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High Throughput Assay for the Simultaneous or Separate Detection of Human Immunodeficiency Virus (HIV) and Hepatitis Type C Virus (HCV)

Key Words
Blood screening · Nucleic acid amplification · HIV · HCV · High throughput

Summary
Background: Current nucleic acid detection formats are too time-consuming for application to individual donor screening. Assays for separate or multiplex detection of HIV and HCV RNA applicable to screening large numbers of samples were designed. **Materials and Methods:** A one-tube assay was developed for detection of HIV/HCV RNA using integrated target capture/magnetic microparticle-based sample preparation, transcription-mediated amplification (TMA), and hybridization protection assay (HPA) technologies. All steps were performed in the same tube and were automation-compatible. The assay was examined for sensitivity, specificity, and throughput in a manual mode. **Results:** Individual and multiplex assays showed analytical sensitivity ≤100 copies viral RNA/ml specimen and reduced the length of the detection window relative to antibody detection. Representatives of HIV type M (A–H) and divergent type O were detected, as were all major HCV genotypes. Specificity at ≥99.5% was observed in a small study of normal specimens. No interference was noted in normal plasmas or plasmas which contain potentially interfering substances such as lipid or bilirubin. No reactivity was observed with samples from patients infected with other blood-borne agents.
Conclusions: Separate or simultaneous detection of HIV and HCV RNA was demonstrated with adequate sensitivity and specificity for large-volume screening needs in an integrated, automation-compatible format.

Schlüsselwörter
Blutspender-Screening · Nukleinsäure-Amplifikation · HIV · HCV · Hoher Probendurchsatz

Zusammenfassung
Hintergrund: Die gegenwärtig verwendeten Tests zum Nachweis von Virus-Nukleinsäure sind zu zeitaufwendig für das Screening von einzelnen Blutspenden. Daher wurden Methoden für den separaten oder gleichzeitigen Nachweis von HIV- und HCN-RNA entwickelt, die für die Testung großer Probenzahlen geeignet sind. **Material und Methoden:** Die Testschritte umfassen eine Probenpräparation durch die Bindung der Viruspartikel an magnetische Kügelchen, eine Transkriptions vermittelte Amplifikation (TMA) und zum Nachweis des Amplifikates einen Hybridisierungs-Protektions-Assay (HPA). Alle Schritte werden im gleichen Reagenzgefäß durchgeführt und eignen sich für eine Automatisierung. Der Assay wurde auf Sensitivität, Spezifität und Probendurchsatz bei manueller Anwendung untersucht. **Ergebnisse:** Sowohl die Einzeltests als auch der kombinierte Test hatten eine analytische Sensitivität von 100 Kopien virale RNA pro ml Probe und verkürzten die Länge des diagnostischen Fensters im Vergleich zur Antikörpertestung. Nachgewiesen werden konnten repräsentative Proben von HIV Typ M (A–H) sowie verschiedene Proben von HIV Typ O sowie alle HCV-Genotypen. In einer kleinen Studie mit normalen Proben war die Spezifität besser als 99,5%. In normalen Plasmen sowie in lipämischen oder bilirubinhaltigen Plasmen wurde keine Interferenz beobachtet. Eine positive Kreuzreaktion mit anderen Vireninfektionen wurde nicht beobachtet.
Schlußfolgerung: Die Methode ist geeignet, getrennt oder gleichzeitig HIV- und HCV-RNA mit adäquater Sensitivität und Spezifität nachzuweisen. Das Testformat ist für große Probenzahlen und Automatisierung geeignet.

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Introduction

Significant improvements in blood safety have been effected by implementation of increasingly more sensitive serological tests for HIV and HCV and by improved donor selection. Transmission of viral infections by donor blood still occurs, however. Failure to detect viral infections by current serological methods may be due to donation while a donor is in the preseroconversion window phase, infection with immunovariant viruses, or donation by nonseroconverting chronic carriers. Data from a number of laboratories have shown that the detection window can be substantially closed and perhaps eliminated by sensitive nucleic acid tests. Evidence for both immunovariant viruses and serologically silent carriers of HIV and HCV has been reported [1]. Intuitively, testing directly for viral nucleic acid should offer significant advantages over antibody and antigen serological tests, which are indirect or surrogate measures of active viral infection.

Antibody testing for HCV and HIV is unlikely to be replaced by nucleic acid testing because of the demonstrated utility in identification of the majority of infected donors and a desire to maintain multiple layers of safety. The HIV p24 antigen test, however, may be replaced with nucleic acid testing, since viral RNA is detected earlier and persists longer than the HIV antigen during the acute phase of the infection [2, 3]. Furthermore, confirmed HIV antigen-positive, antibody-negative results do not always correlate with future seroconversion or conversion to RNA positivity [4]. In 1996, the National Institutes of Health began funding the development of nucleic acid assays appropriate for individual donor testing in accordance with FDA Commissioner statements to reduce the residual risk of viral transmission in the blood supply. In 1996–1997, several plasma manufacturers in the USA and Europe implemented voluntary nucleic acid testing on pools of plasma. In October 1997, a European mandate for nucleic acid testing of all blood was released. The mandate required the test format to have sufficient sensitivity to give a positive result with a 10-fold dilution of a WHO HCV-infected plasma standard, estimated at 10,000 copy level detection [5].

Pooling appears to be the only practical option for screening at this time, as the need for fewer tests leads to a perceived lower cost. Even so, there is an expressed preference for individual donor testing because i) it is consistent with individual donor testing already in place, e. g., licensed serological tests, ii) it obviates the need to validate a pooling algorithm, iii) it inherently gives better sensitivity due to dilution of rare positive samples with predominantly negative specimens in pooling schemes, and iv) it allows the release of product in a timely fashion, leading to control of inventory consistent with current testing procedures.

For the above reasons, our efforts have focused on developing a practical, high throughput system for individual unit testing for HIV and HCV RNA. The assays are designed to allow analytical sensitivity of at least 95% positivity at 100 copies/ml HIV and 100 copies/ml HCV, ≥99.5% specificity, use of an internal control to verify RNA capture, amplification and detection in each reaction, and throughput in the semi-automated mode of at least 200 reactions per day per technician.

Materials and Methods

EDTA plasma or serum (typically 0.5 ml) was added to a 12 × 75-mm polypropylene tube containing 0.5 ml of a buffered detergent solution with magnetic microparticles coupled to oligonucleotides which allow specific RNA capture. In some experiments, a control transcript was added with the detergent solution at about 250–750 copies per reaction. Experiments in which assay conditions and reagent volumes were varied showed that the internal control identified assay inhibition of HIV-1 and HCV amplification at viral RNA levels even below that of the internal control. Samples were incubated at 60 °C to lyse virions and to stabilize, and hybridize oligonucleotides to, the viral RNA. After cooling to room temperature, reaction tubes were placed in a magnetic rack to concentrate microparticles and the bound RNA, and wash steps were performed to remove plasma components.

After completion of washing steps, amplification reagent containing primers, Tris and/or HEPES buffer, salts and cofactors such as potassium chloride and magnesium, 4 deoxynucleotide triphosphates, and 4 ribonucleotide triphosphates required for transcription-mediated amplification (TMA) [6] was added to the same reaction tubes containing microparticles. Following addition of an oil layer, the reaction tubes were incubated at 60 °C to anneal primers and then cooled.

An enzyme mix containing reverse transcriptase and T7 RNA polymerase was added and the tubes incubated for 1 h at 41–42 °C.

Detection using hybridization protection assay (HPA) has been previously described [7]. Oligonucleotide probes specific to viral amplicon and labeled with chemiluminescent acridinium ester (AE) were added to the reaction tubes followed by a hybridization step at 60 °C. Incubation at 60 °C with an alkaline selection solution (0.15 *M* sodium tetraborate pH 8.5–9.0, 1% Triton X-102) chemically reduced the background from unhybridized probe, and no washing steps were required. Tubes were then loaded into a LEADER 450i luminometer (Gen-Probe Incorporated, San Diego, CA, USA) with >200 tube capacity. The instrument performed injection of 0.2 ml of detection reagent I with 0.1% hydrogen peroxide in 1 m*M* nitric acid and detection reagent II, a solution of ≥1 N NaOH. The chemiluminescent signal detected by the instrument is reported as relative light units (RLU), a measure of the photons produced by the chemiluminescent reaction. In some assays, probes labeled with modified AEs with either slow or fast chemiluminescent kinetics were used. Kinetic discrimination of the two signals allowed simultaneous analysis of two hybridization reactions [8]. For the assays using an internal control, the internal control probe was labeled with an AE with fast chemiluminescent kinetics. Probes for both HIV and HCV were labeled with AE with slower chemiluminescent kinetics. Thus, a single readout could indicate an internal control signal and a signal for HIV and HCV probes. HIV and HCV signals were not distinguished. The assay was performed in a manual mode using racks which hold 100 12 × 75-mm reaction tubes. An individual can easily perform 200 assays, including controls and specimens, in a day. Time required for specimen pipetting is reduced in a semi-automated mode. In a fully automated format, an instrument will perform the steps noted in table 1.

Carry-over was addressed by several measures. Unidirectional flow of the personnel and equipment involved in performing the assay reduces the potential for amplicon carry-over. In addition, sample processing was designed to capture only preamplification target sequences. RNA transcripts synthesized during amplification do not contain sequences needed for specific capture and therefore should not be carried into a new reac-

Table 1. Assay timing in a semi-automated HIV/HCV RNA detection system

Assay step	Time to perform step min	Running time h
Pipet controls and specimens with automated pipetting device	20	0.3
Lysis/target capture	45	1.0
Wash steps	35	1.6
Amplification reagent addition and annealing	35	2.2
Amplification	60	3.2
Detection	50	4.0
Luminometer reading	75	5.5

tion. An oil overlay reduces the chance of aerosol contamination during the amplification reaction. The HPA procedure requires no transfer (and therefore minimizes aerosol) of amplicon after amplification. Following detection, sufficient bleach was added to each tube to effectively eliminate further amplification of both RNA and DNA species (data not shown). Bleach was also used to decontaminate work surfaces.

Samples used in the experiments shown were provided by Guadalupe Ercilla (Barcelona, Spain), Lutz Gürtler (Munich, Germany), Angelo Hatzakis (Athens, Greece), Michael Kew (Witwatersrand, South Africa), Ken Kuramoto (Sacramento, CA, USA), Julie Overbaugh (Seattle, WA, USA), Francois Simon (Paris, France), and Mark de Souza (USA Medical component, AFRIMS, Bangkok, Thailand). HIV culture supernatants were provided by Julie Overbaugh, Francois Simon, and Lutz Gürtler. HIV IIIB virion obtained from the NIH repository was grown using standard methods, and cell-free tissue culture supernatant was used as a standard. This virus was quantitated relative to an HIV standard [9] obtained from the Viral Quality Assurance (VQA) Laboratory (James Bremer, Chicago, IL, USA), using an in-house quantitative assay. HCV virion-infected plasma was obtained from Ken Kuramoto at the Sacramento Blood Center and quantified relative to the HCV international standard candidate of the WHO using an in-house quantitative assay. The lyophilized HCV standard AA was received from John Saldanha (NIBSC), dissolved in water to obtain a concentration of 100,000 genome equivalents/ml, and diluted in 1 or 1.5 log steps. Seroconversion panels were obtained from NABI, (Boca Raton, FL, USA) and Boston Biomedica Inc. (W. Bridgewater, MA, USA).

Results

Time to complete steps of the separate HIV, separate HCV, or multiplex HIV/HCV assay in a semi-automated mode is shown in table 1.

In order to determine the analytical sensitivity of detection of HIV RNA, HIV tissue culture supernatant was quantified using a VQA standard and diluted into normal, anti-HIV-1/-2-, anti-HCV-antibody-negative plasma at 90 and 300 copies/ml. 25 replicates of each dilution and the negative plasma were run. Table 2 shows that the analytical sensitivity of the HIV/HCV assay exceeded our initial goal of 95% positivity at 200 copies/ml. Sensitivity in the HIV-1 assay was comparable (data not shown).

Table 2. Sensitivity of detection of HIV-1 virion RNA in the HIV/HCV multiplex format

Copies of HIV RNA/ml	% positive	Number positive/ number tested
0	0	0/25
90	100	25/25
300	100	25/25

Table 3. HIV type O and M subtypes testing positive in separate or multiplex HIV/HCV assays

Type or subtype	Viral isolates	Specimens	Total
O	34	19	53
A	12	25	37
B	10	>100	>100
C, C/E	12	11	23
D	11	13	24
E	4	43	47
F	3	6	9
G	2	1	3
H	1	0	1

In order to assess the ability of the test to detect HIV RNA prior to seroconversion, 27 seroconversion panels were obtained. 100 µl of each member of the panels was tested using the HIV assay as described in Material and Methods. In 20 of 27 panels, RNA was detectable in blood prior to the first antibody-positive sample. In the remaining 7 panels, RNA was first detected at the same time as antibody. 18 of 27 panels contained blood that was HIV p24 antigen-positive prior to the first antibody-positive sample, and in 5 more, antigen was first detected at the same time as antibody. Thus, in 9 of 27 cases HIV RNA was detected before p24 antigen. In 4 of 27 panels (14.8%), no p24 antigen-positive samples were ever observed. Similar results were obtained with the multiplex assay (data not shown). These data confirm the utility of RNA testing for reducing the HIV detection window compared to antibody testing.

HIV subtype, including type O, detection has been an issue for currently available nucleic acid tests [10–13]. To assess HIV subtype detection, in vitro transcripts, tissue culture supernatants from viral isolates, and patient specimens were tested, both undiluted and diluted in normal HIV/HCV antibody-negative plasma. Table 3 summarizes the viral isolates and specimens tested. In all cases tested, type O and type M subtypes including A, B, C, D, E, F, G and H, were clearly detected in the multiplex or individual assays. Viral isolates from tissue culture supernatants were positive at a dilution of 10^{-6}

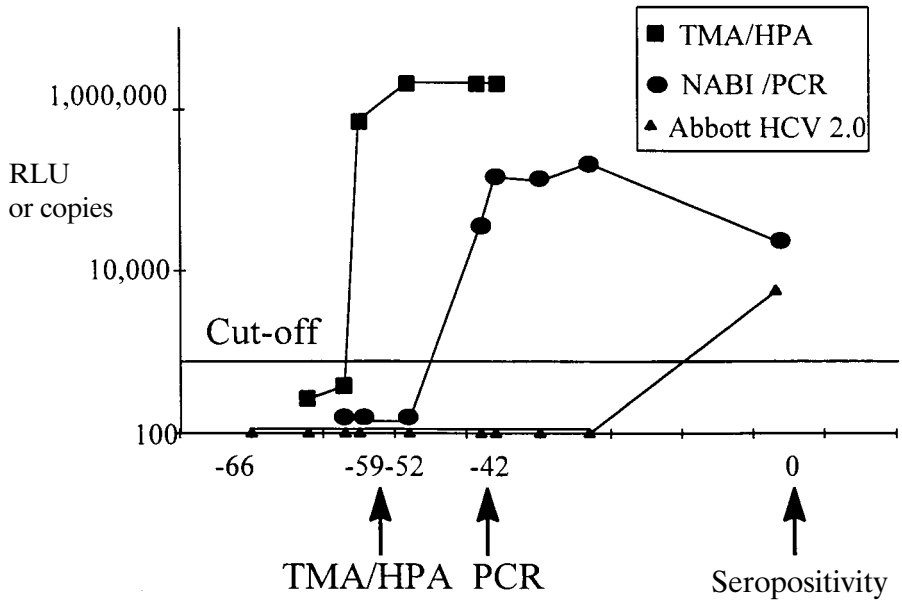

Fig. 1. 100 µl of each panel member was tested in triplicate using the multiplex HIV/HCV assay. The average of 3 values is reported as RLU. Results for in-house PCR from NABI and Abbott HCV2.0 testing were obtained from NABI or from the seroconversion panel SV-0090 package insert.

Table 4. Analytical sensitivity of detection of HCV RNA in plasma using the HIV/HCV multiplex assay

HCV type 1a genome equivalents/ml	% positive	Number positive/ number tested
0	0	0/25
18	77	23/30
180	100	30/30
600	100	30/30
1,800	100	30/30

or lower. All patient specimens were positive when tested undiluted, and most specimens were positive even after serial dilution, depending on the original viral RNA titer of the sample.

To establish the analytical sensitivity of the HCV assay, a high titer HCV infected plasma (calibrated with the WHO HCV standard) was diluted in normal, anti-HIV-1/-2-, anti-HCV-antibody-negative plasma to 1,800, 600, 180, or 18 copies/ml. Replicates of each dilution or the negative plasma were tested using the multiplex format in the presence of internal control with probes directed to HCV. As shown in table 4, detection was 100% at 180–1,800 copies HCV genome equivalents/ml and 77% at 18 genome equivalents/ml, exceeding initial goals for this assay.

Dilutions of a reconstituted HCV standard from NIBSC were also tested in the HIV/HCV multiplex assay. The results were used to calculate the point at which 63% positivity occurred, the estimated point at which one copy of RNA was present per assay. An assumption was made that a single copy of RNA resulted in a positive result. The HIV/HCV multiplex assay showed >50% positivity at a greater than 1:40,000 dilution. Assuming that the undiluted, reconstituted HCV material contained 100,000 HCV genome equivalents/ml, the HIV/HCV multiplex assay had >50% positivity at about 5 WHO genome equivalents/ml with this standard or approximately 5 RNA copies/ml.

The window period between infectivity and seroconversion has not been firmly established for HCV. Testing of multiple seroconversion panels has shown that RNA is frequently detected prior to HCV antibody. As an example, figure 1 shows the results obtained with seroconversion panel SV-0090. The results of the Abbott HCV 2.0 antibody assay (Abbott Park, IL, USA), the results of a NABI in-house RT PCR assay (Boca Raton), and TMA results from the manual HIV/HCV multiplex assay are shown. The results indicated that RNA was detected 42 days prior to antibody detection using the RT PCR assay and 52 days prior to antibody detection using TMA. Two of 3 TMA replicates were positive 59 days prior to antibody positivity. Because seroconversion may have occurred between the last antibody-negative and first antibody-positive samples, the reduction of the detection window may be overestimated. These data confirm work by others that the RNA-positive, antibody-negative window for HCV is quite wide and may be reduced by several weeks with a nucleic acid test [2, 3].

Sensitive detection of all available HCV genotypes was recognized as an important requirement for this assay. Samples of HCV genotypes were obtained from several geographical locations and tested in the single or multiplex format. In a series of experiments, at least 5 different isolates of HCV genotypes 1a, 1b, 2a + 2a/c, 2b, 3a, 4, and 5 were shown to give strong positive signals in the HIV/HCV assay (data not shown).

In order to determine specificity of the multiplex assay, several hundred normal (anti-HIV-1/-2, anti-HCV antibody-nega-

Table 5. Specificity testing with potentially problematic samples

Type of specimen	Number of specimens tested	Spiked control signal	HIV + HCV signal
Bilirubinemic	5	+	–
Hemolyzed	20	+	–
Lipemic	10	+	–
Diagnosis rheumatoid arthritis	5	+	–
Rheumatoid Factor positive	5	+	–
Antinuclear antibody positive	5	+	–
Diagnosis of Lupus	5	+	–
Diagnosis of Cirrhosis	2	+	–
Diagnosis of Liver Cancer	2	+	–
HIV-2	8	+	–
HTLV-1	6	+	–
HTLV-2	5	+	–
HAV	5	+	–
HBV	5	+	–
HGV	5	+	–

tive) plasmas were assayed by 4 operators on 2 days. Reactions were spiked with an RNA internal control which confirmed that target capture, amplification, and detection steps had taken place. Signals from the internal control or the HIV/HCV probe mix were distinguished using kinetic signal analysis. Of 499 plasmas assayed with a mixture of HIV and HCV probes, 2 were positive accounting for an initially reactive rate (IR) of 0.4%. Both samples were retested in duplicate and shown to be true negative samples. Thus, the current manual multiplex assay has a repeatably reactive rate (RR) of 0%. This RR rate has been confirmed in separate studies including more than 1,500 specimens. None of the negative plasmas was inhibitory to amplification of the internal control.

Specificity was also examined using samples containing potentially reactive substances. Table 5 shows results with several different types of specimens, including those from individuals with liver disease. None of the 93 samples were reactive with HIV or HCV probes in the multiplex assay. In addition, no specimens were inhibitory to internal control amplification in the assay.

Discussion

Nucleic acid detection has clear advantages over other testing methodologies for early detection of active viral infection. Adaptation of the technology to the blood bank setting has been slowed by the complexity and cost of standardized tests. We have applied 3 compatible, hybridization-based technologies to develop a rapid, high throughput, sensitive test in a multiplex and automation-compatible format.

The extreme sensitivity of nucleic acid amplification requires special attention be given to prevention of carry-over of amplicon from positive reactions to negative samples prior to amplification. The use of specific RNA capture, unidirectional flow, minimal aerosol-generating steps after amplification, and bleach decontamination all contribute to obtaining accurate results.

The combination HIV/HCV format has the advantage of reducing the number of tests that must be performed and validated by the blood center and of decreasing the cost per result for reagents and technician time. The result is a multiplex assay appropriate for single unit testing with excellent sensitivity and specificity for both viruses, reactivity with all available subtypes and nonreactivity with other infectious agents. The sample processing, amplification and detection methods are applicable to other targets, including other viral RNA such as hepatitis A virus and DNA such as hepatitis B virus and parvovirus.

The assay is currently performed in a manual mode with automated detection. A fully automated instrument system, TIGRIS™, is under development to perform all steps of the assay, from specimen pipetting and processing, to amplification, detection and amplicon deactivation.

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