An oligonucleotide microarray for multiplex real-time PCR identification of HIV-1, HBV, and HCV

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BioTechniques 44:241-248 (February 2008)
doi 10.2144/000112628

We describe a novel microarray-based approach for simultaneous identification and quantification of human immunodeficiency virus type 1 (HIV-1) and hepatitis B and C viruses (HBV and HCV) in donor plasma specimens. The method is based on multiplex real-time RT-PCR performed within the microarray hydrogel pads. Double-stranded amplification products are simultaneously detected using nonspecific SYBR Green I dye due to the reaction run in separate pads bearing 5′-immobilized specific primers. Both the sensitivity and specificity of the assay, based on 132 blood specimens analyzed, were 100% (56, 26, and 8 specimens were seropositive to HBV, HCV, and HIV-1, respectively; 22 were positive to both HIV-1 and HCV, and 2 positive to all three viruses; 18 samples were pathogen-negative). The dynamic range of the quantitative analysis covered a six-order interval ranging from 10^0 to 10^6 genome equivalents per assay. The 95% detection limits were 14 gEq for HIV-1, 10 gEq (1.7 IU) for HBV, and 15 gEq (7.5 IU) for HCV per assay. The proposed approach is considered to be versatile and could be adapted for simultaneous identification and quantification of numerous genetic targets.

INTRODUCTION

The risk of transfusion-transmitted infection with hepatitis B and C viruses (HBV and HCV, respectively), and human immunodeficiency virus type 1 (HIV-1) can be significantly decreased if fast, sensitive, and reliable methods are used to identify these pathogenic viruses in donor blood. Although essential progress has been made in the development of immunological methods (1,2), their application remains limited during the seronegative window period as well as in cases of delitescence and immunovariant and nonimmunogenic forms of infections (3), which are inherent, in particular, in HBV, HCV, and HIV-1.

Methods based on nucleic acid testing (NAT) seem to be preferable for diagnostic applications since they are more sensitive and allow direct identification of specific fragments of infectious agent genome (4,5). Besides, the NAT methods could be performed in multiplex format to identify several agents simultaneously. At present, a number of commercial test systems are available for simultaneous detection of HIV-1, HBV, and HCV in donor blood (5–10). These systems are based on various technical approaches using several methods for sequence-specific amplification of viral nucleic acids, such as PCR (or RT-PCR), ligase chain reaction, nucleic acid sequence-based amplification (NASBA), and transcription-mediated amplification (TMA) (4).

The most advanced techniques are based on real-time detection, which essentially shortens the duration of analysis, improves its sensitivity, and at the same time allows quantitative determinations. Such systems do not require any post-amplification detection of the reaction products (electrophoresis and hybridization) and, thereby, substantially reduce the risk of contamination. There are several commercial systems developed for real-time NAT-analysis (11).

Most of these real-time systems detect amplification products using either nonspecific DNA binding fluorophores or specific fluorescently labeled oligonucleotide probes (5′-endonuclease, adjacent linear and hairpin oligoprobes, and self-fluorescing products) (11,12). Unfortunately, the real-time PCR used in multiplex reactions has an essential limitation: the interference of absorption and fluorescence spectra of simultaneously used fluorophores allows reliable detection of no more than four reactions in one assay (7). The use of nonspecific DNA binding fluorophores, such as SYBR Green I, is also limited, allowing for real-time detection of the total amount of all amplification products synthesized in in-tube reactions (13). This type of dye is commonly used for melting curves measurements providing qualitative analysis (14).

In an effort to further improve the real-time multiplex reaction systems, several variants of oligonucleotide immobilization on solid phase have been proposed, in particular, probe-coated microspheres (6, 15) and DNA microarrays (16–21).

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Oligonucleotide microarrays are powerful tools for parallel, high-throughput detection of numerous nucleic acid targets. Originally developed for the analysis of whole genome gene expression and micro-sequencing, DNA microarrays have considerable potential for microbial and viral diagnostics (22–24). A number of various approaches from routine hybridization to sophisticated enzymatic assays have been developed on microarray platforms (25). One of the most promising approaches to pathogen detection and identification seems to be real-time multiplex PCR performed on a microarray.

DNA microarray with immobilized probes can increase the number of simultaneously analyzed targets (26). At the same time, all microarrays with immobilized primers developed so far use post-amplification detection of results only (19–21).

In this study, an original on-chip real-time PCR approach for simultaneous identification of several genetic targets is described. We have developed a procedure of the on-chip quantitative amplification and applied it to the identification of blood-borne viruses in plasma specimens. This approach is fundamentally different from other multiplex systems since it provides real-time PCR with primers immobilized on a microarray and use a single detector dye. The developed approach combines high analytical sensitivity with multiplex format and can be considered as a versatile tool for further development of other test systems for simultaneous identification of various genetic targets.

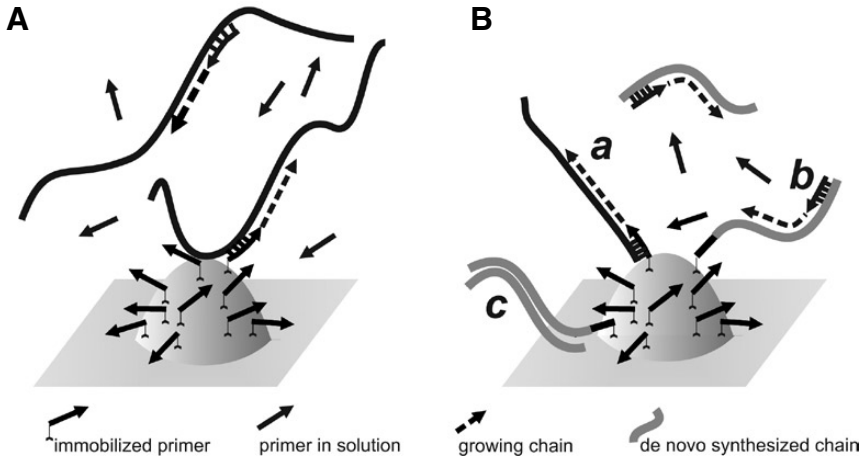

Figure 1. Scheme of real-time PCR inside amplification volume of a gel-based microarray. Forward primers are immobilized, while reverse primers are free in surrounded solution. (A) At the initial reaction stage when the concentration of the target molecules (shown by black bold lines) is low, specific amplification products are formed through the extension of both free and immobilized primers. (B) At the exponential stage, the extended immobilized primers (*a*) form the additional anchored templates to be amplified with free reverse primers (*b*). Double-stranded de novo synthesized product shown by gray lines (*c*) binds SYBR Green I. The accumulation of the dye in the gel pad is detected during each cycle of amplification.

MATERIALS AND METHODS

Primer Design and Microarray Fabrication

The HIV-1 *gag* gene, HBV *X*-gene, and HCV 5′-untranslated region (5′-UTR) sequences available from public databases were analyzed with BioEdit (www.mbio.ncsu.edu/BioEdit/bioedit.html) software. Based on multiple sequence alignments, PCR primers (Table 1) were derived from these regions using the Oligo 6 software (Molecular Biology Insights, Cascade, CO, USA). The sequence identities between the designed primers and the genes of interests were analyzed with a BLAST search (www.ncbi.nlm.nih.gov).

Primers were synthesized on an ABI-394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA, USA) using standard automated phosphoramidite chemistry. To immobilize primers inside hydrogel pads, an amino group was introduced during synthesis using 5′-Amino-Modifier C6 (Glen Research, Sterling, VA, USA). Primers were purified by reverse phase HPLC

Table 1. Specific Primers Used in Two-stage PCR of HIV-1, HBV, and HCV

Primer[a]	Positions[b]	Sequence 5′→3′
HIV_OUT_F	609	AGAACCG(G/A)TCTACATA(G/A)TCTCTAA(G/A)G
HIV_OUT_R	872	TGAGGA(G/A)GCTGCAGAATGGGA
HIV_IN_F_I	643	TGG(C/T)CCTTGT(C/T)TTATGTCCA(G/A)AATG
HIV_IN_R	801	AGAACCAAGGGGAAGTGACATAG
HBV_OUT_F	1373	TCCATGGCTGCTAGGCTGTG
HBV_OUT_R	1584	GTGCAGAGGTGAAGCGAAGTG
HBV_IN_F_I	1431	GTCCCGTCGGCGCTGAATC
HBV_IN_R	1528	CGCGTAAAGAGAGGTGCGCC
HCV_OUT_F	69	CAGAAAGCGTCTAGCCATGGCGT
HCV_OUT_R	290	ACTCGCAAGCACCCTATCAGGCA
HCV_IN_F_I	146	GTCTGCGGAACCGGTGAGTACA
HCV_IN_R	268	GTACCACAAGGCCTTTCGCGAC

[a] OUT, first-stage PCR primers (outers); IN, second-stage on-chip PCR primers (inners); F, forward; R, reverse; I, immobilized.
[b] The number indicates nucleotide position, counting from the 5′ terminus of the gene-encoding sequence according to Gen Bank accession no. AY206658 (HIV-1, *gag* gene, cDNA), AB126581 (HBV, *X*-gene), and AF009606 (HCV, 5′-UTR region).

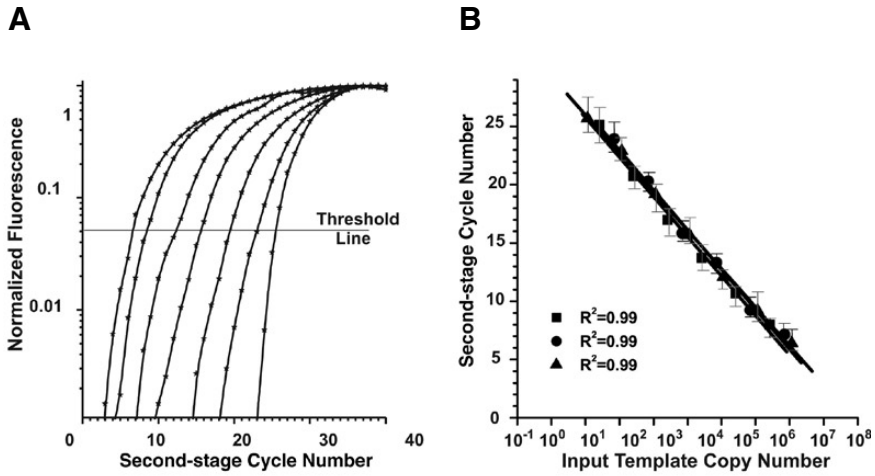

Figure 2. Second stage of on-chip real-time amplification of standard plasma samples. (A) Plot of fluorescence accumulation and threshold cycle number (C_T) for HBV DNA serial 10-fold dilutions (log10) ranging from 10^6 to 10^0 gEq/mL (from left to right). Threshold fluorescence is shown as the horizontal line. (B) Calibration curves to calculate initial nucleic acid concentrations of HIV-1, HBV, and HCV in plasma samples (curves marked with squares, triangles, and circles, respectively). Regression coefficient for each target is indicated.

on C18-Nucleosil (5 µm, 4.6 × 250 mm) columns (Sigma-Aldrich, St. Louis, MO, USA).

Microarrays for real-time PCR were manufactured as described earlier (27). High porosity hydrogel M18 (Biochip-IMB Ltd, Moscow, Russia) was used to form microarray hydrogel pads 600 µm in diameter. The primers were immobilized in the pads at their 5′ ends at 200 µM.

Reference Specimens and Clinical Samples

Reference serology positive plasma samples, in which HIV-1, HCV, and HBV viral loads had been quantified, were kindly provided by Jacques Izopet (Institut Fédératif de Biologie—Hôpital Purpan, Toulouse, France). The quantitative plasma standards contained 3.85 × 10^6 gEq/mL of HIV-1, 1.73 × 10^7 gEq/mL (2.89 × 10^6 IU/mL) of HBV, and 1.04 × 10^7 gEq/mL (5.22 × 10^6 IU/mL) of HCV.

The plasma standards and their serial dilutions prepared using seronegative plasma were used to generate calibration curves and evaluate the 95% detection limits. To produce calibration curves, 10-fold dilutions were performed to cover the range from 10^6 to 10^0 gEq per assay. For concentrations of 10^2 gEq/10 µL and higher 4 to 8 replicates were used; lower concentrations were tested in 25 replicates.

To calculate the 95% detection limits, serial dilutions were prepared in concentrations ranging from about 10^2 to 1 gEq/10 µL by means of 2-fold dilutions. Each point was tested in 25 replicates.

Plasmids pBluescript II (Stratagene, La Jolla, CA, USA) with cloned fragments HG00-HG01 of the HIV-1 *gag* gene (strains VI310 and TB132) were used as control templates to explore the PCR assay characteristics.

Anonymous plasma samples were collected at the Moscow Hematological Research Center and serologically characterized. In total, 132 randomly selected samples were tested: 18 seronegative, 56 HBV-positive, 26 HCV-positive, 8 HIV-1–positive; 22 samples were positive to both HIV-1 and HCV and 2 were positive to HIV-1, HBV and HCV.

Viral DNA/RNA Isolation

Viral nucleic acids were isolated from 200 µL of plasma samples with QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany). Nucleic acids were eluted with 30 µL of AVE buffer, and then were either directly analyzed or stored at -70°C until further use.

Multiplex RT-PCR

A reaction mixture for the first stage of multiplex RT-PCR contained 1× StrataScript buffer (Stratagene); 0.2 mM each dATP, dCTP, and dGTP; 0.6 mM dUTP; 0.2 µM of each first-stage primer (Table 1); 5 U SureStart Taq polymerase (Stratagene); 10 U StrataScript Reverse Transcriptase (Stratagene); 20 U RNasin (Fermentas, Vilnius, Lithuania); and 10 µL of a template nucleic acid preparation per 25 µL reaction. The RT-PCR was performed as follows: the RT reaction at 55°C for 30 min, Taq polymerase activation at 95°C for 10 min, 25 three-step cycles (20 s at 95°C; 30 s at 62°C; and 30 s at 72°C), and final incubation at 72°C for 5 min.

Real-time PCR on Microarray

The second-stage real-time PCR was performed on the microarray with the corresponding forward immobilized primers (Table 1, Figure 1) as described earlier (28) with some modifications. In brief, amplification was performed in the presence of Brilliant SYBR Green Quantitative PCR Core Reagent kit (Stratagene). A reaction mixture (25 µL) contained: the core PCR buffer; 4 mM $MgCl_2$; 0.2 mM each dATP, dCTP, and dGTP; 0.6 mM dUTP; 0.2 µM of each second-stage reverse primer (Table 1); 5 U SureStart Taq polymerase; 1× SYBR Green I; 0.01% acetylated BSA (New England BioLabs, Ipswich, MA, USA); and 1 µL of the first-stage product as a template. The reaction was carried out in a microchip chamber formed between the microchip slide, a cover glass, and a Frame-Seal (Bio-Rad Laboratories, Hercules, CA, USA) between them. After an initial incubation at 95°C for 10 min, 30–50 three-step cycles were performed as follows: 30 s at 95°C, 30 s at 55°C, and 45 s at 68°C.

A homemade experimental setup was used for fluorescence measurements. The setup consisted of fluorescence microscope assembled with thermocycler, a charge-coupled device (CCD), camera, and the specialized

software ImageExpress (Biochip-IMB). Fluorescence signals in gel pads were measured in real-time format at 3 s before the end of each elongation step.

The post-PCR melting curves were obtained by increasing temperature from 68° to 95°C at the rate of 3°C/min. To obtain the melting curves, the fluorescence was measured at 0.5°C interval each 10 s during the melting process.

The rough fluorescence data (F) were analyzed by ImageExpress software and normalized according to the formula $(F\text{-}F_{min})/(F_{max}\text{-}F_{min})$, where F_{min} and F_{max} are minimal and maximal fluorescence values registered in a gel pad.

On-chip Quantification of Viral DNA/RNA in Clinical Samples

To quantify HBV DNA, and HCV and HIV-1 RNA in clinical plasma samples using on-chip real-time PCR, the C_T (threshold cycle) value determined for each sample was interpolated to the calibration curves generated as described above. At least three replicates of each specimen were tested.

RESULTS

Design of the Assay

An original approach based on real-time PCR on oligonucleotide microarray of isolated gel drops of nanoliter volume was developed to identify and simultaneously quantify HIV-1, HBV, and HCV in blood samples. The microarray consisted of hemispheric hydrogel pads with 5′-immobilized forward primers. Primer sequences are listed in Table 1. The HIV-1 *gag* gene, HBV *X*-gene, and HCV 5′-UTR were selected as specific targets to be identified.

The developed technique included two stages: (*i*) pre-on-chip multiplex RT-PCR (see Materials and Methods) and (*ii*) multiplex on-chip PCR combined with real-time fluorescence measurements and data acquisition. The first RT-PCR stage performed in a tube was aimed at obtaining cDNA, increasing the reaction sensitivity and specificity due to the nested format, and optimizing the dynamic range of quantitative analysis that could be shifted by variation of RT-PCR stage cycle number (see Quantitative On-chip Real-time PCR section, below).

The use of specific primers immobilized in hydrogel pads to perform on-chip PCR was described earlier (27). In this study, another approach is proposed. It supposes no specific fluorescent labels are incorporated into the in-target molecules. Identification of double-stranded amplification products in hydrogel pads is carried out via fluorescence detection of nonspecific dye SYBR Green I that binds and intercalates into the products. The scheme of the reaction is illustrated in Figure 1.

Products created at the RT-PCR stage are used as templates for real-time on-chip PCR. Target DNA molecules anneal to specific primers immobilized in individual gel pads. Figure 1B shows how the immobilized primers are extended enzymatically (*a*), forming new target chains anchored in the gel via their 5′- termini. These chains serve as targets for further amplification (*b*), and eventually form tethered double-stranded hybridization complexes (*c*) that could bind fluorescent dyes. Since each gel pad contains primers specific to one target only, the kinetics of fluorescence in each gel pad reflects the accumulation of the corresponding amplification product.

Fluorescence signals in gel pads were measured at the end of each elongation step, when specific double-stranded hybridization complexes remain stable due to their high melting temperature, in contrast to shorter nonspecific products. As a result, only specific double-stranded products bind the SYBR Green I dye. To make sure that amplification products produced by on-chip PCR are specific, additional melting curve analysis inside individual gel pads was carried out. The emergence of complexes with high melting temperature within the gel pad indicates that the reaction of amplification was successful and specific.

Quantitative On-chip Real-time PCR

To produce calibration curves, serial 10-fold dilutions of the standard plasma samples were tested (see Materials and Methods). The correlation between the threshold cycle number (C_T) and template copy number is shown in Figure 2. The C_T values determined from the plots similar to those shown in Figure 2A were used to obtain the calibration curves presented in Figure

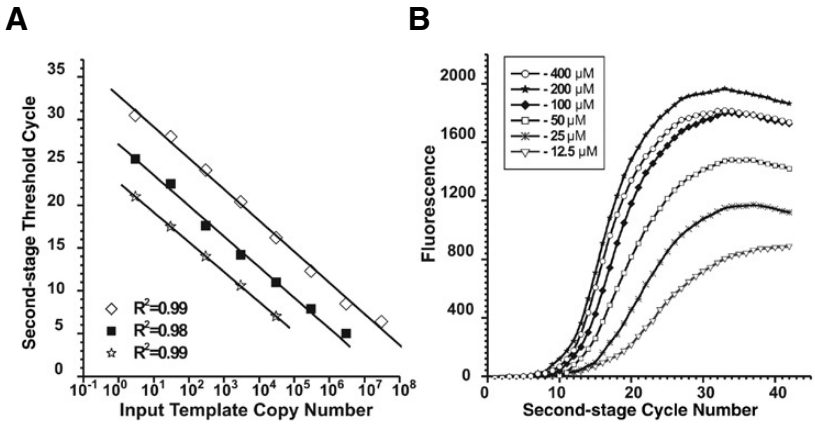

Figure 3. On-chip real-time PCR. (A) Effect of the cycle number at the first RT-PCR stage on calibration curves characteristics. Plot of C_T vs. serial dilutions obtained after 20 (open stars), 25 (closed squares), and 30 (open diamonds) cycles of the first-stage PCR. The *gag* gene of HIV-1 (strain TB132) was cloned in Bluescript II (Stratagene, La Jolla, CA, USA) vector and 10-fold serial dilutions ranging from 3 to 3×10^7 gEq per assay were used as targets. (B) Influence of immobilized primer concentration on the on-chip amplification efficiency. Amplification curves were obtained at indicated concentrations of immobilized primer. The *gag* gene of HIV-1 (strain VI310) cloned in Bluescript II vector (10^5 gEq per assay) was used as a target.

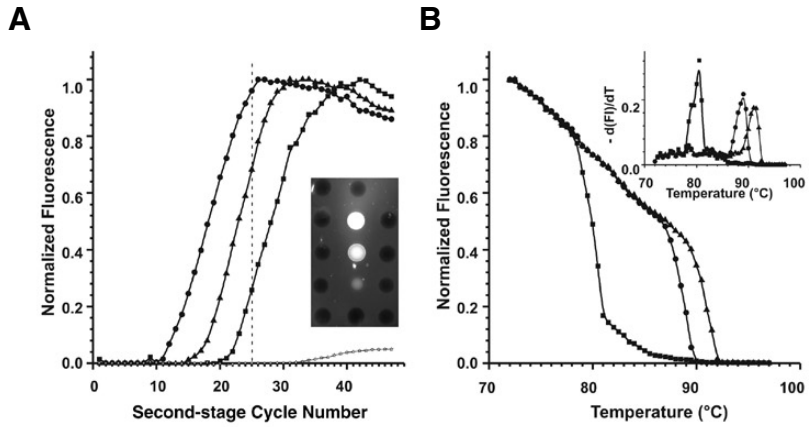

Figure 4. Second-stage real-time on-chip amplification of HCV, HBV, and HIV-1 targets and melting of the amplification products. (A) Amplification plot produced with ImageExpress software (Biochip-IMB Ltd, Moscow, Russia) and fluorescence image of the microarray. The plasma sample contained a mixture of 9×10^3 gEq/mL of HCV (circles), 10^2 gEq/mL of HBV, and 10^1 gEq/mL of HIV-1 (triangles and squares, respectively). The insert shows fluorescence accumulation in gel pads corresponding to HIV-1 (top bright gel element), HBV (middle element), and HCV (bottom bright element) after 25th cycle. Gel pads with immobilized primers are surrounded by background control gel pads. The increase of fluorescence in control pads is marked by the curve with open stars. (B) Melting of duplexes formed between immobilized extended primers and single-stranded amplification products. Melting peaks are shown on the corresponding differentiation plot in the insert.

2B. The 95% detection limits were 14 gEq for HIV-1, 10 gEq (1.7 IU) for HBV, and 15 gEq (7.5 IU) for HCV per assay. The results were reproducible: the deviations in the logarithmic concentration values were within 1Log when four to eight replicates were tested for each concentration.

The dynamic range of quantitative analysis covered six orders of concentrations from 10^0 to 10^6 gEq of HIV-1, HBV, and HCV per reaction if the number of cycles of the first RT-PCR stage was 23–28. Decreasing the number of preliminary stage cycles, one could shift the dynamic range toward quantifying higher concentrations. If the number of pre-on-chip amplification cycles was more than that mentioned above, the dynamic range got narrower but the sensitivity did not increase (see Figure 3A).

The concentration of immobilized primer was shown to be essential for both on-chip PCR efficacy and absolute fluorescence signal intensity. Figure 3B demonstrates the results obtained for various primer concentrations ranging from 12.5 to 400 µM. No significant difference in amplification efficacies was found when 100, 200, or 400 µM concentrations were used. Meanwhile definite decrease of absolute fluorescence signals and increase of C_T value were observed at concentrations below 100 µM. Thus, the concentration of 200 µM was chosen for further assays.

The SYBR Green I concentration suggested by the manufacturer (1×) proved to be optimal to monitor on-chip PCR. Although a 3-fold increase in the dye concentration led to some growth of absolute fluorescence, the efficacy of PCR decreased significantly due to inhibition of the enzyme activity (data not shown).

As a result of optimization, we defined conditions for highly effective on-chip PCR. Overall, its sensitivity did not concede that performed in tubes.

Simultaneous On-chip Real-time Amplification of HIV-1, HBV, and HCV

Figure 4A represents simultaneous on-chip real-time PCR of HIV, HBV, and HCV targets. To demonstrate various kinetics of product accumulation, the three targets were tested in different concentrations. The microarray image was taken at the end of 25th elongation cycle. The T_m values calculated by the melting curve analysis (Figure 4B) were 80°, 92°, and 89°C for HIV-1, HBV, and HCV amplification products, respectively. The obtained temperatures coincided with those calculated theoretically.

To evaluate the ability of the multiplex assay to determine the concentration of each target in the presence of excessive amounts of other viral nucleic acids, serial dilutions of each target (ranging from 10^1 to 10^5 gEq) were analyzed in the presence of about 10^5 gEq of two other viruses. The assays were performed using all possible combinations of the three viruses. No interference between the unrelated viruses and the calibration curve obtained for the tested virus was observed in any combination (Figure 2B). The calibration and melting curves remained unchanged regardless of whether one, two, or three targets were tested in the reaction simultaneously.

Testing Clinical Plasma Samples by Real-time On-chip PCR

A total of 132 serologically characterized blood samples (see Materials and Methods) were analyzed by the developed method. The viral load values indicated below correspond to quantities per 1 mL of initial clinical specimens.

The estimated range of viral loads in 32 HIV-1 serologically positive samples was from 60 to 4.2×10^7 gEq/mL. Five of them contained <1000 gEq/mL.

Among 58 HBV positive samples, 11 had viral loads <1000 gEq/mL and 7 samples had loads $>1.0 \times 10^7$ gEq/mL. The minimal determined load was 50 gEq/mL (8.3 IU/mL).

The minimal load identified in HCV-containing specimens ($n = 50$) was 550 gEq/mL (275 IU/mL); 9 samples had values <1000 gEq/mL; and 4 had loads $>1.0 \times 10^7$ gEq/mL.

In 4 out of 22 samples containing both hepatitis viruses and in 1 out of 2 specimens containing all 3 viruses (HIV, HBV, and HCV), viral loads within 1 sample varied in a range of up to 4 orders of magnitude. With at least 3 replicates of each sample isolated and analyzed separately, the deviations for identical sample portions remained within 1Log of concentrations, thus confirming the reproducibility of the quantitative results.

Eighteen Seronegative Samples Were Tested as Negative Controls, and No False-positive Results Were Obtained

Twenty randomly selected samples were encrypted and tested in a blind format. All positive specimens were correctly identified by on-chip PCR, while no specific amplifications were observed for seronegative samples.

The results of qualitative analysis obtained using serological methods and on-chip PCR techniques were in full concordance. In this study, we had no aim to compare quantitative results for clinical specimens.

DISCUSSION

The paper describes an original approach for simultaneous quantitative identification of HIV-1, HBV, and HCV in blood plasma specimens. The method uses a gel-based oligonucleotide microarray platform to run multiplex PCR in real time. The use of three-dimensional (3-D) polyacrylamide provides a number of advantages (29,30); in particular, it may enhance DNA polymerase activity with immobilized primers relative to primers immobilized on a two-dimensional (2-D) surface (20).

Due to complete physical isolation of individual gel elements, it is possible to use a single nonspecific DNA binding dye (SYBR Green I) to detect all of the multiplex reaction products simultaneously and independently using a microarray consisting of a set of primers immobilized inside separate gel pads. Spatial separation of the immobilized specific primers on a microarray allows the real-time identification of specific targets by measuring the fluorescence emanated from individual gel pads in the course of PCR. This feature distinguishes the proposed approach from other multiplex systems that use SYBR Green I for indirect melting curves analysis of the total amplification product (14). Besides, this technique does not require the preparation of additional specific probes, such as TaqMan (26) or dyes varied in their emission spectra (12).

The technique was developed for the detection of HIV-1, HBV, and HCV because of the necessity of their rapid and reliable identification in blood plasma for early recognition of the socially and epidemiologically dangerous diseases and for large-scale analysis of blood donations. According to the evaluations developed in the UK, USA, and Japan, the use of the NAT-methods for testing donor blood has decreased the risk of HBV, HCV, and HIV-1 transmitting by 90%, 95%, and 80%, respectively (3,31,32). Although several commercial systems are available for simultaneous NAT-testing of HIV-1, HBV, and HCV (5–10), further development of responsive, specific, and reliable multiplex techniques remains an important goal for their wide routine application.

In this study, the design of primers set was carried out to take into account every known polymorphism in the genomes of the viral subtypes. To succeed in amplification of the majority of virus subtypes, the standard primer degeneration tactics was applied. It is admitted that some modifications in primer sequences could be required after testing more various genotypes. Nevertheless, these minor modifications should not affect the general concept of this work.

A nested PCR strategy was applied to increase the sensitivity and specificity of the developed approach. In the course of our experiments, we found that by varying the first-stage PCR cycle number the dynamic range of the method could be easily shifted to desirable range without other key modifications of the procedure. Excellent accordance of the calibration curves obtained for HIV-1, HBV, and HCV when the quantities of the targets are expressed in genome equivalents strongly suggests equal efficacy of amplification of all three targets.

During the optimization of the described assays, we encountered consistent and significant shifts of the optimal annealing temperatures relative to theoretical calculations, when PCR is performed within the microarray gel pads. Adjustment of the theoretically calculated annealing temperature of the immobilized primers has resulted in increased efficiency of real-time PCR. As we described earlier, the annealing temperatures of immobilized primers are approximately 5°C lower than those calculated for primers in solution (28). Although the chemistry of the gel used for the manufacturing of microarrays in this work is different from our previous publications, and the sequences of the primers are completely new, the temperature shift remains similar. As a result, the yield of on-chip PCR products was higher if the annealing temperature was decreased by 5°–6°C in comparison with that calculated theoretically.

The data obtained in this study concerning the identification and quantification of viruses in standard specimens, as well as in clinical plasma samples, show that the developed biochip-based approach is comparable in sensitivity to existing systems built on other principles and used for testing donor blood for HIV-1, HBV, and HCV (5–10). In contrast to a recently developed microarray (24), our approach allows simultaneous identification and quantification of three different viral targets. The assay can be applied for the analysis of samples from patients who have high co-infection rates of blood-borne viruses.

We conclude that the described method is sensitive, specific, and relatively easy to use. The biochip-based analysis can serve as a versatile basis for future development of diagnostic systems for large-scale analysis of clinical blood samples. The developed conception can also be applied for accomplishment of a wide spectrum of tasks related to the multiplex identification and quantification of other genetic targets.

ACKNOWLEDGMENTS

This work was supported by the International Science and Technology Center, grant no. 2906. We are grateful to Sergei Pan'kov and Edward Kreindlin for the manufacturing of microarrays; Sergei Surzhikov and Irina Grechishnikova for synthesis of primers; Alexander Turygin and Roman Urasov for their help with the mathematical calculations and analysis of experimental data; Viktor Barsky

for help with hardware setup; Max Donnikov for his assistance in control experiments; and Alexei Drobyshev for useful discussion. We are very thankful to Jacques Izopet, Tatiana Garanzha, Boris Shevelev, Nataliya Grigortsevich, Dmitry Tikhomirov, and Elena Ignatova for providing plasma samples. The assistance of Elena Novikova and Alexander Kolchinsky (Health Front Line, Ltd., Champaign, IL, USA) in the preparation of this manuscript is appreciated.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Received 25 July 2007; accepted 1 October 2007.

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