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A Rapid and Simple Method for Extracting Human Immunodeficiency Virus Type 1 RNA from Plasma: Enhanced Sensitivity

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We present here a rapid and simple technique for processing human immunodeficiency virus (HIV)-infected plasma by high-speed centrifugation. HIV type 1 virions are pelleted from up to 1 ml of plasma, gently lysed with a nonionic detergent, and directly amplified. The procedure has few manipulations and requires approximately 1.5 h for the processing of 24 samples. Viral recovery ranges from 80 to 90%, with an analytical sensitivity approaching 20 copies/ml.

One of the challenges to nucleic acid-based technologies is to develop rapid and efficient methods for recovery of nucleic acids from test specimens. While procedures have been developed for rapid extraction of DNA from whole blood (6), the extraction of viral RNA from plasma has traditionally required the use of chaotropic agents followed by phenol-chloroform extraction and subsequent precipitation (3, 4). We describe here an alternative procedure (the Ultra-Direct method) for extracting human immunodeficiency virus type 1 (HIV-1) RNA from plasma that is simple to perform, requires minimal hands-on manipulation, and can be used to concentrate virus from a larger volume of plasma.

The procedure uses high-speed centrifugation to pellet the virions. The pelleted virions are lysed with a solution containing a nonionic detergent (2, 7) and RNasin, and the lysate can be added directly to an amplification reaction mixture without further processing. We demonstrate that the procedure can be used for quantitative recovery of viral RNA not only from plasma collected in acid citric dextrose (ACD) and EDTA but also for plasma collected in heparin. Additionally, virions from larger volumes of plasma can be concentrated by high-speed centrifugation, thereby further increasing the sensitivity of the detection assay.

Assays with improved sensitivity for plasma HIV-1 RNA are becoming increasingly important with the introduction of more effective antiretroviral drugs. Whereas earlier drugs provided initial modest reductions in viral loads followed by subsequent return to baseline levels, recent combination therapies have provided dramatic and sustained reductions in viral load (1, 5, 9, 10). In some cases, the viral loads were reduced to below the detection limit of 400 copies/ml of the AMPLICOR HIV-1 MONITOR test (Roche Diagnostic Systems). Assays with greater sensitivity are required to better monitor the effects of the new antiviral drugs; however, development of a more sensitive assay requires increasing the plasma input. The procedure we developed can be used to readily concentrate virions from a larger volume of plasma.

Sample preparation was performed by centrifugation of up to 24 200-μl to 1-ml aliquots of plasma in a Heraeus 17RS tabletop centrifuge (fixed angle rotor 3753 HFA 22.1) in 1.5-ml microcentrifuge tubes, each containing 20 μl of inert, red polystyrene microspheres (0.214-mm diameter; 0.025% [wt/vol]; Bangs Laboratories), for 1 h at 25,500 × *g* and 4°C. Following centrifugation, the supernatants were carefully removed with transfer pipets and the pellets were resuspended in a Roche Molecular Systems (RMS) lysis buffer consisting of nonionic detergent, RNase inhibitor, and an internal quantitation standard. The internal quantitation standard is coamplified with the target sample and is used to adjust for reaction variabilities due to interfering substances (8). The amounts of RNasin and internal quantitation standard used depend on the final resuspension volume. In the absence of concentration, virus from 200 μl of plasma was resuspended in 200 μl of the RMS lysis buffer containing 200 U of RNasin and 400 copies of the internal quantitation standard. For an eightfold concentration of virus from 1 ml of plasma, the pelleted virions were resuspended in 120 μl of lysis buffer containing 200 U of RNasin and 200 copies of the internal quantitation standard. Fifty-microliter volumes of lysate were amplified in duplicate under standard AMPLICOR HIV-1 MONITOR test conditions, but 32 cycles were used in order to boost low-titer signal detection by microwell plate analysis. The red polystyrene microspheres form tight pellets and significantly improved pellet visualization when used with ACD or EDTA plasma. However, in heparinized plasma, the pellets frequently clump and smear and the utility of the microspheres as a tracer is less obvious.

The efficiency of RNA recovery with the method described above was compared to that of the AMPLICOR HIV-1 MONITOR extraction protocol using guanidinium isothiocyanate (GuSCN)-isopropanol precipitation. HIV-1 dilution panels were prepared by serial two- or fivefold dilutions of a well-characterized HIV-1-containing plasma into either seronegative ACD plasma or heparinized plasma. Each ACD sample

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TABLE 1. Analysis of a serially diluted HIV-1-infected plasma panel

Input HIV copies/ml	Ultra-Direct			Supernatant[a] HIV copies/ml	HIV-1 recovery (%)
	HIV copies/ml	SD	CV, % (n = 8)		
125,000	181,305	17,651	10	ND[b]	ND
50,000	54,130	4,246	8	3,368	94
25,000	31,217	4,122	13	ND	ND
10,000	11,955	2,149	18	2,306	81
5,000	4,456	703	16	ND	ND
2,000	1,754	582	33	377	79
1,000	1,286	480	37	ND	ND
500	520	152	29	ND	ND
400	384	111	29	ND	ND

[a] Plasma supernatant processed by GuSCN-isopropanol precipitation.
[b] ND, not determined.

was first extracted by GuSCN-isopropanol precipitation to obtain a copy number reference.

The efficiencies of virus pelleting and virion lysis were evaluated. To assess the efficiency of virus pelleting, following high-speed centrifugation, the plasma supernatants were subjected to GuSCN extraction and RNA was quantified. As evident from Table 1, the amount of RNA recovered from the supernatant was about 10 to 20% of the total signal, indicating that 80 to 90% of the virions were pelleted by high-speed centrifugation. The efficiency of viral lysis was determined by dividing the resuspended virions into two equal volumes and extracting them with either the RMS lysis buffer or GuSCN-isopropanol. The amount of RNA recovered was comparable with both procedures, suggesting that lysis with the RMS lysis buffer is complete (data not shown).

The sample preparation method was evaluated for its ability to quantitatively recover HIV-1 from heparinized plasma. The GuSCN-isopropanol method currently used in the AMPLICOR HIV-1 MONITOR test cannot be used on heparinized plasma samples due to inhibition of DNA polymerase by heparin. Viral recovery from heparinized panels was compared with recovery from matched viral dilution panels prepared in ACD plasma. HIV-1 signals from heparinized plasma have typically been 30% lower than those from matched ACD plasma; however, recovery was reproducible, with coefficients of variation (CVs) of <30% (Table 2).

To evaluate the feasibility of using this method to concentrate virions in plasma, six 1-ml aliquots of a dilution panel similar to that described above were pelleted and each was extracted with 120 μl of the RMS lysis buffer. Fifty microliters

TABLE 2. Effect of anticoagulants on the detection of recoverable HIV-1 RNA

Control[a] HIV copies/ml	ACD plasma			Heparinized plasma		
	Ultra-Direct HIV copies/ml	SD	CV (%)	Ultra-Direct HIV copies/ml	SD	CV (%)
47,171	54,130	4,246	8	38,709	4,739	12
13,100	11,955	2,149	18	8,453	2,586	30
2,807	1,754	582	33	1,266	407	32
570	384	111	29	347	69	20

[a] Treatment consisted of GuSCN lysis-alcohol precipitation (AMPLICOR HIV-1 MONITOR).

TABLE 3. Detection of low levels of viral RNA in serially diluted HIV-1-infected plasma

Input copies/ml	Ultra-Direct HIV copies/ml[a]	SD	CV, % (n = 6)
25,000	20,618	1,183	6
5,000	6,575	1,277	19
2,000	1,742	278	16
1,000	1,284	286	22
400	304	91	30
200	218	16	7
160	188	41	22
80	112	19	17
40	38	25	66
20	22	14	64

[a] Viral concentration (1 ml processed; 417-μl equivalent plasma volume).

of each extraction sample, representing the RNA extracted from the equivalent of 417 μl of plasma, was amplified in duplicate. The results suggest that pelleting HIV virions from larger volumes of plasma can increase the clinical sensitivity of the AMPLICOR HIV-1 MONITOR test to approximately 20 copies/ml (Table 3). In addition, the overall precision and resolution of the method were comparable to those of the standard AMPLICOR HIV-1 MONITOR test. CVs of <30% and twofold resolution were observed for all but the lowest-titered samples. The increased variability with low-copy-number input may be a result of stoichastic sampling and/or recovery differences.

The analytical sensitivity of the assay was determined to be about 20 copies/ml based on the following reconstruction experiment. Twenty 0.5-ml aliquots of a negative plasma were centrifuged, supernatants were aspirated, and the resulting pellets were each resuspended in 55 μl of lysis buffer containing 10 copies of a well-characterized HIV-1 RNA transcript (AMPLICOR HIV-1 MONITOR test control RNA). Fifty microliters of each lysate was amplified, and RNA was detected in all 24 replicates, thus demonstrating that the assay has the sensitivity to detect 10 copies of HIV RNA in 450 μl of plasma equivalent, or about 20 copies/ml.

We applied this procedure to clinical specimens collected

TABLE 4. Detection of HIV-1 RNA in AMPLICOR HIV-1 MONITOR-negative plasma

Sample	Equivalent plasma volume/PCR (μl)	Ultra-Direct HIV copies/ml
24-5-94[a]	333	336
21-6-94	417	124
19-7-94	417	792
16-8-94	417	<20 (~15)
A195615[b]	417	<20 (~7)
A314489	417	22
A384966	417	97
A412696	417	<20 (~11)
A457868	417	389
A193377	417	48
EVC9405051	375	27
1,000-copy/ml HIV-1 plasma	417	1,231

[a] Longitudinally collected plasma from a patient with an undetectable viral load at onset of study.
[b] Samples A and E were collected from individuals with detectable viral loads at the onset of the clinical study but for whom levels rapidly decreased to undetectable; all specimens were HIV-1 Western blot positive.

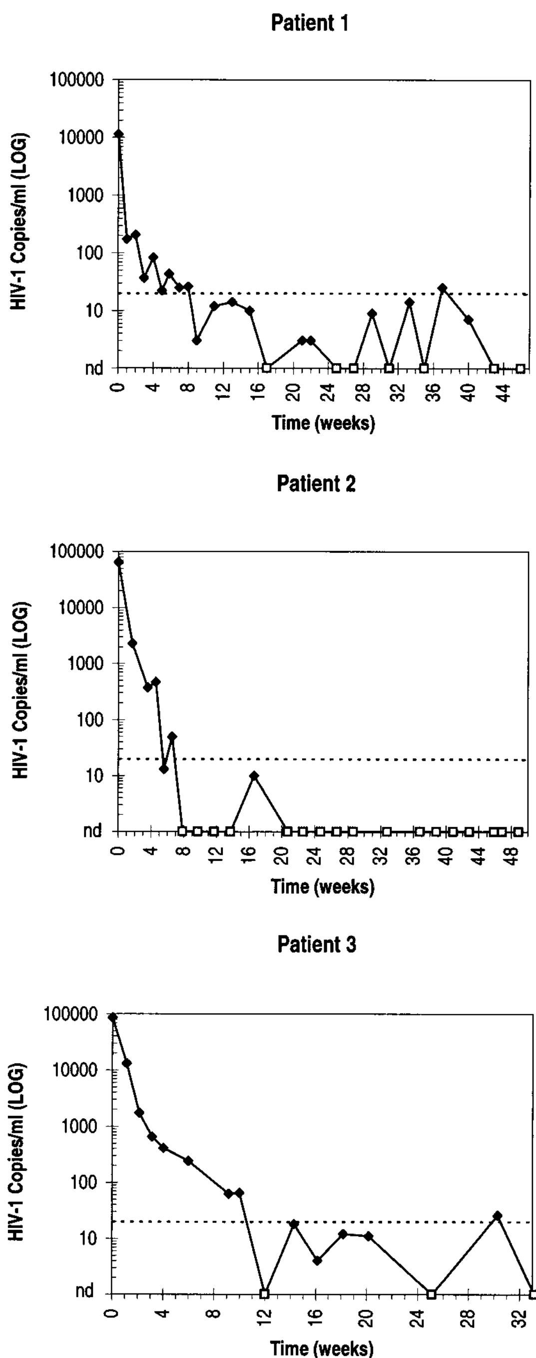

FIG. 1. HIV-1 viral loads of three HIV-infected patients on multidrug therapies. The new sample preparation method was performed on specimens collected after 4 to 6 weeks of therapy. RNA levels below 20 copies/ml were reported as either low positive (qualitative result only) or undetectable. Closed diamonds represent confirmed positives, while open squares represent samples in which HIV was undetectable by all methods.

from individuals who either entered a clinical study with undetectable virus levels by the standard AMPLICOR HIV-1 MONITOR test or had detectable virus at the onset of the clinical study but soon had undetectable levels. EDTA-treated plasma samples from these individuals were centrifuged, and the RNA extracted from the equivalent of 417 μl was assayed. As shown in Table 4, viral RNA was detected at less than 125 copies/ml in all but three specimens. In addition, we analyzed sequential samples from three HIV-infected patients, two of whom were treated with zidovudine, lamivudine, zalcitabine, and alpha interferon and a third who received the same drug regimen plus ritonavir. In all three patients, HIV-1 RNA dropped to below the detection limit of the standard assay after 4 to 6 weeks of therapy (Fig. 1). The new sample preparation procedure enabled these patients to be further monitored. Viral load was observed to drop another log (10-fold) and, at times, to levels even below the sensitivity of the improved method (20 copies/ml).

The sample preparation method described here provides a simple, rapid, and reliable means of processing ACD, EDTA, and heparinized plasma specimens. In preliminary studies, the procedure performs as well as the current GuSCN-OH method but is much simpler and more convenient to use. More importantly, the procedure also provides an effective means of concentrating virions for low-titer analysis.

For example, as much as a 20-fold concentration of virions can be achieved with this procedure to allow high-sensitivity detection by either the quantitative or qualitative assay. Although the clinical significance and utility of quantifying low viral levels are not yet known, the availability of a more sensitive detection assay provides a tool to gain insight into the dynamics of infection at low HIV-1 levels. Furthermore, clinicians are already beginning to aggressively treat infected individuals with combination therapies early in infection.

The sample preparation procedure described here affords several advantages. The greater sample input afforded by this extraction protocol will facilitate the monitoring of a broad spectrum of infected patients throughout therapy, particularly those who are asymptomatic and have low viral burdens. In addition to improving HIV-1 detection, the concentration step provided by this procedure may also improve the detection of HIV-2, for which low viral load is speculated to be responsible for reduced sensitivity.

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