

Abstract

In resource-limited areas, care for patients with human immunodeficiency virus (HIV) is often limited by lack of easy access to a quantitative viral load test. A low-cost, point-of-care diagnostic test that could quickly and efficiently detect HIV would improve testing standards and have the potential to impact patient care. When developing a test suitable for this application, sensitivity is often sacrificed to favor low cost, portability and speed. To address the issue of sensitivity while preserving low cost, we present a closed microfluidic system that will concentrate a human blood plasma sample to ensure that the HIV concentration is above the lower limit of detection of the sensing technique. Our system consists of two modules: (1) a microfluidic chip for sample concentration via evaporation and dialysis and (2) a solid phase extraction column for extraction of HIV RNA following virion lysis. The system uses minimal grid power by replacing in house components with portable versions. We have demonstrated a working prototype that concentrates plasma to a volume factor of ten and extracts HIV RNA for quantification. The low-cost, portable components of the microfluidic system are a step towards developing a more accessible HIV diagnostic platform.

Background

Human immunodeficiency virus (HIV) is a virus that decreases the immune system's ability to fight infections. The virus attacks disease-fighting CD4 cells, resulting in a weaker immune system and an increased risk of developing cancer or contracting infectious diseases [1]. An HIV infection may progress and reach its most advanced stage, which is known as acquired immunodeficiency syndrome (AIDS) [2]. In the past three decades, more than 25 million people have died from AIDS [2, 3]. As of 2012, more than 34 million people are infected with HIV worldwide [3, 4]. The 2010 edition of the UNAIDS Report on the global AIDS epidemic reported that, as of 2009, sub-Saharan Africa remained the region most affected by HIV with 68% of the total HIV population and 72% of deaths due to AIDS [5]. Lack of access to HIV diagnostic tests in low-resource areas such as this leads to undiagnosed individuals with HIV who could potentially transmit the disease. Care for patients who have been diagnosed with HIV is also restricted in many regions due to lack of easy access to a quantitative test; such a test is required to monitor progression of the disease and make important treatment decisions.

According to the U.S. Department of Health and Human Services' Guidelines, the most common method to detect HIV is the HIV antibody test [1]. When the body is infected with HIV, it produces HIV antibodies that can be detected in a patient's urine, blood, or fluid from the mouth [1]. Blood and bodily fluids containing blood have the highest concentrations of HIV [6], but the antibody test can avoid the use of blood by using saliva or urine. This test is performed in an enzyme-linked immunosorbent assay (ELISA) format [7]. An enzyme in a solution links to the targeted HIV antibodies and results in the

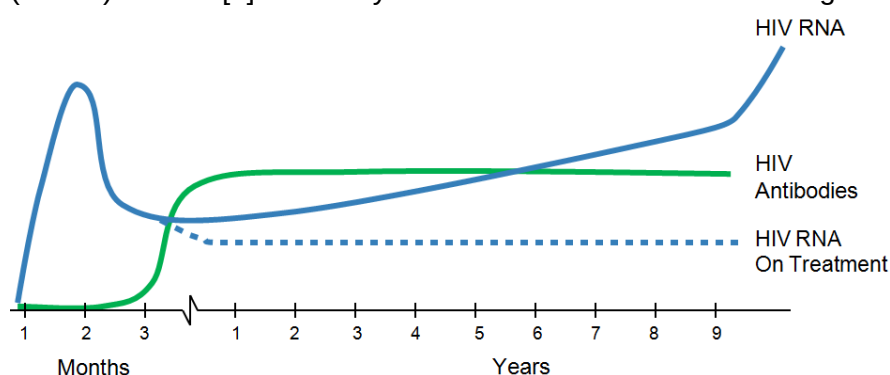


Figure 1: The HIV RNA levels vary with disease progression and response to treatment. Currently available low-cost viral load tests are unable to detect the dashed line (as low as 200 copies/mL). Our device enables low-cost detection of these low concentrations.

solution changing color [8]. If there are no antibodies present, the solution will not change color and the patient is considered HIV negative. Due to the chance of false positives, a Western blot test is performed to confirm that a patient is HIV positive [7, 8]. Since the results of the HIV antibody test are qualitative, they can be used to diagnose HIV but not to make accurate treatment decisions. In addition, there is a three to six month "window period" after infection in which the concentration of antibodies is too

low to be detected [1]. During this window period, the disease may progress further or be transmitted to other people. In the case of mother-to-child transmission, antibody tests are also ineffective during the first 15-18 months after birth because maternal antibodies may still be present and can lead to false-positives [9]. Treatment during these early months is critical to infant survival.

Another available test is the HIV ribonucleic acid (RNA), or viral load, test. This test can be used to detect HIV in a blood sample as soon as nine days post-infection [1]. This method diagnoses HIV directly, rather than diagnosing based on the presence of antibodies. A reverse transcription polymerase chain reaction (RT-PCR), performed after nucleic acid isolation, is the basis of the viral load test [10]. Quantifying the increase in fluorescence after each cycle of PCR enables virus concentration to be determined. Increased virus concentration corresponds to progression of the disease.

While the viral load test has several advantages over HIV antibody testing, this test is not easily accessible in developing countries due to the expense and necessary personnel training [11-15]. A qRT-PCR machine can normally detect 1,000 copies of virus per milliliter. However, to make appropriate treatment decisions for patients on antiretroviral therapy (ART), the test must be able to detect concentrations as low as 200 copies/mL (Figure 1). For this to occur, the sample must be pre-amplified using expensive reagents, which can significantly increase the cost of the test. In addition, the blood sample must be sent to a lab for analysis, which increases the cost and turnover time. Days could pass before results are received, increasing the difficulty of maintaining contact with the patient.

Due to the lack of infrastructure needed to perform viral load tests in resource-limited settings, HIV antibody tests are the standard diagnostic technique. CD4 T-cell counts are used almost exclusively for patient monitoring because of the correlation between decreasing CD4 cell counts and treatment failure. However, this method is inexact and has been shown to lead to unnecessary treatment modifications in nearly 50% of cases [12]. By monitoring viral load directly, a clinician would know which patients were not responding or adhering to treatments and could intervene. They would also be able to spend more time attending to patients identified as high-risk. With a viral load test, infants could be diagnosed shortly after birth, and detection could occur more closely following infection. Thus, there is a desire for a point-of-care, quantitative HIV viral load test, particularly one that is low-cost and will be able to detect the virus at low concentrations.

For a viral load test to be successfully implemented in a resource-limited setting, it must be designed with the inherent restrictions of such a setting in mind. Many virus sensing techniques with the potential for inclusion in low-cost, point-of-care diagnostic platforms are now being researched [16]. However, when developing a biosensor, a compromise must be made between sensitivity, portability, and speed. For point-of-care sensing techniques, portability and speed take priority because tests are often performed at moving clinics and patients must receive results within a few hours. The sensitivity of these techniques consequently suffers. Sample preparation, specifically pre-concentration, prior to the detection step can increase the sensitivity of the sensing technique and thus increase the chance that a viral load will be quantified above the detection threshold. For nucleic acid-based sensors, sample preparation typically involves cell culture, lysis and RNA or DNA isolation. These are lengthy processes that include several handling steps, which reduce the safety of the process and require an increased level of expertise on the part of the technician. In addition, the commercial kits that exist for nucleic acid isolation necessitate the use of expensive equipment and are difficult processes to automate. Automation is likely to make the process less expensive and reduce the amount of time necessary to learn the required skills [17]. Therefore, a low-cost, safe and efficient complete sample preparation system must be developed for global health applications.

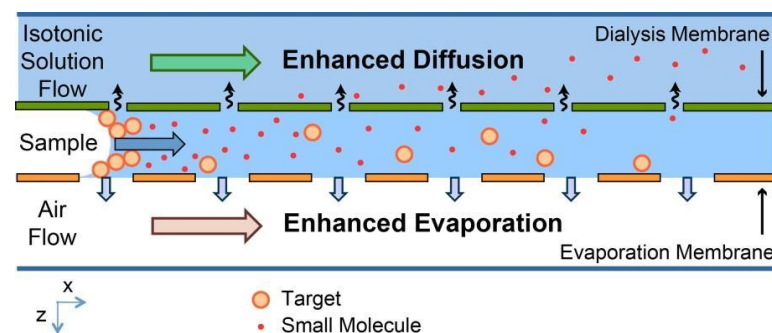


Figure 2: Cross-section of the combined concentration/dialysis device. Courtesy of Mario Cabodi.

Sample concentration can be defined as “the actual increase in target concentration in a fixed sample volume” [18]. Increasing the concentration of virus in a sample improves the performance of biosensors, which have a lower limit of detection. A combined microfluidic device for virus sample enrichment has already been demonstrated by this group [18]. This device concentrates and purifies the sample simultaneously. Evaporation serves as the concentration mechanism.

As the sample volume decreases, the number of virus particles remains constant, and the virus concentration is thereby increased. Purification occurs through dialysis of proteins and electrolytes out of the sample. The dialysis of proteins is necessary to prevent protein dehydration; dehydration, which in this case would be caused by an increase in protein concentration during enrichment, corresponds with an increase in sample viscosity. Once the viscosity reaches a certain level, formation of a gel prohibits further concentration. The initial device, which did not include dialysis, could only concentrate samples by a factor of 3. After adding dialysis, concentration could proceed to a factor of 7 [18].

The sample enrichment device, known as the concentrator, is intended for blood plasma samples and consists of a microfluidic chip with two porous membrane surfaces—one for evaporation and one for dialysis (Figure 2). As evaporation occurs and the sample volume decreases in size, the meniscus moves along the length of a serpentine channel.

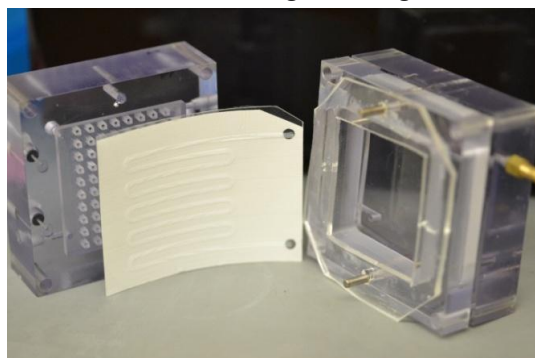


Figure 3: The cartridge and fixture components, evaporation chamber (left) and dialysis chamber (right).

remove proteins in the plasma, resulting in a gel due to protein buildup. This gel problem must be resolved to enable higher concentration factors and facilitate further sample processing. The peristaltic pump must be substituted with a component that does not require grid power to operate. The volume of dialysis fluid must be minimized to improve operator safety. The house vacuum that is used for evaporation must be replaced with a more portable source. For reduced costs and increased safety, the Qiagen kit must be replaced with another extraction technique that can be integrated into a closed system with the concentrator.

The chip is placed inside of a fixture with a vacuum chamber on one side and a chamber for the dialysate on the other (Figure 3). A vacuum is attached to the first chamber to drive evaporation. A peristaltic pump is used to circulate the dialysate, 1x phosphate buffered saline (PBS) into and out of the dialysis chamber.

After enrichment, the sample is removed from the chip, and RNA isolation is performed using a QIAamp Viral RNA Mini Kit, or Qiagen kit (Dusseldorf, Germany), costing about \$230 [19]. The virus concentration is quantified using qRT-PCR.

Before this system can be used in a global health setting, changes and additions must be made to its design.

The peristaltic pump used for dialysis does not efficiently remove proteins in the plasma, resulting in a gel due to protein buildup. This gel problem must be resolved to enable higher concentration factors and facilitate further sample processing. The peristaltic pump must be substituted with a component that does not require grid power to operate. The volume of dialysis fluid must be minimized to improve operator safety. The house vacuum that is used for evaporation must be replaced with a more portable source. For reduced costs and increased safety, the Qiagen kit must be replaced with another extraction technique that can be integrated into a closed system with the concentrator.

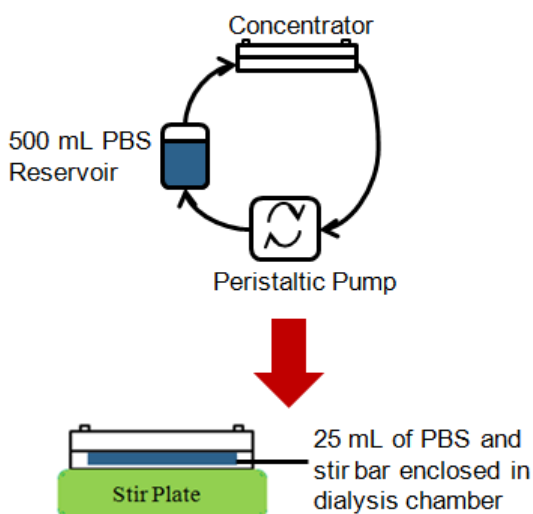


Figure 4: The new method of dialysis to mitigate gel formation and minimize dialysis fluid.

Design

The new integrated system uses a battery operated stir plate, a minimized volume of dialysis fluid, and a low cost method of extraction. Previously, the concentration process used a peristaltic pump to circulate 500 mL of PBS, but it did not efficiently purify the sample and was not practical for use in resource limited settings. A new method of circulation using a stir bar and stir plate was explored. A stir bar was enclosed in the dialysis chamber of the concentrator along with 25 mL of PBS. The concentrator was then placed on a stir plate to spin the stir bar and circulate the PBS (Figure 4). This new method of dialysis successfully mitigated the gel formation while minimizing the volume of dialysis fluid. Although the stir bar method of dialysis was successful, the stir plate still required electricity. To make the system more applicable in resource limited settings, a battery operated stir plate was built (Figure 5). The stir plate was comprised of a motor (computer fan), stir bars, a power switch, a potentiometer to change the speed, and a 9V battery. The use of a battery operated stir plate instead of a peristaltic pump increased dialysis efficiency while significantly

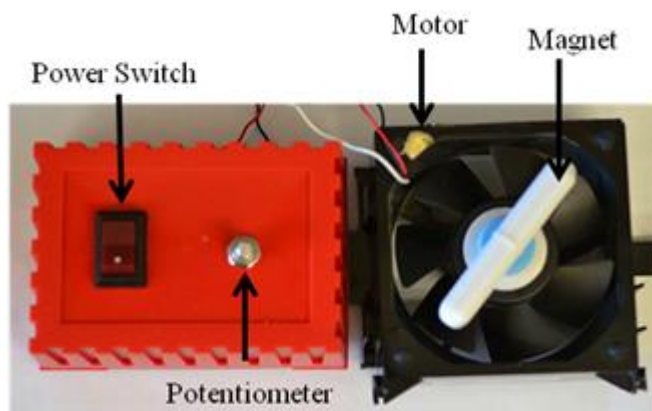


Figure 5: The battery operated stir plate for dialysis. Battery is in control box (left).

preparation platform. The column itself is a porous polymer with porosity optimized to allow for the capture of RNA via the size-exclusion principle. After chemical lysis of HIV, RNA is co-precipitated with glycogen to form particulates too large to pass through the polymeric column. Pressure is applied to push the plasma sample through the column while retaining the HIV RNA. After several wash steps to remove any remaining proteins and other debris, the RNA is eluted from the column through dissolution in nuclease-free water (pushed through the column at 5 psi).

The column, also known as a porous polymer monolith (PPM), is thermally polymerized in situ in a blunt 16-gauge syringe needle. The inner surface of the needle is functionalized through incubation in a solution of 30% 3-(Trimethoxysilyl)propyl methacrylate and 70% methanol. The chosen polymer formulation has a 2:3 monomer-to-cross-linker ratio (butyl methacrylate: ethylene glycol dimethacrylate) and 75% porogen (1-dodecanol). It was the second most porous of the four compositions tested.

Table 1: Extraction optimization parameters. Final selections highlighted in yellow.

Polymer composition	1 (porous)	2	3	4 (dense)
Polymer volume	5 μ L	10 μ L	15 μ L	20 μ L
Precipitating Agent	1-Butanol	Isopropanol		
PPM wash	Ethanol	Acetone		
Plasma volume	100 μ L	200 μ L		
Pressure	20 psi	40 psi		
Temperature	Room Temp (25°C)	Heated (30°C)		

Next, a portable pressure system was designed to push the lysate, wash buffers, and water for elution through the extraction column. Pressure is supplied by a pressure pump (Rietschle Thomas) that is connected to a pressure regulator/gauge and brass manifold with five outlets. This setup distributes pressure to a set of five interconnected syringes holding the lysate and extraction reagents (Figure 7). Valves and 3-way stopcocks control the flow of air and reagents to the column.

decreasing costs.

The house vacuum also made the concentration platform less accessible in resource limited settings. Therefore, a portable vacuum system was needed to increase accessibility. The portable vacuum was comprised of a stainless steel canister and a bicycle hand pump (Figure 6). The piston of the hand pump was inverted to produce negative pressure instead of positive pressure. The portable vacuum system needed to maintain 12 psi for a minimum of 30 minutes to successfully concentrate a sample.

A column-based extraction protocol was implemented to enable integration of the extraction system into the microfluidic sample



Figure 6: The portable vacuum comprised of a canister (left) and a hand pump (right), constructed to replace the house vacuum.

The column volume is 10 μ L. This combination of composition and volume was chosen based on total

extraction time and the relatively high reproducibility and success rate of columns. Several other extraction parameters were tested with the goal of obtaining the best combination of extraction efficiency and speed. The full list of tested parameters is in Table 1. Testing was performed using a multiplex pressurizing system previously developed in the Klapperich Laboratory.

After demonstration of working concentration and extraction modules, the two were combined into a closed system. Three-way stopcocks were used to form a connection between the concentrator and extraction system (Figure 8). After concentration, the dialysate is removed and lysis buffer is added to the channel. A syringe attached to the concentrator output is used to pull both the plasma sample and lysis buffer out of the concentrator. Then, switching a stopcock redirects flow into the extraction system.

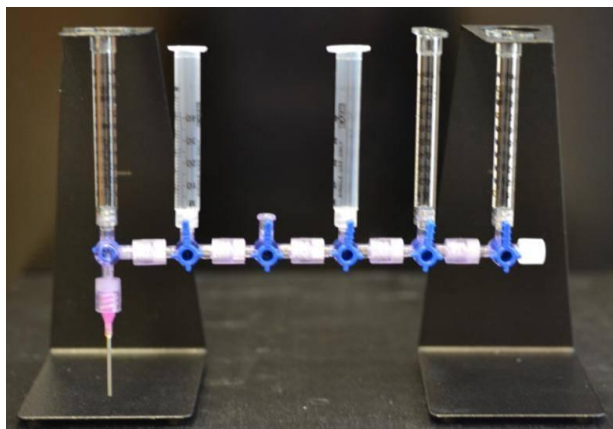


Figure 7: A portion of the portable pressure system. Extraction column in the lower left corner. Syringes from left to right hold water, precipitating agent, and three wash buffers.

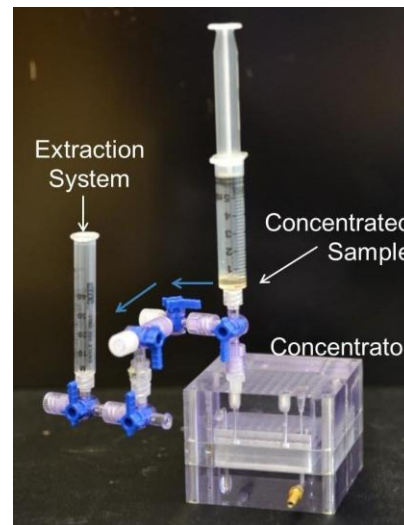


Figure 8: Connection between the concentration and extraction modules.

Results

A Bicinchoninic acid (BCA) assay was performed to measure the protein concentration of samples after the pump and stir bar methods of dialysis. Figure 9 shows the protein concentrations before and after concentrating plasma by a factor of seven. The goal was to prevent gel formation to allow for a more purified sample and further concentration. It was determined that the stir bar method of dialysis was more efficient than the peristaltic pump.

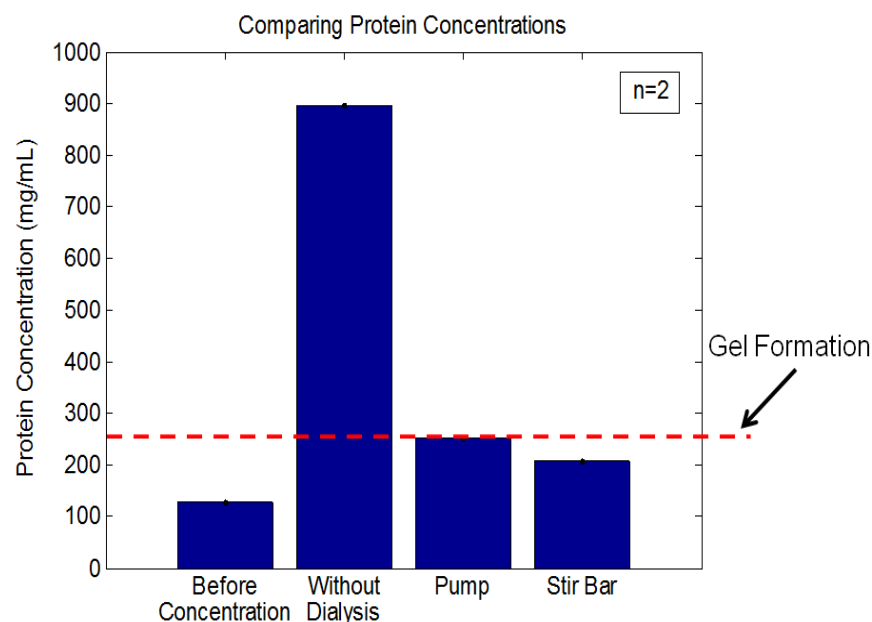


Figure 9: Comparing dialysis efficiency of the peristaltic pump and stir bar. Second bar from the left shows the theoretical protein concentration that would be expected without dialysis.

The portable vacuum for evaporation was able to sustain 15 in Hg (7.35 psi) for a short period of time, but it was not able to meet the minimum requirements for concentration. Although this portable vacuum was not suitable for the concentrator, this proof-of-principle study showed that a portable vacuum system could be developed in future research.

Initial extractions were unable to recover a detectable concentration of RNA. However, completed system optimization has yielded an average RNA recovery percentage of $12 \pm 3\%$. As an illustration, Figure 10 shows the results of the pressure and temperature optimization on extraction efficiency and time.

Initial extractions were performed at 40 psi and room temperature. The optimized extraction procedure is performed at 20 psi and a temperature of $\sim 30^{\circ}\text{C}$. As can be seen in the graph, using this lower pressure and higher temperature resulted in a higher RNA recovery percentage. This corresponds to an increased PCR signal, thereby enabling detection of lower concentrations of HIV. The total extraction time has been increased by approximately 15 minutes on average, but at 46 ± 13 minutes, is still feasible for integration into a point-of-care diagnostic.

A preliminary test of the pressure system demonstrated that it is capable of extracting RNA for quantification. The recovery percentage was low at 0.5% in the elution, but could be due to partial extrusion of the polymer. The total extraction time was only 6.4 minutes at a pressure of 20 psi. These results suggest that recovery percentages in the system may be higher for polymers without defects.

Preliminary tests of the connection between the concentrator and extraction system were successful. Mixing the plasma sample with the lysis buffer and RNA precipitation reagent (isopropanol)

did not cause protein precipitation within the syringes and stopcocks. In addition, a preliminary test proved that the extraction column was capable of extracting RNA from concentrated plasma samples. The RNA recovery percentage was high at 21%, and the total extraction time was two hours. This extraction took longer to complete due to the increased protein concentration of the concentrated sample. However, these tests demonstrate the potential for the combination of the concentration and extraction modules into a closed system. Modifications must now be made to further optimize the system for extraction of RNA from concentrated samples.

A low-cost point-of-care sample preparation platform has the potential to increase worldwide access to HIV viral load tests. By reducing costs and increasing portability, we have demonstrated the potential for our system to be used for this application. We have reduced the use of grid power by designing a battery run stir plate to drive dialysis and have eliminated the need for high-speed centrifugation through the development and integration of extraction in a polymeric column. In addition, we have improved operator safety by implementing a closed dialysis bath and forming a closed-system to minimize sample handling steps. Although the integrated system must undergo further optimization, the successful proof-of-principle experiments show the potential for this point-of-care system.

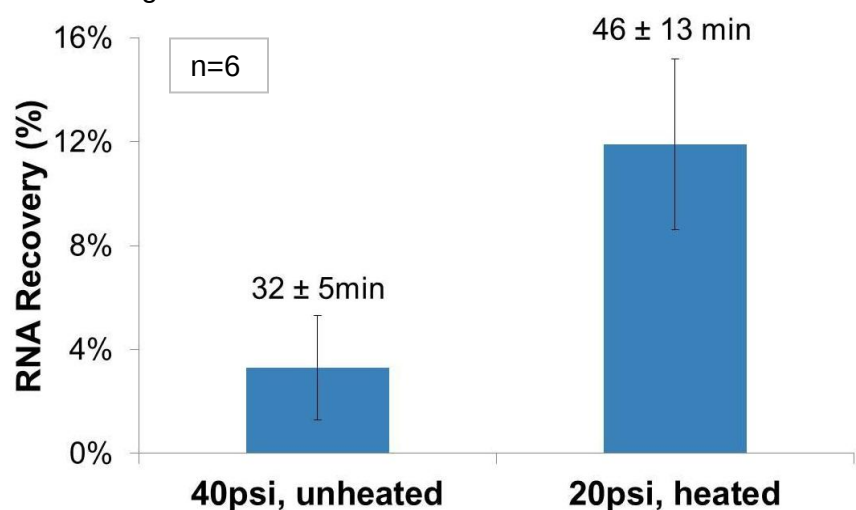


Figure 10: Effect of pressure and temperature optimization on RNA recovery in elution.

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