Branched DNA Technology in Molecular Diagnostics

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Key Words: Microbiology; Virology; Molecular diagnostics

DOI: 10.1309/90BU6KDXANFLN4RJ

Abstract

Viral quantification or viral load testing has become part of the routine management of patients infected with HIV-1 or hepatitis C virus (HCV). There are currently several molecular technologies that are available for use in the clinical laboratory setting. Of these, only the branched DNA (bDNA) assays are FDA-approved for HIV-1 and HCV viral load testing. This signal amplification technology is built on a series of hybridization reactions that are highly amenable to full automation and thus lessen the amount of labor required to perform this type of analysis. This article provides a historical perspective of bDNA and its clinical applications.

The introduction of new therapeutics in the late 1980s and early 1990s to combat the HIV-1 virus also introduced the need for viral quantification assays to be performed by the clinical laboratory. Therapeutic response then could be monitored as the virus decreased or increased in copy number owing to the effectiveness of the treatment. Two HIV-1 viral load assays emerged that used different amplification technologies. The first was the branched chain DNA (bDNA) signal amplification technology followed by the reverse transcriptase–polymerase chain reaction (RT-PCR) sequence amplification assay. These 2 technologies continue to be the most prominent for viral quantification testing. This article provides an overview of bDNA technology and clinical applications.

Molecular diagnostic assays using bDNA technology for detection of nucleic acid target molecules are sensitive, specific, and reliable tools in the diagnosis of viral and bacterial infections and for monitoring disease progression during the course of therapy. bDNA tests have evolved from developmental stages in the research laboratory to US Food and Drug Administration–approved quantitative assays with valuable clinical applications. The bDNA assays are less labor-intensive than many molecular-based procedures because they are highly amenable to total automation. Using bDNA, amplification of a target sequence is not required, and, thus, cross-contamination between replicate samples due to excessive amplicons or carryover is less likely in bDNA assays. In addition, because bDNA is a signal amplification technology, the assay is able to quantify with less than a 0.5 log or 3-fold variability for its entire dynamic range.

bDNA technology has proved versatile because methods have been developed for the detection of infection by a wide

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range of microorganisms, including the parasite *Trypanosoma brucei,*[1] cytomegalovirus,[2] antibiotic-sensitive and antibiotic-resistant *Staphylococcus* bacteria,[3] human papillomavirus,[4] and hepatitis B virus.[5] However, more recent efforts have focused on the development of bDNA assays for the quantification of HIV-1 and hepatitis C virus (HCV) RNA, leading to the routine application of bDNA methods in the clinical molecular diagnostics laboratory. In describing the advancement of bDNA methods, this review emphasizes bDNA assays for HIV-1 and HCV.

How bDNA Works

In contrast with techniques that rely on in vitro amplification of the target sequence (ie, PCR, transcription-mediated amplification, nucleic acid sequence–based amplification, and strand displacement amplification), the sensitivity of bDNA methods is achieved by signal amplification on the bDNA probe after direct binding of a large hybridization complex to the target sequence.[6,7] This series of hybridization steps results in a "sandwich" complex of probes and target sequence. These unusual synthetic oligonucleotides are composed of a primary sequence and secondary sequences that result in a branched structure extending from the primary sequence.[8]

The initial step in a bDNA assay is to ensure that viral particles have been disrupted and that viral RNA is present for analysis **Figure 1A**. In the most recent third-generation bDNA assays, target-specific oligonucleotides (label extenders and capture extenders) then are hybridized with high stringency to the target nucleic acid **Figure 1B**.[7] Capture extenders are designed to hybridize to the target and to capture probes, which are attached to a microwell plate. Label extenders are designed to hybridize to contiguous regions on the target and to provide sequences for hybridization of a preamplifier oligonucleotide. Signal amplification then begins with preamplifier probes hybridizing to label extenders. The preamplifier forms a stable hybrid only if it hybridizes to 2 adjacent label extenders. Other regions on the preamplifier are designed to hybridize to multiple bDNA amplifier molecules that create a branched structure **Figure 1C**. Finally, alkaline-phosphatase (AP)-labeled oligonucleotides, which are complementary to bDNA amplifier sequences, bind to the bDNA molecule by hybridization. The bDNA signal is the chemiluminescent product of the AP reaction **Figure 1D**.

The signal in the bDNA assay is proportional to the number of AP-labeled probes that hybridize to bDNA secondary sequences. Because bDNA assays are based on a series of hybridization reactions, the inhibition of enzymatic steps (AP) by endogenous or exogenous sources is not a significant source of analytic error. Also, these assays have not been shown to be affected by the use of collection devices such as the BD Vacutainer PPT (Becton Dickinson, Franklin Lakes, NJ). Absolute quantification is accomplished by establishing a standard curve for each run. In addition, negative and positive control samples are included in each run.

First-Generation HIV-1 bDNA Assays

Accurate, reproducible first-generation bDNA assays were developed for the detection of HIV-1 RNA and HCV RNA in human plasma (Quantiplex HIV-1 or HCV RNA 1.0 assay, Bayer, Tarrytown, NY).[9-11] In one of the first reports of the Quantiplex bDNA assay, no reactivity using plasma samples from seronegative donors was observed using the first-generation bDNA assay, indicating excellent specificity.[9] Positive results in the bDNA assay were observed in 83% of samples from 348 patients who were seropositive for HIV-1.[9] The dynamic range for quantification using the Quantiplex HIV-1 RNA 1.0 assay extended from 10^4 (lower limit of detection) to more than 10^6 HIV RNA copies/mL.[9,11] Changes in viral load of 2- to 3-fold were statistically significant using the bDNA test, indicating that the Quantiplex HIV-1 RNA 1.0 assay was highly accurate and reproducible.[11] By comparison, changes in viral load of at least 3.7- to 5.8-fold were necessary before results were statistically significant using the earliest versions of the RT-PCR assay.[11] Thus, bDNA became the method of choice for most clinical trials evaluating viral load testing and the clinical efficacy of new HIV-1 reverse transcriptase and protease inhibitors.

The sensitivity of the bDNA detection method was enhanced in the second-generation HIV-1 assay (Quantiplex HIV-1 RNA 2.0 assay, Bayer) by changing the design of the target and capture probes and by the addition of preamplifier oligonucleotides.[12,13] The improved design of the target and capture probes allowed an increase in the stringency of hybridization, thereby decreasing the assay background. Preamplifiers dramatically increase signal intensity because each preamplifier molecule has multiple regions for hybridization to many bDNA molecules. In addition, each bDNA molecule has multiple, repeat sequences for hybridization of AP-labeled probes. The signal output using the Quantiplex HIV-1 RNA 2.0 assay showed linearity from approximately 500 copies/mL to 1.6×10^6 copies/mL (stated dynamic range was 500 to 8×10^5 copies/mL).[11,12] The sensitivity of the second-generation HIV-1 bDNA assay was increased by 20-fold compared with the first-generation assay (lower limits of detection were 500 copies/mL and 10×10^4 copies/mL, respectively).

In an extensive analysis of precision, initial test results and retest results in the Quantiplex HIV-1 RNA 2.0 assay were compared. The HIV-1 RNA 2.0 assay was found to be highly reproducible.[14] Of 174 samples with viral loads of more than

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DOI: 10.1309/90BU6KDXANFLN4RJ

5,000 copies/mL, 96% had differences of less than 0.3 $\log_{10}$ in copy number between initial results and retest results. Of 69 samples with viral loads between 500 and 5,000 copies/mL, 86% had less than 0.3 $\log_{10}$ differences between initial and retest results. However, among 5,339 patients who were tested in routine clinical testing during a 1-year interval, viral loads of fewer than 500 copies/mL were observed in 41.6% of samples. Therefore, a bDNA assay with higher sensitivity (ie, lower limit of detection of <500 copies/mL) was needed for a substantial proportion of patients.

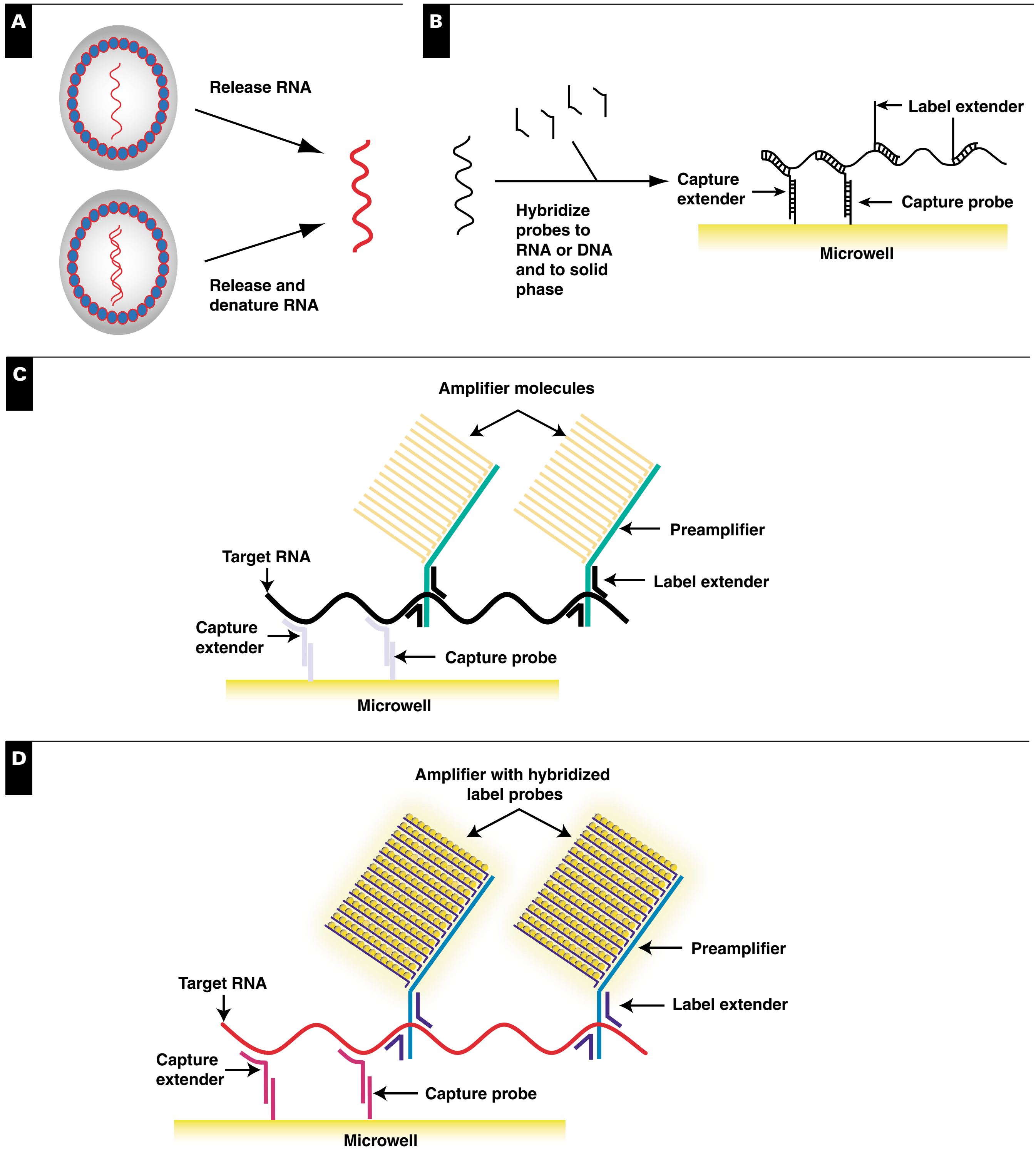

Figure 1 **A**, Branched chain DNA (bDNA) specimen processing. Capture stage: disruption of organism. **B**, bDNA target capture. **C**, bDNA signal amplification. **D**, bDNA detection.

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The performance characteristics of the Quantiplex HIV-1 RNA 2.0 assay and an RT-PCR test (Amplicor HIV-1 Monitor 1.0 assay, Roche Diagnostics, Indianapolis, IN) were compared using dilutions of standard samples that had known HIV-1 virus copy numbers.[13] When dilutions of the same standards were tested in the 2 assays, HIV-1 copy numbers generally were higher from the RT-PCR assay than from the bDNA assay. For example, the ranges of RNA copy numbers were 900 to 7.68×10^5 copies/mL in the bDNA test and 3,360 to 1.88×10^6 copies/mL in the RT-PCR assay. Both assays were linear for the stated dynamic ranges.

Comparison of the slopes of HIV-1 copy number vs signal output regression lines suggested that the bDNA test had less proportional systematic error. Between-run variability, using a standard sample with 1,650 HIV-1 copies/mL, was lower in the bDNA test than in the RT-PCR assay; coefficients of variations for the bDNA and RT-PCR assays were 24.3% and 34.3%, respectively, indicating that the bDNA assay was slightly more precise at this HIV-1 RNA copy number. Compared with using the 1,650-copies/mL standard, the between-run coefficients of variation were higher for a sample containing 165 HIV-1 RNA copies/mL (44.0% and 42.7% for the bDNA and RT-PCR assays, respectively). Assay results were similar when equal HIV-1 copy numbers were compared across HIV subtypes A through F by using the bDNA test. However, by this version of RT-PCR, subtypes A, E, and F were detected less efficiently than the B, C, and D subtypes. The differences between the second-generation bDNA test and the Amplicor Monitor RT-PCR test for HIV-1 quantification indicated that consistency in testing method was required for each individual patient throughout diagnosis and treatment.

HCV bDNA Assays

While continuing to develop an improved third-generation HIV-1 bDNA assay, Bayer recognized the need to develop an HCV viral load assay with an emerging clinical usefulness similar to that for HIV-1 testing. For the detection of HCV, the first-generation bDNA assay (Quantiplex HCV RNA 1.0 assay, Bayer) had a dynamic quantification range in human plasma from 3.5×10^5 to 1.2×10^8 HCV RNA copies/mL.[10,15] Genotypes 1 through 6 were detected by using this assay, although the sensitivity was lower for genotypes 2 and 3 (67% detection rate: positive signal for 60 of 89 serum samples known to contain HCV genotypes 2 or 3) compared with genotype 1 (97% detection rate: positive signal for 67 of 69 serum samples known to contain the HCV genotype 1). A comparison of the Quantiplex HCV RNA 1.0 assay and a research laboratory–developed RT-PCR test for HCV showed greater sensitivity in the RT-PCR test, which had a lower limit of detection of 2.5×10^4 HCV RNA copies/mL. However, the HCV bDNA test had greater reproducibility and was less time-consuming than the laboratory-developed HCV RT-PCR test.[16]

To improve the detection rate for HCV genotypes 2 and 3, a second-generation assay was developed (Quantiplex HCV RNA 2.0 assay, Bayer).[15] The major design change in the second-generation HCV RNA assay was to use probes to sequences in the HCV genome that were more highly conserved across genotypes. These conserved regions were 5' untranslated sequences and sequences in the core gene of the HCV genome. The result of changes to the target and capture probes was to dramatically reduce the variation in detection rate among HCV genotypes in the second-generation assay. Each of the 6 HCV genotypes had a high detection rate, and there was marked improvement in the detection of HCV genotypes 2 and 3 (detection of 93% vs 67% of samples known to contain HCV genotypes 2 or 3 in the HCV 2.0 and HCV 1.0 assays, respectively). Also, the sensitivity of the second-generation bDNA assay was slightly enhanced compared with the HCV 1.0 assay (lower limits of quantification were 2.0×10^5 [HCV 2.0] versus 3.5×10^5 [HCV 1.0]).

Third-Generation bDNA Assays for HIV-1 and HCV

In the first- and second-generation bDNA assays, nonspecific hybridization of oligonucleotide probes to nontarget sequences limited assay sensitivity. The critical technological improvement in the third-generation bDNA assay (Quantiplex [also referred to as VERSANT] HIV-1 RNA 3.0 assay, Bayer) was to use nonnatural bases, 5'-methyl-2'-deoxyisoguanosine (isoG) and 5'-methyl-2'-isodeoxycytidine (isoC), in the synthesis of all probes in the bDNA system, with the exception of capture extenders that mediate capture of the target viral nucleic acid to the plate surface. Because oligonucleotides containing isoG and isoC are not present in nature, nonspecific hybridization is reduced significantly.[7] Thus, probes modified with the nonnatural bases do not form stable hybrids with the capture probe in the absence of target RNA. In the initial description of the third-generation assay, the limit of detection of HIV-1 in plasma samples from 11 patients was 50 copies per mL.[7] This represents a 10-fold improvement in the limit of detection compared with the second-generation assay. During treatment with highly active antiretroviral therapy, HIV-1 viral load decreased to below the limit of detection for all 11 patients. The VERSANT HIV-1 RNA 3.0 assay has a dynamic range of 75 to 5×10^5 HIV RNA copies/mL. Version 3.0 also has been approved to a lower limit of detection equal to 50 copies/mL in several other countries.

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DOI: 10.1309/90BU6KDXANFLN4RJ

When matched samples were compared in the second-generation HIV-1 bDNA assay (Quantiplex version 2.0) and the Amplicor Monitor 1.0 RT-PCR assay, consistently lower HIV-1 copy numbers were obtained using the bDNA test.[13,17] However, a close quantitative correlation in HIV-1 RNA copy numbers was observed between the third-generation (version 3.0) assay and the Amplicor Monitor 1.5 RT-PCR test.[18] Viral load results in the version 3.0 bDNA assay and in the Amplicor RT-PCR assay were approximately 2-fold higher than in the version 2.0 assay. Quantitatively similar results in the version 3.0 bDNA test and the RT-PCR test are important in patient care because of the likely possibility that different methods will be used in testing samples from the same patient for the course of anti-HIV therapies. Recent data suggest that rebaselining for patients tested with an RT-PCR assay may not be necessary.

Similar to the HIV-1 version 3.0 bDNA assay, the third-generation bDNA assay for HCV (VERSANT HCV RNA 3.0, Bayer) also used isoC- and isoG-substituted oligonucleotides to reduce nonspecific hybridization.[19] The use of isoC- and isoG-substituted oligonucleotides increased assay sensitivity approximately 62-fold. The HCV RNA 3.0 assay lower limit of detection was 3.2×10^3 copies/mL compared with 2×10^5 copies/mL in the HCV RNA 2.0 assay. The dynamic linear quantification range of the HCV RNA 3.0 assay extended from 3.2×10^3 copies/mL (615 IU/mL) to 4×10^7 HCV RNA copies/mL (7.7×10^6 IU/mL). The HCV RNA 3.0 assay had a high specificity (98.2%) and, similar to the second-generation assay, was equally effective in the quantification of HCV RNA across all genotypes. Between-run and within-run standard deviations for replicate samples were 0.2 $\log_{10}$ and 0.14 $\log_{10}$, respectively, indicating that the third-generation assay was highly reproducible.

In addition to the eradication of HCV in serum, an important goal of anti-HCV therapies is to reduce HCV levels in the liver. The usefulness of the HCV RNA 3.0 assay for the detection and quantification of HCV RNA in liver biopsy specimens was studied in 25 patients coinfected with HCV and HIV.[20] The reproducibility of the third-generation HCV bDNA assay was similar between liver biopsy specimens and serum samples. Also, detection of HCV RNA in liver specimens from patients infected with genotypes 1, 3, and 4 by the HCV RNA 3.0 assay was highly specific and sensitive. In this study of 25 patients, high pretreatment levels of intrahepatic HCV correlated with a low frequency of response to anti-HCV therapy. Pretreatment intrahepatic HCV levels were highest in patients infected with HCV genotype 1. Results of this study demonstrated that important markers of HCV disease progression, HCV levels in liver and in serum, can be quantitated reliably by bDNA analysis during treatment.

The methods for third-generation bDNA assays include sample preparation, hybridization, and signal detection for HIV-1 RNA and HCV RNA. All 3 steps are performed in the microwells on the System 340 platform **Image 1** for the HCV RNA 3.0 assay that does not require a separate extraction step. In the version 3.0 HIV-1 RNA assay, sample preparation is different from the HCV RNA method and is performed outside the System 340 platform. A recent study evaluated an adaptation of the version 3.0 HIV-1 RNA method in which the HIV-1 sample processing step was modified to accommodate simultaneous testing for HCV and HIV-1 on the System 340 platform.[21] The HIV-1 method was modified by omitting the 2-hour incubation at 63°C for viral lysis. Instead, HIV-1 and HCV lysis was performed on the System 340 platform. The HCV bDNA test methods were unchanged in the combined assay. The specificity and quantification by the combined bDNA method were within specifications for the individual HIV-1 and HCV assays. Simultaneous testing for HIV-1 and for HCV improved workflow in the clinical laboratory and resulted in lower costs. Because HIV-1 RNA and HCV RNA detection and quantification are crucial for diagnosis and for the evaluation of responses to therapy, the ability to carry out simultaneous testing for both viruses represents a significant advance in molecular diagnostics.

Summary

bDNA methods have progressed from first-generation assays, which were accurate and reproducible but relatively insensitive, to third-generation bDNA tests that are accurate, reproducible, highly sensitive, and automated laboratory tests for more optimal patient management. Essential technological discoveries that resulted in significantly better assay performance and permitted the evolution from first- to third-generation bDNA assays are as follows: (1) increased hybridization stringency of target and capture probes, (2) increased signal amplification by using preamplifier oligonucleotides, and (3)

Image 1 Bayer System 340 bDNA [branched chain DNA] Analyzer.

DOI: 10.1309/90BU6KDXANFLN4RJ
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greatly reduced nonspecific hybridization to nontarget sequences by using isoC- and isoG-substituted oligonucleotides for all probes in the bDNA system, with the exception of probes that mediate capture of the target RNA to the microwell. The results of this evolution in bDNA technology are Food and Drug Administration–approved methods for the detection and quantification of HIV-1 and HCV (VERSANT HIV-1 RNA 3.0 assay and the VERSANT HCV RNA 3.0 assay).

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Financial support for preparation of this article was provided by Bayer Healthcare.

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Acknowledgment: David Sorscher, PhD, is gratefully acknowledged for preparing a working draft of this manuscript.

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DOI: 10.1309/90BU6KDXANFLN4RJ