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Evaluation of a new molecular assay for detection of human immunodeficiency virus type 1 RNA, hepatitis C virus RNA, and hepatitis B virus DNA

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Abstract

Background: Rapid, sensitive, specific, and cost-effective screening of donated blood to prevent transmission of infectious agents remains challenging. In recent years, incorporation of nucleic acid testing for HIV-1 and HCV RNA improved blood safety by reducing the window period between infection and serologic detection. For HBV infection, this window period with most serologic assays is 50–60 days. Adding a nucleic acid test (NAT) for HBV DNA with existing NATs for HIV-1 and HCV RNA would further improve blood safety and blood screening efficiency.
Objective: To evaluate the Procleix Ultrio Assay for simultaneous detection of HIV-1 and HCV RNA and HBV DNA and corresponding discriminatory assays.
Study design: The performance of these assays, which utilize the same technology and assay format as the Procleix HIV-1/HCV assay, was determined using relevant clinical specimens and analytical sensitivity and specificity panels.
Results: The Procleix Ultrio Assay demonstrated specificity of ≥99.5% in healthy donor blood specimens and in plasma containing potentially interfering substances or other blood-borne pathogens. Assay sensitivity demonstrated >95% detection of 100 copies/mL, 30 IU/mL, and 15 IU/mL for HIV-1 and HCV RNA, and HBV DNA, respectively. The assay detects all known HIV-1 subtypes and HCV and HBV genotypes and is highly reproducible. Statistical analysis using receiver operating characteristic plots demonstrated wide analyte cutoff values for each assay associated with assay specificity and sensitivity of ≥99.5%.
Conclusions: In this investigational study, the Procleix Ultrio Assay sensitivity and specificity were similar to existing NATs used in blood-bank settings to detect HIV-1 and HCV RNA and provided equivalent sensitivity and specificity for detection of HBV DNA. Using this combination assay, blood safety may be improved and the multiplex format enhances blood screening efficiency. The throughput capability of this assay is compatible with large volume processing and the chemistry is adaptable to full automation.

Keywords: HIV-1; HCV; HBV; TMA assay; NAT

1. Introduction

The residual risk of hepatitis B virus (HBV) transfusion-transmitted infection is 1 in 30,000 to 1 in 140,000 in the United States (Chiavetta et al., 2003; Lublin, 2002; Schreiber et al., 1996) and Germany (Roth et al., 2002), and 1 in 74,000, 1 in 470,000 and 1 in 242,000 in Spain, France and The Netherlands, respectively (Koppelman et al., 2005). This is due to the 50–60 day window period (Biswas et al., 2003; Mimms et al., 1993) between HBV infection and detection by serology-based screening tests. Current screening tests

Abbreviations: HIV-1, human immunodeficiency virus type 1; HCV, hepatitis C virus; HBV, hepatitis B virus; NAT, nucleic acid test; TMA, transcription-mediated amplification; RLU, relative light units; ROC, receiver operating characteristic; CV, coefficient of variation; S/CO, signal to cutoff; WHO, World Health Organization; S.D., standard deviation; CI, confidence interval; Procleix Ultrio Assay, refers to the multiplex Procleix Ultrio Assay

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in the United States are based on the presence of HBV surface antigen (HBsAg) and/or antibodies to HBV core antigen (anti-HBc). Consequently, donors in the early stage of viremia might test negative for HBV. In addition, there is also a risk of transmission from donations by chronic low level HBV carriers in countries where anti-HBc testing has not been introduced. Minipool NAT screening has been shown to reduce the residual risk of HBV transmission from pre-seroconversion donors as well as from chronically infected carriers. Specifically, a NAT for HBV has detected yield cases at a rate of 1 in 290,895 in the United States (Roche Molecular Systems, 2005c). In Europe and the Asia-Pacific region, 4 HBV window period cases and 59 chronic carriers were identified from 417,000 donations with the Procleix Ultrio Assay (Linnen, 2005). The Procleix HBV Discriminatory Assay also demonstrated sufficient sensitivity to detect HBV infection during the window period in an individual repository sample (Smith et al., 2005). It is possible that the maximum risk reduction would be gained by screening donations for anti-HBc and with a very sensitive HBV NAT.

FDA-licensed tests that detect human immunodeficiency virus type 1 (HIV-1) and hepatitis C virus (HCV) nucleic acids are currently used in the United States blood-bank setting. Similar systems are available in Europe along with validated in-house developed nucleic acid tests (NATs). The NATs are used to test sample pools of variable sizes or individual donor samples. The Prodeix® HIV-1/HCV Assay, the first approved NAT for blood screening, provides a screening efficiency by combining HIV-1 and HCV RNA detection in one test and significant improvement in blood safety by detecting HIV-1 and HCV infection earlier than serologic tests. The Procleix HIV-1/HCV Assay detects HIV-1 infection 8–14 days earlier than approved HIV-1 antibody or HIV-1 p24 antigen tests (Kolk et al., 2002), respectively, and detects HCV infection approximately 59 days earlier than approved antibody tests (Kolk et al., 2002). Since the implementation of NATs in the United States in 1999, 12 HIV-1 confirmed yield cases in approximately 37 million donations and 170 HCV confirmed yield cases in approximately 40 million donations have been identified (Stramer et al., 2004). Consequently, the residual risk of HIV-1 and HCV transfusion-transmitted infection is estimated to be as low as 0.5 per million units (Biswas et al., 2003). In Europe, the residual risk of HIV-1 and HCV transfusion-transmitted infections are estimated to be 0.37 and 0.93 per million donations, respectively, since implementation of NATs for these viruses (Coste et al., 2005).

Several NATs for HBV DNA detection have been developed. Depending on the specific NAT, pool size and HBsAg serologic test used, NATs detect HBV infection several days to several weeks earlier (Biswas et al., 2003) than serologic tests. Nevertheless, a multiplex HIV-1, HCV, and HBV NAT would greatly increase the screening efficiency. Some HIV-1, HCV, and HBV NATs have been described; however, they are not as sensitive for HIV-1 and HCV RNA detection as the approved Procleix HIV-1/HCV Assay (Biswas et al., 2003; Meng et al., 2001; Mine et al., 2003). Therefore, the Prodeix® Ultrio™ Assay was developed for the simultaneous detection of HIV-1 and HCV RNA and HBV DNA based on the technology used for the Procleix HIV-1/HCV Assay. The Procleix Ultrio Assay maintains high sensitivity for HIV-1 and HCV RNA detection and is more sensitive than next generation HBsAg serologic tests for HBV DNA detection. In addition, discriminatory NATs were developed for the detection of HIV-1 and HCV RNA, or HBV DNA individually. In the present report, the evaluation of these four assays is described.

2. Materials and methods

2.1. Procleix Ultrio Assay technology

The Procleix Ultrio Assay utilizes three proprietary technologies: (1) target capture-based sample preparation, (2) transcription-mediated amplification (TMA), and (3) chemiluminescent detection via a hybridization protection assay (Giachetti et al., 2002; Kacian and Fultz, 1995; Nelson et al., 1996). The discriminatory assays utilize the same three main steps as the multiplex assay (target capture, TMA, and hybridization protection assay), follow the same basic assay procedure and use the same reagents with the exception that individual HIV-1, HCV and HBV-specific probe reagents are used in place of the multiplex probe reagent used in the Procleix Ultrio Assay. The assays use a specimen input volume of 0.5 mL (Procleix Ultrio Assay Package Insert, 2004).

2.2. Assay run, calibrators, and internal control

Each assay run accommodates up to 100 reactions, including positive and negative assay calibrators placed at the beginning of each run (9 assay calibrators for the multiplex assay and 10 assay calibrators for the discriminatory assays). The positive and negative calibrator results are used to determine the validity of the run and to establish the assay cutoff values as described below. The positive calibrator is also used to determine the effectiveness of viral lysis.

All four assays incorporate an internal control to validate each reaction. The internal control is an RNA transcript that serves as a general monitor of assay performance for each specimen tested because it is added to each test specimen and assay calibrator reaction. Prior to performing target capture, the internal control is added to a newly opened target capture reagent bottle and is used to control for false negative results due to operator errors or potential sample inhibition.

2.3. Formula-derived assay cutoff values

To determine reactivity in the assays, two cutoff values are determined. These cutoff values are derived from the assay calibrator analyte and internal control relative light unit (RLU) values.

Formulas were established to determine a floating analyte cutoff value in each assay within the range of RLU values indicated by receiver operating characteristic (ROC) analysis to achieve greater than or equal to 99.5% sensitivity and specificity. Specimens exhibiting an analyte signal to analyte cutoff (S/CO) ratio of greater than or equal to 1.0 are considered to be reactive, or positive for HIV-1 RNA, HCV RNA, and/or HBV DNA, while those with an analyte S/CO ratio less than 1.0 are considered non-reactive, or negative for HIV-1 RNA, HCV RNA, and/or HBV DNA.

The internal control cutoff value is also established for each individual assay run, with the cutoff calculation selected to minimize the number of invalid results while at the same time preventing the occurrence of false negative results. A cutoff calculated as 50% of the mean negative calibrator internal control signal met these conditions for the Procleix Ultrio Assay and the HIV-1, HCV and HBV discriminatory assays. If the internal control S/CO ratio is greater than or equal to 1.0, the reaction is considered valid, while any result less than 1.0 is interpreted as invalid result. When samples are reactive (analyte S/CO of 1 or higher) for analyte signal, the result is treated as valid regardless of internal control signal.

2.4. Instrumentation

The testing system is comprised of the following components: a TECAN® GENESIS™ series – RSP® Model 150/8 Instrument pipettor, a GEN-PROBE® target capture system, a GEN-PROBE® LEADER® HC+ luminometer, a dedicated personal computer with Procleix Ultrio Assay data reduction software, circulating water baths for the incubation steps, and multi-tube vortexers for the mixing steps. Data were transferred from the LEADER HC+ luminometer instruments to a structured query language server database from which the data were retrieved and analyzed using Microsoft Excel™ 2000 software.

2.5. Study design

Samples were tested in accordance with the Procleix Ultrio Assay package insert instructions (Procleix Ultrio Assay Package Insert, 2004).

2.5.1. Assay specificity in healthy blood donor specimens

Specificity data were generated by testing previously frozen individual plasma samples that were seronegative for HIV-1, HCV, and HBV from healthy donors obtained from Cruz Roja Española Centro de Donacion de Sangre (Madrid, Spain) and the Community Blood Center of Greater Kansas City (Kansas City, MO). About 2945 samples were tested on specimens from 1240 donors using two lots of reagents.

2.5.2. Analytical sensitivity

Analytical sensitivity panels comprised of HIV-1 subtype B, HIV-1 World Health Organization (WHO) International Standard (97/656), HCV genotype 1 WHO International Standard (96/790), and HBV genotype A WHO International Standard (97/746) were used to evaluate assay sensitivity.

The HIV-1 subtype B panel members were prepared by serial dilution to concentrations of 300, 100, 30, 10, 3, 1, and 0 viral copies/mL of an isolate from a tissue culture supernatant. The viral stocks used to make the panel were quantified using a validated TMA-based HIV-1 quantitative assay that was calibrated against the Virology Quality Assurance Laboratory standard (Virology Quality Assurance Laboratory, Rush-Presbyterian St. Luke's Medical Center, Chicago, IL). HIV-1 WHO International Standard (97/656), HCV genotype 1 WHO International Standard (96/790), and HBV genotype A WHO International Standard (97/746) were serially diluted to concentrations of 600, 200, 60, 20, 6 and 0 IU/mL; 100, 30, 10, 3, 1, and 0 IU/mL; and 45, 15, 5, 1.67, 0.56, and 0 IU/mL, respectively. Panel dilutions were prepared in negative human serum (negative for anti-HIV 1/2 and HIV Ag by US FDA licensed test procedures and/or non-reactive by licensed NATs for HIV-1 RNA; negative for anti-HCV and/or non-reactive by licensed NATs for HCV RNA; and negative for HBsAg by US FDA licensed test procedures).

Forty replicates of each preparation were tested with each of two reagent lots in the Procleix Ultrio Assay and appropriate discriminatory assay with the exception of the HIV-1 WHO standard. This panel was tested with the HIV-1 discriminatory assay only, to ensure reactive results were due to detection of HIV-1 RNA and not the known HBV DNA reactivity of the HIV-1 WHO standard (Shyamala et al., 2004). Probit analysis was used to predict the virus copy levels at which the detection probability was 50% or 95%.

2.5.3. Detection of genetic variants

Multiple, independent isolates of each variant were tested, with the exception of HIV-1 Group N and HBV genotype G, for which only one isolate was available due to their low prevalence.

HIV-1, HCV, and HBV genetic variant panel members were made by serial dilution of patient plasma samples or tissue culture-derived virus in negative human serum. Panel members were created at concentrations of 300, 100, and 30 copies/mL, stored frozen ($\leq -65\,^{\circ}C$) in single-use aliquots, and thawed at room temperature (22–25 °C) immediately prior to testing. Two replicates of each target copy level for each specimen were tested using the Procleix Ultrio Assay and the appropriate discriminatory assay. If one or both replicates of a specimen were reactive, the specimen was assigned a reactive result. Results are reported for the unique number of specimens tested.

2.5.3.1. Detection of HIV-1 subtypes. Specimens were either from patients infected with different HIV-1 subtypes, (obtained from Boston Biomedica, West Bridgewater, MA), or from tissue culture supernatant, obtained from the National Institutes of Health AIDS Reagent Program. The concen-

trations of HIV-1 Group M (subtypes A through G) in the plasma samples from Boston Biomedica were determined by value assignments obtained from the vendor's Certificates of Analysis, from Roche® Amplicor® (version 2.0) or Bayer® Quantiplex™ bDNA (version 2.0) quantitative testing performed by Quest Diagnostics (San Juan Capistrano, CA), or from a validated, proprietary PCR quantification (SuperQuant) performed by National Genetics Institute (Los Angeles, CA). The concentrations of HIV-1 Groups N and O from NIH were determined using a validated TMA-based HIV-1 quantitative assay performed at Gen-Probe (Emery et al., 2000). In addition, all HIV-1 positive specimens with sufficient volume were tested for HIV-1 p24 antigen (Beckman Coulter, Fullerton, CA).

2.5.3.2. Detection of HCV genotypes. Specimens from patients infected with different HCV genotypes (genotypes 1 through 6) were obtained from Boston Biomedica, Intergen Company (Purchase, NY), Teragenix (formerly Millennium Biotech Inc., Ft. Lauderdale, FL), ProMedDx LLC (Norton, MA), and Dr. Ching-Lung Lai (University of the Hong Kong, Hong Kong, China). The concentrations of HCV genotypes in the plasma samples were determined by value assignments obtained from the vendors' Certificates of Analysis, Roche® Amplicor (version 2.0) or Bayer® Quantiplex bDNA (version 2.0) quantitative assays performed by Quest Diagnostics (San Juan Capistrano, CA), or, a validated proprietary PCR quantification (SuperQuant) performed by National Genetics Institute (Los Angeles, CA). Quantitated values were reported in copies/mL. A conversion factor of 1 IU equal to 2.7 copies can be used for comparison of HCV results in copies/mL and IU/mL (23).

2.5.3.3. Detection of HBV genotypes. Specimens from patients infected with different HBV genotypes (genotypes A through G) were obtained from Boston Biomedica, Teragenix and ProMedDx LLC. The concentrations of HBV genotypes in the plasma samples were determined by value assignments obtained from the vendors' Certificates of Analyses, which used the Roche Amplicor (version 2.0) assay. Quantitated values were reported in copies/mL.

2.5.4. Effects of donor and donation variables on assay performance

The effects of donor and donation factors on assay performance were evaluated using various types of specimens and two reagent lots. Conditions, factors, and sample characteristics that were evaluated included the following: (1) viral infections other than HIV-1, HCV, or HBV (herpes simplex virus types 1 and 2, human T-cell lymphotrophic virus types I and II, hepatitis A virus, hepatitis G virus, HIV-2, cytomegalovirus, Epstein-Barr virus, rubella virus, and parvovirus B19) and influenza and HBV vaccination effects; (2) specimens contaminated with bacterial, yeast, or fungal pathogens (*Staphylococcus epidermidis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Corynebactehurn diphtheriae*, *Propionibacterium acnes*, *Candida albicans*, and *Pneumocystis carinii*) (healthy blood donations from the Community Blood Center of Greater Kansas City spiked to 10^6 CFU/mL of each microorganism); (3) plasma samples from donors with autoimmune and other diseases (rheumatoid arthritis, rheumatoid factor, antinuclear antibody, lupus, multiple sclerosis, multiple myeloma, hypergammaglobulinemia [IgG and IgM], alcoholic cirrhosis, and elevated alanine aminotransferase levels); (4) specimens containing potentially interfering substances (hemolyzed, icteric, and lipemic specimens; and a custom analytical panel containing known high levels of bilirubin (2.5 mg/dL), lipids (3000 mg/dL), hemoglobin (500 mg/dL), or serum albumin (6 g/dL)); (5) serum and plasma specimens collected in various anticoagulants (acid citrate dextrose, plasma preparation tube with dipotassium EDTA as the anticoagulant, dipotassium EDTA and tripotassium EDTA, sodium citrate, sodium heparin, citrate phosphate dextrose, and serum tube).

Overall, approximately 470 HIV-1, HCV, and HBV antibody-negative specimens of various donor and donation factors were tested in the four assays for specificity analysis; approximately 10 individual specimens were tested in each category. Aliquots of each specimen were also spiked with HIV-1 target (200 copies/mL), HCV target (60 IU/mL), or HBV target (30 IU/mL) for analysis of assay sensitivity.

Ninety control specimens from healthy blood donors (Community Blood Center of Greater Kansas City) were used as HIV-1, HCV, and HBV antibody-negative specimens for specificity analysis. For the sensitivity analysis, healthy donor specimens were spiked with HIV-1 (targeted input of 200 copies/mL, $n = 90$), HCV (targeted input of 60 IU/mL, $n = 90$), or HBV (targeted input of 30 IU/mL, $n = 102$).

2.5.5. Assay reproducibility

The reproducibility of the Procleix Ultrio Assay was evaluated with respect to variations introduced by laboratory, operator, equipment (LEADER HC+ luminometer system), and assay run. Three operators performed the assay using three luminometers and one reagent lot. The HIV-1, HCV, and HBV samples evaluated were panels containing HIV-1 subtype B, HIV-1 Group O, or HCV genotype 1a at 100 copies/mL, or HBV genotype A at 20 IU/mL. A total of 720 samples were tested and 711 valid results were used in the analysis.

2.5.6. Statistical determination of assay cutoff values

Data from the specificity and sensitivity studies were used for the ROC analysis. Positive samples containing at least 100 copies/mL of HIV-1 subtype B RNA (or 300 copies/mL of other HIV-1 subtypes), 100 copies/mL of HCV genotype 1a RNA (or 300 copies/mL of other HCV genotypes), or 15 IU/mL of HBV genotype A DNA were included in the ROC analysis of the Procleix Ultrio Assay and the appropriate discriminatory assay. Negative samples (unspiked samples) and specimens from healthy blood donors were included in the ROC analyses as specificity samples.

The parameters of sensitivity and (1 – specificity) were plotted for each cutoff value as a ROC curve (Zhou et al., 2002). From these analyses, a range of cutoff values was determined that allows for sensitivity and specificity estimates of 99.5% or higher. The point at which the sensitivity and the specificity were mutually maximized was also determined.

Formulas were derived to calculate a floating cutoff value designed to fall within the range of analyte RLU defined by ROC analysis to achieve greater than or equal to 99.5% sensitivity and specificity. The cutoff formula for each assay averages the analyte signal from the negative calibrators and then, depending on the assay, adds a fraction of the analyte signal values obtained for the HIV-1, HCV, and/or HBV positive calibrators. To maximize sensitivity and reduce the chance of false negative results, the formulas were designed to achieve analyte cutoff values in the lower end of the range defined by ROC curves for each assay.

3. Results

3.1. Assay specificity in healthy blood donor specimens

Data from the specificity study is presented in Table 1. Invalid results due to initial internal control failure were rare (0.82% or lower; Table 1). In all cases, retesting of these samples resulted in valid internal control amplification.

One of 736 specimens tested with the Procleix Ultrio Assay was initially reactive. This specimen was reactive when retested and was reactive when tested with the HCV discriminatory assay (Table 1). This sample was confirmed to be HCV positive using a quantitative HCV NAT and was excluded from this analysis. Of the remaining 735 specimens, none were reactive for a specificity rate of 100% (Table 1). When specimens were tested using the HIV-1, HCV, and HBV discriminatory assays, none of the seven initially reactive samples were reactive upon retest (Table 1), indicating initial false positive results, and therefore no additional testing of these samples was performed. False positive results can occur due to processing errors, such as technique during the selection step, or specimen contamination.

3.2. Analytical sensitivity

Assessment of the analytical sensitivities of the Procleix Ultrio Assay and the HIV-1, HCV, and HBV discriminatory assays is shown in Table 2. There was 100% detection of HIV-1 subtype B, HCV WHO standard, and HBV WHO standard samples with greater than or equal to 100 copies/mL, 10 IU/mL, and 15 IU/mL, respectively, using both the Procleix Ultrio Assay and appropriate discriminatory assay. When HIV-1 WHO standard was tested with the HIV-1 discriminatory assay, there was 100% detection of samples with concentrations of 60 IU/mL or higher.

Results from the probit analysis are shown in Table 3. The predicted 50% and 95% detection probabilities were similar between the Procleix Ultrio Assay and appropriate discriminatory assay. The 95% detection rates for HIV-1 subtype B, HCV genotype 1a and HBV genotype A samples were at approximately 21 copies/mL, 3 IU/mL and 8 IU/mL, respectively.

3.3. Detection of genetic variants

The sensitivities of the Procleix Ultrio Assay and discriminatory assays for detection of genetic variants of HIV-1, HCV, and HBV are summarized in Table 4. Occasionally, the number of specimens tested differed between the Procleix Ultrio Assay and appropriate discriminatory assay because some specimens were co-infected and were not tested with the Procleix Ultrio Assay. Sensitivities of the Procleix Ultrio Assay and discriminatory assays were greater than 96.4% for all HIV-1, HCV, and HBV preparations with the exception of HIV-1 and HBV samples containing at least 100 copies/mL (Table 4). In addition, all HIV-1 positive specimens tested with the HIV-1 p24 antigen test (Beckman Coulter, Fullerton, CA) were seronegative, confirming that the Procleix Ultrio Assay and HIV-1 discriminatory assay are more sensitive than HIV-1 p24 antigen testing (not shown).

Table 1
Specificities of the Procleix Ultrio and HIV-1, HCV, and HBV discriminatory assays in samples from healthy donors

Parameter	Procleix Ultrio	HIV-1 discriminatory	HCV discriminatory	HBV discriminatory
Number tested	736	730	740	740
Initial reactive	0[a]	1	4	2
Initial reactive rate (%)	0.00	0.14	0.54	0.27
Repetitive reactive rate (%)	0.00	0.00	0.00	0.00
Specificity (%)[b]	100	99.86	99.46	99.73
Mean analyte S/CO (S.D.)	0.09 (0.07)	0.13 (0.08)	0.11 (0.07)	0.06 (0.08)
Initial IC failures	6	3	3	6
Initial IC failure rate (%)	0.82	0.41	0.41	0.81
Repetitive IC failure rate (%)	0.00	0.00	0.00	0.00
Mean IC S/CO (S.D.)	2.01 (0.17)	1.98 (0.16)	1.96 (0.11)	2.03 (0.16)

IC: internal control.

[a] One specimen repeat tested positive using the Procleix Ultrio and dHCV assays only. This sample was confirmed to be HCV positive using quantitative HCV NAT and was excluded from analysis.

[b] Specificity rates are based on initial reactive results.

Table 2
Procleix® Ultrio Assay – detection of HIV-1 subtype B, HIV-1 WHO standard, HCV WHO standard, and HBV WHO standard in analytical sensitivity panels

Target	Procleix Ultrio Assay						Assay	Discriminatory assays					
	Number of samples (reactive/tested)	%Positive	95% Confidence limits		Average S/CO	%CV		Number of samples (reactive/tested)	%Positive	95% Confidence limits		Average S/CO	%CV
			Lower	Upper						Lower	Upper		
HIV-1 subtype B (copies/mL)													
300	80/80	100	95	100	16.2	12	HIV-1	80/80	100	95	100	27.4	13
100	80/80	100	95	100	13.8	11		80/80	100	95	100	22.0	18
30	77/79[a]	97	91	100	10.4	33		79/80	99	93	100	16.3	37
10	55/79[a]	70	58	79	7.8	40		55/80	69	57	79	12.8	50
3	24/80	30	20	41	6.0	57		16/79[a]	20	12	31	12.5	60
0	0/79[a]	0	0	4	0.1	65		0/80	0	0	4	0.1	80
HIV-1 WHO (IU/mL)[b]													
600			N/A				HIV-1	80/80	100	95	100	29.4	18
200			N/A					80/80	100	95	100	24.1	17
60			N/A					79/79[a]	100	95	100	21.9	22
20			N/A					73/80	91	83	96	16.8	38
6			N/A					46/80	58	46	68	12.1	59
0			N/A					0/77[a]	0	0	4	0.1	92
HCV WHO (IU/mL)													
100	80/80	100	95	100	8.5	9	HCV	80/80	100	95	100	24.4	4
30	80/80	100	95	100	7.5	7		80/80	100	95	100	22.4	6
10	80/80	100	95	100	7.5	7		80/80	100	95	100	21.9	8
3	74/80	93	84	97	7.0	15		79/80	99	93	100	20.5	19
1	49/80	61	50	72	7.1	13		47/80	59	47	70	18.0	36
0	0/80	0	0	4	0.1	63		0/78[a]	0	0	4	0.1	162
HBV WHO (IU/mL)													
45	80/80	100	95	100	16.1	4	HBV	80/80	100	95	100	25.5	4
15	80/80	100	95	100	14.9	12		80/80	100	95	100	23.1	14
5	59/80	74	63	83	13.7	19		60/80	75	64	84	21.4	27
1.67	28/80	35	25	46	10.3	51		35/80	44	33	55	17.9	48
0.56	9/80	11	5	20	12.2	42		10/80	13	6	22	20.9	27
0	0/80	0	0	4	0.1	42		0/80	0	0	4	0.0	144

N/A: not applicable.
[a] Invalid reactions were not included.
[b] Due to the presence of HBV in this standard (Shyamala et al., 2004), only the Discriminatory HIV-1 Assay was tested. For further information, contact National Institute for Biological Standards and Control (NIBSC), Blanche Plane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, U.K. Enquiries@nibsc.ac.uk or http://www.nibsc.ac.uk. Phone: 44 (0) 1707 641000. Fax: 44 (0) 1707 646854.

3.4. Effects of donor and donation variables on assay performance

The reactivity rates obtained with samples representing various donor and donation variables tested using the Procleix Ultrio Assay are shown in Table 5. All HIV-1-, HCV-, and HBV-seronegative samples, regardless of specimen type, exhibited reactivity rates of 0%, except the autoimmune and other disease specimen categories for which the initial reactive rate was 4.8% (Table 5). However, the overall reactivity rate of 0.8% (CI = 0.2–2.0%) was not statistically different from that determined with the control specimens (0%

Table 3
Probit analysis of Procleix Ultrio and HIV-1, HCV, and HBV discriminatory assays

Target	Assay	Detection probabilities[a]	
		50% (95% fiducial limits)	95% (95% fiducial limits)
HIV-1 subtype B	Procleix Ultrio	8.5 (7.2–10.2)	20.7 (17.7–25.4)
	HIV-1 discriminatory	8.5 (7.3–9.9)	17.8 (15.4–21.6)
HIV-1 WHO (97/656)	HIV-1 discriminatory	8.1 (6.7–9.6)	19.6 (17.2–23.2)
	Procleix Ultrio	1.2 (1.0–1.4)	2.8 (2.4–3.3)
HCV WHO (96/790)	HCV discriminatory	1.0 (0.9–1.2)	2.0 (1.7–2.4)
	Procleix Ultrio	3.2 (2.7–3.8)	7.5 (6.4–9.0)
HBV WHO (97/746)	HBV discriminatory	3.0 (1.7–6.2)	7.4 (6.4–9.0)

[a] Detection probabilities are reported as copies/mL for HIV-1 and IU/mL for HIV-1 WHO, HCV, and HBV.

Table 4
Detection of HIV-1 subtypes and HCV and HBV genotypes with the Procleix Ultrio Assay and the HIV-1, HCV, or HBV discriminatory assays

Variant	Concentration[a] (copies/Ml)	Ultrio[b] (reactive/tested)	Discriminatory assay	
			Specific assay	Reactive/tested
HIV-1 Group M subtype A	300	7/7	HIV-1	7/7
	100	7/7		7/7
	30	7/7		7/7
HIV-1 Group M subtype B	300	5/5	HIV-1	7/7
	100	5/5		7/7
	30	4/5		7/7
HIV-1 Group M subtype C	300	8/8	HIV-1	8/8
	100	7/8		8/8
	30	5/8		8/8
HIV-1 Group M subtype D	300	7/7	HIV-1	7/7
	100	7/7		6/7
	30	7/7		7/7
HIV-1 Group M subtype E	300	6/6	HIV-1	7/7
	100	6/6		7/7
	30	6/6		7/7
HIV-1 Group M subtype F	300	4/4	HIV-1	6/6
	100	4/4		6/6
	30	4/4		6/6
HIV-1 Group M subtype G	300	2/2	HIV-1	3/3
	100	2/2		3/3
	30	2/2		3/3
HIV-1 Group N	300	1/1	HIV-1	1/1
	100	1/1		1/1
	30	0/1		1/1
HIV-1 Group O	300	7/7	HIV-1	7/7
	100	7/7		7/7
	30	7/7		7/7
HIV-1 variants total	300	47/47	HIV-1	53/53
	100	46/47		52/53
	30	42/47		53/53
HCV genotype 1	300[c]	7/7	HCV	7/7
	100[c]	7/7		7/7
	30[c]	7/7		7/7
HCV genotype 2	300[c]	13/13	HCV	14/14
	100[c]	13/13		14/14
	30[c]	13/13		14/14
HCV genotype 3	300[c]	6/6	HCV	6/6
	100[c]	6/6		6/6
	30[c]	6/6		6/6
HCV genotype 4	300[c]	6/6	HCV	7/7
	100[c]	6/6		7/7
	30[c]	6/6		7/7
HCV genotype 5	300[c]	3/3	HCV	3/3
	100[c]	3/3		3/3
	30[c]	3/3		3/3
HCV genotype 6	300[c]	5/5	HCV	5/5
	100[c]	5/5		5/5
	30[c]	5/5		5/5
HCV variants total	300	40/40	HCV	42/42
	100	40/40		42/42
	30	40/40		42/42

Table 4 (*Continued*)

Variant	Concentration[a] (copies/Ml)	Ultrio[b] (reactive/tested)	Discriminatory assay	
			Specific assay	Reactive/tested
HBV genotype A	300[d]	15/15	HBV	15/15
	100[d]	14/15		15/15
	30[d]	12/15		14/15
HBV genotype B	300[d]	7/7	HBV	7/7
	100[d]	7/7		7/7
	30[d]	7/7		7/7
HBV genotype C	300[d]	9/9	HBV	9/9
	100[d]	9/9		9/9
	30[d]	8/9		7/9
HBV genotype D	300[d]	10/10	HBV	10/10
	100[d]	9/10		9/10
	30[d]	6/10		7/10
HBV genotype E	300[d]	5/5	HBV	5/5
	100[d]	5/5		5/5
	30[d]	4/5		4/5
HBV genotype F	300[d]	9/9	HBV	9/9
	100[d]	9/9		8/9
	30[d]	8/9		7/9
HBV genotype G	300[d]	1/1	HBV	1/1
	100[d]	1/1		1/1
	30[d]	1/1		1/1
HBV variants total	300	56/56	HBV	56/56
	100	54/56		54/56
	30	46/56		47/56

[a] Copy levels were chosen to represent levels near the assay sensitivity claim for each variant.
[b] Results are reported for the number of independent isolates tested. A isolate was reactive if one or both replicates were reactive.
[c] 1 IU is equivalent to 2.7 copies.
[d] 1 IU is equivalent to 4 copies.

Table 5
Effect of donor and donation factors on sensitivity and specificity of Procleix Ultrio

Condition	Number reactive/number tested[a] (% positivity)			
	HIV-1, HCV, and HBV antibody negative	HIV-1 positive[b]	HCV positive[b]	HBV positive[b]
Viral infections (not HIV-1, HCV, or HBV)[c]	0/114 (0)	106/106 (100)	108/108 (100)	113/113 (100)
Microbe contamination[d]	0/70 (0)	70/70 (100)	70/70 (100)	72/72 (100)
Autoimmune and other diseases[e]	4[f]/84 (4.8)	90/90 (100)	90/90 (100)	71/72 (98.6)
Potentially interfering substances[g]	0/123 (0)	132/132 (100)	134/134 (100)	129/131 (98.5)
Various anticoagulants[h]	0/80 (0)	80/80 (100)	80/80 (100)	80/80 (100)
Overall % positivity[i] (95% CI)	0.8 (0.2–2.0)	100 (99.4–100)	100 (99.4–100)	99.3 (98.1–99.9)
Control ($n = 90$)[i] (95% CI)	0 (0–3.2)	100 (96.7–100)	100 (96.7–100)	96.1 (90.3–98.9)

[a] Variable sample volume availability resulted in slightly different numbers of replicates tested within categories of specimens.
[b] Aliquots of each specimen were also spiked with HIV-1 target (200 copies/mL), HCV target (60 IU/mL), or HBV target (30 IU/mL) for analysis of assay sensitivity.
[c] Herpes simplex virus types 1 and 2, human T-cell lymphotrophic virus types I and II, hepatitis A virus, hepatitis G virus, HIV-2, cytomegalovirus, Epstein-Barr virus, rubella virus, and parvovirus B19 (ProMedDx, LLC; BioClinical Partners, Inc.; and Boston Biomedica).
[d] *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Candida albicans*, *Pneumocystis carinii*, *Micrococcus luteus*, *Propionibacterium acnes*, *Corynebacterium diphtheriae* (10 donors per microbe).
[e] Lupus, antinuclear antibody, rheumatoid arthritis, rheumatoid factor, multiple myeloma, elevated ALT, alcoholic cirrhosis ($n = 5$), multiple sclerosis ($n = 5$), hyperglobulinemia IgG ($n = 9$), hyperglobulinemia IgM ($n = 3$) (10 donors per type, unless noted; ProMedDx, LLC and BioClinical Partners Inc.).
[f] A 4.8% (4/84) false positive and had insufficient volume for further testing. The overall reactivity rate of 0.8% (CI = 0.2–2.0%) was not statistically different from that determined with the control specimens (0% [CI = 0–3.2%]). Similar results were obtained using the discriminatory assays with 0% reactivity (CI = 0–0.8%; data not shown).
[g] Lipids, hemoglobin, bilirubin, albumin (12–13 replicates per type); icteric, lipemic, and hemolyzed (five donors per type; ProMedDx, LLC).
[h] Dipotassium and tripotassium EDTA, plasma preparation tube, sodium citrate, sodium heparin, acid citrate dextrose, serum, and citrate phosphate dextrose (10 donors per type; ProMedDx, LLC).
[i] Values in parentheses are 95% confidence intervals.

[CI = 0–3.2%]) based on 95% confidence intervals for the control sample size of 90. Similar results were obtained using the discriminatory assays with 0% reactivity (0.0%; 95% CI = 0.0–0.8%; data not shown).

When aliquots of the samples spiked with HIV-1, HCV, or HBV were tested, the overall reactivity rates were 100% (95% CI = 99.4–100%) for HIV-1 and HCV spiked samples and 99.3% (95% CI = 98.1–99.9%) for HBV spiked samples. The rates for detection of the three targets were statistically indistinguishable based on 95% confidence intervals and were similar to reactivity rates reported for the control samples (Table 5). Similar results were obtained using the discriminatory assays (data not shown).

3.5. Assay reproducibility

A total of 711 valid reaction results were analyzed for assay reproducibility using the Procleix Ultrio Assay to determine the influences of laboratory, operator, equipment (luminometer), and run on assay variability. The reproducibility of the Procleix Ultrio Assay was evaluated in terms of positivity rates (agreement with expected results) and coefficients of variation (CV). Overall results indicated that the HIV-1 subtype B and HCV genotype 1a panels containing 100 copies/mL (1 IU = 2.7 copies of HCV) achieved 100% detection rates and the HBV genotype A panel containing 20 IU/mL resulted in a 98.9% reactive rate. In negative panel members, the positivity rate was 0.56% due to one false positive result. Across factors, CV values were 14.1% or lower. Overall, intra-run variance, also known as random error, introduced more variability than the other factors assessed. The effects of the potential variability-inducing factors were determined by calculating the mean, S.D., and CV of the signal among laboratories ($n = 2$), operator ($n = 3$), LEADER HC+ luminometer system ($n = 3$), runs ($n = 18$), and of the signal within runs ($n = 18$). For HIV-1 subtype B detection, CV values ranged from 0% to 4.7% for the factors analyzed. For HCV genotype 1a detection, CV values ranged from 0% to 3.7%. CV values ranged from 0% to 14.1% when specimens containing HBV genotype A were tested. Overall, intra-run variance, also known as random error, introduced more variability than the other factors assessed, with CV values ranging from 3.7% to 14.1%.

3.6. Statistical determination of assay cutoff values

Conventional ROC plots showed that in all cases, even with very high sensitivity (99.5% or higher), specificity also remained very high at 99.5% or higher (data not shown). For the Procleix Ultrio Assay, the cutoff values that maximized sensitivity and specificity for detection of each target ranged from 74,952 to 81,445 RLU. These optimal cutoff values were also similar between all assays, ranging from 42,862 to 108,874 RLU.

The calculated cutoff values, determined from the same runs that were included in the ROC analyses, showed that for all four assays, the highest average and lowest average calculated cutoff values were within the ranges determined by ROC analysis to achieve greater than or equal to 99.5% sensitivity and specificity in the detection of HIV-1 and HCV RNA, and HBV DNA, confirming the cutoff formulas.

4. Discussion

The results of the present investigational study demonstrate that the multiplex Procleix Ultrio Assay provides a highly specific, sensitive and reproducible assay for all three target viruses: HIV-1, HCV, and HBV in various blood specimens and under a variety of collection conditions. The individual HIV-1, HCV, and HBV discriminatory assays, which may be used to determine the identity of the virus present in a specimen testing positive using the Procleix Ultrio Assay, exhibited similar specificity, sensitivity and reproducibility as the multiplex Procleix Ultrio Assay. The Procleix Ultrio Assay and the HIV-1, HCV, and HBV discriminatory assays demonstrated specificity of greater than or equal to 99.5% and sensitivity of detection greater than or equal to 99.5% in samples containing HIV-1 subtype B RNA at greater than or equal to 100 copies/mL, HCV genotype 1a RNA at 30 IU/mL, and HBV genotype A DNA at 15 IU/mL. This was achieved when testing samples from normal blood donors, positive samples containing various genetic variants of each target, and with specimens of varied characteristics, including potentially interfering donor and donation factors. Among the various donor and donation factors evaluated, some initial false positive results were observed when testing specimens from patients with autoimmune diseases (four specimens out of 84 tested). Unfortunately, there was insufficient volume to investigate these results further and although this rate was higher than expected it was not statistically different from the false positive rate in the control specimens.

The high specificity and analytical sensitivity of the FDA-licensed Procleix HIV-1/HCV Assay was maintained in the Procleix Ultrio Assay and probit analysis predicted 95% detection rates in the WHO International Standards for HIV-1 subtype B, HCV genotype 1a, and HBV genotype A samples at 21 copies/mL, 3 IU/mL, and 7 IU/mL, respectively. In comparison, when evaluated using probit analysis, 95% detection rates in the WHO International Standards of 78.4 IU/mL (50.2 copies/mL), 28.8 IU/mL, 4.41 IU/mL were reported for the COBAS AmpliScreen™ HIV-1, HCV, and HBV assays, respectively, using the more sensitive Multiprep Specimen Processing Procedure. When using the Standard Specimen Processing Procedure, 95% detection rates in the WHO International Standards of 323.4 IU/mL (207.3 copies/mL), 41.9 IU/mL, 15.99 IU/mL were reported for the COBAS AmpliScreen™ HIV-1, HCV, and HBV assays, respectively (Roche Molecular Systems, 2005a,b,c).

Furthermore, the Procleix Ultrio Assay detected all genetic variants of each target with similar sensitivities, including the most divergent HIV-1, HCV and HBV vari-

ants present worldwide. Therefore, the Procleix Ultrio Assay and the corresponding discriminatory assays are suitable for worldwide use. These results for detection of HIV-1 and HCV RNA from genetic variants with the Procleix Ultrio Assay are similar to those obtained with the Procleix HIV-1/HCV Assay (Linnen et al., 2002). Although the Procleix Ultrio Assay appears to be more sensitive for detection of HCV genotype 2 RNA (100% positivity at 30 copies/mL and 100 copies/mL) than the Procleix HIV-1/HCV assay (60% and 89% positivity at 30 copies/mL and 100 copies/mL, respectively) the relatively small sample sizes tested do not support a robust statistical comparison.

The advantages of the multiplex format of the Procleix Ultrio Assay are similar to those for the Procleix HIV-1/HCV Assay. The time required to complete initial screening for three targets with the Procleix Ultrio Assay is reduced by approximately two-thirds compared to a single target format due to the specimen processing, amplification, and detection of multiple targets simultaneously. The batch specimen processing protocol allows extraction of nucleic acids from 200 specimens (either individual donor samples or plasma pool samples) and calibrators by a single operator in less than 2 h and completion of 182 test results for each target in 6 h on a semi-automated platform. Given a pool size of 16, throughputs up to 2912 samples yielding 8736 NAT results can be achieved in 6 h by one operator. These throughput capacities meet NAT screening requirements of blood testing centers.

Like the Procleix HIV-1/HCV Assay, the Procleix Ultrio Assay will allow continued detection of HIV-1 and HCV NAT-positive, antibody-negative donations. The Procleix Ultrio Assay expands this capability to the detection of HBV NAT-positive, antibody-negative donations. The analytical sensitivity of the Procleix Ultrio Assay for HBV DNA detection is substantially higher than that of licensed and next generation HBsAg tests, which are reactive at estimated viral loads of 363–1069 IU/mL and 102–267 IU/mL, respectively (Biswas et al., 2003).

Finally, the technology is readily adaptable to include other targets such as West Nile virus, hepatitis A virus, and parvovirus, and the assay chemistry is adaptable to full automation.

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