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Comparison of Packed Beds and Qiagen Columns for Recovering Trace Amounts of *B. anthracis* DNA from Liquid Suspensions

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Auspices Statement

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Preface:

This report describes work funded by the Department of Homeland Security as part of the Forensics and Attribution Thrust Area in DHS' Biocountermeasures Portfolio. It is submitted to the sponsoring DHS program office in fulfillment of FY05 deliverable 1.2.2.1.5.3.3.4.5, "Report with SOP" on High-Yield Purification of Trace DNA Using a Packed Bed with In-Bed Amplification. The corresponding authors are Elizabeth Wheeler (technical) and David Camp (programmatic).

Executive Summary

The goal of this work was to optimize and evaluate LLNL's in-bed amplification technology to improve the level of detection for suspensions containing trace amounts of anthracis DNA. The binding/cleaning performance of the packed bed is compared to the conventional commercial approach; Qiagen column cleanup and elution, followed by detection through an ex-situ amplification process.

Five liquid suspensions were spiked with *B.anthracis* DNA in concentration series. These suspensions were: 1) water, 2) water with EDTA, 3) dirty water from carpet extraction, 4) dirty carpet extraction with phosphate buffered saline (PBS) plus 0.1% Tween 20 plus 0.1% gelatin, and 5) a subway aerosol collected in water. Each suspension matrix was spiked with DNA and injected (in replicate) into either Qiagen Microcolumns (using the kit processing instructions) or LLNL's packed bed (using the LLNL in-bed purification and amplification protocol). The process output was assayed by quantitative polymerase chain reaction (QPCR). Table ES-1 shows the level of DNA (pg per 100 uL of input suspension) that resulted in successful amplification for all reactions (X=Y), and the level for which at least one of the reactions was successful (X>0). For each suspension and DNA concentration, there were Y QPCR assays of which X showed successful amplification.

LLNL's packed bed technology outperformed Qiagen Microcolumns for all five suspensions, typically by one order of magnitude in both the limit of assured detection (all reactions positive), and the lower limit of detection (some reactions positive).

DNA titrations performed in the following liquids	Limit of assured detection (all PCR rxns successful)		Lower limit of detection (X successful / Y rxns)	
	Qiagen column	Packed bed	Qiagen column	Packed bed
Water	100pg	10 pg	0.1 pg (6/30 rxns)	0.01 pg (5/8 rxns)
Water + EDTA	Limited testing gave qualitatively similar results			
Dirty carpet extracted w/ water	100 pg	10 pg	1 pg (6/24 rxns)	0.1 pg (3/8 rxns)
Dirty carpet extracted w/ PBS + Tween20 + gelatin	100,000 pg	10 pg	1,000 pg (7/16 rxns)	0.1 pg (3/16 rxns)
Subway aerosol collected into water	100 pg	100 pg	1 pg (10/48 rxns)	0.1 pg (4/8 rxns)

Table ES-1. Qiagen Microcolumns and LLNL's packed bed were fed 100 uL of liquid suspension spiked with various masses (10^{-2} to 10^{5} pg) of B. *anthracis* DNA. Prescribed Qiagen cleanup protocols (100-uL output volume) and LLNL cleanup/amplification protocols were followed. From each of the replicate 100µL output volumes of each Qiagen column, 3 to 8 aliquots were assayed by QPCR. The LLNL system has just one QPCR assay per sample, and typically 8 replicate samples were run.

Background

Law enforcement personnel and other customers need efficient, validated methods for collecting, extracting, and concentrating or purifying biothreat agents and their signatures for subsequent analysis. Liquid samples and unprocessed extracts from solid samples often are dilute and/or contain inhibitors to cell growth and/or DNA amplification. Validated lab methods are needed for concentrating and purifying signatures from these samples in preparation for analyses. When only trace quantities of target DNA may be present, highly efficient methods that minimize losses and have low detection limits are needed. Promising novel methods must be evaluated in comparison to widely used standard methods.

Current techniques for forensic analysis of DNA can be broken into several steps: sample collection, purification and concentration of DNA from the sample, and analysis [1]. Since sample collection is typically done at the crime scene and is out of the control of the lab, we will not focus on this step. Clean-up and isolation is typically done using one of several methods: organic extraction, column extraction and Chelex extraction [6-9]. Once cleaned and isolated, analysis is typically performed by PCR. PCR is an enzyme-driven process for exponentially amplifying specific DNA sequences of interest by repetitive temperature cycling. Forensics laboratories typically use PCR to look for unique identification signatures present in DNA sequences [7, 9]. Recently, investigators have begun to look at sequences of mitochondrial DNA, which are more abundant in a given sample [11, 12].

When the quantity of DNA in a sample is limited, contamination becomes a significant issue. Contamination can come from many sources including the environment, poor techniques during collection, the forensic lab, the technician, and the sample itself. Many of these contaminants can inhibit PCR amplification and diminish the analytical success [13]. While some contamination can be controlled by good collection and lab techniques, others are inherent in the system and must be dealt with by the technician, through DNA extraction and purification of the sample. This purification is typically done by classical procedures, which include detergent-mediated lysis, proteinase treatment, extractions with organic solvents, and ethanol precipitation [14]. These types of purifications can be labor intensive, time-consuming, and most result in substantial loss of starting material [6].

Many PCR reactions require a cleanup step *a priori*, since the DNA to be amplified frequently contains contaminants that inhibit the enzymes necessary for PCR amplification. Cleanup is typically done using a two-step process, in which the sample is passed over a sorbent bed containing oxides of either silicon or aluminum, in the presence of a chemical that binds DNA to the sorbent, and then the sorbent is washed to remove the contaminants, while the DNA remains attached (U.S. patent 5,234,809 issued to Boom *et al.*). Finally, the DNA is eluted using a different chemical, and is then amplified. Three major issues are apparent in this extraction process: (1) the loss of DNA in the cleanup process that remains bound to the solid phase, (2) the cost of the process, and (3) the speed with which the process occurs. The loss of DNA in the cleanup process can approach 50% at moderate loadings, and may be more at trace loadings. LLNL's packed bed with on-sorbent amplification technology is designed to reduce the loss of DNA.

To avoid the problem of sample loss, methods of DNA purification have been proposed that utilize certain materials separating DNA by adsorption [14, 15]. Such DNA binding matrices avoid physical and biochemical degradation problems during purification. One of the most common methods utilizes chemical lysis followed by DNA purification using silica resins [14, 15]. In this method, DNA is selectively absorbed by the surface of silica in the presence of

chaotropic agents such as guanidinium thiocyanate [14, 15]. The chaotrope is a powerful agent for purification of DNA and RNA due to its ability to lyse cells and inactivate nucleases [14]. The purification of DNA by silica-based solid-phase extraction is accomplished by elution using solutions of low ionic strength or water [14, 15].

This type of purification is advantageous when combined with PCR due to the removal of inhibitors and protein. However, DNA recovery is highly variable in the hands of different researchers. One group has reported double-stranded DNA recovery in the size range of 48kb to approximately 60bp at an efficiency of 50% [14]. Carter and Milton have developed a method that is suitable for DNA recovery with an estimated efficiency of 60-80% [16]. Another group found that with their method, DNA is recovered at an efficiency of 80% [15]. Solid phase extraction is simple and kits are commercially available to achieve these results. Fragments of DNA smaller than 4kb are recovered with an elution efficiency of 70-95% by using a QIAEX II DNA Extraction Kit. (http://www1.qiagen.com) However, all of these efficiencies are based upon particular matrices with orders of magnitude more DNA than our application.

Another simple, rapid method for DNA extraction and purification utilizes immobilized silica beads. The silica beads compose a cheap, convenient DNA binding matrix. Most cells are expected to lyse when introduced to a chaotrope-containing lysis buffer. Nucleic acids are released and eventually attach to the silica beads in the presence of a chaotropic agent, such as guanidinium thiocyanate. The matrix is then washed with washing buffer, followed by ethanol. After drying, the nucleic acids are subsequently eluted with a low-salt buffer [14]. The eluted DNA is available for a variety of applications without additional precipitation or concentration steps [15]. This method yields highly purified DNA through a silica-based solid-phase material.

In the last few years, great strides have been made in amplifying limited amounts of starting material using a method known as whole genome amplification [17-19]. The technique involved a new method of multiple displacement amplification (MDA) utilizing Φ29 polymerase, known for its high strand displacement capabilities and low error rate (1 in 10^6-10^7) [20]. The technique has been shown to provide complete, non-biased amplification of the starting material, producing as much as 20-30 μg of DNA from as few as 10 cells. We have used the Φ29 polymerase, with slight modifications to the reaction mix producing as much as several μg DNA, while maintaining the quality of the initial sample. Previous work in our lab demonstrated that this technique produces enough DNA for as much as 1000 PCR reactions from a single sample [2].

We have developed a technique that couples the use of amplification with a packed silica column. The sample is loaded and bound to the column. While bound, the DNA is then washed and amplified, making use of all the available starting material. After amplification, the sample can be eluted for further analysis. Although some material may be lost during elution, this is no longer a problem because of the large amount of DNA available following amplification. In our hands, successful PCR analysis following clean-up can still be performed from as little as 1 pg of starting DNA.

Approach

The approach used in this project is to optimize LLNL's packed bed purification with in-bed amplification technology. The first-year scope was for the detection of *Bacillus anthracis* DNA in liquid suspensions, ranging from clean to real-world dirty. The in-bed amplification technology was compared to the standard method of Qiagen column purification followed by ex-situ PCR. Two modes of amplification were tested in early work: QPCR for target marker; and Whole Genome Amplification (WGA) followed by QPCR for target markers.

We report on this technique to clean and isolate successively more dilute samples, and then amplify them for successful PCR analysis. Our technique is compared with currently available methodologies such as standard Qiagen column purification prior to PCR. To do this, we tested the following conditions (schematically illustrated in Figure 1):

- bound sample is amplified by PCR and eluted for analysis
- sample is washed in Qiagen column, eluted for analysis and amplified by PCR
- sample is amplified by PCR
- investigated use of WGA to aid in low copy number applications

Note the initial proposal called for a detailed analysis of WGA in combination with the packed bed technology. Following program sponsor guidance at the January 2006 technical review emphasis was placed on the QPCR only work, deferring WGA work to out-years.

It is anticipated that our methods will be able to amplify smaller quantities of DNA in larger volumes than other current methods of purification and analysis. The nature of the column to trap passing DNA can be combined with the power of the Φ29 polymerase to clean and amplify even trace amounts of DNA that have not successfully been analyzed. The most important aspect of the DNA capture and cleanup process is to recover maximum amounts of DNA, which can then be used for analysis. There exists a limit of DNA input mass below which no DNA is recovered from the conventional cleanup process. The goal of this project is to lower the limit of input target DNA mass that can be recovered by using the packed bed for sample cleanup and amplification

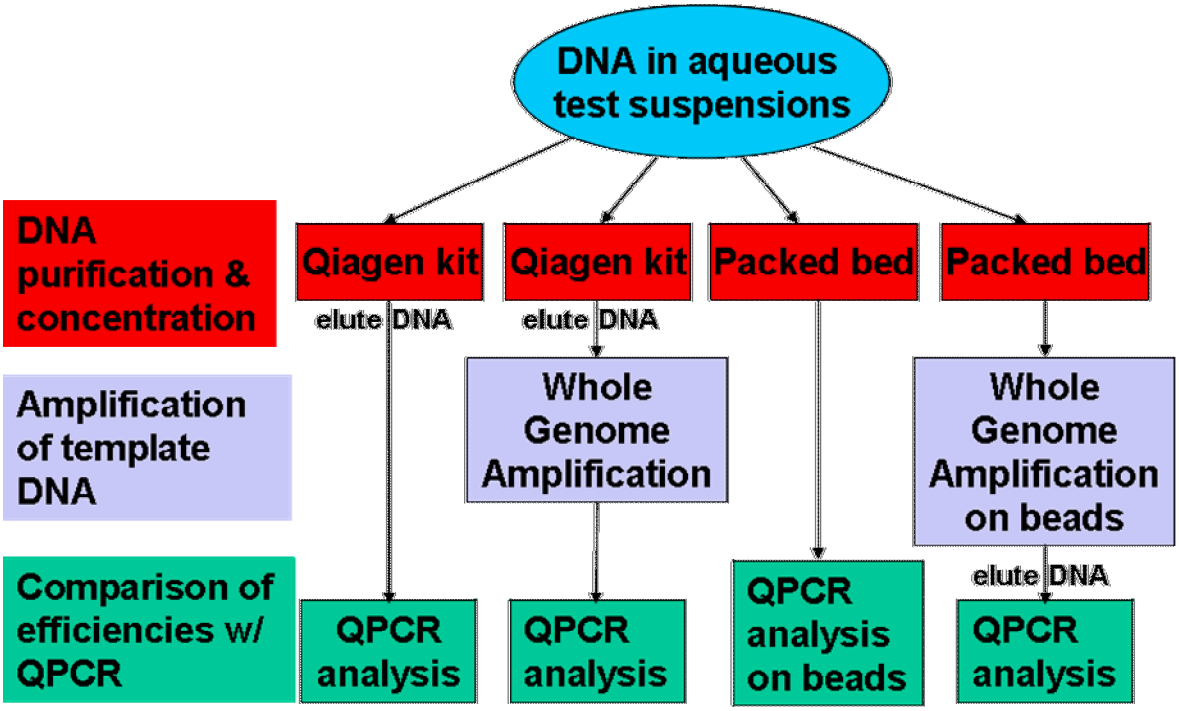


Figure 1. Graphic representation of experiments performed in which in-bed amplification technology is compared to standard Qiagen purification followed by ex-situ amplification. Whole Genome Amplification was tested on both approaches as a method for non-specifically amplifying all the DNA before doing QPCR amplification for target markers but not in an exhaustive study.

Materials and Methods

Liquid Test Suspensions

Water

Distilled water was spiked with B.*anthracis* DNA.

Water with EDTA

The aqueous suspensions of DNA in water were spiked with EDTA to a final concentration of 4mM prior to processing. 4mM was chosen because it strongly inhibited PCR (see Water with EDTA Results Section).

Dirty Carpet Extracted with Water

A 1.5-inch square sample was cut from a very dirty area of rubber-backed nylon commercial carpet that had been removed from an office building. The sample was placed in 50 ml of water and agitated. 45 ml of the resulting extract suspension was drawn off and stored at 4°C for use in these experiments. The photograph in Figure 2 shows that this solution is relatively clear (as compared to the other carpet extract and the subway aerosol suspension). Spectrophotometric characterization of this suspension is reported in the results section. Testing confirmed that the sample is PCR inhibitory (see Results Section).

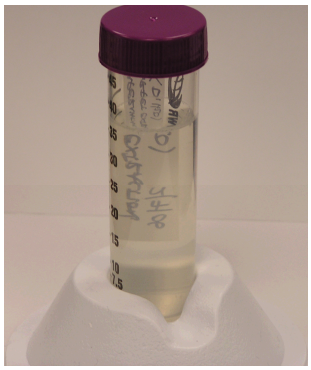


Figure 2. Water extract of dirty carpet

Dirty Carpet Extracted with PBS, Tween20, and Gelatin (PTG)

A 1.5-inch square sample was cut from a very dirty area of rubber-backed nylon commercial carpet that had been removed from an office building. The sample was placed in 50 ml of extraction buffer, and agitated. The extraction buffer was phosphate buffered saline (PBS) plus 0.1% Tween 20 plus 0.1% gelatin and abbreviated here as PTG buffer. 45 ml of the resulting extract suspension was drawn off and stored at 4°C for use in these experiments. As can be seen in Figure 3 this solution was much dirtier than the carpet extract in DI water. Spectrophotometric characterization of this suspension and the difficulties it poses for PCR are reported in the results section.

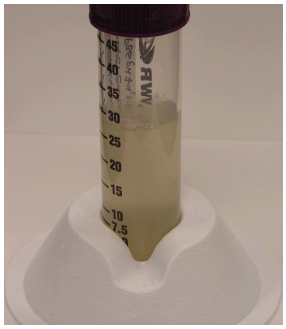


Figure 3: PBS+Tween20+gelatin (PTG) extract from dirty carpet

<u>*Subway Aerosol Suspension*</u>

An aerosol collector was operated in a subway in which aerosol particulates centrifugally impinged into water. Figure 4 shows photographs of the liquid. With time the heavy particulate sediments to the bottom of the tube. Prior to taking samples for analysis the sample was mixed to allow better sampling.

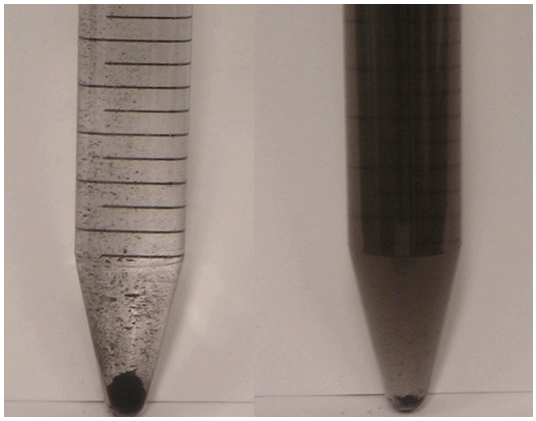


Figure 4: Photographs of liquid from aerosol collector after operation in a subway. a) Particulate sediments out of solution. b) Solution from photograph a) after vortexing.

B. anthracis DNA Spiking

B. anthracis DNA was obtained from another group on site at LLNL that specializes in growing and characterizing different agents. The *B. anthracis* DNA was extracted by them using an Epicentre kit which results in high purity DNA. The DNA was then quantified using a Nano Drop spectrophotometer. Serial dilutions of the stock DNA were tested on all the suspensions.

A 100µl aliquot of the suspension to be tested was removed after gently centrifuging the stock solutions (Figures 2-4) to ensure mixing. The 100µl test suspension was then spiked with DNA at the appropriate concentration. The spiked suspension was then processed either through a Qiagen spin column or a packed bed.

DNA Purification Methods

Standard Qiagen spin column

The experimental methods are detailed in the Appendices and summarized here. The process for sample purification using the Qiagen spin columns is shown directly from their protocols in Figure 5. The spin columns used in this project were QIAamp MinElute Columns (QIAamp DNA Micro Kit (50) Cat. no. 56304; included QIAamp MinElute Columns, buffers, collection tubes).Buffers are proprietary but include: a binding buffer that contains guanidine hydrochloride, a wash buffer and an eluting buffer. The general steps include capturing the DNA on a solid phase in the column by spinning a solution of sample with the capture buffer for 1 minute at 8,000rpm, washing matrix again with spinning, and then retrieval of the DNA by adding elution buffer and spinning at 14,000prm for 1 minute. PCR can then be performed on the eluted DNA.

Packed bed technology

The experimental methods are detailed in the Appendices and summarized here. The packed bed consists of acid washed silica beads (<106microns) held in place in a piece of tubing by a frit. The frits used for this project were stainless steel with a porosity of 10µm. (Appendix B describes alternate manifestations for performing purification, concentration, and amplification on beads if a flow through device or insertable frits are not available). For all experiments except the carpet extract with tween and gelatin we used 5mg of beads. The average size of these beads as measured by SEM was 79µm with a standard deviation of 17µm. Characterization of these beads showed that the DNA binding capacity saturates between 20 and 45ng of DNA / mg of beads.

The process for using a packed bed to purify and concentrate a sample is summarized in Figure 6. The key advance of this technology is Step 3, amplification of DNA directly on the beads.

Step 1, the dirty sample is mixed with a chaotropic salt / binding agents (guanidine isothiocyanate was used in this project) and introduced into the packed bed. DNA binds to the packed bed matrix.

Step 2, contaminants are washed away using ethanol.

Step 3, amplification mix is introduced to the beads and thermally cycled.

Step 4, amplification markers are released for detection.

Step 5, amplified DNA is eluted from the packed bed matrix.

PCR Assays

QPCR Assays

For PCR controls and elutants from Qiagen spin columns the following PCR was used:

12.5uL Plat UDG
1uL 10uM F&R
1uL 5uM Probe
8.5uL ddH_2O
2uL Template

(After the purified DNA is eluted from the spin columns in 100µl TE buffer, 2µl of this was added as sample to the PCR mix. Replicate reactions were run.)

For samples with DNA bound to silica beads extra enzyme was needed:

12.5uL Platinum UDG Mix
1uL 10uM forward and reverse primers
1uL 5uM Probe
10.2uL ddH2O
0.3uL Platinum Taq enzyme
(No template is added since the DNA is bound to the beads. The beads with bound DNA are added to the PCR mix.)

Thermal cycling was performed on a BioRad iCycler IQ Optical System. The software provided with this system was used to perform QPCR analysis (determining the critical threshold value as well as quantification of DNA in an unknown sample from a standard dilution curve). The manual provided by the instrument manufacturer was followed.

The beads from packed bed concentration and purification were amplified on the same iCycler instrument as the elutants. Real time detection of the fluorescence from the probe was performed during thermal cycle (Steps 3 and 4 of the packed bed protocol occur simultanteously when using a Taqman assay)

WGA Amplification

For controls and elutants from Qiagen columns the following mix was used for WGA:

4uL 1x Phi Polymerase Buffer (10uL 10x Phi Buffer + 88uL ddH2O + 2uL 100x BSA)
5uL 10mM dNTPs
1uL 2mM Random Hexamers
1uL 4mM DTT
0.5uL Phi Polymerase

For DNA bound to beads in the packed bed the WGA mix was modified to include more enzyme:

4uL 1x Phi Polymerase Buffer (10uL 10x Phi Buffer + 88uL ddH2O + 2uL 100x BSA)
5uL 10mM dNTPs
1uL 2mM Random Hexamers
1uL 4mM DTT
1uL Phi Polymerase

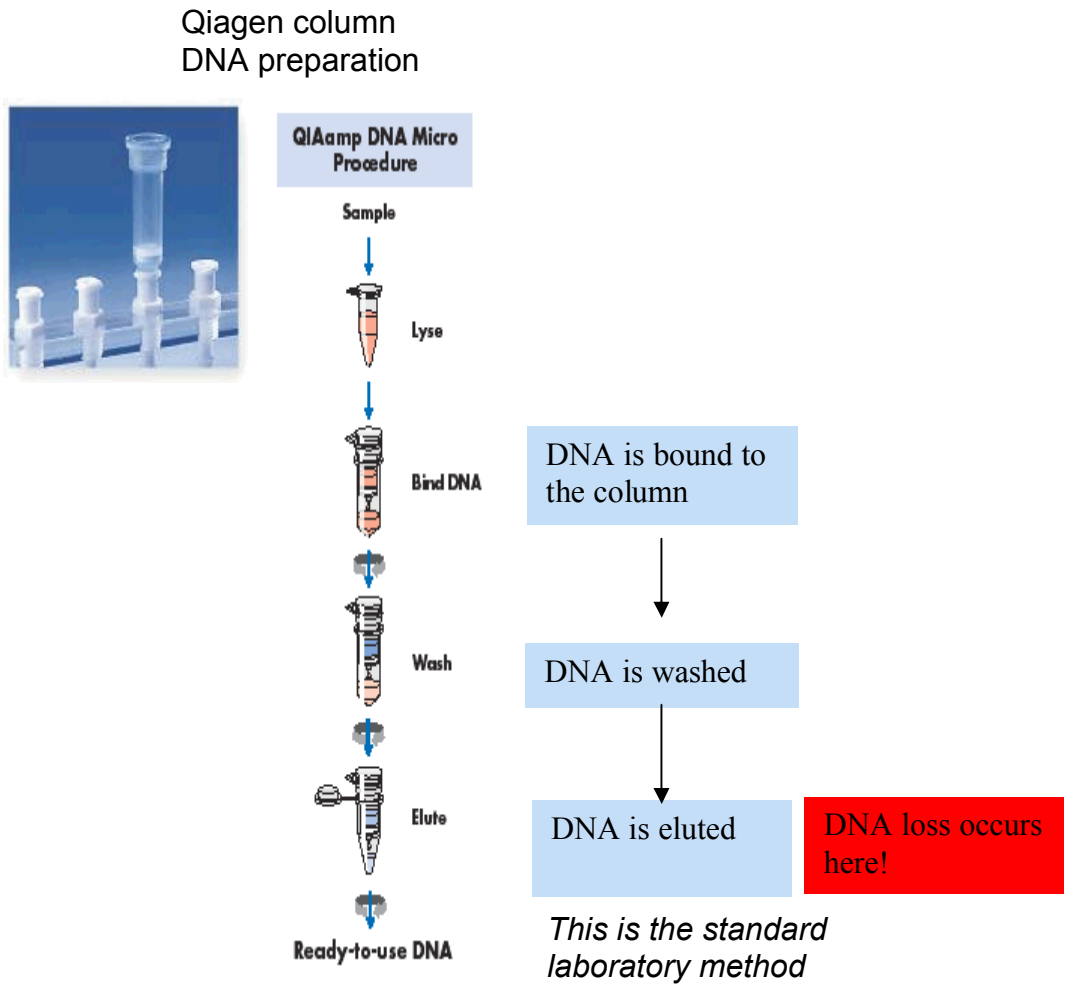


Figure 5. Standard method of packed bed purification and elution, followed by separate DNA amplification can lose 60% or more of the initial DNA due to inefficient elution. From Qiagen web page.

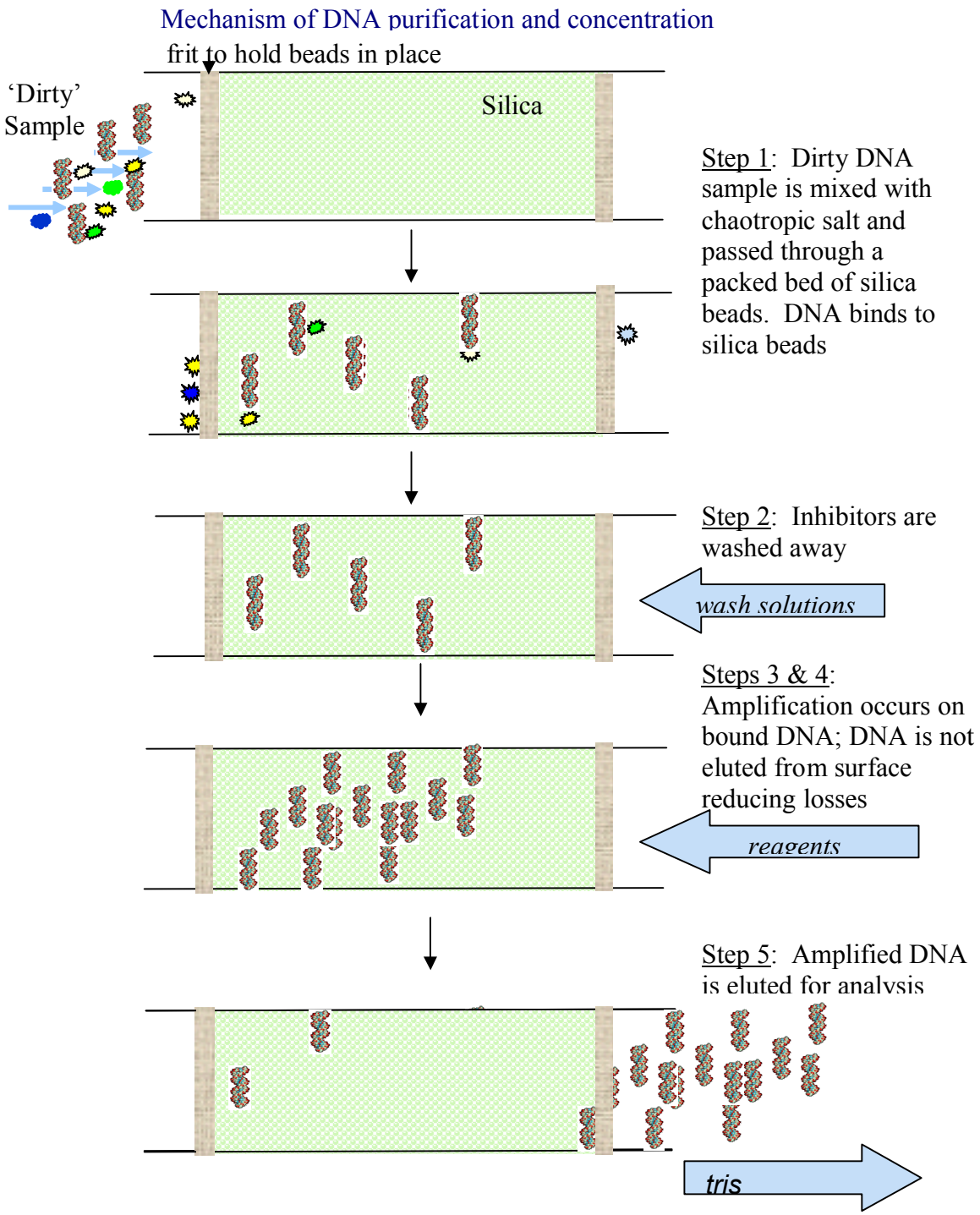


Figure 6. LLNL's packed bed purification followed by in-bed amplification will amplify DNA before elution, thereby avoiding/minimizing the elution losses.

Results

The following environmental samples were assessed to determine their effect on the lower limit of detection:

1. Water
2. Water spike with 4mM EDTA
3. Carpet extract in DI water
4. Carpet extract in 1xPBS with 0.1% tween20 and 0.1% gelatin
5. Sample from an aerosol collector operated in a subway

Both the standard Qiagen spin column and LLNL's packed bed technology were evaluated by a DNA titration series in each of the matrices listed above. After the discussion of the different environmental samples, preliminary results are shown for WGA, as well as a brief description of automation of the packed bed technology.

1. DNA in water

Analysis of Qiagen MicroColumn

Aqueous solutions of DNA were processed through Qiagen MicroColumns to establish a baseline for comparison of LLNL's new approach to sample purification and extraction. Discussions with the company as well as experimental characterization led us to the optimum protocol for this application. The protocol supplied with the columns for purification of genomic DNA was followed. Samples were eluted into 100µl of TE buffer. This volume was chosen because it yielded the highest percent recovery. After elution, a 2µl aliquot was added to 23µl of PCR master mix (details in Appendix A). For each column that quantitative PCR reactions were performed in triplicate. Ten-fold dilutions in the mass of starting DNA ranged from 100ng to 10fg (approximately one copy of DNA).

Using quantitative PCR the cycle threshold (CT) correlates with the initial of mass of DNA in the reaction prior to PCR. Therefore, a sample with a higher starting mass of DNA will reach the fluorescence threshold earlier (lower cycle number) than a lower mass of DNA. Figure 7 shows part of the titration curve for the Qiagen columns. For the higher masses of DNA the data show CT values with low standard deviations.

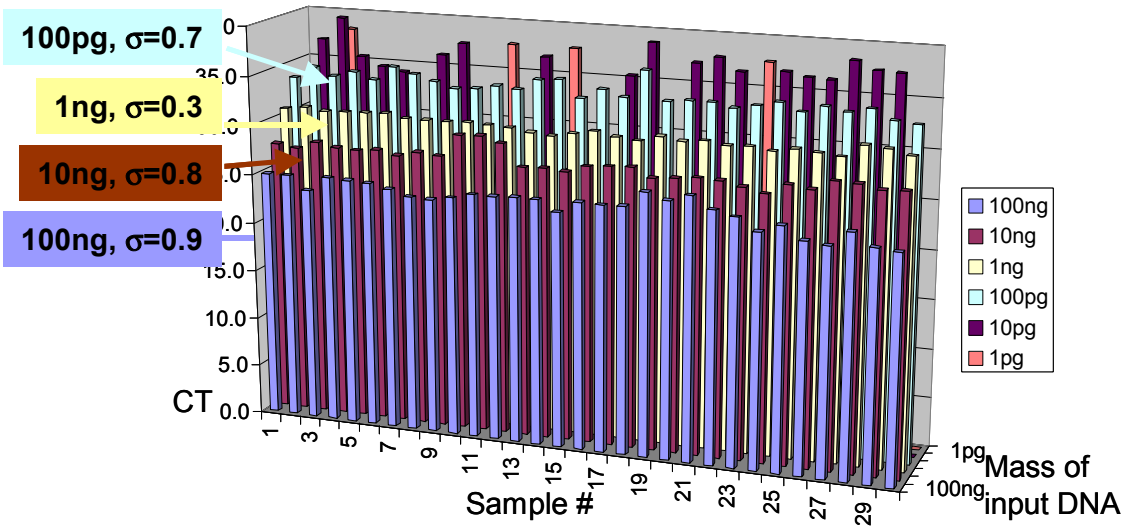


Figure 7: DNA titration curve (100ng-10fg) for Qiagen Microcolumn shows column DNA mass recovery limited when <100pg. Data from 10 columns with triplicate PCR reactions from aliquots of elutants.

Figure 8 summarizes the percent recovery of starting material for the Qiagen column. The blue diamonds represent the average of 10 columns (each with triplicate QPCR reactions from the elutant). The error bars are the standard deviations of each concentration of DNA.

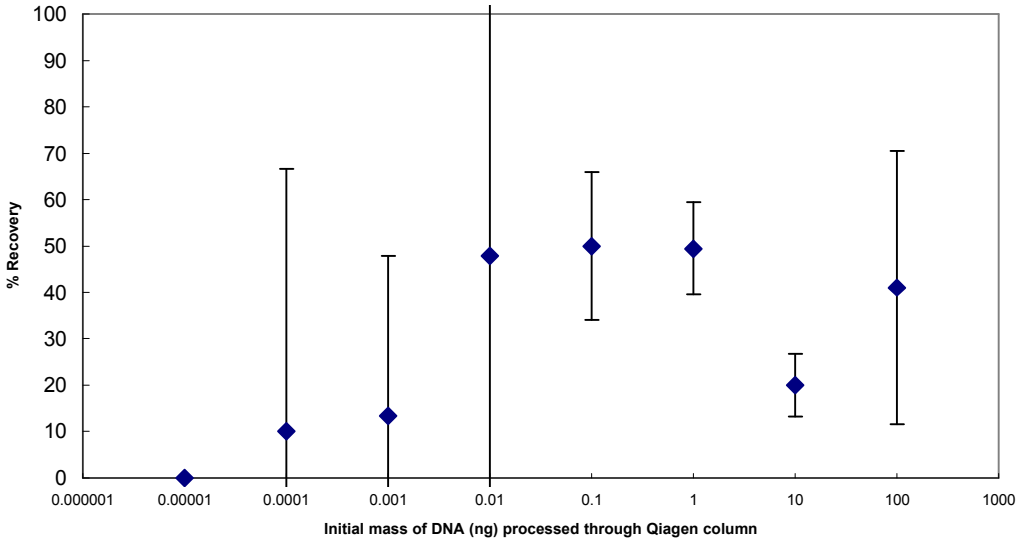
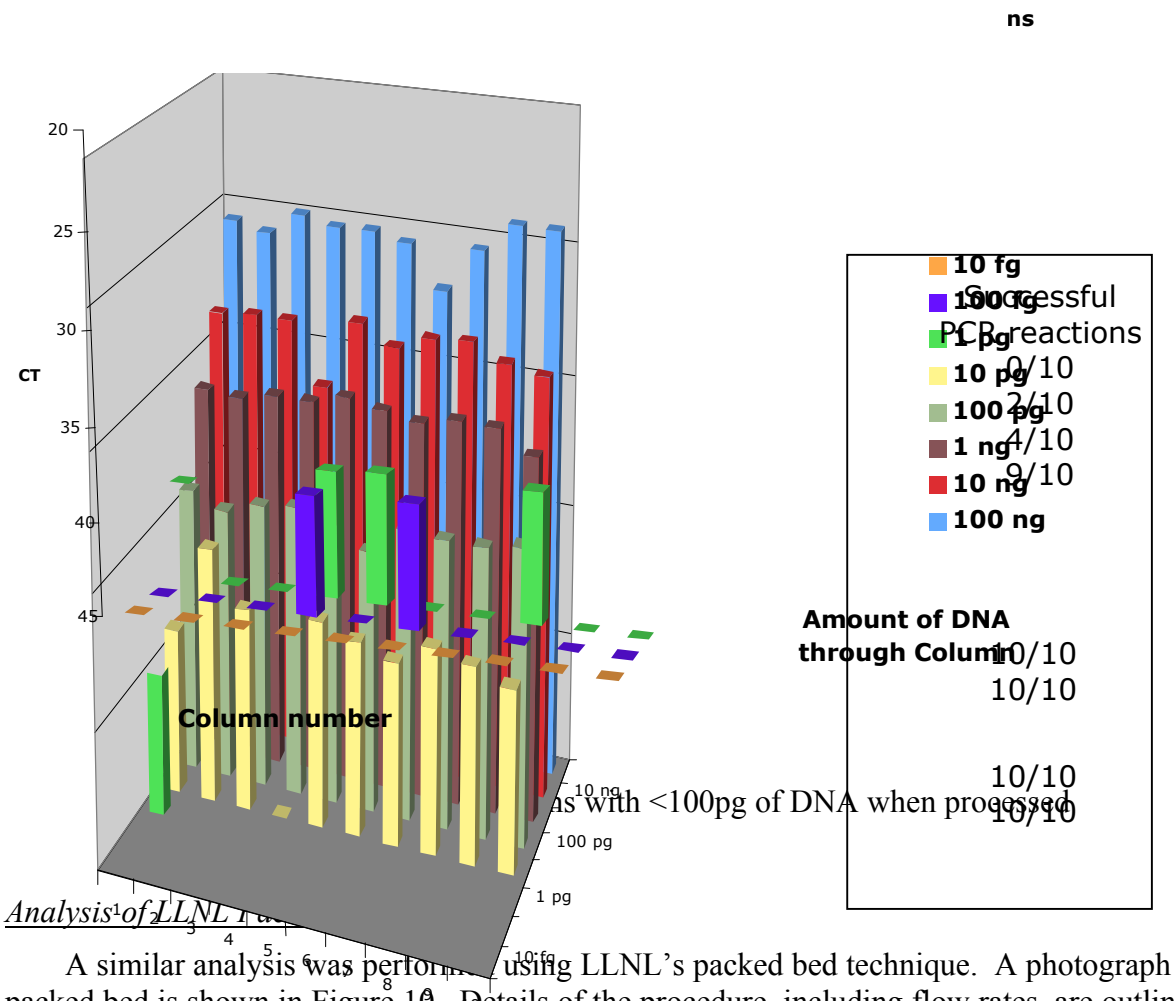


Figure 8: Percent recovery of the Qiagen column

The DNA titration curve for the Qiagen Microcolumn shows DNA mass recovery limited to <100pg, Figure 9. When the mass of DNA processed through the column is below 100 pg many of the PCR reactions for each column fail. For example, of the 10 columns processed with 100pg only 2 of the columns had all 3 of the replicate PCR reactions be positive. Of the other 8 columns only 1 or 2 of the PCR reactions were positive. Similarly, when 1pg (~1 x 10^4 copies) of DNA are processed through the columns only 4 out of 30 QPCR reactions were positive. It is at these lower amounts of DNA that the packed bed can help increase not only the limit of detection but also the probability of a successful PCR at the lower limit.



Analysis of LLNL Ra

A similar analysis was perform[illegible] using LLNL's packed bed technique. A photograph of a packed bed is shown in Figure 10. Details of the procedure, including flow rates, are outlined in Appendix C.

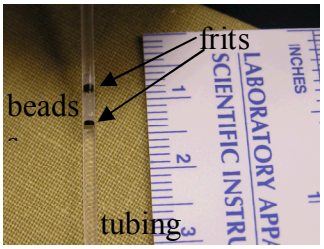


Figure 10: Photograph of packed bed in tubing used for processing samples. Tubing has an outer diameter of 1/16inch and inner diameter of 0.040".

The aqueous solutions of DNA were mixed with a chaotropic salt and flowed through a packed bed (flow rate is important for binding trace amounts). Ethanol was then flowed over the packed bed to wash inhibitors (including the chaotropic salt, which has been shown to inhibit PCR at very low concentrations) away. As outlined in Figure 6 the DNA is captured on the beads and then amplified while bound to the silica surface. Amplification on the surface minimizes losses of DNA. To eliminate any discrepancy in PCR thermal cylcers, the beads in the packed bed were then transferred into PCR tubes, PCR master mix was added, and they were thermal cycled. Additional units of polymerase and Taqman probe were utilized based on previous PCR optimization experiments. Since the DNA is bound to the surface of the beads it is not possible to perform replicate experiments from a single packed bed experiment. (Performing a whole genome amplification of the beads will yield enough DNA for subsequent reactions – preliminary WGA results are shown in later section.) The same DNA titration curve was generated for the packed bed technique, Figure 11. For each mass, 8 different packed beds were processed.

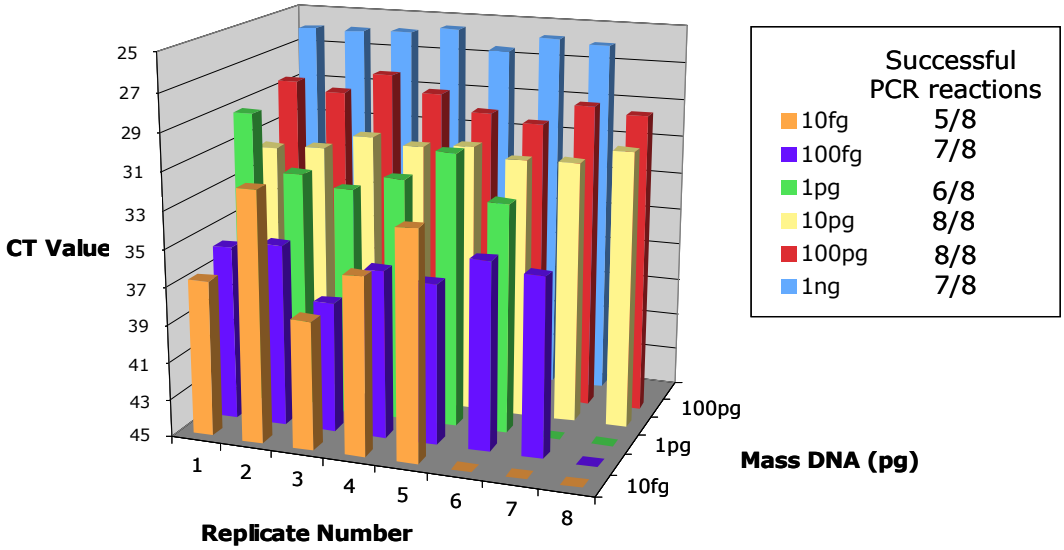


Figure 11: DNA titration curve for purifying DNA using LLNL's packed bed technique.

The packed bed technique has a limit of detection of 10pg of starting DNA in aqueous solution. This is an order of magnitude improvement over the Qiagen column.

Comparison of LLNL Packed Bed and Qiagen MicroColumn

Table 1 shows a direct comparison of the two sample purification methods for an aqueous solution containing DNA. The packed bed improves the limit of detection by an order of magnitude for DNA in aqueous solutions. It should also be noted that at masses of DNA lower than 10pg there is a higher percentage of successful PCR reaction than the corresponding Qiagen data. Also the Qiagen column had no successful PCR reactions for 10fg of DNA, whereas the packed bed had 5 out of 8 successful.

Amount DNA	Qiagen Column		Packed Bed
	Successful columns (Successful Reactions)	Success rates of triplicate rxns per column	Successful beds (one PCR rxn each)
1 ng	10 / 10 (30 / 30)	3 / 3 (10 cols)	7 / 8 (7 / 8)
100 pg	10 / 10 (30 / 30)	3 / 3 (10 cols)	8 / 8 (8 / 8)
10 pg	8 / 9 (16 / 27)	3 / 3 (1 col)	8 / 8 (8 / 8)
		2 / 3 (6 col)	
		1 / 3 (1 col)	
		0 / 3 (1 col)	
1 pg	4 / 10 (4 / 30)	3 / 3 (0 cols)	6 / 8 (6 / 8)
		2 / 3 (0 cols)	
		1 / 3 (4 cols)	
		0 / 3 (6 cols)	
100 fg	2 / 10 (3 / 30)	3 / 3 (0 cols)	7 / 8 (7 / 8)
		2 / 3 (1 cols)	
		1 / 3 (1 cols)	
		0 / 3 (0 cols)	
10 fg	0 / 10 (0 / 30)	0 / 3 (10 cols)	5 / 8 (5 / 8)

Table 1: Comparison of successful PCR reactions per column or per packed bed. Of the 9 Qiagen columns processed at 10pg of DNA only 1 column had all 3 PCR positive.

2. Water with EDTA

Determination of the Concentration of EDTA that Inhibits PCR

Guanidine isothiocyanate, the chaotropic salt used to bind the DNA to the silica surfaces, is known to inhibit PCR at very low concentrations. Since PCR amplification was successful as described in the previous section, it is expected that the packed bed technology will be able to remove other chemical inhibitors as well. To confirm this hypothesis we spiked our PCR reactions with ethylenediaminetetraacetic acid (EDTA). EDTA is a chelating agent and is commonly used in buffer solutions. At high concentrations EDTA binds the [Mg^{2+}] present in the PCR solution, resulting in inactivation of polymerase enzyme (depends on Mg^{2+} for functionality/binding).

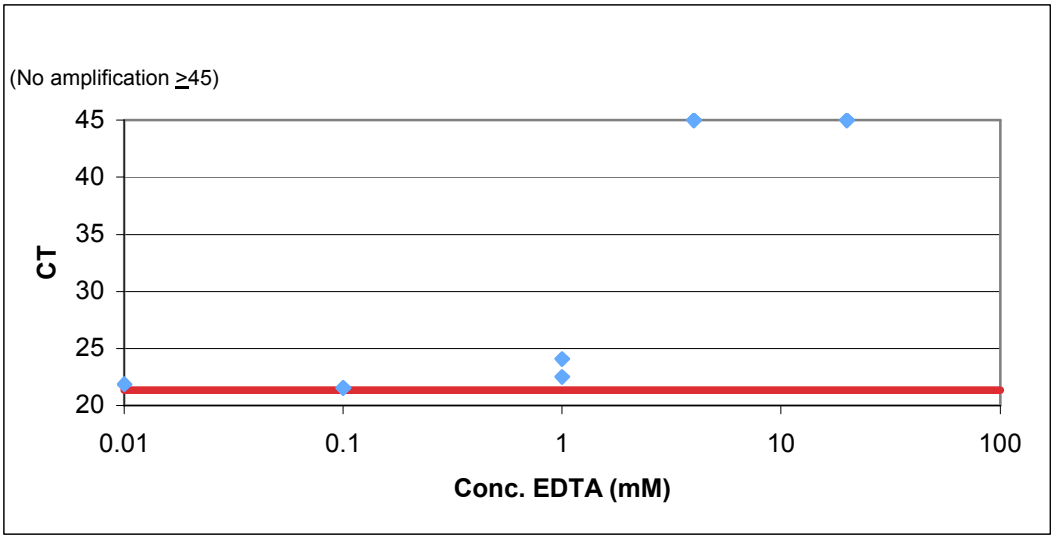


Figure 12: Demonstration that EDTA inhibits PCR at 4mM.

PCR amplification in the presence of various amounts of EDTA was performed to determine the concentration ofEDTA that would inhibit our standard PCR reaction conditions. A dilution series of EDTA was performed using a standard DNA concentration of 20ng. The red line in Figure 12 shows the standard CT value without addition of EDTA to the reaction. As the concentration of EDTA increases, the CT also increases indicating that less amplification is occurring and being detected in these reactions. Slight inhibition of the PCR occurs as indicated by the attenuation of the fluorescent signal intensity (CT shift from standard 21.4 to 24 or ~1 log value). Tests were also run at 4mM and 20mM, neither of which resulted in amplification. Since 4mM EDTA concentration completely attenuated our fluorescent signal (CT value) this concentration was utilized for the confirmation testing of the packed bed removal procedures.

Testing with EDTA Suspensions: Qiagen Spin Columns and Packed Bed Technology

Aqueous solutions of *Bacillus anthracis* DNA were then spiked with EDTA (to a final concentration of 4mM) and processed through both LLNL's packed bed technology as well as the Qiagen spin columns to demonstrate chemical clean up. The procedures for both the packed bed and the Qiagen spin columns were identical to those described used for DNA in water (detailed in Appendix C). For the spin columns 8 replicate PCRs were performed on the elutant from each spin column.

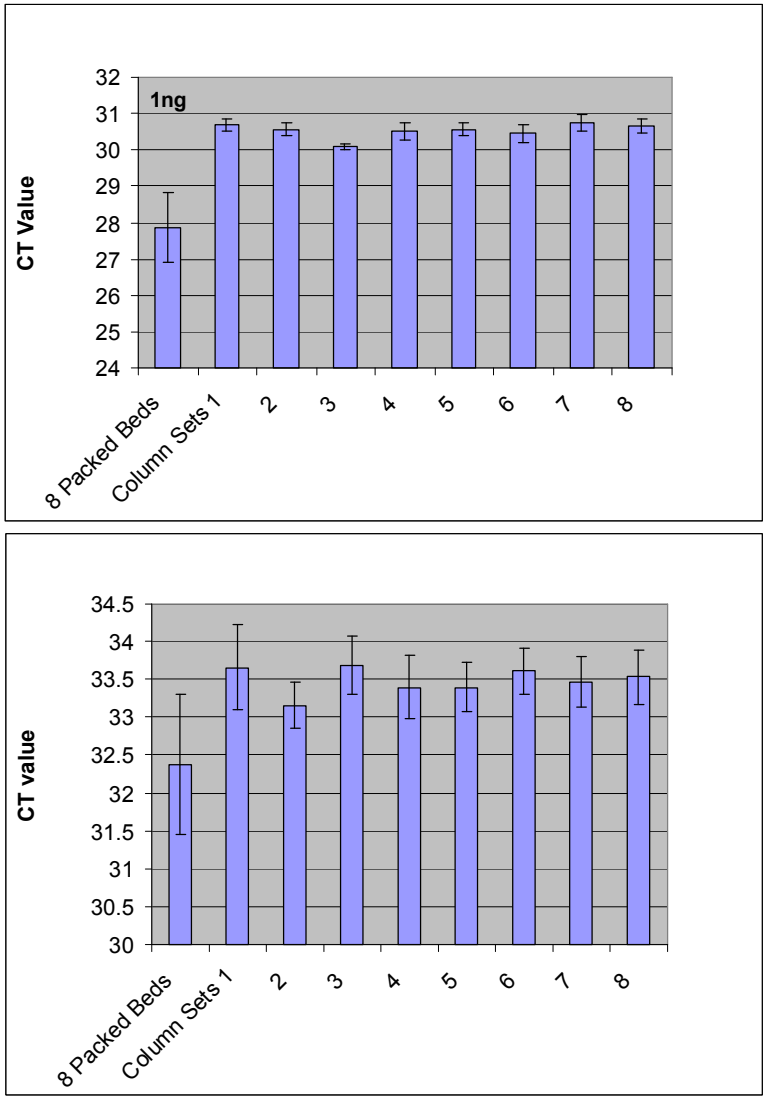


Figure 13: Both spin columns and packed beds are able to remove EDTA from aqueous suspensions of DNA. Results are shown for 1ng (top) and 100pg (bottom) initial mass of DNA.

As Figure 13 shows both the packed bed and the spin columns (for 100pg) were able to remove EDTA and allow successful amplification. All packed beds and all replicates of each spin column resulted in positive PCR amplification. Had the spin columns or packed beds not removed the EDTA no amplification would have been detected. Although only two concentrations of DNA (1ng and 100pg) are shown here it is expected that other concentrations of DNA would perform similarly.

3. Water Extract of Dirty Carpet

Dirty carpet was extracted with water as described in Materials and Methods section to produce an aqueous suspension. When 5ul of this carpet extract was added to a PCR reaction containing 1ng of DNA, a shift of 2 CTs was observed. This attenuation of fluorescent signal indicates a decrease in the efficiency of PCR amplification or inhibition of PCR amplification.

DNA titration series were performed by spiking DNA into 100ul of sample from Figure 11. These samples were then processed in both a Qiagen spin column as well as LLNL's packed bed technology. The protocols are outlined in Appendix C.

Carpet extract in DI Water: Qiagen spin columns

At the higher concentration of DNA all PCR reactions are positive, Figure 14. The Qiagen columns were able to purify the carpet extract sample with minimal losses of DNA. The low standard deviation in the fluorescent detection (CT) values across multiple Qiagen columns indicates a consistent ration of sample binding and purification. The unanimous lower limit of detection for DNA spiked into carpet extract in DI water is 100pg (30 out of 30 PCR reactions worked). DNA is detected below 100pg but with less success.

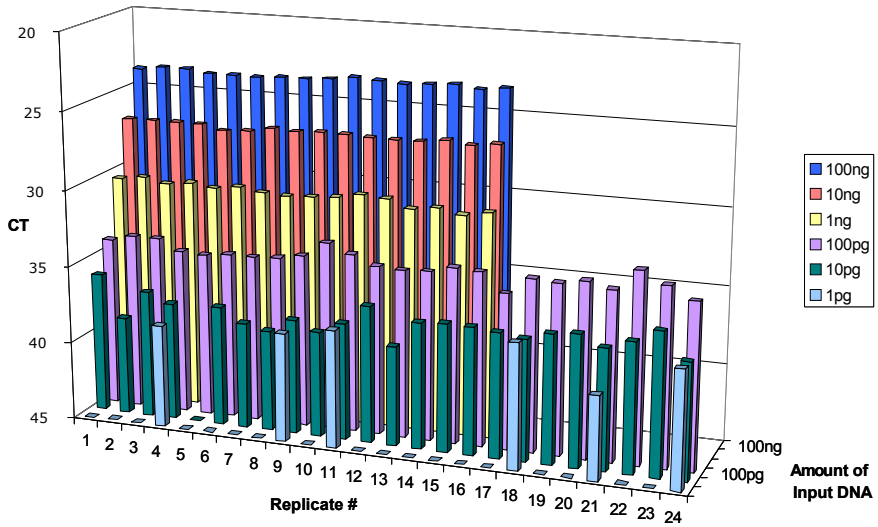


Figure 14: DNA titration curve in carpet extract in DI water for Qiagen spin column. For 1ng and greater 4 replicates per column were amplified, whereas for100pg and below 6 replicate PCR reactions were run. At 1pg only 6 of 24 reactions worked.

Focusing on the lower amounts of DNA, more failed PCR reactions are apparent. At 1pg concentration, 4 samples were processed through spin columns. Six PCR reactions were performed on the elutant from each column (total of 24 reactions). However, only 6 PCR reactions were positive.

Carpet extract in DI Water: LLNL's packed bed

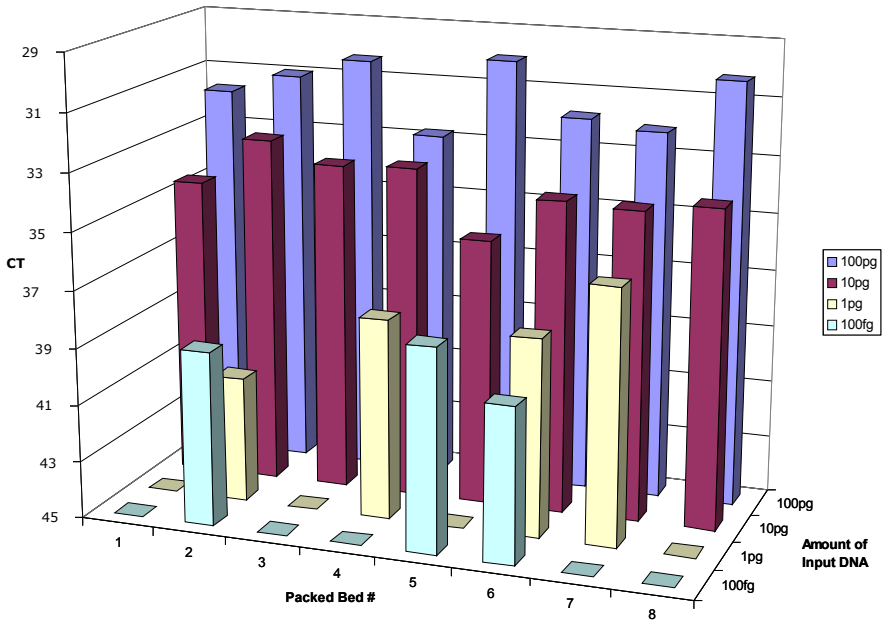


Figure 15: DNA titration series in carpet extract in DI water processed through packed bed technology.

As the data in Figure 15 show the packed bed works 100% of the time down to 10pg (8 out of 8 replicate packed beds). DNA was detected down to 100fg but with a lower success rate (3 out of 8 packed beds.

Comparison of LLNL's packed bed with Qiagen spin column

Table 2 below compares the two technologies for sample clean up. The undisputed limits of detection are 100pg for the Qiagen spin columns and 10pg for the packed bed. The limit of detection improved by an order of magnitude improvement using the packed bed technology. As the DNA concentration decreases below the undsiputed limit of detection, the packed bed shows a higher success rate than the spin columns. For example at 1pg none of the spin columns had all 6 replicate PCRs work. Two columns had 2 out of 6 PCR reactions succeed, while 2 columns had only 1 out of 6 succeed. By comparison the packed bed had 4 out of 8 reactions successful.

Amount DNA	Qiagen Column		Packed Bed
Carpet Extraction Sample in DIH_2O	Overall successful columns	Success rates of replicate rxns per column	
100 ng	4 / 4	4 / 4 (4 cols)	
10 ng	4 / 4	4 / 4 (4 cols)	
1 ng	4 / 4	4 / 4 (4 cols)	
100 pg	4 / 4	6 / 6 (4 cols)	8 / 8
10 pg	3 / 4	6 / 6 (3 cols)	8 / 8
		5 / 6 (1 col)	
1 pg	0 / 4	2 / 6 (2 cols)	4 / 8
		1 / 6 (2 cols)	
100 fg			3 / 8

Table 2: Comparison of Qiagen spin columns with LLNL's packed bed for carpet extracts in DI water.

4. PTG Extract of Dirty Carpet

Dirty carpet was extracted with the PTG extraction buffer (Phosphate buffered saline with 0.1% Tween20 and 0.1% gelatin) as described in Materials and Methods section to produce an aqueous suspension.

Initial characterization and testing

Initial packed bed experiments conducted on this sample, showed no amplification even at the higher concentration of spiked DNA. We hypothesized that the binding capacity of the beads must be reaching saturation. To investigate, we used 4 sequential packed beds for this suspension.

The following experiments were performed using the carpet extract in PTG (Table 3). For the spin columns at the higher concentrations only 4 replicate PCRs were performed. As the amount of DNA decreased we increased number of PCR replicates. Once the lower limit was detected, no lower dilutions were performed. For each packed bed sample processed the sample was flowed through 4 successive packed beds to improve the capture/binding probability.

	Qiagen Spin Columns		Packed Bed	
Amt DNA	# columns	# PCR rxns/col	# replicates	# packed beds per sample
100 ng	8	4	4	4
10 ng	8	4	4	4
1 ng	4	4	4	4
100 pg	4	6	4	4
10 pg	4	6	4	4
1 pg			4	4
100 fg			4	4
10 fg			2	4

Table 3: Number of replicates processed for carpet extract in PTG buffer with varying DNA concentrations.

Qiagen spin column analysis

Figure 16 shows the CT values for 100ul of carpet extract in 1xPBS with tween and gelatin spiked with DNA ranging from 100ng to 10pg. The standard deviation for 7 columns with 4 replicate PCR reactions spiked at 100ng (column 3 consistently had a lower CT value, indicating that perhaps this column had more DNA was excluded from this analysis) was 0.8, comparable to the standard deviation for the aqueous solutions of DNA (Figure 7).

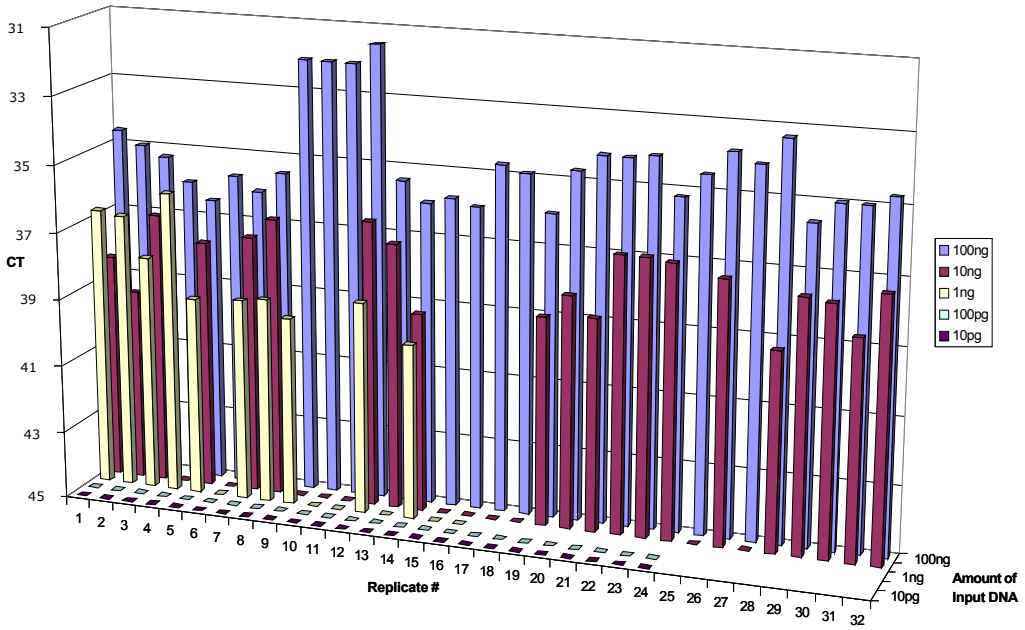


Figure 16: DNA titration curve performed in the presence of carpet extract in PBS with tween and gelatin, purified using Qiagen spin columns.

Even at 10ng of input DNA (approximately 10^8copies), the column is unable to clean up the DNA for PCR with 100% efficiency, Figure 16. With 10ng of input DNA only 2 of the eight columns had all 4 PCR replicates positive. But all eight columns had at least one of the replicates of the elutant PCR positive. At 100pg of input DNA no PCR could be detected. For consistent comparison these experiments were performed using the same protocol as for all spin column data presented so far. It has recently been suggested that there may be a different protocol that could be used with the spin columns for samples containing surfactants. No other protocol was found in the handbook supplied by the vendor for these particular columns. Perhaps a conversation with Qiagen's technical support could help improve the spin columns efficiency for this dirty environment sample. However, it should be noted that no changes were made to either protocol in terms of the solutions that were used.

Analysis in Packed Bed

Significant changes have been made to the packed bed protocol. The amount of DNA that can be captured prior to bed saturation is 100 to 255 ng of DNA using 5mg of beads. Since the extracted carpet sample has more DNA in the environmental sample (100ul of 53ng/ul) we had to either put the sample through multiple packed beds or make packed beds with larger amounts of beads to prevent saturating the bed's binding capacity. We chose to use multiple packed beds in succession to prevent any sample loss due to bed saturation by the environmental samples. Had we decided to increase the amount of beads in a single packed bed we would have needed to re-optimize the PCR assay given the increased amount of surface area for the enzyme adherence. Figure 17 shows a schematic of the revised protocol. Details are given in Appendix D.

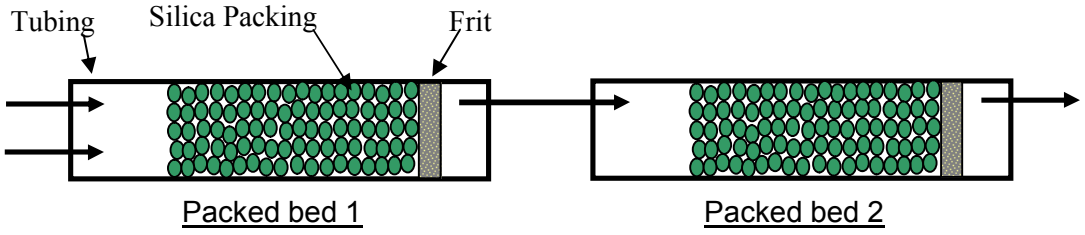


Figure 17: Schematic of procedure for samples containing large amounts of DNA or cellular debris.

DNA was mixed with the chaotrope and flowed through the first packed bed. The waste stream was collected and used as the input to the next packed bed. No additional chaotropic salt was added to the mix. This process was repeated for a total of four packed beads. Each packed bed was then washed with ethanol. The beads with DNA attached were then collected in a PCR tube, PCR mix was added and then thermal cycled. Each packed bed was amplified individually. For each target DNA concentration, experiments were performed in 4 replicates. Each sample was flowed through 4 packed beds. Figure 18 shows the resulting CT values for each packed bed. Each plot in Figure 18 represents a different DNA concentration. Each color within a plot is representative of a given sample.

For example, looking at the data for 100pg of input DNA, none of the DNA was captured in the first packed bed for any of the four samples. In the second packed bed, positive results were

seen for samples 1, 2, and 3 (blue, maroon, and yellow). In the third and fourth packed beds all samples showed positive results.

Interestingly, only 3 of the first packed beds showed positive results. It appears that the environmental contaminants may be preferentially adhering to the silica beads. Clearly for this type of sample more surface area is needed to detect trace amounts of DNA. At the spiked concentration level of 10pg each sample (4 out of 4) was detected. Had we performed this experiment with one large bed of 20mg of beads the 10pg concentration would have definitively been the unanimous lower limit of detection. Below 10pg not all samples were detected. At 10fg (~1copy, using the approximation of 10fg for the mass of a single genome) duplicate samples were tested using the four successive packed beds. For each duplicate run only 1 of the packed beds detected DNA. Since the amount of DNA input into the system is approximately one copy of DNA, you would not expect more than 1 packed bed to capture the genome and produce a positive detection.

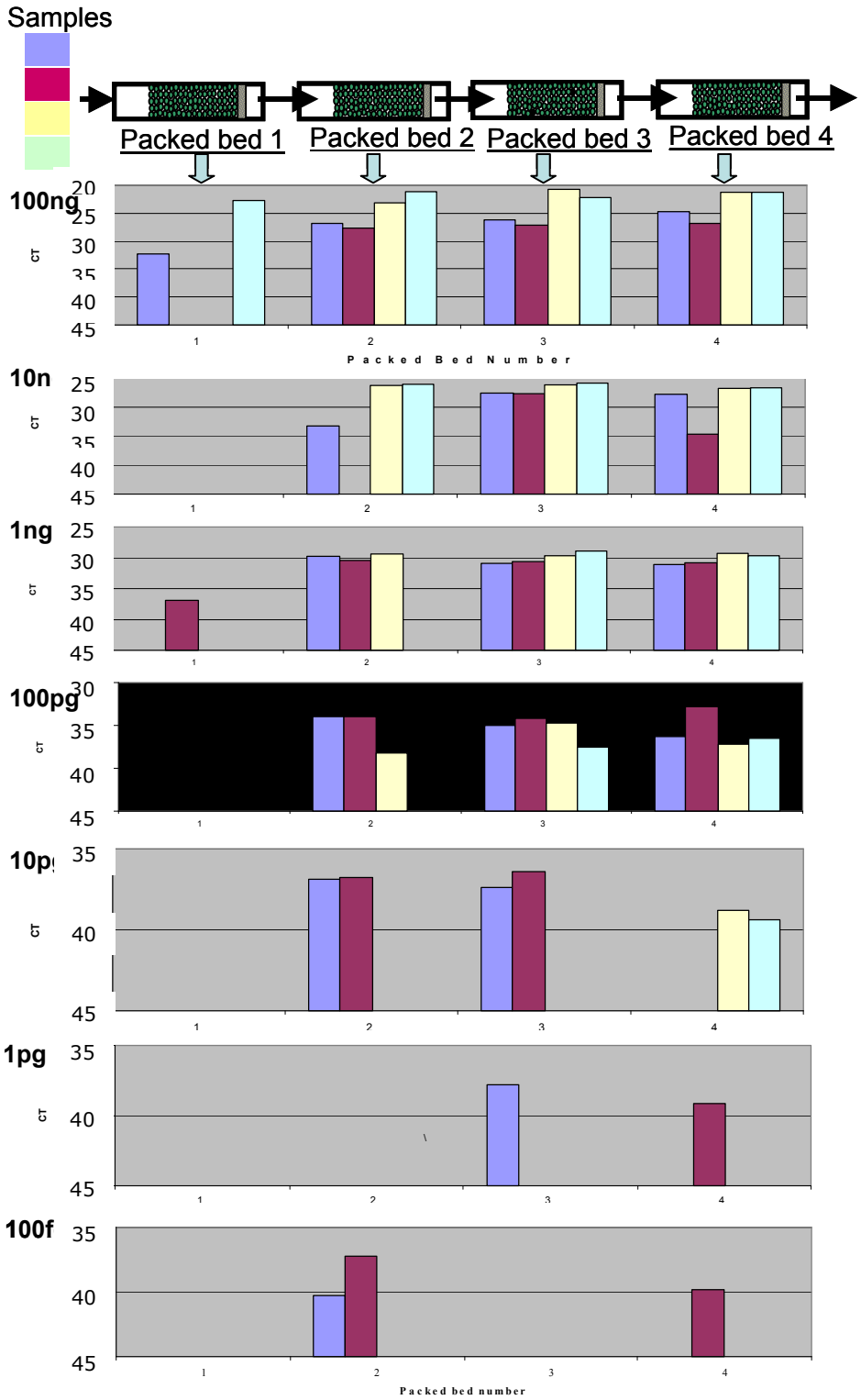


Figure 18: DNA titrations processed through 4 successive packed beds. Y-axis is fluorescent detection, therefore, no value indicates no detection. Colored bars represent a given sample in each packed bed.

Comparison of Packed bed and spin column

Table 4 compares the Qiagen spin columns and packed beds when DNA is spiked into a 1xPBS buffer with carpet extract, 0.1% tween20 and 0.1% gelatin. The unanimous limit of detection for the Qiagen kit is 100ng. For the packed bed the limit of detection is 10pg. DNA can be detected for both the Qiagen and packed beds at 1ng and 10fg, respectively.

Amount DNA	Qiagen Column		Packed Bed
Carpet Extraction Sample in 1XPBS, .1% Tween, .1% Gelatin	Overall successful columns	Success rates of replicate rxns per column	Successive PB # detected / four duplicates (need only be detected in one packed bed)
100 ng	8/8	4 / 4 (32 rxns)	4 / 4
10 ng	2 / 8	4 / 4 (2 cols)	4 / 4
		3 / 4 (2 cols)	
		2 / 4 (3 cols)	
		1 / 4 (1 col)	
1 ng	1 / 4	1 / 4 (2 cols)	4 / 4
		2 / 4 (1 col)	
		3 / 4 (1 col)	
100 pg	0 / 4	0 / 6 (4 cols)	4 / 4
10 pg	0 / 4	0 / 6 (4 cols)	4 / 4
1 pg			2 / 4
100 fg			2 / 4
10 fg			2 / 2

Table 4: Comparison of Qiagen and packed bed technologies for carpet extract in 1xPBS with tween and gelatin.

5. Results for Subway Aerosol Suspension – A Sample with Heavy Particulates

The next sample matrix investigated was a liquid aerosol sample collected from a subway. Figure 4 shows photographs of the liquid. With time the heavy particulate sediments to the bottom of the tube. Prior to taking samples for analysis the sample was mixed to allow better sampling.

As performed for all other environmental samples, the spin column was compared to the packed bed technology. Due to limited time and resources the scope of investigation into this matrix was slightly reduced. Table 5 summarizes the experiments performed in this section. Preliminary scoping of the problem indicated that the packed beds only needed to run in 8

replicates for the lower concentrations of DNA. Similarly fewer experiments were performed at the higher concentrations for the spin columns.

	Qiagen Spin Columns		Packed Bed
Amt DNA	# columns	# PCR rxns/col	# packed beds
100 ng	4	4	
10 ng	4	4	
1 ng	4	4	
100 pg	4	6	
10 pg	8	6	8
1 pg	8	6	8
100 fg	4	6	7

Table 5: Experiments performed for subway aerosol collector sample

Subway sample – Qiagen Spin Column Analysis

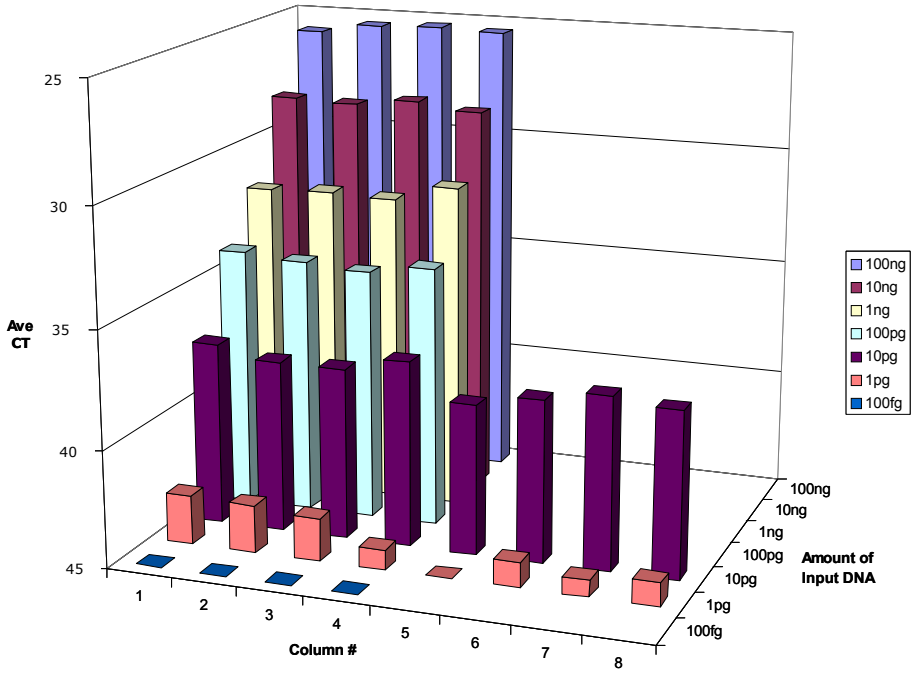


Figure 19: Average CT values of varying DNA amounts in subway aerosol collector samples cleaned with spin columns.

Figure 19 shows the titration experiments using the spin column. Again, 8 replicate PCR reactions were performed on the each elutant. The bars in Figure 19 represent an average of the successful PCR reactions. When 1pg of DNA was added to 100ul of subway aerosol collected solution only column five had absolutely no successful PCR reactions. However, from all 8

columns for 1pg only 10 reactions (out of 48) showed amplification. So although it appears in Figure 21 that 7 out of 8 columns worked, the success rate for the total number of reactions was only 10 out of 48. Note that for 100fg in 100ul of subway sample no amplification could be detected.

Subway sample – packed bed analysis

For the subway sample changes to the protocol had to be made. Due to the pore size in the frit as well as the interstitial spacing of the beads, this sample tended to clog the packed bed. We alleviated this problem by flowing the sample in smaller aliquots through the packed bed. When the packed bed became clogged we backflushed the tubing to remove the clog and then continued loading the sample, Figure 20. To perform the backflushing required that we use 2 frits, one on either end of the bed to hold it in place. Appendix E outlines the steps used for this type of sample.

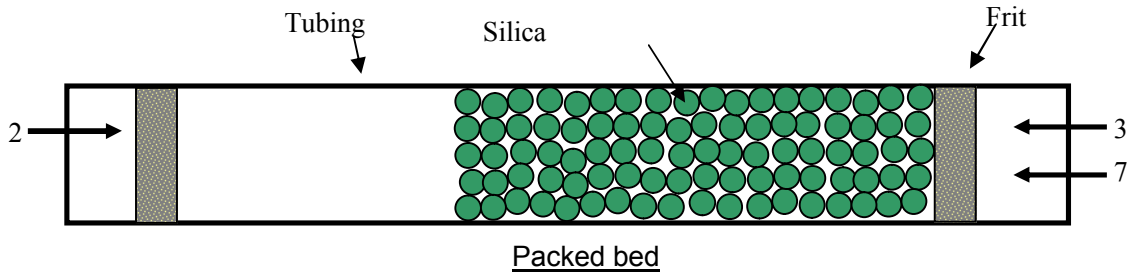


Figure 20: Schematic of modified packed bed with 2 frits to allow backflushing in the case of clogging by large particulate.

The unanimous detection limit for processing 100ul of sample from Figure 21 with DNA is greater than 10pg. However, even down at 100fg of spiked DNA four out of eight packed beds showed successful PCR, Figure 21.

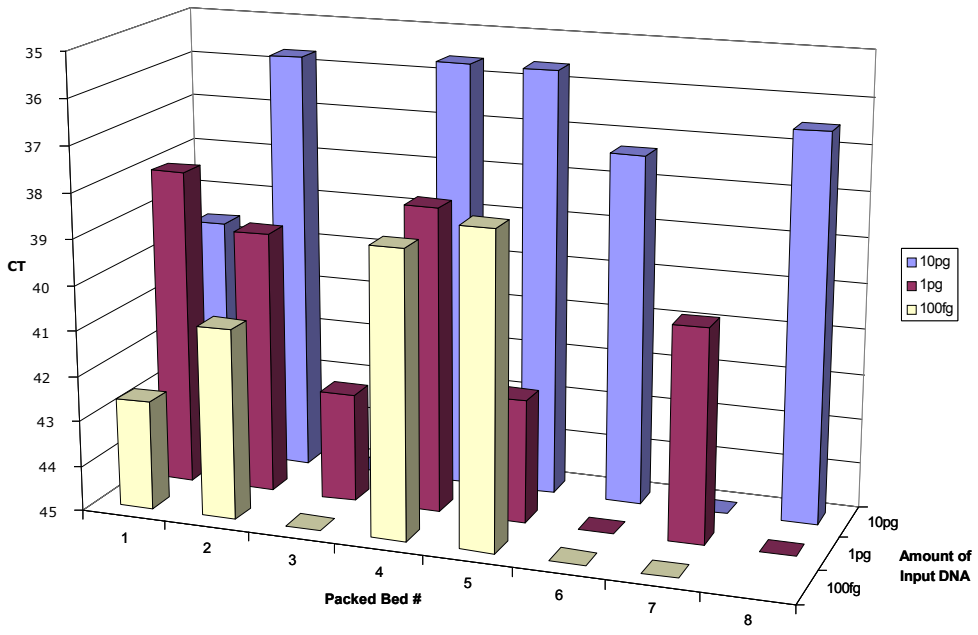


Figure 21: DNA titration curve for purifying DNA using LLNL's packed bed technology when DNA is in liquid from an aerosol collector operated in a subway.

Subway Sample – packed bed vs spin column comparison

Table 6 compares the Qiagen spin columns to the packed bed. As presented earlier, Qiagen data is split into successful columns and successful reactions. A column is deemed successful if all replicate PCRs are positive. Column 3 shows the break down of successful replicate reactions. For example, at 1pg of DNA spiked into the aerosol collected subway sample zero spin columns had all replicate PCRs positive. However, 3 columns had 2 of the 6 replicate PCRs positive. By comparison at 1pg of DNA the packed bed had 6 out of 8 successful PCR reactions. The unanimous limit of detection for this environmental matrix is 100pg. However, the packed bed had a 50% success rate at 100fg of DNA whereas the spin columns were unable to detect any DNA at 100fg.

Amount DNA	Qiagen Column		Packed Bed
Aerosol Subway Sample	Overall successful columns	Success rates of replicate rxns per column	
100 ng	4 / 4	4 / 4 (16 rxns)	
10 ng	4 / 4	4 / 4 (16 rxns)	
1 ng	4 / 4	6 / 6 (24 rxns)	
100 pg	4 / 4	6 / 6 (24 rxns)	
10 pg	7 / 8	6 / 6 (7 cols)	6 / 8
		5 / 6 (1 col)	
1 pg	0 / 8	2 / 6 (3 cols)	6 / 8
		1 / 6 (4 cols)	
		0 / 6 (1 col)	
100fg	0/4	0 / 6 (4 cols)	4 / 8

Table 6: Comparison of Qiagen spin column and packed bed for DNA spiked aerosol subway sample. For example, at 10pg 7 columns had all 6 replicates positive for PCR while 1 column only had 5 of 6 replicates positive.

Results from Whole Genome Amplification Testing

Initial amounts of DNA in an aqueous solution (ranging from 100ng to 10pg) were processed through a Qiagen column (see schematic in Figure 5). QPCR was then performed on the elutant. Using a calibration curve the quantity of DNA was determined. Figure 22a shows the resulting DNA after processing through the column.

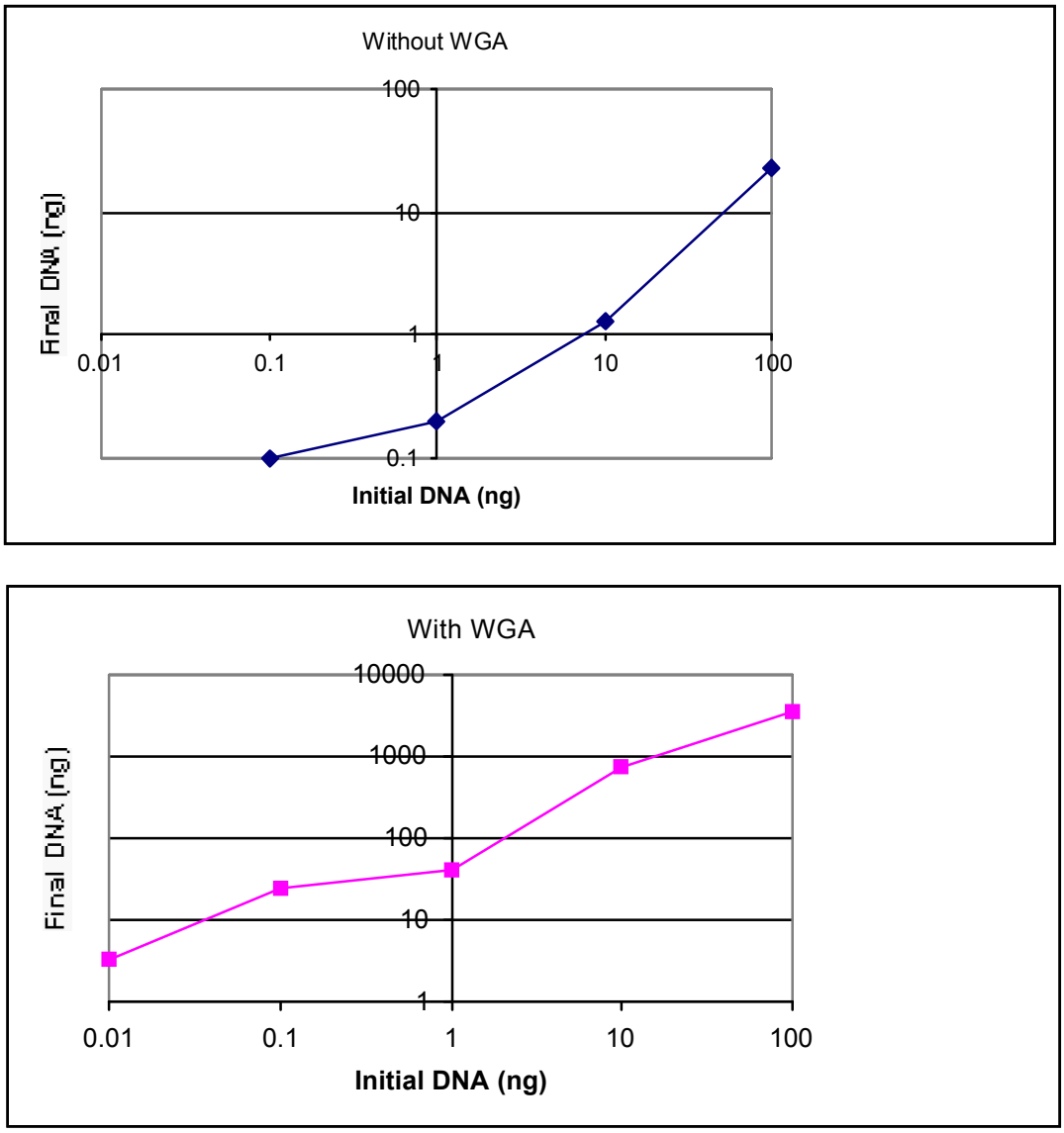


Figure 22: Varying initial amounts of DNA were processed through a Qiagen column. The resulting amount of DNA was quantified using QPCR. a) Shows a QPCR of the eluatant, note that the 0.01ng sample was not detectable with just QPCR b) Shows QPCR after first performing a WGA to increase the amount of DNA

Figure 22b shows the final amount of DNA after a whole genome amplification. Whole genome amplification was performed using the Phi 29 polymerase with random hexamers and incubating the sample at 30°C for 16 hours. Substantial increases in the amount of DNA result.

Future Research

Areas of research that warrant further investigation include:

- More systematic study of WGA in packed bed
- Incorporating the packed bed into Qiagen 8000 automated platform

Based on surveying Qiagen's website it should be relatively straight forward to incorporate the key aspects of the packed bed technology into this system

- All results presented here were performed in singleplex reactions. Similar studies for multiplexing should be performed.
- Extend to other matrices and agents such as: wider range of environmental sample suspensions, R.*communis* DNA from suspensions of castor leaves, beans, or ricin.
- More formal, multi-site validation testing

Summary and Conclusions

In summary, when tested with trace quantities of B.*anthracis* DNA in water, LLNL's packed bed technology outperformed Qiagen Microcolumns by one or more orders of magnitude in the mass of DNA that can be detected. The limit of unanimous detection (all PCR reactions positive) was reduced from 100 pg to 10 pg; the limit of majority detection (majority of PCR reactions positive) was reduced from 10 pg to 0.01 pg; and the ragged edge of detection (some PCR reactions positive) was reduced from 100 fg to below 10 fg.

Significant losses in the amount of DNA available for analysis frequently occur during purification and concentration. LLNL's technology for doing both packed bed purification of DNA and amplification of the DNA within the bed is a promising approach for reducing the limit of detection and recovering trace DNA in higher yields.

A comparison between Qiagen Microcolumns and the packed bed has been performed for five environmental samples. The limit of detection for DNA in aqueous solution where all PCR reactions are positive is 100pg for the microcolumns. Using a packed bed improves this an order of magnitude. Below these limits of detection successful PCR reactions occur but with decreasing probability. For example the packed bed detected 5 / 8 replicates of 10fg of input DNA. Whereas, the microcolumn had zero positive hits at this amount of DNA. These purification techniques are now being evaluated with "dirty" samples.

Table 7 summarizes the unanimous (or assured) limit of detection as well as the lower limit (or "ragged edge") of detection. For the unanimous detection there is an order of magnitude improvement when using the packed bed for aqueous solutions and carpet extract in DI water. The unanimous limit of detection is the same for the subway aerosol sample. There is a 4 order of magnitude improvement when using the packed bed for the carpet extract in 1xPBS with tween and gelatin. 100fg of DNA (approximately 100 copies) was detected (with less than 100% success rate) for all environmental samples tested when analyzed in the packed bed.

DNA titrations performed in the following liquids	**Limit of assured detection (all PCR rxns successful)**		**Lower limit of detection (X successful / Y rxns)**	
	Qiagen column	**Packed bed**	**Qiagen column**	**Packed bed**
Water	**100pg**	**10 pg**	**0.1 pg (6/30 rxns)**	**0.01 pg (5/8 rxns)**
Water + EDTA	**Limited testing gave qualitatively similar results**			

Dirty carpet extracted w/ water	**100 pg**	**10 pg**	**1 pg (6/24 rxns)**	**0.1 pg (3/8 rxns)**
Dirty carpet extracted w/ PBS + Tween20 + gelatin	**100,000 pg**	**10 pg**	**1,000 pg (7/16 rxns)**	**0.1 pg (3/16 rxns)**
Subway aerosol collected into water	**100 pg**	**100 pg**	**1 pg (10/48 rxns)**	**0.1 pg (4/8 rxns)**

Table 7. Qiagen Microcolumns and LLNL's packed bed were fed 100 uL of liquid suspension spiked with various masses (10^{-2} to 10^{5} pg) of B. *anthracis* DNA. Prescribed Qiagen cleanup protocols (100-uL output volume) and LLNL cleanup/amplification protocols were followed. From each of the replicate 100-uL output volumes of each Qiagen column, 3 to 8 aliquots were assayed by QPCR. The LLNL system has just one QPCR assay per sample, and typically 8 replicate samples were run.

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Appendix A – PCR and WGA recipes and thermal protocolss

PCR

For controls and elutants from spin columns

12.5uL Plat UDG
1uL 10uM F&R
1uL 5uM Probe
8.5uL ddH_2O
2uL Template

For samples with DNA bound to silica beads (extra enzyme was added)

12.5uL Plat UDG
1uL 10uM F&R
1uL 5uM Probe
10.2uL ddH2O
0.3uL Pt Taq (3X)

Thermal Protocol
Performed on a BioRad IQ

Step 1:	50.0ºC for 02:00
Step 2:	94.0ºC for 02:00
Step 3:	94.0ºC for 00:30
Step 4:	55.0ºC for 00:30
Step 5:	68.0ºC for 00:15
Step 6:	go to step 3 for 45 cycles
Step 7:	4.0ºC HOLD

WGA

<u>Recipe for controls and elutants from Qiagen spin columns</u>

4uL 1x Phi Polymerase Buffer (10uL 10x Phi Buffer + 88uL ddH2O + 2uL 100x BSA) (NEB)
5uL 10mM dNTPs (Roche)
1uL 2mM Random Hexamers (Genosys: 5'-nnnnnn-3' with n= A,T,C,or G)
1uL 4mM DTT (NEB)
0.5uL Phi Polymerase (New England Biolabs = NEB)

<u>Recipe for DNA bound to beads (packed bed)</u>

4uL 1x Phi Polymerase Buffer (10uL 10x Phi Buffer + 88uL ddH2O + 2uL 100x BSA) (NEB)

5uL 10mM dNTPs (Roche)
1uL 2mM Random Hexamers (Genosys: 5'-nnnnnn-3' with n= A,T,C,or G)
1uL 4mM DTT (NEB)
1uL Phi Polymerase (New England Biolabs = NEB)

Thermal Profile:

95C - 1 min.
30C - 16 hrs.
65C - 10 min.
4C - forever

Appendix B – Packed bed alternate manifestations

Material for packed beds used in this project:

1/16″ OD FEP tubing with 0.040″ ID
2µm stainless steel frits
5mg of <106µm diameter acid washed glass beads from Sigma

Other bench top equipment used

Syringe pump to flow at 1µl/sec
Bio-Rad thermal cycler

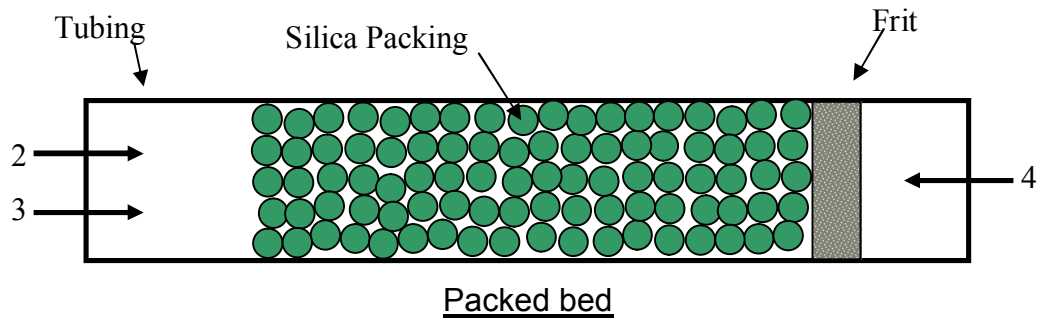
The frits used in this project were custom made to fit inside the tubing. If custom frits are not available there are a couple of alternatives:

1. Standard bench top equipment
   DNA can be captured on the beads in a PCR tube. The desired amount of beads is weighed out into the tube. The sample of interest is then mixed with guanidine isothiocyanate and added to the tube. The sample/GuSCN/beads are vortexed and allowed to incubate at room temperature. The beads are then washed with Ethanol by adding to the tube and then pipetting off the supernatant (multiple times). PCR mix can then be added to the washed beads and thermal cycled. This technique has been performed in our lab and works. However, we have found the flow through packed bed to be more efficient.

2. Flow through configuration
   If insertable frits are not available Frit-in-a-Ferrule™ Filters from Upchurch Scientific (Cat # P-272) or similar external frits can be connected to the end of the tubing used for the packed bed. Protocols can then be modified based on those given in later Appendices. It should be noted that this modified flow through packed bed configuration has not been implemented by this group but should be feasible.

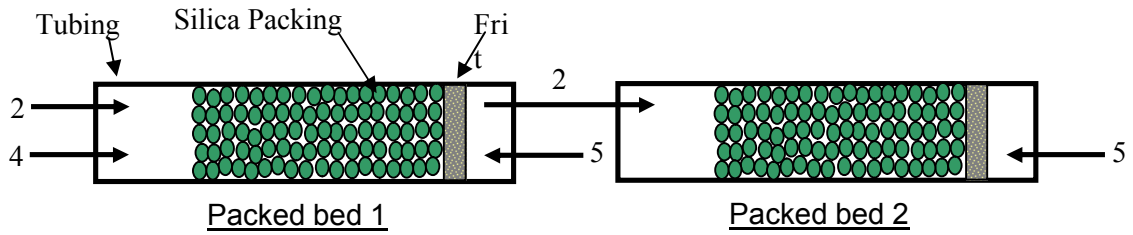
Appendix C – Standard Operating Procedure for Aqueous solution
Procedure for using packed bed technology (details are given for flow through configuration; procedure can easily be translated for use with standard bench top equipment, if no syringe pump is available)

The schematic below shows the flow direction of steps.



1. Sample is mixed with equal volume of 6M guanidine isothiocyanate (GuSCN)
2. Sample and GuSCN mix are flowed through packed bed at 1ul/s. The residence time in the bed is important. Note no optimization of the flow rate was performed in this project. It may be possible to process the sample faster, but the loss in capture efficiency has not been investigated. If performing on the benchtop (not flow through) then the appropriate incubation time will need to be determined.
3. Flush 1.5mL of 70% ethanol over the beads. Flow rate is not important in this step.
4. 100ul is back flushed through the system. Beads and ethanol slurry are collected in the Eppendorf tube that will be used for PCR amplification.
5. The ethanol supernatant is removed, and the beads are allowed to dry. Note it is important not to overdry the beads, since the DNA becomes more difficult to amplify from the surface.
6. PCR (or WGA) mix is then added to the beads from step 5.
7. The tube with beads and PCR mix is then thermally cycled on a standard PCR thermal cycler.

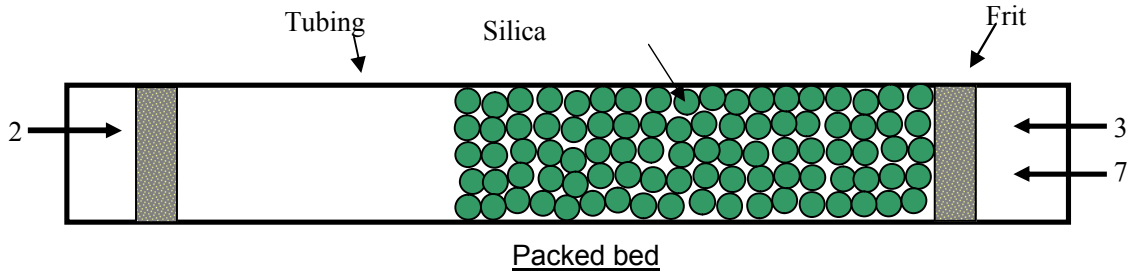
Appendix D – Standard Operating Procedure for Large amount of cellular debris



1. Sample is mixed with equal volume of 6M guanidine isothiocyanate (GuSCN)
2. Sample and GuSCN mix are flowed through packed bed at 1ul/s. The residence time in the bed is important. Note no optimization of the flow rate was performed in this project. It may be possible to process the sample faster, but the loss in capture efficiency has not been investigated.
3. Since the surface area available is potentially saturated, multiple packed beds need to be used. The GuSCN waste stream coming out of the first packed bed becomes the input stream for packed bed 2. No additional GuSCN need be added to the output from the first packed bed. This feeding into a subsequent packed bed continued for 4 packed beds for the samples described in this project. These packed beds can either been in one continuous length of tubing or individual lengths of tubing.
4. Each packed bed is then washed with 2.0mL of 70% ethanol flows over the beads. Flow rate is not important in this step.
5. 100ul is back flushed through the system. Beads and ethanol slurry are collected in the Eppendorf tube that will be used for PCR amplification.
6. The ethanol supernatant is removed, and the beads are allowed to dry. Note it is important not to overdry the beads, since the DNA becomes more difficult to amplify from the surface.
7. PCR (or WGA) mix is then added to the beads from step 5.
8. The tube with beads and PCR mix is then thermally cycled on a standard PCR thermal cycler.

Appendix E – Standard Operating Procedure for Large amount of particulate
Procedure for using packed bed technology (details are given for flow through configuration). Note in this SOP the packed bed now contains 2 frits with the beads loosely packed. During flow the beads will pack against the frit in the flow direction. Both frits are necessary to facilitate backflushing the system.

The schematic below shows the flow direction of steps.



1. Sample is mixed with equal volume of 6M guanidine isothiocyanate (GuSCN)
2. Sample and GuSCN mix are flowed through packed bed at 1ul/s. The residence time in the bed is important. Note no optimization of the flow rate was performed in this project. It may be possible to process the sample faster, but the loss in capture efficiency has not been investigated.
3. When the frit clogs with particulate then 70% ethanol is flowed from the downstream direction. This unclogs the frit.
4. Steps 2 and 3 are iterated until all sample has been flowed through the packed bed.
5. In total, 2.0mL of 70% ethanol flows over the beads.
6. Immediately after flowing the ethanol, with the beads all packed near the downstream frit, the tubing is cut between the beads and upstream frit.
7. 100ul is back flushed through the system. Beads and ethanol slurry are collected in the Eppendorf tube that will be used for PCR amplification.
8. The ethanol supernatant is removed, and the beads are allowed to dry. Note it is important not to overdry the beads, since the DNA becomes more difficult to amplify from the surface.
9. PCR (or WGA) mix is then added to the beads from step 5.
10. The tube with beads and PCR mix is then thermally cycled on a standard PCR thermal cycler.

Appendix F – Decontamination Routine for packed bed

1. Fill 1mL syringe with 1mL fresh 20% Bleach
2. Flush 20% Bleach through tubing and leave excess bleach in tubing
3. Heat tubing with 20% Bleach at 95°C for 60 seconds
4. Rinse tubing with 3mL dH_2O
5. Flush 2mL 70% EtOH through tubing
6. Dry tubing with air prior to loading beads
7. Load 5mg beads with 70% EtOH
8. Dry bed