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A Portable, Pressure Driven, Room Temperature Nucleic Acid Extraction and Storage System for Point of Care Molecular Diagnostics

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Abstract

Many new and exciting portable HIV viral load testing technologies are emerging for use in global medicine. While the potential to provide fast, isothermal, and quantitative molecular diagnostic information to clinicians in the field will soon be a reality, many of these technologies lack a robust front end for sample clean up and nucleic acid preparation. Such a technology would enable many different downstream molecular assays. Here, we present a portable system for centrifuge-free room temperature nucleic acid extraction from small volumes of whole blood (70 μ L), using only thermally stable reagents compatible with storage and transport in low resource settings. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis of simulated samples demonstrate a lower limit of detection of 1000 copies/ml, with the ability to detect differences in viral load across four orders of magnitude. The system can also be used to store extracted RNA on detachable cartridges for up to one week at ambient temperature, and can be operated using only hand generated air pressure.

Keywords

DNA; RNA; blood; HIV; point of care; diagnostics; PCR; solid phase extraction

INTRODUCTION

The U.S. President's Emergency Plan for AIDS Relief, in conjunction with the Global Fund to fight AIDS, Malaria, and Tuberculosis, has dramatically increased the availability of antiretroviral therapy (ART) for patients in the developing world, with as many as 5.6 million people in low and middle-income countries receiving treatment as of 2011¹. While widespread treatment availability has done much to alleviate patient suffering, the distribution of therapy has outpaced the ability of many developing countries to monitor patients for treatment failure and adherence, thus increasing the risk for the emergence of drug resistant strains^{2–6}. Two diagnostic tests are performed to assess therapy effectiveness: CD4⁺ cell counts and HIV viral load tests. Although still debated, several studies have concluded that for patients on ART, CD4⁺ counts alone are not sufficient to justify therapy

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modifications, and when feasible, a combination of either both tests or viral load tests alone are favored^{7–10}.

While the initial detection of HIV infection can be performed with inexpensive, qualitative assays, the monitoring of patient response to a treatment regimen requires a quantitative viral load measurement. The most commonly used HIV viral load tests detect HIV RNA genome copies circulating in plasma through an RT-PCR based nucleic acid amplification test (NAAT), which typically requires complex and time consuming sample preparation protocols prior to analysis^{2,4}. In the context of low resource countries, the infrastructural requirements of these techniques limit the proliferation of viral load-based ART monitoring to well equipped, centralized laboratories. While centralization of treatment monitoring allows for the application of economies of scale (through optimal batch sizing), as well as standardization and quality control mechanisms, the populations to which these facilities can service is limited by the availability of cold chain storage and transport. As a result, the monitoring of treatment regimens in populations that are geographically distant from testing facilities often results in significant travel and opportunity costs to the patient¹¹.

Several research efforts have focused on the development of a “sample in, answer out” microfluidic total analysis system (μ TAS) designed to move an entire assay to the point of care (POC), thus eliminating the need for sample storage and transport, and avoiding the occasionally challenging process of returning test results to patients at a later date^{12–14}. While these research efforts have often resulted in μ TAS platforms with impressive analytical capabilities, and represent a potentially paradigm-shifting long term solution, they remain an area of active research and may not be applicable to all settings. More specifically, the use of these systems in low resource settings is inhibited by their continuing need for permanent instrumentation and upstream sample preparation while being unable to take advantage of the cost and quality control advantages of centralized batch processing^{5,15,16}.

The use of dried blood spots (DBS) has been thoroughly investigated as a potential sample collection method for increasing the capacity for viral load testing in low resource areas. In the first approach, small volumes of blood (50 – 100 μ L) are absorbed onto a chemically treated paper and dried in place over several hours, stabilizing the sample for storage and deactivating any pathogen present¹⁷. Although this process results in an increase in the complexity of sample processing and purification once samples are received by a central laboratory, the use of dried blood spots (DBS) offers a simple and robust sample collection mechanism at the point of care. While dried blood spots have been successfully used for the diagnosis of perinatal HIV infection and viral load monitoring of HIV patients with high viral loads, the absolute lower limit of detection (LLOD) of DBS prepared samples is still under evaluation, with several studies indicating poor sensitivity for viral loads less than 4,000 copies/mL^{18–20}.

As an alternative to DBS techniques, which stabilize blood samples but require additional downstream purification before qPCR analysis, a minimally instrumented sample preparation technology that outputs a purified and thermally stabilized nucleic acid sample would allow for cold chain elimination without reduced sensitivity or an increase in downstream processing infrastructure. The ideal POC sample prep technology for HIV RNA viral load testing would be able to purify RNA from whole blood without the need for permanent instrumentation, electricity, temperature sensitive reagents, or trained operators^{5,13,15,16}. In this context, microfluidic solid phase extraction (μ SPE) techniques represent an attractive platform for the development of POC compatible RNA and DNA purification.

Solid phase extraction utilizes a high affinity surface to preferentially bind nucleic acids from a lysed sample, which can later be eluted in a small volume after a washing step removes residual salt and protein contaminants. Most approaches utilize the binding of nucleic acids to a silica solid phase under chaotropic conditions, as the chaotropic lysis chemistry lyses cells, denatures proteins, DNases, and RNases, and provides high affinity binding conditions in a single step. Unlike traditional liquid-liquid extraction techniques, which utilize phase separation to isolate hydrophilic nucleic acids from proteins and contaminants, SPE techniques work with homogeneous solutions that are more compatible with automation and miniaturization. While the use of commercially available SPE solutions have been investigated and validated for HIV RNA viral load sample prep, none are currently directly compatible with point of care use.⁵

A variety of solid phases have been developed for the microfluidic purification of nucleic acids from biological samples, taking the form of patterned silicon surfaces²¹, packed beds of silica particles^{22,23}, sol-gels and sol-gel immobilized silica beads^{24–27}, and organic polymer monoliths with immobilized silica particles^{28–31} or functionalized surfaces^{32,33}. Alternative solid phases for non-chaotropic nucleic acid purification have been reported with chitosan coated particles and surfaces^{34–36} and photoactivated polycarbonate surfaces³⁷. Of these platforms, organic porous polymer monoliths benefit from an in-situ fabrication process that is most compatible with inexpensive and high throughput manufacturing. However, while these systems have been successfully demonstrated for the purification of RNA and DNA in laboratory settings, they have largely been focused on the maximization of analytical sensitivity through miniaturization and sample preconcentration, and have typically shown limited compatibility with unprocessed whole blood samples. In contrast, the ideal low resource POC sample preparation technology would be focused on the minimization of infrastructural and operator requirements while maintaining a clinically relevant limit of detection in fingerprick compatible volumes of whole blood.

There are two major challenges in the adaptation of SPE sample preparation from a laboratory process to a low resource setting compatible POC technology. The first challenge stems from the fact that the binding capacity of affinity based analyte capture scales with increases in the effective surface area of the solid phase, which corresponds in turn to increases in the fluidic resistance of the system. While this does not typically pose a problem in a laboratory setting, where the use of high pressure syringe pumps or spin columns can easily provide the high pressures required, the development of a POC compatible low pressure SPE process requires the careful optimization of solid phase geometry, permeability, operating pressure, sample volume, and assay run time.

The second challenge encountered in adapting a SPE technique for a POC device is the question of how to deal with the large amounts of protein present biological samples. Endogenous proteins, which dramatically outnumber nucleic acids in biological samples, compete with nucleic acids for binding sites in surface based affinity capture (reducing capture efficiency), and can form protein aggregates with the potential to clog highly porous fluidic systems. While this phenomenon is encountered in purification of many types of biological samples, it is greatly exacerbated in the solid phase extraction of nucleic acids from whole blood, and has been shown to dramatically reduce the extraction efficiency of microfluidic SPE systems^{22,25,27,32}. To combat this problem, many SPE techniques employ the enzyme proteinase K to digest denatured proteins following chaotropic lysis of a blood sample. Although effective, the need for incubation steps at elevated temperature drives up the cost and complexity of the technology, while the need for refrigerated reagent storage limits the technology to cold chain accessible locations. Alternatively, Wen et al.³³ investigated the use of a two column system incorporating a reverse phased monolith to capture hydrophobic proteins from whole blood, followed by a downstream tetramethyl

orthosilicate grafted organic polymer monolith for DNA capture. While this approach was able to effectively screen ~80% of the protein from a blood sample, the whole capacity of the system was still limited to 10 μ L, as unfiltered protein continued to compete for binding sites on the downstream monolith. In another approach, Cao³⁴ and Hagan^{23,35} reported that pH dependent capture of DNA and RNA using a chitosan solid phase displayed increased compatibility with whole blood, as the aqueous binding conditions and hydrophilic solid phase surface minimized the binding of hydrophobic conditions whole blood proteins. While this approach shows promise for integration in μ TAS systems, as the aqueous binding chemistry eliminates the need for PCR-inhibiting chaotropic and organic reagents, it is of limited use in the stabilization and storage of HIV samples, as the high pH conditions required for elution (pH 9) have been shown to rapidly degrade RNA through hydrolysis³⁸.

The development of a whole blood compatible, enzyme-free, ambient temperature, low pressure nucleic acid extraction process would enable the creation of a completely powerless nucleic acid sample preparation technology well aligned with the needs of HIV viral load monitoring in low resource areas. To this end, a novel hybrid alcohol precipitation and solid phase extraction process is presented, utilizing glycogen carrier particles to increase the effective hydrodynamic radius of precipitated nucleic acids aggregates, and a thermally polymerized acrylate based solid phase as a weir for analyte capture. This process avoids the protein induced efficiency losses reported for silica based SPE systems through aggressive protein solubilization and selective alcohol induced analyte precipitation, and provides a platform upon which extracted nucleic acids can be stored at room temperature. Additionally, an air pressure driven mechanical system was developed to perform this process in a semi-automated fashion, in order to insulate the process from variations in operator technique. Together, they form the System for Nucleic Acid Purification (SNAP).

RESULTS AND DISCUSSION

Nucleic Acid Extraction Chemistry

The SNAP system is based upon a three step alcohol precipitation process utilizing a novel lysis buffer we have named BoomD, adapted from the work of Boom³⁹ and Chomczynski⁴⁰. Initially, a blood sample is mixed with a chaotropic lysis buffer which utilizes guanidinium thiocyanate (GuSCN), N-lauroylsarcosine, and 2-mercaptoethanol to aggressively lyse cells and virions, denature and solubilize proteins, and irreversibly deactivate RNases. As nucleic acids (NA) are released into solution, they bind with glycogen carrier particles present in the lysis buffer to form glycogen-NA complexes. The subsequent addition of an isopropanol precipitation buffer increases the hydrophobicity of the solution, causing the highly hydrophilic glycogen-NA complexes to precipitate while leaving the more hydrophobic denatured proteins solubilized. As this resulting solution is passed through an acrylate based solid phase, precipitated glycogen-NA complexes become trapped within the porous polymer, while the remainder of the solution flows through to a waste collection reservoir. An ethanol wash step removes residual salts and contaminants from the solid phase, and a final air drying step dehydrates the glycogen-NA complexes in place, stabilizing the sample for transport and storage. Elution of samples from the SPE column with nuclease free water results in the dissociation of the glycogen-NA complexes, yielding a qPCR compatible sample without the need for further purification.

While organic alcohol precipitation techniques are widely used to purify nucleic acids from heavily proteinacious samples,⁴⁰ a typical alcohol precipitation would not normally be performed until after contaminating proteins had been removed via centrifugation, phase separation, and supernatant removal. The addition of glycogen, a branched polysaccharide that is well documented as a high affinity carrier in the recovery of small amount of nucleic acids,⁴¹ acts to reduce the solubility of bound nucleic acids in organic solutions due to the

large number of exposed hydroxyl groups. When combined with the increased protein solubility provided by high concentrations of chaotropic guanidine and thiocyanate ions, this solubility difference can be exploited for the selective precipitation of glycogen-NA complexes. Nonetheless, careful buffer optimization is required in order to minimize protein co-precipitation, and it is possible that some small amount of protein co-precipitation does occur. In any event, it is unlikely that any residual denatured protein will dissociate upon elution in a low-ionic strength buffer, a hypothesis which is corroborated by the lack of PCR inhibition in our SNAP purified samples.

The RNA extraction process described above differs from traditional SPE extraction processes, in the sense that analyte sequestration occurs in the bulk solution through selective binding and subsequent co-precipitation with glycogen molecules, instead of through a two dimensional adsorption mechanism on the solid phase surface. This sequestration reduces the dependence of extraction efficiency on the microstructure of the solid phase, as increases in pore size (and corresponding decreases in effective surface area) do not negatively affect performance as long as pores remain small enough to mechanically capture precipitated glycogen-NA complexes (See Supplemental Information). The use of a solid phase with relatively large pores limits the vulnerability of the system to clogging events caused by protein aggregation and reduces the fluidic resistance of the system, enabling low pressure device operation via an inexpensive bicycle pump. Additionally, as the downstream elution of purified nucleic acids is based on the dissolution of accumulated precipitates, the system exhibits a temperature dependent elution profile. Finally, because the SNAP system stores extracted nucleic acids in a protecting matrix of dehydrated glycogen, it provides enhanced resistance to sample degradation.

Mechanical Design

The SNAP mechanical platform (Figure 1) is designed to encapsulate the Boom D extraction chemistry, insulating the process from variations in operator technique and minimizing avenues for environmental contamination. While future iterations of this technology will likely use injection molded microdevices due to advantages in geometric control and high throughput manufacturing, this initial proof-of-concept platform was developed with off the shelf fluidic tubing and components, due to the relative simplicity of the fluidic system and the milliliter scale fluid volumes involved. Solid phase columns are thermally polymerized inside polypropylene pipette tips, which are then mounted inside a milled acrylic housing to form a detachable cartridge onto which purified RNA is captured, dehydrated, and transported (Figure 2). Samples and reagents are introduced to the system via preloaded 3 mL plastic syringes, which double as mixing chambers for the initial lysis and alcohol precipitation steps. After the sample has been lysed, and glycogen-NA complexes precipitated with an alcohol buffer, the resulting solution is injected through a one way valve into a long buffer tube (5 m), followed by an ethanol wash. The buffer tube is wrapped in a tight coil to minimize its footprint. The small cross sectional area of the buffer tube minimizes diffusion between the sample and washing solutions, and subsequent pressurization of the system with an external bicycle pump sequentially forces the two solutions through the detachable SPE cartridge and into a waste collection reservoir. Excess air pressure follows the wash buffer through the column, dehydrating the captured analytes and stabilizing them for transport. When not in use, the system can be collapsed into a compact and easily transported configuration. (For a detailed description of device design and operating procedure, refer to the Supplemental Information.)

SPE Column Optimization

Pressure controlled flow was used to power the SNAP system, due to its relative simplicity and the availability of hand powered bicycle pumps in low resource areas. As a result, flow

rates and cycle times vary between extractions due to fluctuations in hydraulic resistance between different SPE cartridges. While cycle times could be decreased by increasing the porosity of the SPE, resulting in higher flow rates, excessively high porosities could result in both a drop in extraction efficiency and a loss of structural integrity. Porogen concentrations of 65% or lower resulted in a polymer that was too dense for fluid flow, while concentrations of 85% or higher resulted in a loss of structural integrity (crumbling of the SPE column) upon pressurization. SPE cartridges made using porogen concentrations of 70%, 75%, and 80% were evaluated with respect to cycle time, extraction efficiency, and efficiency loss due to uncaptured RNA (Figure S3a). These experiments were performed at 50 psi.

Flow test results indicated a nonlinear increase in flow rate when the porogen concentration reaches 75% (Figure S3b). Although the mechanism for this change was not investigated here, it has been hypothesized to be the result of a change in the regime of pore distribution, resulting in shunting of the fluid through low resistance pathways that form as a result of smaller pores coalescing during the phase separation process. By utilizing SPE columns with porogen concentration just over the 75%, it was found that relatively short cycle times (8.23 ± 1.85 min, $n=6$) could be achieved without significant decreases in RNA recovery. Further increases in porogen concentration resulted in a drop in recovery efficiency, and corresponding increases in the RNA content of the flow through volumes.

Additional experiments were performed to quantify the effect of changes in pressure on recovery efficiency (Figure S4). Only SPEs made with 75% and 80% porogen were used in these experiments. While the 75% porogen SPE displayed a similar extraction efficiency ($53.0 \pm 20.0\%$ at 50 psi, 53.8 ± 21.2 at 20 psi, $n=5$) at both pressures, the 80% porogen SPE displayed reduced recovery at the higher operating pressure ($30.7 \pm 8.1\%$ at 50 psi, $47.8\% \pm 12.1\%$ at 20 psi, $n=5$). Additionally, the flow-through solution for each condition was analyzed to determine the portion of RNA that was not captured by the column. Increasing the porogen concentration increased the average loss of RNA in the flow-through (Figure S4). These results again indicate that the 80% SPE may be too porous to accurately and reliably capture RNA from the input sample. In terms of ease of use, while the 50 psi extractions required half the time as those performed at 20 psi, the lower operator pressure made the device significantly easier to pressurize via bicycle pump. All further work was performed using SPE columns with 75% porogen content.

Elution Profiles and Temperature

With the optimal SPE formulation and run-pressure determined, the next step was to determine the elution profiles of RNA from the SPE columns. Extractions were run using 75% porogen SPE columns at 20 psi. Four 70 μ L fractions were collected from each SPE in the elution step. These extractions were performed at two elution temperatures (20 °C and 40 °C). The elutions at 40 °C resulted in significantly higher RNA recovery ($85.6\% \pm 20.8\%$) than elutions at 20 °C ($47.4\% \pm 19.2\%$) (Figure S5). For both elution temperatures the majority of RNA was recovered during the first two elutions. Due to this data, all subsequent experiments utilized two sequential 70 μ L elutions.

Detection of HIV Virions in Whole Blood

Extractions of RNA from HIV-1 LAV/8E5 virions in whole blood were performed at concentrations ranging from 1×10^6 copies of HIV RNA per ml (c/ml) down to 1×10^3 c/ml using the Boom D lysis system. Quantification of the input samples of HIV-1 was based on the number of HIV-1 genomes, rather than the number of virions, to account for the fact that each virion contains two copies of RNA. Further, we assumed that our lysis system achieved 100% lysis of the virions in each sample. This range compares favorably to ranges of RNA

recovery achieved using dried blood spots (DBS) or dried plasma spots (DPS) for viral load determination. Here, our goal was to demonstrate that SNAP with Boom D would be able to reliably extract RNA from whole blood down to 10^3 c/ml.

RNA was extracted from each sample using the SNAP system and was then quantified using qRT-PCR as described above. Note that the QIAamp extractions were performed using virions spiked into serum, while the SNAP extractions were performed using virions spiked into whole blood. Serum was used instead of whole blood for the QIAamp extractions because the manufacturer's protocol recommends against extractions directly from whole blood. Figure 3 shows the absolute and relative recovery of HIV RNA from human whole blood over four orders of magnitude for the SNAP system. Recovery performance of the SNAP system was compared to a gold standard RNA extraction performed according to the manufacturer's protocol (QIAamp Viral Kit, Qiagen, Valencia, CA). For both extraction methods, an input sample of 70 μ L was used in order to simulate a typical sample volume obtainable via finger prick. Two 70 μ L elution fractions were taken from each experiment and 5 μ L of each fraction was used in a RT-qPCR to quantify the extracted RNA. It was possible to quantitatively distinguish differences between viral loads over 4 logs (Figure 3A). SNAP extractions were comparable to those performed using the QIAamp Viral Kit for viral loads between 10^4 and 10^6 c/ml, although RNA extracted using the QIAamp Kit at 1000 c/ml was not amplifiable in our qRT-PCR assay. Our in house qRT-PCR assay for HIV is only sensitive down to 1000 c/ml, so we were unable to test the performance of the SNAP system below this value.

Cleaning Studies

To verify the effectiveness of the cleaning procedure in between samples and ensure no contamination between samples, device wash studies were performed with HIV spiked blood samples followed by blood only negative controls. Using a water only flush resulted in 1% ($\pm$ 0.5%) recovery of HIV RNA (n=6) from "blank" whole blood samples, when compared to the initial positive sample concentration. By adding a 10% bleach wash, followed by a water wash, the recovery of HIV RNA from blank samples was consistently less than 0.01% of the initial sample concentration via qRT-PCR analysis (data not shown).

Temperature and Storage Studies

To demonstrate the ability of the SNAP SPE cartridges to store extracted RNA, samples of HIV virions spiked into whole blood were extracted without performing the final elution step. The SPE cartridges containing the extracted RNA were stored at two temperatures (20 °C (RT) and 37 °C) for up to two weeks before elution and quantification using qRT-PCR. SPE cartridges were stored in zip top bags containing a desiccant packet (either in a bio-hood for 20 °C (RT) storage or a dry incubator for 37 °C storage). The SNAP system was able to preserve RNA extracted from virions in whole blood for up to one week at 37 °C. Significant sample degradation was observed at the two week time point for both temperatures. In this work, no RNA stabilization reagent was used. Although one week of storage without a cold chain is a significant improvement over the complete cold chain required for transporting blood samples, it still may be too short for low resource settings where transport can often take more than one week. Longer term storage stability may be possible through the use of additional sample stabilization chemistries (e.g. RNastable, Biomatrix, Inc.), but was not investigated in the course of this work.

MATERIALS and METHODS

Reagent Preparation

All reagents and buffers used for the experiments were prepared with sterile molecular biology grade water (Thermo Fisher Scientific, Waltham, MA). To prepare the Boom D lysis buffer, 35.4 g of guanidine thiocyanate (GuSCN, Sigma-Aldrich, St. Louis, MO) was dissolved in 14 mL of sterile RNA grade water in a 50 mL conical tube. The solution was then heated in a 50°C bath for 10 minutes followed by vortex mixing for 1 minute. Next, 5 mL of 1 M MOPS (pH 7.0 titrated from the free acid with 10 M NaOH, Sigma-Aldrich, St. Louis, MO) and 1.36 mL of 20% N-lauroylsarcosine (sodium salt) solution (Sigma-Aldrich, St. Louis, MO) were added to the GuSCN solution. The solution was allowed to cool to room temperature for 20 minutes; during this time, GuSCN crystals form inside the tube. Finally, 360 µL of 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) and 500 µL of glycogen (20 mg/mL, Fermentas Molecular Biology Tools) were added to the buffer. The GuSCN crystals were separated from buffer and the supernatant was used in the SNAP device. The precipitation buffer was comprised of 550 µL 100% isopropanol, while 1 mL of 75% ethanol (Sigma-Aldrich, St. Louis, MO) was used as a wash buffer. Pipette tips and centrifuge tubes were DNase-, RNase-, and pyrogen free (Denville Scientific, Metuchen, NJ). Fresh human whole blood (less than 5 days old) was obtained commercially and pooled from three different donors with the same blood type (Research Blood Components, Brookline, MA).

Fabrication of SPE Columns

The SPE columns were polymerized and housed in unaltered FinnTip Universal 250 µL filter-less pipette tips (Thermo Fisher Scientific, Waltham, MA). Ethylene dimethacrylate (98%, EDMA), 1-dodecanol (98%) and 1,1- azobis(cyclohexanecarbonitrile) (98%, ACHC) were purchased from Sigma-Aldrich. The pre-polymer monomer solution was prepared by mixing EDMA (350 µL), 1-dodecanol (1050 µL), and solid ACHC (8.00 mg). This solution was thoroughly vortexed and 50 µL was pipetted into each pipette tip. The pipette tips containing the pre-polymer solution were held in a pipette tip tray during polymerization at 100°C for 20 minutes. After polymerization, the tips were washed with 200 µL of methanol followed by 150 µL of 95% ethanol to remove excess unreacted material. Finally, the SPE columns were dried in a fixture under air pressure for two minutes at 20 psi and stored in a pipette tip box with a desiccant packet until use.

RNA Capture, Isolation, Storage and Elution

Noninfectious HIV-1 LAV/8E5 reverse transcriptase mutant virions (PN-227, Seracare Life Sciences, Milford, MA) were diluted in human whole blood (Research Blood Components, Brookline, MA). All samples were handled using standard precautions for blood borne pathogens in a BSL2 facility. Samples were loaded into a 3 mL syringe to allow extra space for in-syringe mixing and attached to the SNAP system. The SNAP lysis process uses a 1:7 sample to buffer ratio (see Supplemental Information for the complete on-device mixing protocol). After a 15 minute incubation, the precipitation buffer (isopropanol 40% final) was added, causing glycogen/RNA complexes to precipitate. The resulting solution was forced under pressure (20 psi) through the SPE cartridge, where the precipitated glycogen/RNA complexes are captured through a size exclusion mechanism. The column was washed with 75% ethanol to remove contaminants, and dried under air pressure for two minutes.

Once dry, the SPE cartridges were detached from the SNAP system and stored at ambient and elevated temperatures for various time periods to determine the stability of the extracted RNA. Purified samples were recovered from the cartridges with two sequential elution volumes of 70 µL of sterile molecular biology grade water heated to 40 °C. The eluents, 5µL

each, were used in qRT-PCR reactions without further purification. The SPE cartridges and reagent syringes are single-use components that are replaced for each sample. Permanent device components were sterilized between extractions by flushing with 10 mL of 10% bleach followed by 10 mL of sterile water.

Flow-Through Analysis

In some experiments, the flow through (waste) from the SPE was collected to quantify the amount of uncaptured RNA and to differentiate between losses in extraction efficiency due to incomplete capture, excessive washing, and RNA degradation. To analyze the flow through, 5 μ L of glycogen (20 mg/mL) was added to the solution and spun down at 13,000 rpm for 15 minutes, after which a glycogen-RNA pellet was visible at the bottom of the tube. After the supernatant was removed, the pellet was washed with 500 μ L of 75% ethanol and spun down at 13,000 rpm for 5 minutes. The resulting supernatant was then removed, and the pellet was left to air-dry for 10 minutes at room temperature in a biosafety hood. After drying, the pellet was re-suspended in 40 $^{\circ}$ C sterile water and analyzed using qRT-PCR.

qRT-PCR

The extraction efficiencies for each experiment were calculated by comparing the known input concentration of HIV virions to the results of a qPCR assay. A Taqman-based qRT-PCR was performed on 5 μ L of purified samples eluted directly from the SNAP system to verify and quantify extraction results. We chose the HIV-gag gene as our target for analysis. The forward primer was 5'-GGCTACACTAGAAGAAATGATGACAGCAT-3', the reverse primer was 5'-GCTCATTTGCTTCAGCCAAAACCTCTTGC-3', and the Taqman probe sequence was 5'-[6FAM]-AGTAGGAGGACCCGGCCATA-[MGB]-3' using the Brilliant II qRT-PCR 1-Step Core Kit (Agilent Technologies, Santa Clara, CA). The PCR reactions were run on an ABI 7300 (Applied Biosystems, Foster City, CA) real-time PCR instrument with the following protocol: a 50 $^{\circ}$ C hold for 30 minutes, a 95 $^{\circ}$ C hold for 10 minutes, 45 cycles of 95 $^{\circ}$ C for 30 seconds, 65 $^{\circ}$ C for 30 seconds and 72 $^{\circ}$ C for 30 seconds. Data was collected during the 72 $^{\circ}$ C step. The PCR results were analyzed using the automated threshold cycle (C_T) value calculation in the ABI 7300 software (Applied Biosystems). This assay is sensitive down to 1000 c/mL. In order to test the SNAP system at lower viral loads, a commercial assay will be necessary.

Titrated Input and Lower Limit of Detection

To determine the lower limit of detection for the SNAP system, titrated concentrations of HIV virions diluted in whole blood were extracted and quantified. As a control, HIV RNA was extracted using the QIAamp Viral Kit (Qiagen, Valencia, CA) and was quantified using qRT-PCR. Extractions performed on this QIAamp system were performed as recommended by the manufacturer (including the use of carrier RNA), with one exception. We used smaller volumes to match the requirements of the SNAP system (substituting a 70 μ L sample input instead of 140 μ L input).

Temperature and Time Storage Studies

To quantify the ability of the SNAP cartridge to store RNA for future use, HIV RNA was extracted from virions in whole blood. Before elution, the SPE cartridges were stored in sealed plastic bags with desiccant packets at either room temperature (20 $^{\circ}$ C) or in an incubator (37 $^{\circ}$ C) for up to two weeks. Following the storage period, the samples were eluted with 40 $^{\circ}$ C sterile molecular biology grade water and analyzed using qRT-PCR.

CONCLUSION

We have described a portable device for nucleic acid extraction and storage using thermally stable reagents that does not require the use of electrical power. The device weighs less than three pounds and is capable of extracting HIV-1 viral RNA directly from virions in whole blood in less than 35 minutes. Samples are stabilized on detachable cartridges, which can be eluted either at the point of care, or transported to a centralized laboratory for later analysis.

The results presented here suggest that the Boom D RNA extraction process is a potentially valuable tool that could be used in conjunction with several emerging HIV viral load assays. Lower limits of detection using qRT-PCR were quantitative across 4 logs, with a lower limit of detection of 1000 c/mL. This LLOD is comparable or better to those achieved with DBS sample collection methods, with significantly reduced time and labor requirements. The LLOD is potentially lower than 1000 c/ml, but we were unable to make this assessment due to the limitations of our in house qRT-PCR assay. In future work, we will test SNAP extracted samples in a clinical setting where commercial assays on automated systems are sensitive down to 100 c/ml or less. In summary, SNAP represents a new tool in the quest for low cost and appropriate molecular diagnostics in low resource settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
A) SNAP device. B) CAD rendering, open configuration. C) CAD rendering, storage condition

Figure 2.
A) SPE column housed in a detachable cartridge with fluidic connections. **B)** SEM image of representative pores in an SPE column.

Figure 3.

Recovery results from SNAP and QIAamp Viral Kit over four orders of magnitude. **A.** Absolute recovery of RNA from the SNAP device (n=6). A clear distinction is observed between each order of magnitude, enabling an accurate viral load determination directly from SNAP outputs. **B.** Comparison of recoveries for SNAP (n=6) and QIAamp QIAamp Viral Kit (n=3). **C.** Extraction efficiency for SNAP (n=6) and QIAamp QIAamp Viral Kit (n=3). Note that the QIAamp extractions were performed using virions spiked into serum, while the SNAP extractions were performed using whole blood. In these experiments, QIAamp Viral Kit extracted samples at 1000 c/ml did not amplify in the qRT-PCR assay.

Figure 4.

Recovery results for SNAP extractions stored on detachable SPE cartridges at two temperatures for up to two weeks. No significant difference in recovery between the 20 °C (RT) and 37 °C storage conditions was observed at any of the time points. Recovery at the two-week time point was poor for both temperatures, likely due to RNA degradation.