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Automation and Analytical Techniques

High-Throughput Purification of Viral RNA Based on Novel Aqueous Chemistry for Nucleic Acid Isolation

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Background: **Extraction protocols using magnetic solid phases offer a high potential for automation. However, commercially available magnetic-bead–based assays either lack the sensitivity required for viral diagnostics or are disproportionately expensive.**

Methods: **We developed an aqueous chemistry for extraction of viral nucleic acids from plasma samples by use of common magnetic silica beads. Nucleic acids were bound to the beads at acidic conditions in the presence of a kosmotropic salt and were eluted at a slightly alkaline pH. The method was implemented on a standard pipetting workstation for fully automated extraction of up to 48 samples of 240 μL plasma in 1 batch.**

Results: **The detection limit of the method was comparable to the spin-column–based QIAamp Viral RNA Mini Kit, which relies on chaotropic salts and binding to a silica membrane, as the comparison method. The 95% detection limit was 23.1 IU per PCR for HIV-1 and 10.7 IU per PCR for hepatitis C virus (HCV). Suitability for clinical routine testing was confirmed in a total of 178 HIV-1- or HCV-positive plasma samples. The method linearity (R^2) was >0.99 for the viruses evaluated.**

Conclusions: **Use of reagents without organic solvents allows simple and cost-effective automation of this method on common pipetting robots with low risk of contamination. Performance characteristics of the novel extraction method make it suitable for use in diagnosis of infectious diseases and viral load determinations.**

The superior sensitivity of nucleic acid amplification technique (NAT)[4] enables diagnosis of infectious diseases at an early stage before positive serologic results indicate an infection *(1)*. NAT has therefore become a standard application in the clinical laboratory in recent years. In addition to diagnosis of infectious diseases, the determination of virus load has gained increasing importance in the clinical virology laboratory. Viral load represents the most accurate prognostic marker for HIV-1 and, with limitations, for hepatitis C virus (HCV) infection *(2, 3)*. Determination of viral load can be used to assess the reduced efficacy of antiretroviral medications, allowing timely adaptation of the medication regimen.

Although the introduction of real-time PCR has led to considerable progress in automating the amplification and detection steps of NAT, nucleic acid isolation remains very labor-intensive when performed manually. Traditional phenol–chloroform extraction and ethanol precipitation methods are complicated, time-consuming, hazardous, and unsuitable for treating high numbers of samples. These methods have therefore been largely supplanted by extraction procedures based on the method described by Boom et al. *(4)*, which uses the principle of adsorption of nucleic acids to silica matrices in the presence of chaotropic salts and alcohol *(5)*.

The term "chaotropic salt" originates from the Hofmeister series *(6, 7)*, which divides chaotropic from kosmotropic salts depending on their influence on the structure of macromolecules and water. According to

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[4] Nonstandard abbreviations: NAT, nucleic acid amplification technique; HCV, hepatitis C virus; HBV, hepatitis B virus; and Ct, cycle threshold.

the literature, the salt guanidine thiocyanate is one of the most powerful chaotropes and is commonly used in the Boom method. Guanidine thiocyanate has both RNase-inactivating and lysing properties for viral particles and cellular structures *(8, 9)* and promotes binding of nucleic acids to silica surfaces *(10)*. The Boom method is most commonly implemented in conjunction with spin columns.

To streamline the NAT process, automated platforms for nucleic acid isolation have been developed *(11, 12)*. Most recent developments use magnetic beads that bind the nucleic acids to their silica surface and transfer the nucleic acids through the various steps of the extraction process *(13–19)*, but automation of the Boom method is complex because of the chemical properties of the reagents used. As a result, the costs for consumables and reagents associated with automated extraction considerably exceed those of manual sample preparation.

In this study we report on a novel aqueous chemistry for purification of nucleic acids that does not involve use of chaotropic salts or alcohol. The technique uses magnetic silica beads and was developed with a focus on sensitivity, cost efficiency, and ease of automation. We also describe a novel binding mechanism that allows simple and rapid automation on common pipetting workstations at low costs without compromising sensitivity.

We compared the detection limits and clinical sensitivities of the proposed method for HIV-1 and HCV with the detection limits and sensitivities of the QIAamp Viral RNA Mini Kit, which uses spin columns and chaotropic salts. We assessed the linearity and precision for the 2 RNA viruses in combination with our in-house TaqMan PCR assays.

Materials and Methods

CLINICAL PLASMA SAMPLES

To investigate the clinical sensitivity of the proposed method, we randomly selected aliquots of EDTA- or citrate-anticoagulated plasma samples submitted to the Institute for Medical Virology for diagnosis of HIV-1 or HCV or viral load determination. Viral loads of HIV-1 samples had been quantified previously by automated, target-specific COBAS AmpliPrep extraction (Roche) *(16)* of 300 or 800 μL of plasma and amplification with COBAS Amplicor HIV-1 Monitor (Ver. 1.5). HCV viral loads had been determined by use of the COBAS Amplicor HCV Monitor (Ver. 2.0) procedure according to the manufacturer's instructions.

CALIBRATOR FOR QUANTIFICATION

We prepared a quantification calibrator by diluting HIV-1- and HCV-positive plasma with HIV-1-, HCV-, hepatitis B virus (HBV)-negative human plasma to concentrations of 8000 IU/mL HCV and 4000 IU/mL HIV-1. The HCV- and HIV-1–positive plasma had been calibrated previously against WHO International Standards 96/790 and 97/656 for HCV and HIV-1, respectively.

MAGNETIC SILICA PARTICLES

For binding of the nucleic acids, MagPrep® silica particles (Merck) were used in this study. Particles consist of a nucleus of paramagnetic iron oxide coated with silica. Particle size ranges between 0.5 and 2.5 μm, and the surface area is ~20 m^2/g. Silica particles are provided as a suspension of 50 mg of particles/mL. The use of silica beads from other suppliers gave variable recoveries in conjunction with our aqueous chemistry.

PREPARATION OF BUFFERS

The lysis/binding buffer (TAAN) was prepared by dissolving 3.96 g of ammonium sulfate (Merck) in 99.2 mL of 0.2 mol/L Tris acetate, pH 4.0 (Merck); 0.8 mL of Nonidet P40 (Calbiochem) was then added, and the solution was homogenized by shaking at room temperature for 20 min. Before the extraction, 3.95 mL of bead suspension was added, and the mixture was homogenized by brief vortex-mixing. The washing buffer (WBN) consisted of 0.5 mL of Nonidet P40 dissolved in 99.5 mL of 0.01 mol/L Tris-HCl (pH 6.8). Before extraction, 2.5 mL of proteinase K solution (20 g/L; Merck) was dissolved in 100 mL of WBN. The elution buffer (EB) was 10 mmol/L Tris-HCl (pH 8.5).

PROTOCOL FOR EXTRACTION OF VIRAL NUCLEIC ACIDS FROM PLASMA SAMPLES

In 1.5-mL microcentrifuge tubes, 240-μL plasma samples were mixed with 760 μL of TAAN containing 30 μL of bead suspension and appropriate amounts of internal controls. After incubation for 8 min, 25 μL of proteinase K was added, and the mixture was incubated for another 8 min. Particles were separated in a magnetic device (Novagen), and the supernatant was withdrawn by a single-use pipette. Subsequently, 500 μL of WBN containing proteinase K was added to the tube, and the pellet was resuspended thoroughly in the wash buffer by pipetting up and down. After 10 min of incubation with WBN, the beads were separated and the supernatant was withdrawn. This washing step was repeated once with 1 min of incubation. After removal of the final washing buffer, 100 μL of elution buffer EB was added and incubated for 10 min at 80 °C and 1400 rpm in a Thermomixer (Eppendorf). In a final step, the beads were separated in the magnetic device, and the eluate was transferred to a fresh RNase-free tube. Twenty-four samples could be processed in ~60 min.

AUTOMATED EXTRACTION PROTOCOL

To permit a higher throughput, the manual protocol described above was implemented on a Genesis robotic workstation (Tecan) equipped with a Tecan magnetic separation module (TeMagS) with an integrated heating block. The workstation allows 48 plasma samples to be processed in 1 batch within ~2 h.

NUCLEIC ACID EXTRACTION WITH QIAamp Viral RNA Mini Kit

To determine the clinical sensitivity of the spin-column–based method, we processed 140-μL plasma samples with the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Calculated amounts of internal controls were added to buffer AVL. Nucleic acids were eluted from the columns with 60 μL of injection-grade water (Braun) at 80 °C. For experiments to determine the detection limit, both the input sample volume and elution volume were 100 μL.

REAL-TIME PCR

PCR assays were performed on an ABI Prism 7000 Sequence Detection System (PE Applied Biosystems). Amplification of HIV-1 and HCV RNA was carried out as described previously *(20, 21)*.

STATISTICAL ANALYSIS

Inter- and intraassay variation was determined with Excel 2000. The detection limits of both extraction methods were calculated by probit analysis using SPSS 11.5 software. Graphic representations were performed with SigmaPlot 2001.

Results

Initial experiments showed that purified DNA binds to silica beads at acidic conditions. On the basis of this observation, we developed a method that is suited to extract nucleic acids directly from clinical specimens.

ENHANCED RNA BINDING BY AMMONIUM SULFATE

To illustrate the effect of ammonium sulfate in binding buffer TAAN, we diluted QIAamp-purified HIV-1 RNA with RNase-free water to a final RNA concentration of 83 000 IU/mL and subjected 240-μL aliquots to our extraction method, as described in the *Material and Methods*, using binding buffer TAAN with or without 300 mmol/L ammonium sulfate. The experiment was performed in triplicate, and the mean values obtained are shown as curves in Fig. 1.

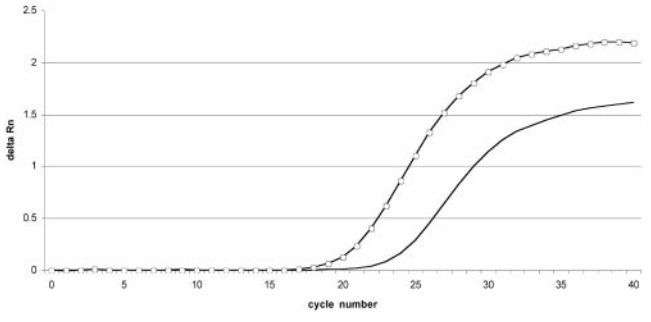

Fig. 1. Enhancement of RNA binding by ammonium sulfate.

Comparison of binding buffer containing ammonium sulfate (○) with binding buffer lacking ammonium sulfate (*line* with *no symbols*). Recovery of HIV RNA was improved by at least one order of magnitude with binding buffer containing ammonium sulfate.

The cycle threshold (Ct) values of the resulting PCR amplification curves differed considerably. The Ct represents the point at which amplification of nucleic acids is detected above background fluorescence and is used for real-time quantification. In this experiment, the buffer with ammonium sulfate gave a mean (SD) Ct of 19.27 (0.07) cycles compared with a mean Ct of 22.91 (0.43) cycles for the buffer without ammonium sulfate. Consequently, RNA recovery was improved by at least 1 order of magnitude (corresponding to ~3 Ct) when binding buffer containing ammonium sulfate was used.

RNase-INHIBITING PROPERTIES OF BINDING BUFFER TAAN

To illustrate the RNase-inhibiting effect of binding buffer TAAN, we added 30 μL of QIAamp-purified HIV-1 RNA (6.8×10^6 IU/mL) to (*i*) 1000 μL of RNase-free water, (*ii*) 240 μL of plasma + 760 μL of TAAN, (*iii*) 240 μL of plasma + 760 μL of RNase-free water, and (*iv*) 240 μL of plasma + 760 μL of 200 mmol/L Tris-acetate buffer (pH 4.0). After incubating the mixtures for 15 min at room temperature, we extracted 100-μL aliquots with the QIAamp Viral RNA Mini Kit and subjected 10 μL of each HIV extract to reverse transcription-PCR. RNA recovery for the RNA in RNase-free water was set to 100%. The RNA diluted in plasma + TAAN and plasma + Tris-acetate buffer yielded recoveries of 93% and 74%, respectively, whereas no signal was detectable for the RNA diluted in plasma + RNase-free water (Fig. 2)

DETECTION LIMIT COMPARED WITH MANUAL QIAamp Viral RNA Mini Kit

To evaluate the limits of detection of both extraction methods, we diluted the quantification calibrator described above 6 times in 2-fold dilution steps in HIV-1-, HCV-, HBV-negative human plasma. For bead extraction, we added 100 μL of the quantification calibrator to 140 μL of HIV-1-, HCV-, HBV-negative human plasma. Samples were processed with the Tecan Genesis pipetting robot.

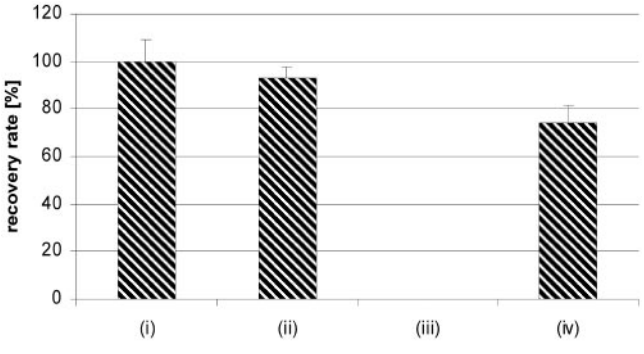

Fig. 2. RNase-inhibiting properties of binding buffer TAAN.

We added 30 μL of QIAamp-purified HIV RNA to 1000 μL of RNase-free water (*i*), 240 μL of plasma + 760 μL of TAAN (*ii*), 240 μL of plasma + 760 μL of RNase-free water (*iii*), or 240 μL of plasma + 760 μL of 200 mmol/L Tris acetate buffer, pH 4.0 (*iv*). RNA recovery in RNase-free water alone (*i*) was set to 100%. All experiments were done in triplicate.

Table 1. Intra- and interassay precision for HIV-1 and HCV.

	Intraassay			Interassay		
	Ct, cycles			Ct, cycles		
	Mean	SD	CV, %	Mean	SD	CV, %
HIV, IU/mL						
10^6	16.63	0.17	1.0	16.29	0.06	0.35
10^3	26.32	0.73	2.8	25.53	0.56	2.2
HCV, IU/mL						
10^6	13.76	0.22	1.6	13.95	0.17	1.2
10^3	23.03	0.69	3.0	22.91	0.08	0.35

For QIAamp extraction, 100 μL of the undiluted quantification calibrator was extracted by a skilled technician. Nucleic acids were eluted from the columns with 100 μL of injection-grade water at 80 °C.

Each dilution of the quantification calibrator was tested in 12 replicates with both methods. From the total eluted volume of 100 μL by each method, 10 μL was subjected to HIV-1 PCR and 15 μL to HCV PCR, respectively. Probit analysis indicated 95% detection limits of 23.1 IU of HIV-1 per PCR and 10.7 IU of HCV per PCR for the bead extraction and 21.6 IU of HIV-1 per PCR and 5.4 IU of HCV per PCR for the QIAamp extraction.

CLINICAL SENSITIVITY

A total of 89 HIV-1–positive plasma samples containing 130 to $>1 \times 10^6$ genome-equivalents/mL and 89 HCV-positive plasma samples containing <600 to $>6 \times 10^5$ IU/mL were analyzed. Clinical samples were extracted in parallel by the automated bead extraction and the QIAamp Mini Kit (comparison protocol). Extracts were amplified with our in-house TaqMan assays as described above. All clinical specimens previously tested positive for HCV with the COBAS Amplicor Monitor procedure were confirmed with both extraction protocols in combination with our in-house PCR assays (Table 1 of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue7/). For HIV-1, the automated bead extraction/TaqMan assay combination failed to detect 1 HIV-1–positive specimen, whereas the QIAamp protocol/TaqMan assay combination failed to detect 2 specimens previously tested positive for HIV-1 (Table 2 of the online Data Supplement). Failed samples were all in the lowest range of viral load (<300 genome-equivalents/mL).

ASSAY PRECISION

We assessed intraassay imprecision by determining the Ct values of samples with low and high concentrations of HCV and HIV-1, with 16 replicates for each concentration of each virus, and interassay imprecision by determining the Ct values for the same concentrations with 12 replicates on 3 consecutive days with 1 lot of reagents. The results are summarized in Table 1.

LINEARITY

We evaluated the linearity of automated bead extraction for HIV-1 and HCV in combination with our TaqMan in-house assays. The parameters of the regression curve of Ct values vs the decimal logarithm of IU/mL were as follows: for HIV-1, $R^2 = 0.9909$; slope, −2.998; for HCV, $R^2 = 0.9903$; slope, −3.033 (Fig. 3).

Discussion

In this report we describe a novel aqueous kosmotropic chemistry for purification of nucleic acids and give examples of its main application. Detection limits and clinical

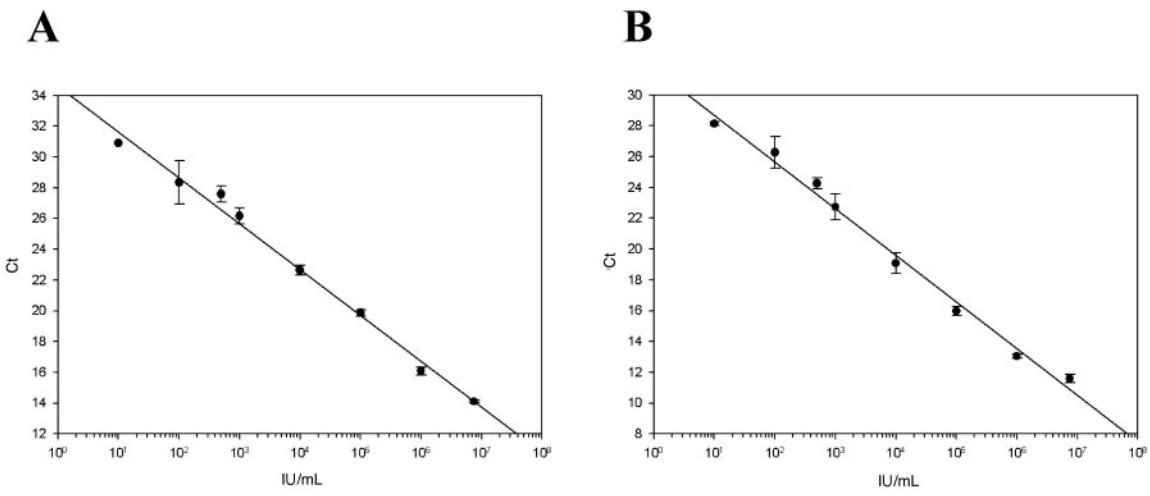

Fig. 3. Calibration curves for HIV-1 (*A*) and HCV (*B*) quantification.

(*A*), dilutions ranging from 7.5×10^6 to 10^2 IU/mL of HIV-1-positive plasma were tested in triplicate, and the mean Ct values were plotted against the viral concentration (IU/mL). $R^2 = 0.9909$; slope = −2.998 (95% confidence interval, ±0.30). (*B*), dilutions ranging from 6.8×10^6 to 10^2 IU/mL of HIV-1-positive plasma were tested in triplicate, and the mean Ct values were plotted against the viral concentration (IU/mL). $R^2 = 0.9903$; slope = −3.033 (95% confidence interval, ±0.285). *Error bars*, SD.

sensitivities were compared with the QIAamp Viral RNA Mini Kit, which is based on the chaotropic Boom method *(4)*. Our results demonstrate that the analytical sensitivity of our method was in the same range as the QIAamp procedure, which is used as a "gold standard" in our laboratory. Whereas the detection limit in the described experiment was slightly higher than the detection limit obtained with the QIAamp extraction, the clinical sensitivity of the fully automated bead extraction was as good or better than that of the QIAamp procedure: the bead extraction procedure detected 1 HIV-1–positive sample with low virus load that was missed by the QIAamp extraction procedure. We assessed the linearity of the automated bead extraction in a dilution series of HIV-1- or HCV-positive plasma samples. The correlation coefficients (R^2) were >0.99, indicating that this method is suitable for virus load determination.

The established Boom method *(4)* is most commonly implemented in conjunction with spin columns and has been acknowledged for its efficiency and efficacy in the removal of inhibitory substances. However, there are some limitations to the use of silica membranes. For example, automation is expensive and elaborate because the method requires vacuum stations, and processing of larger volumes is restricted by the limited capacity of the membranes.

The magnetic bead technology is a more forward-looking approach for nucleic acids purification, as it circumvents the above-mentioned limitations of the membrane-based techniques *(13)*. However, it is conceivable that residual buffer, located in spaces between the particles, would be difficult to remove without use of centrifugation or vacuum. Because alcohol and chaotropic salts are highly inhibitory for enzymatic reactions, residual amounts of these substances in the eluate may interfere with subsequent PCR amplification.

In contrast to the Boom method *(4)*, our novel aqueous chemistry does not contain any chaotropic salts or alcohol. Wash buffer WBN contains no PCR-inhibiting substances other than proteinase K, which is heat-inactivated during the elution step. Thus, no PCR inhibition was observed. Problems with inhibitors, which may be introduced by the purification procedure itself, can therefore be excluded by use of our aqueous chemistry.

The method consists of detergent lysis, acidic binding of the nucleic acids to the magnetic silica beads, 2 washing steps at neutral conditions, and elution of the nucleic acids at a slightly alkaline pH. Binding buffer TAAN contains ammonium sulfate, which according to the literature is a kosmotropic salt *(6, 7)*. We showed that ammonium sulfate in micromolar concentrations enhances the recovery of HIV-1 RNA (Fig. 1). This binding-enhancing effect of ammonium sulfate, however, is inconsistent with the theory that high concentrations of chaotropes (most commonly at concentrations of 5–7 mol/L) are essential for binding of nucleic acids to silica *(10)*.

Furthermore, the acidic conditions of our binding buffer sufficiently inhibited RNase activity (Fig. 2). The observed slight degradation of RNA in a TAAN–plasma mixture may be of minor importance because the adsorption process is completed in <8 min and we expect that RNA bound to the beads is protected from degradation.

Our bead-based extraction protocol could be easily implemented on a standard pipetting robot without the need of custom-made components. For separation of the magnetic particles during the process, we placed a magnetic separation module on the worktable. After the workstation was equipped with extraction reagents and up to 48 plasma samples in 2.0-mL cups, samples could be processed fully automated within ~2 h. After the extraction, PCR master mixtures were placed on the worktable, and the PCR setup was carried out by the robot. The whole procedure markedly reduced the hands-on time needed for extraction and PCR setup compared with manual extraction methods.

In contrast, automation of membrane- or bead-based extraction methods that use chaotropic salts and organic solvents is complicated, in particular because of difficulties in handling of alcohols and the need for heated vacuum stations. Use of organic solvents is generally problematic because of their high vapor pressure. Improper liquid handling is almost unavoidable and must be compensated by the implementation of tip guards and custom-made consumables. These precautions against contamination, however, add substantially to the overall cost per extraction.

Costs for consumables in commercial automated bead-based extraction methods vary between $1.50 and $3.00 (US) per extraction. Together with the costs for reagents, the overall costs per extraction are between approximately $4.00 and $6.00 (US). For our automated extraction method, the costs for consumables are almost identical to the costs for disposable aerosol-resistant pipet tips and do not exceed $1.00 (US) per sample. Together with the low costs for the reagents, the overall costs can be estimated as less than $2.00 (US) per extraction. However, it should be noted that the price calculation for the reagents in this case relies on in-house production of the buffers. The buffers are not hazardous and can be prepared in every laboratory without specific skills and precautions.

In conclusion, the performance characteristics of the novel extraction method, as described in this report, enable its use for diagnosis of infectious diseases and viral load determinations. The detection limit with respect to input copies was comparable to that of the QIAamp Viral RNA Mini Kit that was used as the comparison protocol. We assessed the linearity of the extraction method in combination with our in-house TaqMan assays and obtained correlation coefficients (R^2) >0.99 for both viruses tested. Furthermore, the reagent properties allow simple automation on standard pipetting robots that are already available in most laboratories. Costly consumables and hardware modifications are not required to prevent con-

tamination, and the reagent costs are low. This method, therefore, might be an interesting alternative for cost-conscious clinicians and researchers who need high-throughput purification of nucleic acids.

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