral inactivation-removal steps into manufacturing processes. As antibody screening of single donations is not sufficient to completely eliminate HCV RNA positive plasmas from plasma pools, testing for HCV RNA by gene amplification techniques may be necessary to identify positive donations. Using modern molecular biology techniques, we developed a specific, sensitive and reproducible method for routine PCR screening for HCV RNA in plasma pools. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Hepatitis C virus; Viruses; RNA

1. Introduction

The risk of transmitting viral infection by blood and plasma derived products have long been known and still remain an area of concern. Hepatitis C virus (HCV) is a recently discovered positive-stranded RNA virus which is known to be the major cause of both post-transfusion and community acquired non-A, non-B hepatitis [1]. Of the methods available, amplification of HCV cDNA by polymerase chain reaction (PCR) commends itself by virtue of its extreme sensitivity and its resultant ability to detect the very low copy numbers of HCV RNA that could be present in individual plasma donations [2]. In order to assure the virological safety of blood products, in addition to serological testing of individual donations and virus inactivation steps undertaken during manufacture, routine PCR testing for HCV RNA of source material (small, medium and large plasma pools) would be necessary [3]. The PCR system should meet strict requirements regarding specificity, sensitivity [with a defined cut-off in genome equivalents/ml (gequiv./ml)] reproducibility, standardization and validation. Using modern molecular biology techniques we developed a specific, sensitive and reproducible method for routine PCR screening for HCV RNA in plasma pools.

2. Experimental

2.1. Materials

2.1.1. Plasma

Plasma obtained by collected whole blood donations at blood transfusion centres and plasma obtained by plasmapheresis in the plasmapheresis centre (Institute of Immunology, Zagreb, Croatia) have been pooled to medium size pools (70 l). The plasma pool obtained by plasmapheresis, consists of approx. 120 donations while plasma pool obtained by whole blood collection, consists of approx. 360

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PII: S0021-9673(99)00468-9

donations. All donors were negative for antibodies to hepatitis C virus.

2.1.2. Standards

For nucleic amplification technique detection efficiency of HCV the following standards have been used: (a) the NIBSC HCV working reagent (96/586, National Institute for Biological Standards and Control, UK); (b) Pelicheck HCV RNA sensitivity panel (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands); (c) Internal controls, constructed by inserting [73 base pairs (bp)] and deleting (58 bp) within the target sequence (untranslated 5′ region of HCV genome) and defined for amplification (Institute of Immunology, Zagreb, Croatia).

The Pelicheck HCV RNA is a panel of diluted plasma standards (Eurohep genotype 1 and 3) for the validation of HCV RNA amplification test systems.

2.2. Equipment

Reverse transcription (RT), amplification reaction and cycle sequencing were performed in a GeneAmp system 9600 Thermocycler (Perkin-Elmer, USA).

Vertical polyacrylamide gel electrophoresis (PAGE), DNA sequencing and GeneScan analysis were performed on the ABI PRISM 377 DNA Sequencer (Perkin-Elmer).

The Perkin-Elmer ABI PRISM 377 DNA Sequencer detects DNA fragments by automated fluorescent scanning detection. The GeneScan Analysis software analyzes the data collected by sizing and quantifying the DNA fragments.

2.3. Methods

2.3.1. Isolation of RNA

HCV RNA was isolated by the guanidinium thiocyanate–phenol–chloroform method [4] with minor modifications. For each extraction an appropriate amount of RNA internal controls were used.

2.3.2. Reverse transcription

cDNA synthesis was performed in a 20 μl reaction volume at 42°C for 20 min by using 1×PCR buffer II (50 m*M* KCl, 10 m*M* Tris–HCl, pH 8.3), 5 m*M* $MgCl_2$, 1 m*M* each deoxyribonucleotide (dNTP), 2.5 μ*M* random hexanucleotide primers, 1 U/μl RNAse inhibitor and 2.5 U/μl murine leukaemia virus reverse transcriptase (MuLv).

2.3.3. Gene amplification method (PCR)

Gene amplification method was laboratory nested PCR. Primers were defined in the 5′-noncoding region of the HCV genome. The outer pair of primers were a sense primer HSU (nucleotide positions 1–21) and an antisense primer A1 (nucleotide positions 341–320). The inner pair of primers were a sense KY80 (nucleotide positions 56–79) and an antisense primer KY78 (nucleotide positions 276–299). The inner sense KY80 primer was labelled with fluorescence day 5-carboxyfluorescein (FAM). The first and the second round of nested amplification was carried out in a final volume of 100 μl.

For the first round of amplification PCR-mix consisting of 50 pmol of each outer primer, 1×PCR buffer II, 1.25 m*M* $MgCl_2$ and 2.5 U AmpliTaq DNA polymerase were used. After an initial denaturation step of 94°C for 3 min, 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 3 min, with a terminal elongation step of 10 min at 72°C were done.

The second round of amplification was carried out on 5 μl of the first round PCR product in a final volume of 100 μl containing 34 pmol of each inner primers, 1×PCR buffer II, 2.1 m*M* $MgCl_2$, 0.21 m*M* each of dNTP and 2.5 U AmpliTaq DNA polymerase. After an initial denaturation step of 94°C for 3 min, 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 3 min, with a terminal elongation step of 10 min at 72°C were done.

2.3.4. Detection of PCR products by electrophoresis

Aliquots of 9 μl of the amplification products were subjected to electrophoresis in a 2% agarose gel with ethidium bromide. For detection by PAGE, FAM labelled amplification products were diluted 1:20 by deionized water. In a clean 0.5 ml microcentrifuge tube, we added 1 μl diluted FAM labelled amplification product, 1 μl Genescan-500 TAMRA size standard (DNA fragments of known sizes labelled with ABI PRISM dye TAMRA [*N*,*N*,*N*′-tetramethyl-6-carboxyrhodamine]), 0.5 μl 2×agarose loading buffer and 2 μl deionized formamide. To

denature, we heated the sample at 90°C for 2 min. The sample was then placed on ice until ready to load. Electrophoresis was performed on the ABI PRISM 377 DNA Sequencer using a 4% denaturing acrylamide gel, and a 36 well-to-read plate length.

2.3.5. Cycle sequencing

The cycle sequencing was carried out in a final volume of 20 μl containing 8 μl terminator ready reaction mix (A-dye terminator, C-dye terminator, G-dye terminator, T-dye terminator, dITP, dATP, dCTP, dTTP, Tris–HCl (pH 9.0), $MgCl_2$, thermal stable pyrophosphatase and AmpliTaq DNA polymerase FS), PCR product (30–180 ng), primer 3.2 pmol and water to a total volume of 20 μl.

After rapid thermal ramp to 96°C, 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min were done.

2.3.6. Sequence analysis

After cycle sequencing extension products were purified by the ethanol precipitation method according to Perkin-Elmer ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit protocol. Samples were resuspended in 6 μl loading buffer (deionized formamide and 25 m*M* EDTA [pH 8.0] containing 50 mg/ml Blue dextran in a 5:1 ratio of formamide to EDTA/Blue dextran). Samples were heated at 90°C for 2 min to denature and placed on ice until ready to load. Electrophoresis was performed on the ABI PRISM 377 DNA Sequencer using a 4% denaturing acrylamide gel, and a 36 cm well-to-read plate length.

All contamination prevention measures, suggested by Kwok and Higuchi [5] were strictly followed. HCV RNA isolation, identification by agarose gel electrophoresis and PAGE and characterisation by sequencing for positive samples have been performed on three separate occasions.

3. Results and conclusions

3.1. Control of sensitivity and specificity of the test systems

For control of the sensitivity of HCV RNA amplification test system we used the Pelicheck HCV RNA sensitivity panel. The plasma standards have been characterized in two collaborative Eurohep studies [6,7]. For adequate sensitivity, the 1:1000 dilution and preferably also the 1:4000 dilution of the Eurohep genotype 1 plasma standard, should be reactive. In our laboratory nested amplification test system, the Eurohep genotype 1 plasma standard gave positive results in four dilutions (1:100, 1:1000, 1:4000 and 1:16 000) with a defined cut-off of 225 gequiv./ml. The electrophoresis of the amplification products of the Eurohep genotype 3 plasma standard is shown in Fig. 1a.

The Eurohep genotype 3 plasma standard gave positive results in three dilutions (1:10, 1:100 and 1:1000) with a defined cut-off of 1200 gequiv./ml. The electrophoresis of the amplification products of the Eurohep genotype 3 plasma standard is shown in Fig. 1b.

For control of the specificity of HCV RNA amplification test systems we sequenced PCR products and the result of sequencing is shown in Fig. 2.

In conclusion, the level of detection of our amplification test was 225 gequiv./ml HCV RNA for genotype 1 and 1200 gequiv./ml HCV RNA for genotype 3. By sequencing the amplification products, we proved that the amplification test is specific for HCV RNA.

3.2. Standardization and validation of RT-PCR for HCV RNA by reference preparation and internal controls

The results of a collaborative study [8] to asses the suitability of an HCV RNA reference sample (NIBSC working reagent), indicate that a 10^{-4} dilution of the positive donation in cryosupernatant is a useful standard for monitoring the performance of PCR assays for HCV RNA in plasma pools. For the validation HCV RNA amplification test runs, in each series of the PCR assays we tested the NIBSC working reagent (undiluted and 1:10 dilution) for testing plasma pools. Negative controls were interspersed regularly throughout the reaction series to monitor false-positive results.

A high degree of reliability was achieved by co-processing, co-amplification and co-detection of the internal standards, together with the nucleic acid to be determined. The positive signal from the

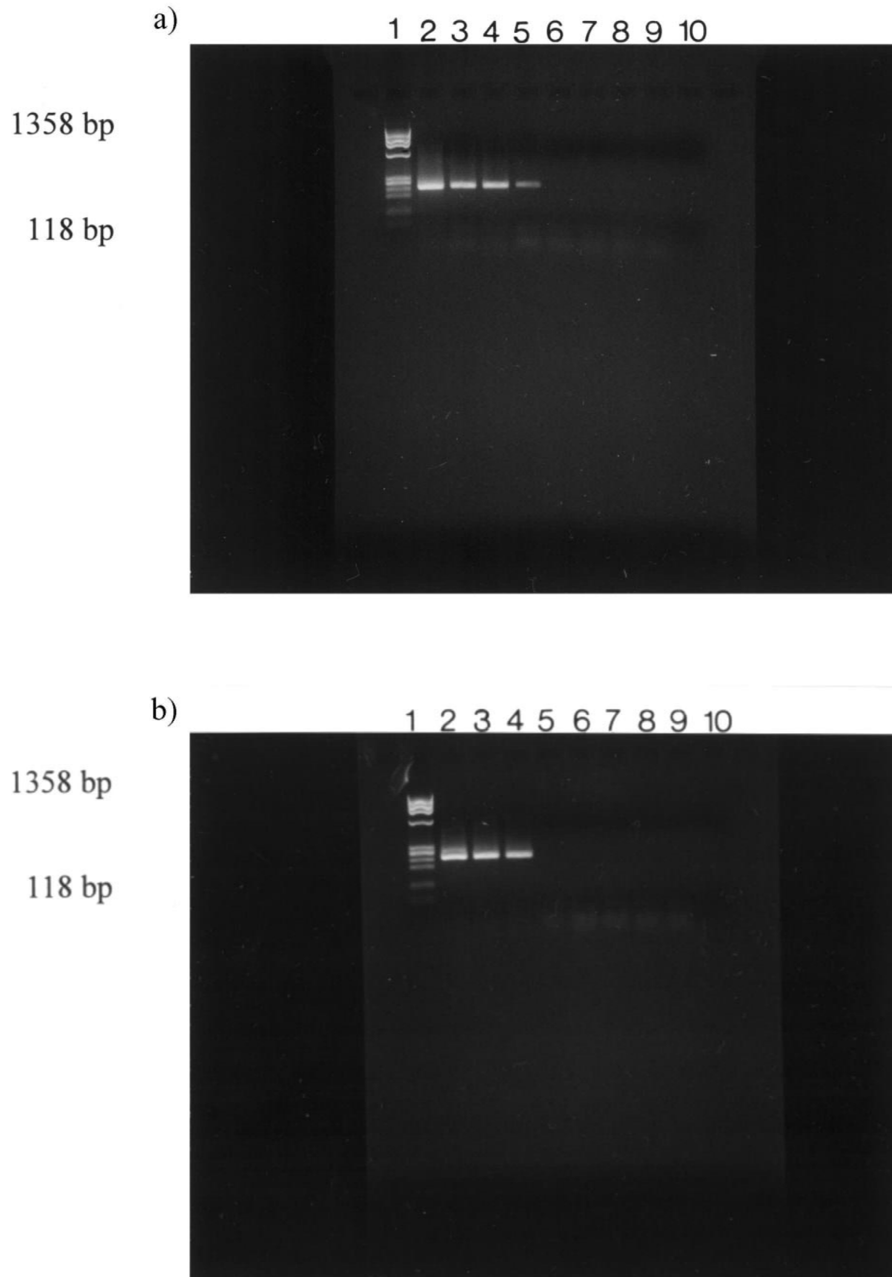


Fig. 1. Analysis of the amplification products from Pelicheck HCV RNA sensitivity panel by ethidium bromide–agarose gel electrophoresis. (a) 9 μl nested PCR product from Eurohep genotype 1 (lanes: 1=molecular mass marker ΦX 174RF DNA/Hae III, 2=36 000 gequiv./ml, 3=3600 gequiv./ml, 4=900 gequiv./ml, 5=225 gequiv./ml, 6=56 gequiv./ml, 7=16 gequiv./ml, 8=4 gequiv./ml, 9=1 gequiv./ml, 10='No DNA' control). Molecular mass marker ΦX 174RF DNA/Hae III consists of: 72 bp, 118 bp, 194 bp, 234 bp, 271 bp, 281 bp, 310 bp, 603 bp, 872 bp, 1078 bp and 1358 bp. (b) 9 μl nested PCR product from Eurohep genotype 3 (lanes: 1=molecular mass marker ΦX 174RF DNA/Hae III, 2=120 000 gequiv./ml, 3=12 000 gequiv./ml, 4=1200 gequiv./ml, 5=300 gequiv./ml, 6=75 gequiv./ml, 7=19 gequiv./ml, 8=5 gequiv./ml, 9=1 gequiv./ml, 10='No DNA' control). Molecular mass marker φX 174RF DNA/Hae III consists of: 72 bp, 118 bp, 194 bp, 234 bp, 271 bp, 281 bp, 310 bp, 603 bp, 872 bp, 1078 bp and 1358 bp.

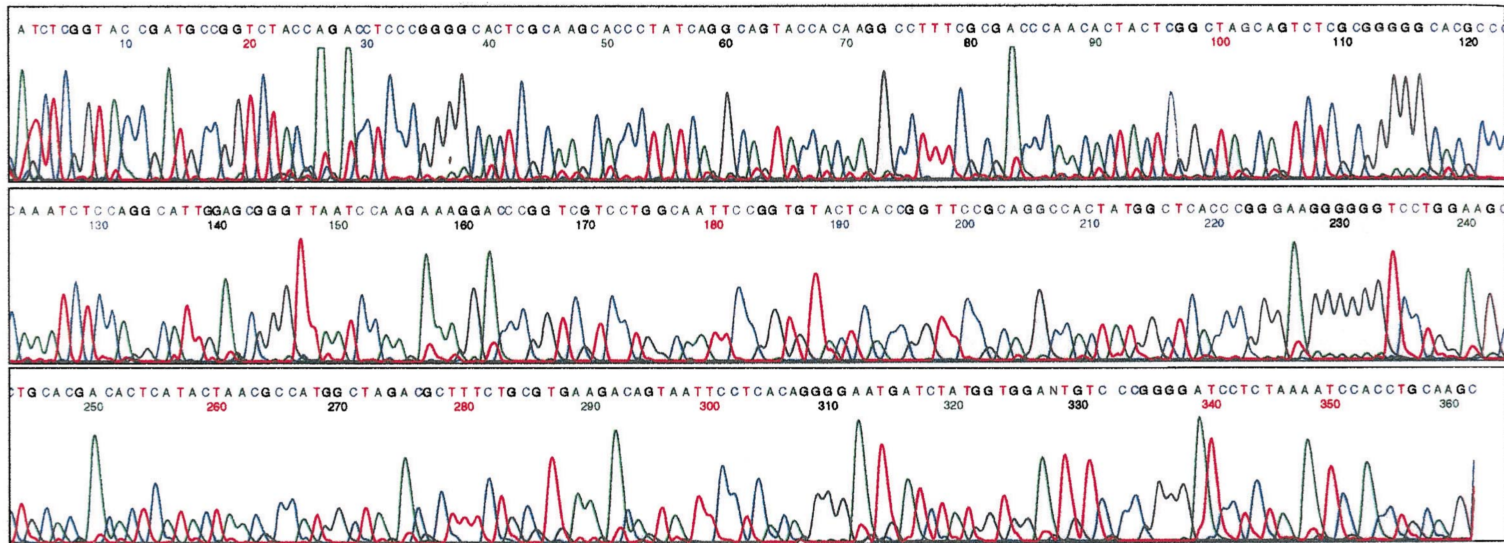


Fig. 2. Electropherogram of sequencing 324 bp PCR product on the ABI PRISM 377 DNA Sequencer.

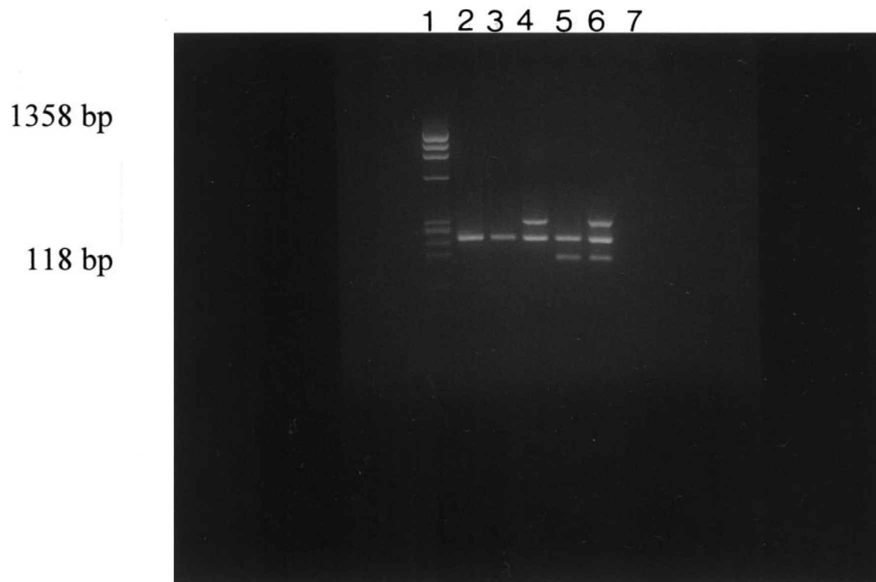


Fig. 3. Analysis of amplification products from NIBSC working reagent undiluted sample (lane 2), 1:10 dilution (lane 3), NIBSC undiluted sample co-amplificated with internal control (deletion [−]) (lane 4), NIBSC undiluted sample co-amplificated with internal control (insertion [+]) (lane 5), NIBSC undiluted sample co-amplificated with internal controls (insertion [+] and deletion [−]) (lane 6) and 'No DNA' control (lane 7) by ethidium bromide–agarose gel electrophoresis. Lane 1 represents molecular mass marker ΦX 174RF DNA/Hae III (72 bp, 118 bp, 194 bp, 234 bp, 271 bp, 281 bp, 310 bp, 603 bp, 872 bp, 1078 bp and 1358 bp).

Table 1
The results of PCR RNA HCV screening in plasma pools

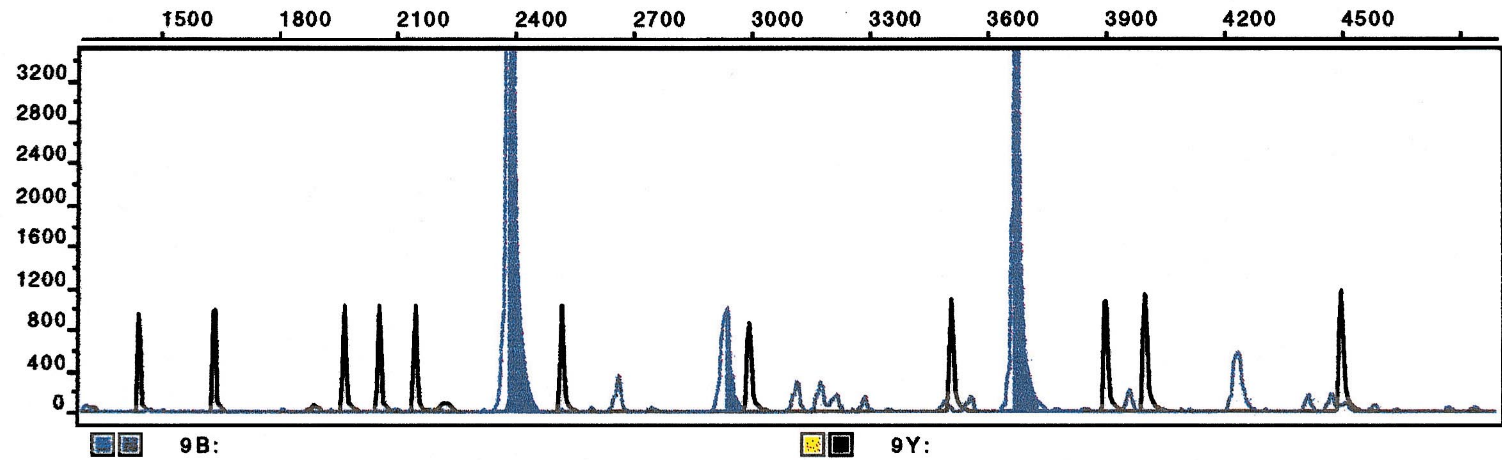
Source of plasma	Total number of plasma pools[a] tested	RT-PCR HCV negative plasma pools	RT-PCR HCV positive plasma pools	% of positive plasma pools
Institute of immunology	283	279	4	1.4
Blood centres	567	554	13	2.3

[a] Medium plasma pool of approx. 70 l were made by pooling the samples of four small plasma pools of approx. 18 l. RT-PCR HCV positive medium plasma pools were subjected to further analysis.

insertions and deletions demonstrates successful amplification, thereby validating a negative result for the target sequence (untranslated 5′ region of HCV genome) [9]. Positive results of amplification of the NIBSC working reagent (undiluted and 1:10 dilutions) alone and with internal standards are shown in Fig. 3.

3.3. Detection of HCV RNA in plasma pools

Two types of source materials were investigated: plasma obtained by collected whole blood donations at blood donor centres and plasma obtained by plasmapheresis in the plasmapheresis centre (Institute of Immunology, Zagreb, Croatia). Table 1 shows the results of PCR screening RNA HCV in plasma pools of 70 l. Our results show that more RNA HCV positive plasma pools were detected among plasma pools from blood transfusion centres then among plasma pools obtained by plasmapheresis at the Institute of Immunology (Table 1). The reason could be less individual donations needed for plasma pools obtained by plasmapheresis.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
9B, 4	63.81	186.23	5412	92991	2393
9B, 10	78.37	244.23	1026	14261	2939
9B, 28	97.97	316.93	5871	71775	3674

Fig. 4. Electropherogram and tabular data GeneScan electrophoresis of amplification products from NIBSC working reagent undiluted sample (244 bp) co-amplificated with internal controls (insertion (+) [317 bp] and deletion (−) [186]).

3.4. Identification of amplification products by gene scanning

The detection efficiency of the viral nucleic acid is determined primarily by the primers and the reaction conditions used during the RT and PCR steps. The important parameter ensuring specificity is the precise determination of the size of the amplification products. We combined the DNA fragments with the internal size standard and electrophoresed them simultaneously. The amplification products detection by laser-induced fluorescence on ABI PRISM 377 DNA Sequencer allow the direct quantification of the amplification products relative to internal standards and include a physical sizing step to ensure specificity [10]. The results of the experiment have been displayed as an electropherogram and as tabular data (Fig. 4). The electropherogram shows fluorescence as a function of time or size. Each electropherogram represents a single lane for ABI PRISM 377 data. The tabular data provides detailed sizing and quantitative information.

We developed a specific, sensitive and reproducible method for routine PCR screening for HCV RNA in plasma pools. Because nested PCR is long-lasting, expensive and with high possibilities of contamination, we are in the process of establishing single PCR for screening RNA HCV with the same sensitivity and specificity.

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