Recovery of Organisms and Nucleic Acids from Complex Samples

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Proper sample preparation, a fundamental step in identifying and responding to potential bioterrorist attacks, is required to isolate biological or chemical targets from the extraneous material in which they may be contained, particularly if the targets are present in very low concentrations. Procedures that are straightforward in the laboratory can pose significant challenges when performed in the field, but they can be facilitated by well-designed tools that are easy to use under stressful conditions. Our goal has been to develop fast, easy techniques for sample preparation prior to analysis for identification. Because our clients consist primarily of soldiers, field inspectors, and first responders, we have focused on protocols and devices that require little or no power, are lightweight and fieldable, and can be carried out by personnel with little or no technical background.

» **The process of recognizing, identifying,** and responding to a biological or chemical attack requires multiple stages of sample and information collection and processing. There are several methods for identifying target organisms and nucleic acids in a sample. These methods can be broadly divided into categories, the most commonly used of which are the polymerase chain reaction (PCR), immunologic assays (immunoassays), and mass-spectrometry-based methods. In addition, Lincoln Laboratory has developed a highly sensitive, rapid cell-based immunoassay called CANARY, which is capable of detecting and identifying a wide variety of bacterial and viral agents. (See the article "Rapid Sensors for Biological-Agent Identification," on page 63.)

Currently, PCR is one of the preferred choices for the confirmatory identification of bacterial, viral, and nucleic-acid targets contained in forensic, clinical, and food samples. Many kits and instruments are available for PCR-based identification of biological agents, including quantitative real-time PCR machines and reagents. The advent of a number of field-portable rapid PCR units in the last several years has reinforced the choice of PCR as the preferred confirmatory analysis technique. What has been lacking, however, is a complementary fast and easy sample cleanup procedure to precede the PCR step. Clean DNA or RNA is crucial to the ability to harness the specificity and sensitivity of PCR, which is very susceptible to the presence of reaction inhibitors that degrade the amplification reaction [1]. These reaction inhibitors are ubiquitous in environmental and clinical materials. A wide range of effective sample-purification kits (e.g., kits made by Qiagen, Ambion, Pierce, and Invitrogen) are available to isolate and purify nucleic acids from a range

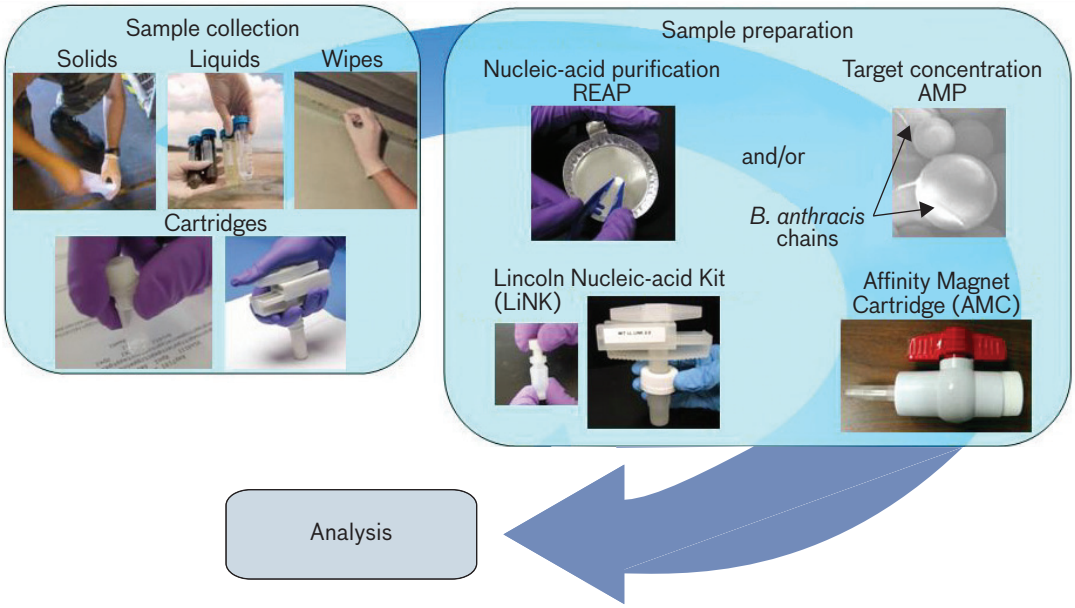


FIGURE 1. Two processing steps prior to analysis are addressed by our work. On the left are various sample-collection tools, including the Lincoln Nucleic-acid Kit (LiNK) version 1.0 and 2.0. On the right are the purification tools, which include LiNK family of cartridges, the Affinity Magnet Protocol (AMP) and the Recovery, Extraction, and Archiving Protocol (REAP).

of materials, including clinical matrices such as blood and urine, foods, animal and plant tissue, and environmental matrices such as soils and water. Generally, these kits are optimized for the matrix, and can be used only in a laboratory setting because they require centrifugation and/or solvent-based reagents, and entail several steps that are generally best performed by technically experienced personnel.

Many organizations, both academic and commercial, have expended a significant amount of effort over the last ten years or more to design smaller, faster, more efficient PCR machines, some of which have been successfully exercised in remote field settings. However, there has been much less emphasis on the development of sample-preparation procedures that can take a raw sample (a lump of soil, a piece of plant matter, a jar of liquid), extract nucleic acids contained within the sample, and present them in a form acceptable to these PCR machines. Figure 1 shows the sample processing procedure broken into several stages: sample collection; sample preparation, consisting of target concentration, extraction (typically of DNA), and purification; and target amplification and identification in an appropriate analysis machine. We have chosen to address the first two stages, for which we have developed methods that are compatible with most current nucleic-acid amplification and analysis tools and equipment. We have developed two sets of protocols—the Affinity Magnet Protocol (AMP) for target concentration and purification, and the Recovery, Extraction, and Archiving Protocol (REAP) for DNA purification. We have implemented these protocols into cartridge formats designed for collection, storage, and processing of samples that non-technical personnel can operate in a field setting. The Affinity Magnet Cartridge (AMC) incorporates the magnetic-bead-based AMP into a fieldable unit; several versions of this cartridge have been developed and tested, and are described in this article. The Lincoln Nucleic-acid Kit (LiNK), designed for collection, storage, and processing of field samples, contains the basic elements of the REAP. This article describes two generations of this device.

Over the past nine years, we have developed and exercised our protocols to extract bacterial and human DNA from a variety of materials and targets, ranging from simpler materials such as water and bacterial growth media to more complex matrices such as soils and clinical and environmental samples. We are also working on extending our protocols to extract RNA, as it is of interest in the detection of viral bio-agents, and proteins such as biological toxins. RNA is more difficult to extract than its counterpart DNA because of its fragility and the ubiquitous

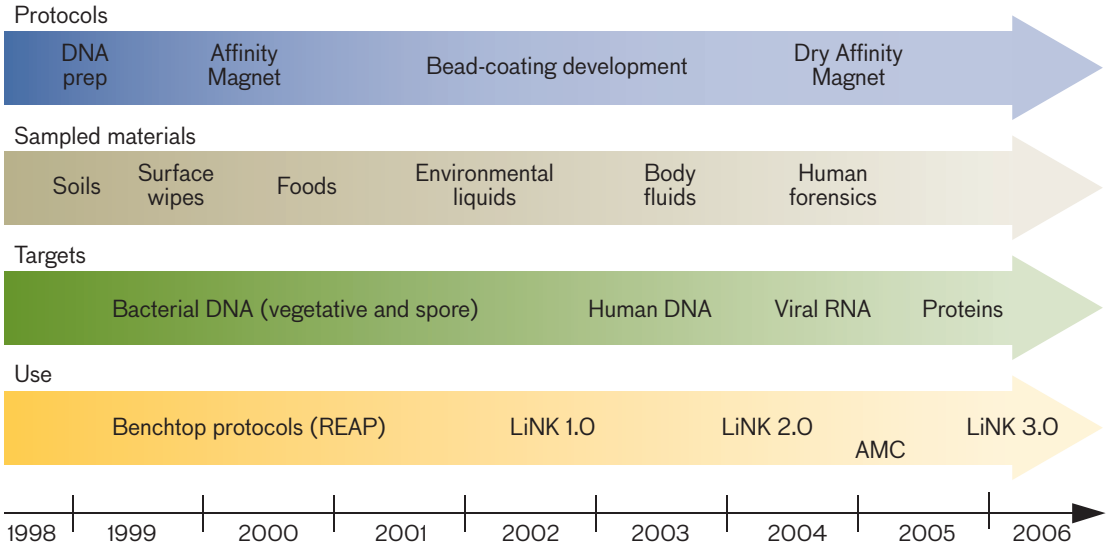


FIGURE 2. The timeline of sample-preparation activities at Lincoln Laboratory shows the evolution of our sample-preparation methods from laboratory-based protocols, highlighted in purple, into field-portable devices, highlighted in gold. The increasing breadth of materials and targets that our work has addressed are highlighted in beige and green, respectively.

presence of RNA-degrading enzymes called RNases in the environment. Our goal continues to be the development of universal sample-preparation devices, through the implementation of effective concentration and purification methods. Figure 2 shows a timeline of our sample-preparation programs.

Sample concentration, with concomitant purification, consists of extracting biological targets of interest from a raw sample and isolating them into a smaller volume of liquid, leaving behind both the rest of the raw sample and any components of the sample that may inhibit PCR or other downstream assays. Sample concentration is important because the input volumes acceptable to most amplification reactions are orders of magnitude smaller than the initial sample volume. Without a concentration step, trace-level detection of target would be highly unlikely.

Sample purification consists of separating DNA from impurities in the raw sample that can interfere with an identification assay such as PCR. It can also include steps to break open or lyse organisms to release the DNA contained within, and separate the DNA from the rest of the cellular components. PCR is highly sensitive to the presence of proteins, ions, and other components that can impede or completely inhibit the enzymatic reactions that occur during the amplification process. Current methods for pre-PCR sample preparation require involved procedures and solvents [2, 3]; our aim was to develop much simpler methods that not only produce clean DNA but also stabilize it for storage if required.

Sample Concentration—Affinity Magnet Protocol

The goal of the Affinity Magnet Protocol (AMP) is to concentrate targets of interest (bacterial vegetative cells, bacterial spores, and nucleic acids) from liquids and semisolid slurries into a PCR-compatible fluid. This concentration should be sufficiently selective that PCR inhibitors are left behind in the raw sample, thus providing purification as well as concentration. The protocol, which has been used in one form or another for decades, utilizes microscopic magnetic beads with coatings that have affinities for the targets. Traditionally, these coatings have been antibodies that are either specific to the target organism of interest or silica-based to capture DNA [4]. Our approach is novel in that the affinity coatings we use are not antibody based, as is the case for almost all commercially available magnetic-bead-based biological assays. Instead they are simple chemical groups that exploit the target's basic surface properties, such as hydrophobicity, hydrophilicity, and surface charge. These simple chemical groups tend to be more robust and less likely than antibodies to degrade in the presence of denaturing components in environmental samples. They also have the advantage of being semiselec-

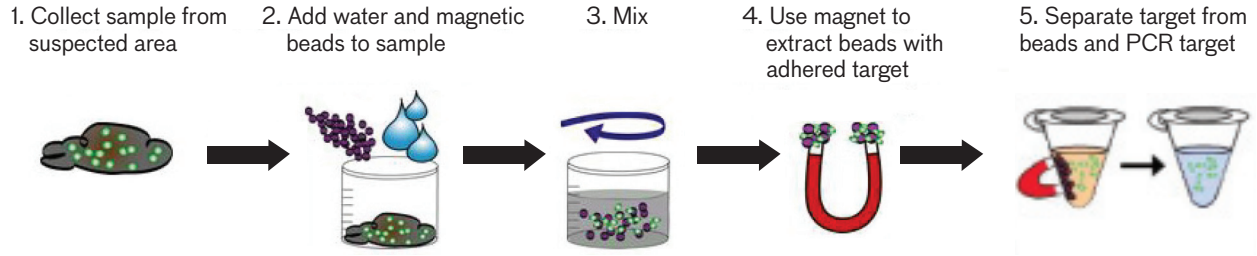


FIGURE 3. The AMP is used to isolate a target from a raw sample. The target is separated from the beads by using elution buffers, and the resulting material can be processed by polymerase chain reaction (PCR). This procedure has been implemented in a cartridge format.

tive, enabling the capture of classes of target that can then be identified by using the highly selective PCR process.

Figure 3 illustrates the basic AMP procedure. A raw sample is collected and, if in solid form, is mixed with water to form a slurry. The magnetic beads are mixed with the raw sample to allow the target to adhere to the bead surfaces. A collection magnet is used to remove the bead-target complex from the sample, thereby separating the target from the rest of the raw sample, and concentrating it into a smaller volume. The bead-target complex can then be processed in one of three ways—it can be directly subjected to PCR amplification, the target can be separated from the beads by using specially designed elution buffers (as shown in Figure 3), or the DNA can be extracted from the target by subjecting the bead-target complex to a purification procedure such as our REAP. We have demonstrated all three of these options, with an emphasis on the second approach, which is also used in the cartridge implementation of the AMP.

Bead-Coating Development

The magnetic beads consist of a magnetic core covered with a polymeric coating that is amenable to chemical functionalization (Figure 4). Tailored functional groups enable the selective capture of targets. We screened a number of commercial-off-the-shelf (COTS) magnetic and nonmagnetic beads for their target capture ability and used the results to guide the synthesis and development of customized coatings by using a range of functional groups, as well as elution buffers that enable effective separation of the target from the magnetic beads. Figure 5 shows the results of a screening of eleven COTS nonmagnetic beads for their ability to capture water-borne DNA (in this case a commercially available preparation of calf-thymus DNA). The results clearly indicated that multiple-amine-based functional groups provided the best capture efficiency.

On the basis of this experiment and other similar screenings, we synthesized a large number of functional groups, some of which are illustrated in Figure 6, and attached them to 50 μm silica beads. A screening of these coatings confirmed the efficacy of amine-based functional groups in DNA adhesion, and some of the customized coatings provided capture efficiencies in excess of 90%, even before protocol refinement.

Further screening experiments revealed distinctive differences in adhesion efficiency between various COTS

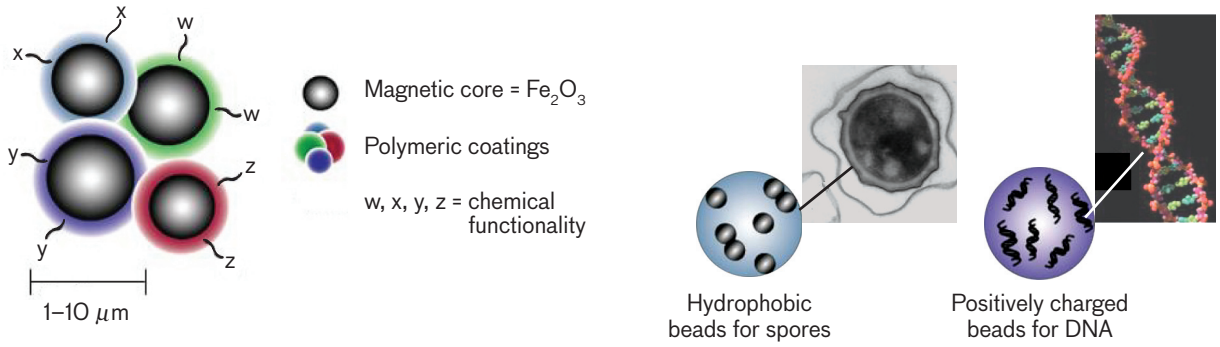


FIGURE 4. Magnetic beads with polymeric coatings are designed to attract and hold certain types of targets. Shown on the left is the structure of several beads, each designed with a specific chemical functionality. On the right are two conceptual images of bead coatings indicating the specificity of the design of the bead coatings to the surface properties of the targets.

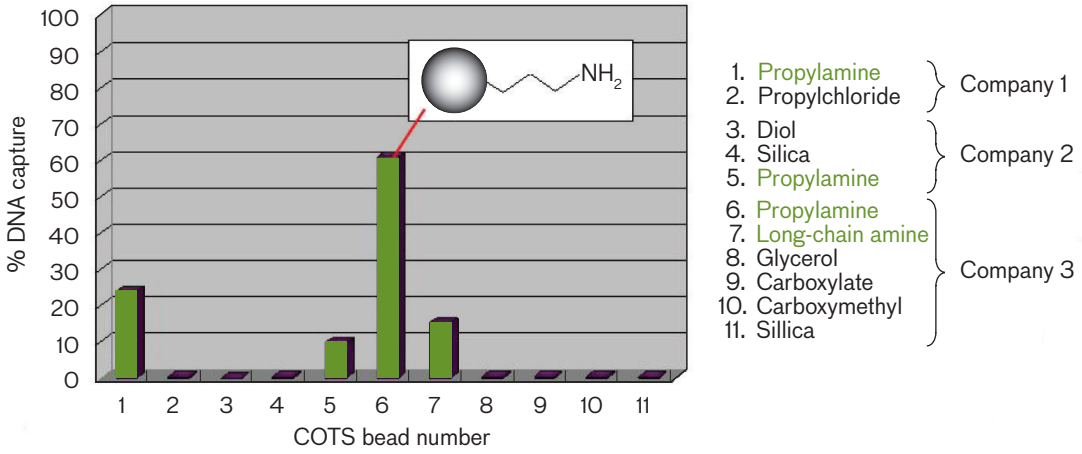


FIGURE 5. Screening of commercial-off-the-shelf (COTS) beads indicates the superior DNA adhesion to amine-based coatings (samples 1, 5, 6, and 7).

and Lincoln Laboratory–synthesized (LLS) beads, even among closely related target species, in this case nonpathogenic *Bacillus anthracis* (*Ba*) Sterne strain and *Bacillus thuringiensis* Kurstaki strain (*Btk*). We found similar results when we compared adhesion of gram-negative species (*E. coli* versus *Y. pestis*), and different forms of the same organism (*Ba* vegetative cells versus spores). In each case, we have found at least one bead functionality that captures almost a hundred percent of the target from solution under the screening conditions. Depending on the application, more or fewer beads can be added to the sample for faster complete adhesion if necessary. Most importantly, these results suggest that several bead types can be combined into a "cocktail" such that all desired targets are removed from a sample, or specific beads can be used that capture one target preferentially over others.

We also found that the beads are not saturated with target, even at the highest target levels tested (Figure 7). In the case of vegetative *Ba* and *Btk*, the chains of bacteria span several beads and cause them to clump together. These images show proof that the bacterial targets are in fact adhered to the bead surface, enabling them to be used as concentrators of both DNA and whole cells.

Not only is target adhesion important, but also equally important is the recovery or release of target from the bead surface after extraction from the sample. Knowing that pH and salt concentrations can be used to control adhesion of DNA to the beads, we conducted a series of experiments with a variety of buffers at a range of pH (2 to 13) and ionic strengths to optimize DNA recovery from the high-capture-efficiency multiple-amine-coated beads. Three sets of conditions provide optimal recovery: (1) NaOH at pH 11 with >2-Molar NaCl, (2) 50-mMolar carbonate buffer at pH 11 with low salt, and (3) 0.01-Molar NaOH at pH 12 with calf-thymus DNA at 100 μg/mL of sample. During these experiments, we found that the type of silica beads used as a base, and the exact process for synthesizing the functional groups and attaching them to the base beads, greatly affect the amount of DNA recovered from the bead surface after capture. We optimized the synthesis procedure to achieve close to 100% capture and elution of DNA.

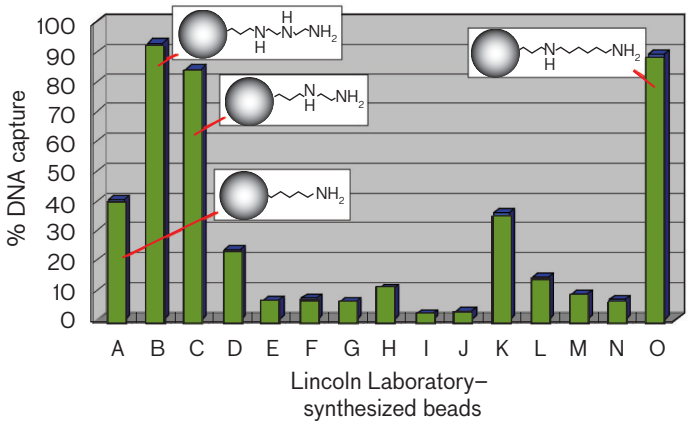


FIGURE 6. The Lincoln Laboratory–synthesized (LLS) bead coatings show similar results as the COTS beads with the best performance coming from amine-based functional groups (samples A, B, C, and O). Screening was done under identical conditions as the COTS beads.

PCR and DNA Replication

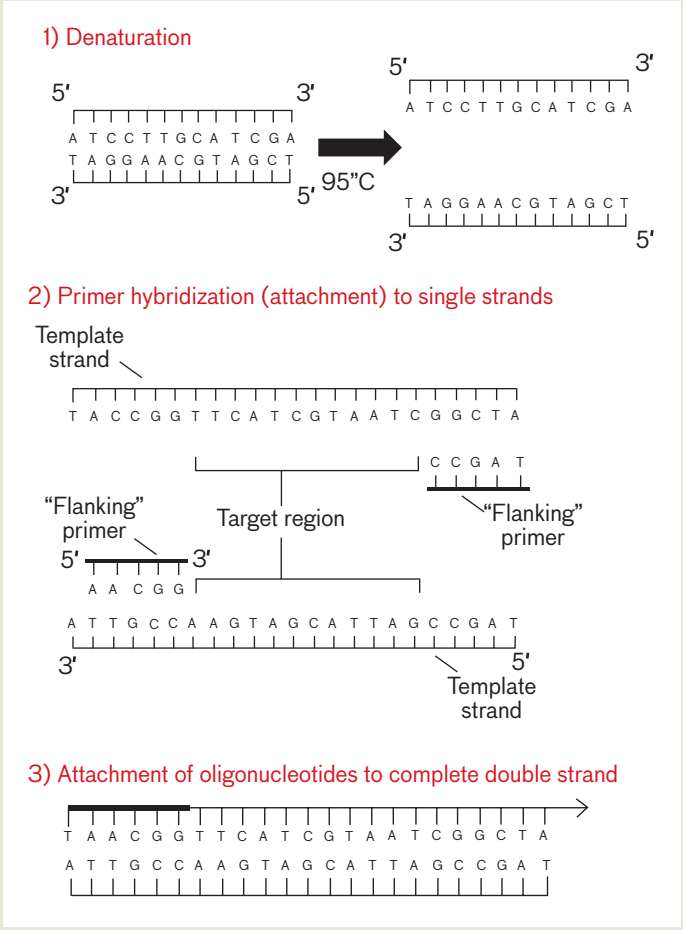
The polymerase-chain-reaction process unzips DNA, locks in the region of interest, replicates the region, and rezips the DNA. Each step progressively doubles the concentration to facilitate low-concentration analysis.

Polymerase chain reaction (PCR) is a biochemical process that uses enzymes and known starter-sequence primers of nucleic acid to amplify a quantity of DNA by orders of magnitude. Fluorescent tags can be added to enable optical detecting and counting of the generated DNA copies. The amplification produces an amount of DNA that is amenable to quantification and additional subsequent downstream analysis [a].

The basic procedure is illustrated in the accompanying figure, and consists of three main steps; a high temperature (>90°C) incubation to denature, or separate, the two strands of the DNA to be amplified, a low temperature (<60°C) step in which short nucleic-acid sequences hybridize (bind) to two different portions of the single DNA strands, and a third step at an intermediate temperature (70 to 75°C) in which an enzyme called polymerase rebuilds a double strand from each primer-modified single strand by using unattached oligonucleotides. The primers are designed to bind exclusively to portions of the target DNA of interest, providing target specificity. Each cycle of these three steps doubles the amount of DNA present in the reaction mixture. Thus 30 cycles can ideally produce about a billion copies of a single starting piece of DNA. Modern-day PCR was made possible by the discovery of the thermostable enzyme Taq polymerase (found in bacteria that live in deep-sea hydrothermal vents or hot springs), which is able to survive the high-temperature denaturation step. The polymerase found in mammalian cells is unstable at temperatures above physiological range.

Real-time PCR incorporates fluorescent reporter groups that are attached to the primers, enabling counting of the DNA strands as they are replicated. For example, Applied Biosystems has developed a system that uses an additional probe molecule, consisting of a short oligonucleotide sequence (also designed to match with a portion of the target DNA of interest)

Archiving target captured from samples is also a desirable attribute that should be included in any sample-preparation protocol whenever possible. Archiving is defined as the preservation of material necessary to perform an identification assay, such as PCR, with no significant loss of signal strength when assaying the preserved sample at some future date. To test the capability of amine beads to archive DNA targets, we adhered DNA to the beads and then stored them dry in a sealed container at room temperature for one week, after which the DNA was eluted from the beads and subjected to PCR. Our experiments show that a large amount of the DNA can be recovered from the dried beads after the one-week storage. The AMP-with-archiving procedure consisted of drying the beads, after target DNA capture, in an 80°C oven for one hour. We used the AMP elution step to elute target from one set of the dried beads immediately, and from another set after one week of room-temperature storage. Both sets of samples showed good signal recovery, indicating that the archiving step did not degrade the quality of the captured DNA. We have also demonstrated the ability to store DNA in the elution buffer in liquid form at room temperature for a period of at least six months, with no degradation of signal in PCR amplification of the aged DNA. Preserving the target DNA dried on the beads or eluted into buffer provides us with two archival formats prior to analysis via PCR. The AMP is highly effective in concentrating all tested targets in all tested matrices. The final optimized AMP is given in Table 1, and the limits of detection (LOD) for various target-matrix combinations are given in Table 2.



flanked by a fluorescent reporter dye molecule on one end and a quencher molecule on the other. This probe hybridizes to the single strand of DNA in step 2, along with the primers. As the polymerase builds up the double strand of DNA in step 3, the probe is broken up and the reporter and quencher are spatially separated, resulting in an increase in fluorescence signal when the sample is interrogated with a light source of the appropriate wavelength. Since each single strand binds to exactly one probe, the signal intensity is directly proportional to the number of DNA strands being amplified, and can thus give a measure in real time of the number of strands present in the sample at each cycle.

Reference

a. K.B. Mullis, "Process for Amplifying Nucleic Acid Sequences," U.S. Patent No. 4,683,202, Oct. 25, 1985.

In addition to concentration, the AMP performs a crucial cleanup function, separating the target from reaction inhibitors that degrade the signal obtained during PCR, as shown in the representative PCR plot given in Figure 8. The plot shows the fluorescence signal intensity during amplification of DNA (extracted from dirty environmental water with the AMP) with the use of the Applied Biosystems TaqMan real-time PCR process in an ABI 7700 system. The increase in fluorescence signal as a function of the number of amplification cycles performed indicates the increase in the amount of DNA produced, and the presence of more nucleic-acid target in the starting sample causes the amplification signal to increase at an earlier cycle. The metric of interest is the lowest starting target concentration that produces a detectable fluorescence signal. Note that the distinctive creep (deviation from a classic sigmoidal curve) associated with dirty water is exhibited by the red control curves but not by the green eluate curves. Since this creep is thought to be caused by contaminants in the sample that degrade the fluorescent probes in the PCR reaction, this creep elimination indicates that a significant number of the PCR inhibitors present in dirty water have been removed by the AMP.

The AMP was also tested with two clinically relevant matrices, blood and saliva. Blood contains PCR inhibitors in the form of various proteins, as well as iron-containing hemoglobin. Saliva also contains many inhibitory proteins. We were able to demonstrate effective concentration and separation of vegetative *Ba* from blood and *Ba* spores from saliva. With the addition of a post-capture wash step to the blood protocol, we showed the ability to concentrate target from blood to a level equal to

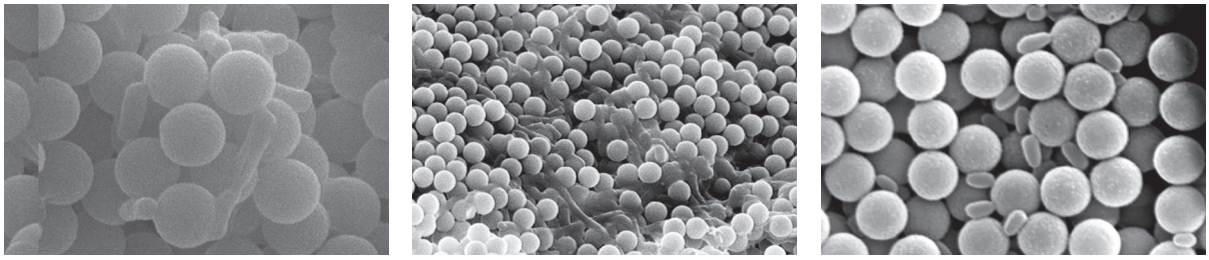


FIGURE 7. Scanning-electron-microscope images show COTS magnetic beads after being used to capture vegetative *Btk* cells (left), vegetative *Ba* cells (middle), and *Ba* spores (right). The bead target complexes were washed three times before imaging to remove non-adhered cells.

that of target concentrated from plain water. The saliva protocol required a pre-capture pH adjustment step, which greatly improved capture.

Fieldable-Device Implementation of AMP

The AM protocol, like most magnetic-bead-based protocols currently in use, requires a laboratory setting for implementation and is not amenable to field use. The AMC was developed in various formats to answer the need for fieldable versions of the protocol. The AMC implements the AMP in a self-contained unit, takes as input a liquid or solid sample containing whole organism (bacteria or viruses) and/or DNA, and produces an output consisting of an eluate that is ready for PCR. The AMC contains all required reagents and components, and can be used to store both the raw sample and the concentrated target indefinitely. In detail, the AMC has been designed to perform the following functions: provide a receptacle for the raw sample, consisting of up to 5 mL of liquid or 1 g of sand; enable mixing of the sample with the magnetic beads, which are preloaded into the cartridge; separate and isolate the unwanted components of the raw sample from the target; separate the target from the magnetic beads with an elution buffer, also preloaded into the cartridge; and provide a target-containing eluate volume of 100 to 500 μL, in a form that is ready for PCR.

Table 1: Affinity Magnet Protocol Parameters

VARIABLE	OPTIMIZED PARAMETERS
Bead type	Amine-coated 2.7 μm particles (Dynabeads® M-270 Amine, Product No. 143.07/08 from Dynal) or Lincoln Laboratory–synthesized amine magnetic beads
Sample volume	1 to 5 mL
Bead quantity	1 to 5 mg/mL
Capture time	1 to 5 min
Elution time	30 sec
Elution buffer pH	11.8
Elution buffer composition	0.01M NaOH with calf-thymus DNA added at 100 μg/mL
Elution volume	150 μL for a 1 mL sample, 150 μL to 1 mL for a 5 mL sample
Target concentration	5-fold to >33-fold
Wash steps	None
Archiving	DNA stabilized on dried beads (stable for at least one week at room temperature, requires further life tests) DNA stabilized in elution buffer (stable for at least six months at room temperature)

Several manifestations of the AMC have been designed and fabricated. The first version is composed of modified COTS components, and is referred to as the two-valve COTS AMC, or the two-valve AMC. The second version uses a single COTS ball valve with added custom components, and is referred to as the one-valve COTS AMC, or the one-valve AMC. The other versions are variations that incorporate added functionality to the one-valve AMC.

Two-Valve COTS AMC

The basic two-valve COTS-based cartridge shown in Figure 9 consists of three chambers separated by two valves. Each chamber is formed from a polycarbonate test tube cut to an appropriate length; the chambers are connected together with COTS polyacetal (Delrin) valves (manufactured by John Guest International Ltd.). All parts in the flow path of the sample are composed of FDA-approved materials that do not bind the target. The left chamber (input chamber) serves initially as the sample collection chamber and subsequently as the waste chamber, and contains magnetic beads with affinity coatings for the targets. It also contains a small Teflon ball to facilitate movement of liquid between the sample chamber and the processing chamber through the relatively small valve opening. The middle chamber (processing chamber) contains a free-floating collection magnet that is used to collect and separate the magnetic beads from the raw sample. The right chamber (eluate chamber) contains the elution buffer used to separate the target from the magnetic beads,

Detection Limits

Limits of detection (LOD) are given in one of two units. Whole target organisms are specified in cells/mL or spores/mL, which refers to the number of target organisms per milliliter of target medium, counted with an optical microscope. Dilution factors are used for supernatants, which are prepared by using a starting overnight culture of the target organism, generally at a cell count of 10^8 to 10^9 cells/mL, centrifuging to remove whole cells and cell fragments, and volumetrically diluting the remaining DNA-containing cell-culture medium in the test medium. The dilution factor defines how dilute a sample we can prepare and still detect the target DNA (the larger the dilution factor, the more sensitive a test is in detecting an organism).

Table 2: Improvements in LOD with AMP

MEDIUM	CLEAN WATER		DIRTY ENVIRONMENTAL WATER		UNDILUTED GROWTH MEDIA (BRAIN–HEART INFUSION)	
TARGET	NO PREP + PCR	AMP + PCR	NO PREP + PCR	AMP + PCR	NO PREP + PCR	AMP + PCR
Ba culture supernatant	$10^4\times$ dilution	$10^5\times$ dilution	Probe degradation	$10^5\times$ dilution	$10^5\times$ dilution	$10^7\times$ dilution
Ba vegetative cells	10^4 cells/mL	10^3 cells/mL	Probe degradation	10 cells/mL	10^4 cells/mL	10 cells/mL
Ba spores	100 spores/mL	10 spores/mL	Probe degradation	10 spores/mL	100 spores/mL	10 spores/mL
Btk culture supernatant	$10^6\times$ dilution	$10^6\times$ dilution	Probe degradation	$10^6\times$ dilution	$10^6\times$ dilution	$10^6\times$ dilution
Btk vegetative cells	100 cells/mL	10 cells/mL	Probe degradation	10 cells/mL	TBD	TBD
Yp vegetative supernatant	10 cells/mL	10 cells/mL	Probe degradation	10 cells/mL	TBD	TBD
Yp culture supernatant	TBD	TBD	Probe degradation	TBD	TBD	TBD

and can be unscrewed from the cartridge body when processing is complete and sealed with an attached cap. The two valves are attached to a plastic backbone, providing rotational rigidity to the cartridge as well as stability when the cartridge is placed horizontally on a flat surface.

The cartridge is provided with all reagents and components necessary for sample processing, and magnetic beads are preloaded into the input chamber of the cartridge. The collection magnet is included in the eluate chamber. The removable eluate chamber enables eluate storage for processing at a later date if desired.

We tested the two-valve AMC by using two sample matrices—dirty environmental water and acid-washed sand—and two targets—*Ba* spores and *Ba* vegetative cells. The magnetic beads we used were the AMP-specified Dynal beads at a concentration of 1 to 5 mg/mL of sample, and we eluted the targets into 500 μL of Lincoln Laboratory elution buffer solution. We diluted various quantities of *Ba* cells or spores into dirty environmental water to prepare liquid samples, and we prepared soil samples by seeding 1 g quantities of acid-washed sand (VWR cat.# AAA19936-0B), with known quantities of *Ba* spores or vegetative cells and allowing the sand to dry. During testing, each sand sample was formed into a slurry by mixing with 5 mL of deionized water in the sample input chamber; the liquid samples were used as prepared. The two-valve AMC was effective in concentrating target from these matrices, and enabled the processing of larger (5 mL) quantities of initial sample. It also provided isolation between the sample and the final elution buffer.

One-Valve COTS-Based Cartridge

While the two-valve COTS AMC performs well and provides several key features such as raw-sample final eluate isolation, the operational procedure is somewhat complicated because of the presence of two valves that must be operated in the correct order. The one-valve COTS AMC addresses this issue by using only a single valve that has been modified to create two chambers. The operation of the COTS-based one-valve AMC is illustrated in

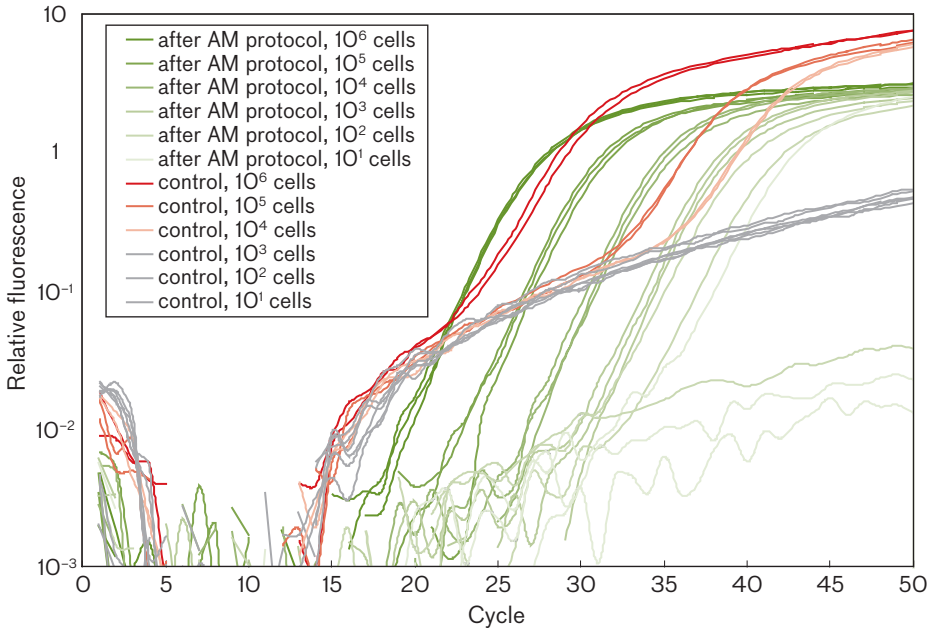


FIGURE 8. PCR amplification curves show detectable concentrations when we observe the sigmoidal increases. In this case the green lines indicate the level of target material in the eluate after using the AM protocol to concentrate *Ba* vegetative cells from dirty environmental water. Each concentration was measured three times, indicated by the triplet curves. Note that control curves for starting concentrations of less than 10^4 cells/mL (in gray color) do not show any detectable signal, whereas the corresponding eluate curves for those concentrations do show detectable signal down to 10 cells/mL (two of the 10^2 and one of the 10 cells/mL concentrations show the sigmoidal curve), indicating a limit-of-detection improvement of three orders of magnitude.

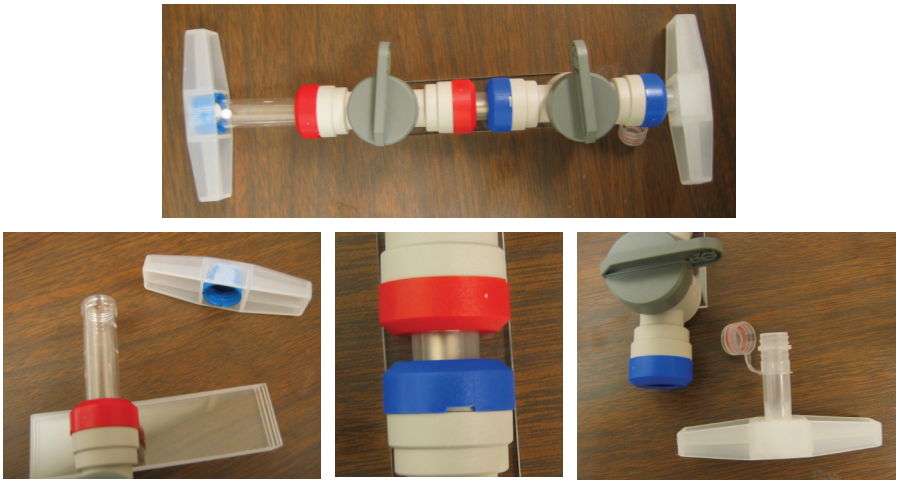


FIGURE 9. The two-valve AMC is based on COTS components. Shown at the top is the assembled cartridge. Below on the left is the polystyrene test-tube input chamber with the cap removed. In the center is the polypropylene test-tube processing chamber with the collection magnet (a radially magnetized chrome-plated neodymium-boron rare-earth cylinder) inside the chamber. On the right is the eluate chamber, a 1.5 mL cryogenic-compatible storage vial, with the attached cap and handle.

Figure 10. Like the two-valve version, the one-valve version contains all required reagents (magnetic beads, elution buffer) and components (collection magnet, eluate vial), and accommodates samples consisting of up to 5 mL of liquid or up to 1 g of solid formed into a slurry with 5 mL of liquid. The input chamber contains a mesh filter to prevent coarse particulates from accessing the collection magnet, which is embedded in the ball of the valve. The eluate vial is identical to that used in the two-valve AMC. The unit is considerably simpler to use than the two-valve COTS AMC, but does have an LOD of about one order of magnitude worse. This limitation is because the one-valve COTS AMC has an embedded collection magnet, whereas in the two-valve COTS AMC the collection magnet can circulate throughout the sample as well as the elution buffer for more effective target capture and DNA elution. Table 3 summarizes the LODs obtained with the two-valve AMC and one-valve AMC when they are tested with various targets and matrices.

AMC Variations

Both the one-valve and two-valve COTS AMC were developed with specific sponsor requirements in mind, and have been tested extensively. Over the course of testing, both we and a number of our sponsors suggested several improvements to increase the efficiency of the device as well as make it more functional. These improvements were incorporated into a series of cartridge variations.

(1) (2) (3) (4) (5)

FIGURE 10. To process a sample with the one-valve AMC procedure we (1) open the cap and load the sample, close the cap and capture the target onto the beads; (2) rotate the valve to expose the collection magnet and collect the beads and target; (3) rotate the valve to expose the magnet and the beads to elution buffer; (4) consolidate the beads suspended in the elution buffer into the eluate vial for target elution; and (5) recollect the beads onto the magnet after elution and remove the eluate vial.

Table 3: LOD for One-Valve and Two-Valve COTS AMC

TARGET	MATRIX	LOD ONE-VALVE COTS AMC	LOD TWO-VALVE COTS AMC
Ba spore	Dirty water	100 spores/mL	10 spores/mL
Ba spore	Sand	1000 spores/mL	100 spores/mL
Ba vegetative	Dirty water	1000 cells/mL	10 cells/mL
Ba vegetative	Sand	1000 cells/mL	1000 cells/mL (lowest level tested)
Yp	Dirty water	$1:10^7$ dilution	
Yp	Sand	$1:10^6$ dilution	
Vaccinia virus	Culture medium	100 plaque-forming units/mL	

One-Valve Custom Cartridge

Because the collection magnet is fixed inside the central rotating ball, the one-valve COTS AMC does not allow the complete release of the magnetic beads into the elution buffer, which is a requirement for some applications. To address this issue, we developed the one-valve custom AMC (shown in Figure 11), consisting of a single valve-like structure that has a sample input chamber incorporated in the unit, a removable eluate vial, and a removable valve handle that incorporates the collection magnet. The one-valve custom AMC provides the ability to reuse the collection magnet. The design provides cost as well as weight advantages when carrying large numbers of cartridges, since only one handle-collection-magnet unit is required for an unlimited number of cartridges.

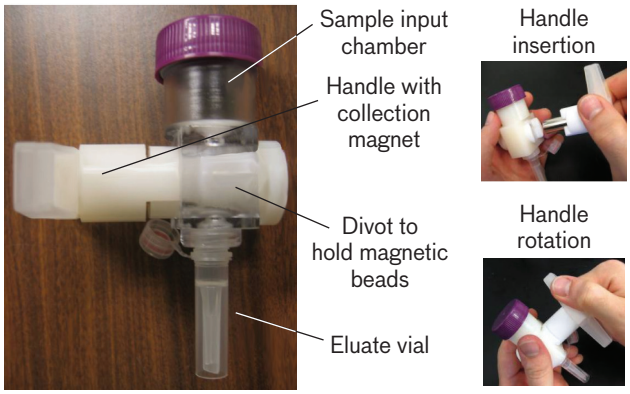


FIGURE 11. The one-valve custom AMC reduces the weight of multiple cartridges by utilizing a single handle that contains the reusable collection magnet.

Attachment for Easier Input and Dispensing

To facilitate input of the sample into the cartridge, we modified the input chamber cap of the one-valve COTS AMC, as shown in Figure 12, to include a dropper for sample collecting and cartridge loading. A flow diverter was designed and incorporated into the base of the dropper sleeve to prevent the dispensed liquid from being re-aspirated when the user releases the dropper bulb. To facilitate eluate removal and subsequent dispense into an analysis unit, we modified the eluate vial to contain a Luer-lok one-way valve that enabled extraction of the eluate by using a Luer-lok syringe.

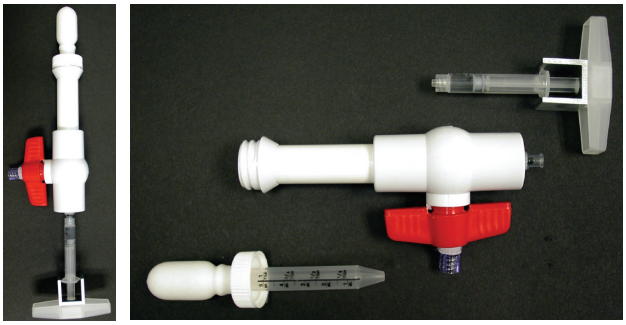


FIGURE 12. The assembled (left) and disassembled (right) AMC shows the dropper attachment to the input chamber, the syringe, and the one-way valve attachment to the output end. The cartridge body has an extension on the input chamber to accommodate the dropper, and the syringe has an attached handle for eluate removal (top right).

FIGURE 13. A grinding and cutting attachment to the cartridge includes the plunger on the right that forces the solids onto and through the blades inside the sleeve in the left.

Attachment for Grinding and Cutting

To be able to extract and concentrate target from raw samples that may contain target DNA within the sample material, such as animal or plant tissue, we constructed a front-end attachment that can crush and grind the sample and render it into a slurry or paste. This releases and provides magnetic-bead access to any target that is embedded in the sample. Figure 13 illustrates a prototype of the grinder-cutter attachment, consisting of a set of plungers with cutting blades and a rigid perforated plate. Figure 14 shows its usage.

Three-Chambered, One-Valve AMC

The two-valve COTS AMC shows superior collection and extraction efficiency compared to the one-valve COTS AMC, primarily because in the two-valve AMC, the collection magnet is free floating and able to access all parts of the sample for magnetic-bead collection, as well as being immersed in the elution buffer for more effective target elution. Additionally, the presence of three chambers in the two-valve AMC isolates the sample from the eluate. We developed a three-chambered single-valve AMC to implement these advantages in a compact, simpler to use cartridge. The body of the unit consists of a COTS three-port ball valve, and the three chambers can be custom fabricated to the desired volumes to mate with the valve ports, or can be formed from COTS sample vials. Figure 15 illustrates this version of the AMC and its usage. Preliminary testing of this device shows that it is effective in concentrating target while providing sample-eluate isolation.

The Affinity Magnet family of protocols and devices has been shown to be effective for concentration of target from liquid or slurry samples. For the collection of wipe samples and for archiving samples in dry form, we have developed the REAP/LiNK family of devices and protocols.

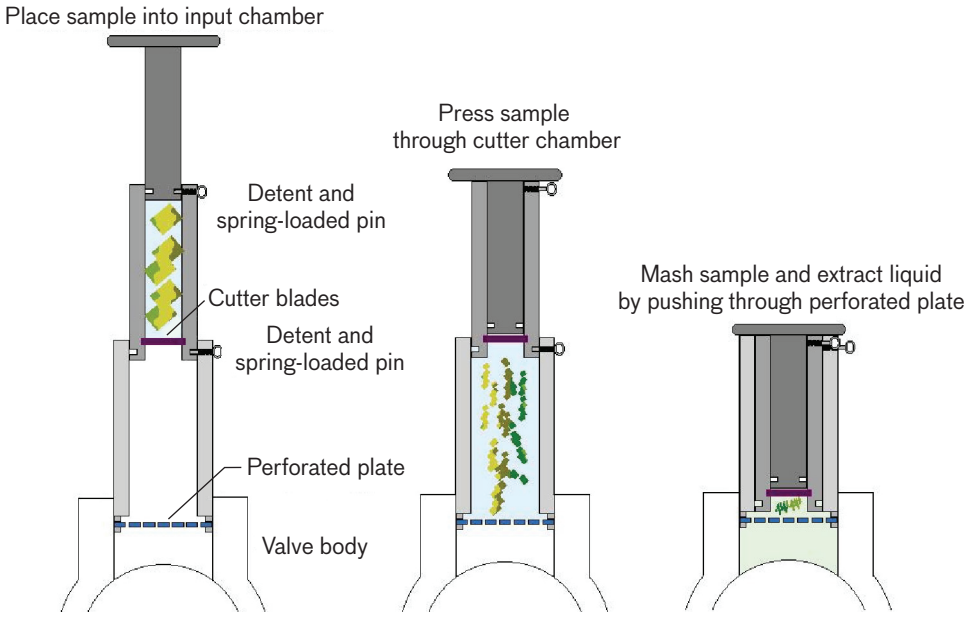


FIGURE 14. The tissue-grinding attachment initially chops the material into fine pieces. The mashing process compresses the solids and extracts the liquid, which proceeds into the sample chamber of the AMC.

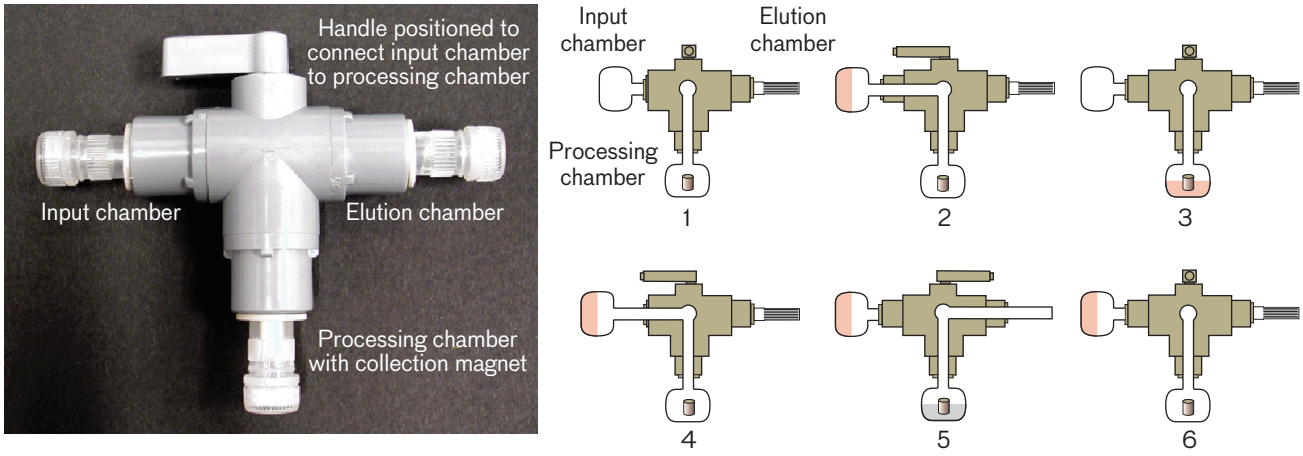


FIGURE 15. The core of the three-chambered AMC is a three-port ball valve shown in the image at left. The procedure followed with this unit, shown on the right, is similar to each of the other AMCs. (1) Load the cartridge with the beads, the elution buffer, and the collection magnet. (2) Load the sample into the input chamber, let sit to collect the target onto the beads, and rotate the valve handle to move the sample into the processing chamber with the collection magnet. (3) Let sit to collect the beads and the target onto the collection magnet. (4) Rotate the handle to move waste material back into the input chamber. (5) Rotate the handle to move the elution buffer into the processing chamber, and let sit to separate target from beads. (6) Move the eluate back into the elution chamber and close the valve. The eluate is in the elution chamber, ready for use.

Sample Purification—REAP

It is notoriously difficult to extract amplifiable DNA from many varieties of soil, plant matter, food, environmental waters, blood, and other bodily fluids and tissues because these materials can contain strong PCR inhibitors or can lead to severe ion imbalance in PCR. We have developed the Recovery, Extraction, and Archiving Protocol (REAP), which uses a chemically treated paper for the rapid and easy separation of amplifiable DNA from difficult matrices. The protocol is exceptionally easy and is based on a commercially available chemically impregnated cellulose paper [5] (manufactured by Schleicher and Schuell, now Whatman) that was originally developed for the isolation and preservation of human DNA derived from blood. Liquid samples are placed directly onto the paper, and solid samples, such as soil, are made into a slurry by adding water, which is then placed on the paper. The chaotropic salts present in the paper lyse bacterial vegetative cells, killing them and releasing their DNA. The paper is then dried, causing many PCR inhibitors to bind to the paper, after which it is washed briefly to remove the majority of the salt and particulate matter. Finally, the paper is placed in hot water, where the DNA is eluted off the paper into the water, leaving the PCR inhibitors behind.

This protocol is easy and fast (approximately thirty minutes), requires no additional reagents except water, and stabilizes the sample, enabling extended room-temperature preservation and archiving of field-collected DNA with the addition of desiccant to keep the paper substrate dry. This stabilization feature is especially important because it prevents degradation of field-collected samples en route to the processing laboratory. Lincoln Laboratory participated in a field exercise in which pond water containing a biowarfare agent simulant was collected either into test tubes or was spotted and dried onto the chemically treated paper, after which both types of samples were mailed back to the Laboratory. After several days, the untreated water sample did not produce any detectable PCR signal, but the sample stabilized with the REAP had detectable levels of the simulant. This protocol is suitable for use with a wide array of sample types, as is illustrated in Table 4, and in many cases affords detection of samples that otherwise would have been impossible to process via PCR.

A further example of the effectiveness of the REAP in enhancing PCR is shown in Figure 16: a liquid containing environmental particulate matter was spiked with a dilution series of *Ba*. Samples subjected to PCR without the REAP showed a detection limit of 10^5 cells/mL, whereas performing the REAP improved the detection limit by two orders of magnitude.

Lincoln Nucleic-Acid Kit

The REAP is highly effective in isolating and purifying DNA from a variety of materials, but is primarily designed for use in a laboratory setting. The Lincoln Nucleic-acid Kit (LiNK) cartridge family of devices incorporates the chemically treated paper that is integral to the REAP into a portable, easy-to-use format, and additionally provides a handle for the paper such that the device is not only a DNA purifier, but also a sample collector. Broadly, the LiNK family of sample-preparation tools meets the following functional requirements. They collect environmental samples (liquids, wipes, sandy soils); purify samples (filter out interferent particles, bind PCR inhibitors); extract DNA (lyse bacteria, do not bind DNA); archive samples without loss of target signal; have minimal to no power requirement; are lightweight and easy to use, even in mission-oriented protection posture (MOPP) gear; use non-toxic, non-flammable reagents incorporated into the cartridge; require no additional reagents or components to accomplish the task (sample collection and purification); and have a fast and simple operating procedure.

Table 4: Recovery, Extraction, and Archiving Protocol (REAP) Effectiveness

MATRIX	LOD WITHOUT SAMPLE PREP.	LOD WITH REAP PREP.
Glass plates	N/A	10^6 cells/mL (dried)
Blood	X	10^4 cells/mL
Urine	X	10^3 cells/mL
Saliva	X	10^2 cells/mL
Serum	10^4 cells/mL	10^3 cells/mL
NIST meat homogenate	–	10^2 cells/50 mg
Whole milk	X	10^3 cells/mL
MRE (clam chowder)	X	10^3 cells/mL
MRE (turkey)	X	10^2 cells/mL
Soil (sandy)	N/A	10^2 cells/g (vegetative)
Soil (sandy)	N/A	10^6 cells/g (spore)
White paper (plain)	10^1 cells/cm^2	10^0 cells/cm^2

X indicates no detection. N/A indicates that PCR is not possible. MREs are military meals ready to eat, and NIST is the National Institute of Standards and Technology.

First Generation: LiNK 1.0

LiNK enables sample collection followed by either immediate extraction of laboratory-PCR-compatible DNA or long-term DNA stabilization [6]. The LiNK 1.0 was initially developed in response to an urgent request from the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) in October 2001 in response to the anthrax letter incidents. The LiNK 2.0, described in the next section, is functionally identical to the LiNK 1.0, but incorporates additional features that make it compatible with the RAZOR field-portable PCR unit.

The LiNK 1.0 is derived from a COTS syringeless filter; the basic unit consists of two parts, an inner plunger into which the chemically treated paper of the REAP has been incorporated, and an outer cylinder. The chemically treated paper is covered with a large-pore nylon mesh to protect it against physical damage when the LiNK is used to collect surface-wipe samples. Usage is very simple, with a total processing time of approximately six minutes.

The LiNK 1.0 has been tested successfully against a wide variety of samples in the laboratory, including environmental, medical, food, and surface wipe samples spiked with *Ba* and *Btk*. It has also been successfully deployed at several field trials, including a Navy trial in September 2002. In this trial, Navy personnel in full MOPP gear (Figure 17) used LiNK 1.0 units to collect environmental samples in an abandoned warehouse. The warehouse surfaces were spiked with *Btk*, which served as a simulant for *Ba*. Even though the warehouse was highly con-

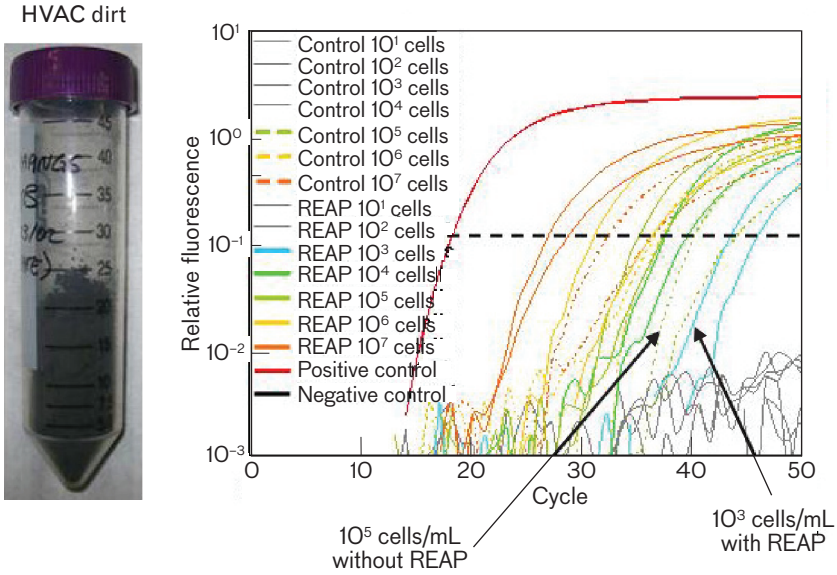


FIGURE 16. We used the REAP to process dirty environmental samples obtained from air intakes at an airport. The conical tube contains particulate material removed from the intake filter. This material was suspended in water to which was added known amounts of *Ba* vegetative cells. The sample was then processed with and without the REAP and subjected to PCR, with the resulting amplification curves shown on the right. REAP-processed samples showed a detection limit 100× better than that of unprocessed samples. The horizontal dashed line indicates the response level necessary for detection.

taminated with pigeon droppings, the LiNK 1.0 enabled detection of the simulant in every instance.

Second Generation: LiNK 2.0

The LiNK 2.0 is functionally identical to the LiNK 1.0, but was developed specifically to mate with the RAZOR field-portable PCR unit. The RAZOR, developed by Idaho Technologies, can run a complete PCR assay in about thirty minutes, is relatively easy to use, and has been ruggedized for use under field conditions, addressing many of the operational issues that have prevented the use of PCR in the field [7]. Further, the RAZOR works with lyophilized (i.e., shelf stable) reagents contained in simplified vacuum-sealed pouch sample holders. However, the reagents themselves can still be inhibited by environmental contaminants. Commercially available sample-purification kits can remove inhibitors but are difficult to operate, require several additional pieces of equipment, and can take one hour or more to complete purification. The lack of suitable rapid fieldable sample preparation negates many of the benefits of the RAZOR.

To provide RAZOR compatibility, the LiNK 2.0 incorporates the following features not found in the LiNK 1.0:

- a controlled-aspiration syringe for sample loading into RAZOR, with a tip compatible with the RAZOR's unique vacuum-sealed pouch. The syringe is packed inside the LiNK and accommodates the vacuum-driven loading into the pouch while ensuring no bubbles are introduced into the pouch;
- an eluate composition and volume that is compatible with the RAZOR lyophilized PCR reagents;
- a resistance to depressurization up to 30,000 feet; and
- a shipping outer cylinder that is packed with desiccant to dry and preserve the collector and target.

Like the LiNK 1.0, the LiNK 2.0 has a very short processing time of under six minutes to match the rapid (less that thirty minutes) analysis time of the RAZOR.

The completed LiNK 2.0 is illustrated in Figure 18, and consists primarily of custom-designed components that were injection molded from medical-grade

FIGURE 17. Navy personnel in full mission-oriented protective posture (MOPP) gear use LiNK 1.0 to collect environmental samples in a field trial. The LiNK is being held in the person's gloved hand, and is circled.

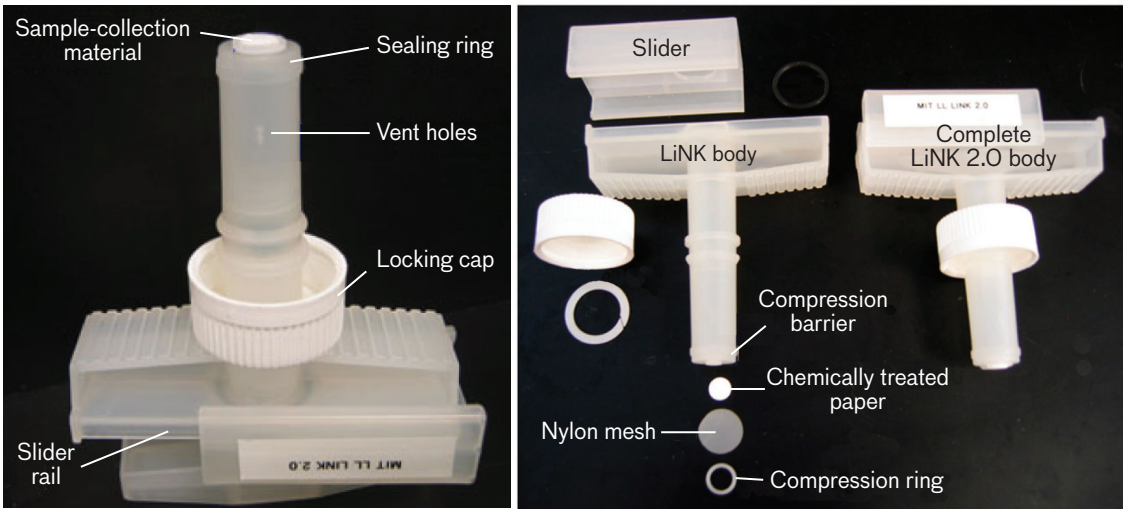


FIGURE 18. The LiNK 2.0 prototype assembly has all the components necessary for processing samples. On the left is the inner body. On the right is an exploded view of the inner body with components and a completely assembled unit.

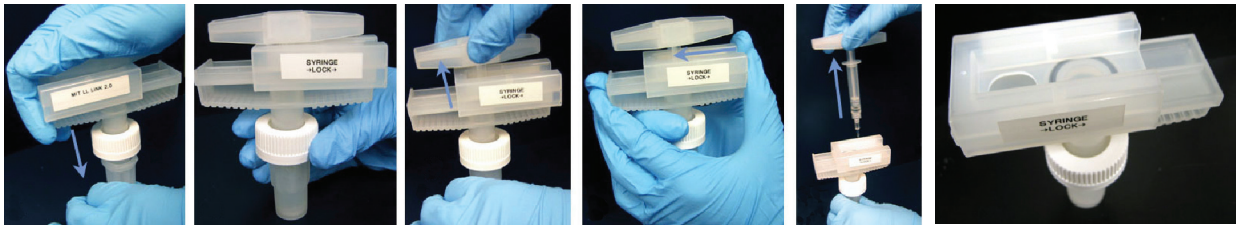


FIGURE 19. The post-sample-collection processing steps for the LiNK 2.0 are (1) insert the inner body into outer cylinder, filled with 2.0 mL water; (2) lock the cap; (3) load the syringe by pulling up on handle; (4) release the syringe by pressing the slider locking mechanism; (5) remove the syringe; and (6) push the slider to put LiNK 2.0 into the final closed position to seal the eluate into the unit for shipping/storage.

polypropylene. COTS components of the assembly include the 0.5 mL controlled-aspiration syringe and accompanying blunt cannula tip. When packaged for field use, the complete kit includes an inner body assembly and an outer cylinder filled with elution buffer, both packaged inside a pouch with instructional labels. A dropper bottle containing PCR-grade water can be used with many LiNK units and is not included in each pouch.

The LiNK 2.0 can be used to collect liquid and wipe samples in the same manner as the LiNK 1.0. The post-sample-collection LiNK 2.0 processing procedure is illustrated in Figure 19. Once we have removed the loaded syringe, we can insert it directly into the RAZOR vacuum pouch, as shown in Figure 20. After loading the pouch, we can place the syringe back inside the LiNK 2.0 body and reload it for a second dispensing, if desired. When we no longer need the syringe, we can seal the LINK 2.0 for shipping by moving the slider completely along the slider rails to the full stop at the end of the rails.

Experimental Testing of Design

We tested the LiNK 2.0 against two different biowarfare-agent simulants, *Ba* and *Btk* spores, in three different matrices: Leighton-Doi (LD) medium (a common growth medium), dirty environmental water, and clean (acid-washed) sand. Samples of matrices spiked with target material were processed with the LiNK 2.0 and the resulting eluates were subjected to PCR in a RAZOR (samples hereafter referred to as RAZOR+LiNK2), or in an ABI 7700 laboratory PCR machine, using TaqMan chemistries (samples referred to as ABI+LiNK2). We also performed PCR on spiked samples that were not processed with LiNK 2.0. Finally, we processed spiked samples with the

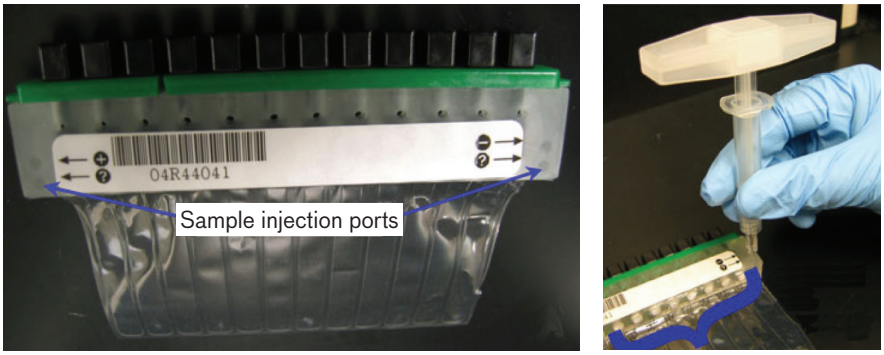


FIGURE 20. The vacuum-sealed RAZOR pouch, shown on the left, contains the thin flexible lower pouch section that is inserted into the RAZOR. Loading a sample into a RAZOR pouch, shown on the right, requires a controlled-aspiration syringe with a blunt cannula tip and a Lincoln Laboratory–designed custom handle.

REAP procedure and analyzed them with the ABI 7700 to provide a benchmark to which the RAZOR results could be compared (samples referred to as ABI+REAP). Recall that the REAP uses the same chemically treated paper that is present in the LiNK. All three analysis methods (ABI+REAP, ABI+LiNK2, RAZOR+LiNK2) were run at both high (10^6 cells/mL) and low concentrations (10^3 cells/mL) for both targets (*Ba* and *Btk)* in all three matrices.

By using the RAZOR+LiNK2 approach, we detected approximately 66% of the *Ba* low-concentration (10^3 cells/mL) samples and 50% of the *Btk* low-concentration samples. As expected, the LiNK 2.0 eluates gave better results when processed with the RAZOR than with the ABI 7700, since the ABI process requires an additional 1:10 dilution not needed by the RAZOR.

Overall, at high concentrations the RAZOR+LiNK2 performed as well as or better than the ABI+REAP benchmark. For high concentrations spiked into dirty water or LD medium, the RAZOR+LiNK2 protocol performed as well as the ABI+REAP benchmark, as both methods successfully detected 100% of the samples. The RAZOR+LiNK2 protocol when used with spiked sandy soils actually performed better than the benchmark, detecting 100% of the spiked samples, while the ABI+REAP missed one of the *Ba*-spiked sand samples.

At low concentrations the RAZOR+LiNK2 performance, as compared to the ABI+REAP benchmark, varied with the environmental matrix tested but overall did nearly as well as the benchmark and in one case better. For low concentrations spiked into LD media, the RAZOR+LiNK2 performed as well as the ABI+REAP. In dirty water at low spiked concentrations, the RAZOR+LiNK2 did not perform as well as the ABI+REAP benchmark. For sandy soils, the RAZOR+LiNK2 outperformed the ABI+REAP, particularly for the *Ba*-spiked

Table 5: Lincoln Laboratory Sample-Preparation Protocols and Devices

	CLINICAL		SURFACE	SOIL	ENVIRON.	ABILITY TO	VENUE		EASE
	BLOOD	OTHER	WIPE	SLURRY	LIQUID	CONCENTRATE TARGET	LAB	FIELD	OF USE
REAP	√	√		√	√		√		Fair
LiNK 1.0		TBD	√	√	√		√	√	Good
LiNK 2.0		TBD	√	√	√		√	√	Excellent
AMP	√	TBD		√	√	√	√		Fair
AMC	TBD	TBD		√	√	√	√	√	Excellent

Table 6: Comparison of LiNK and AMC

CONDITION	LiNK	AMC
Large volumes	No	Yes
Culture from eluate?	TBD	(+)
Cell lysis	Yes	Yes
DNA preservation	Yes, on chemical paper	Yes, in eluate
Sample concentration	No	Yes
Sample purification	Yes	Yes
Easy sample handling	Yes	Yes
SAMPLES	**LiNK**	**AMC**
Clinical samples	TBD	(+)
Food samples	(+)	(+)
DNA-based targets	Yes	Yes
RNA-based targets	TBD	TBD

(+) indicates that this process is untested but highly probable with existing technology.

samples, which were not detected at all by the benchmark protocol. Given the limited number of samples tested so far, all of these results should be considered preliminary.

AMC/LiNK Product Comparison

We developed our suite of sample-preparation protocols and devices to cover a range of venues and sample types and conditions. Table 5 gives a summary and comparison of all of our available tools, and Table 6 gives a comparison of the LiNK and AMC family of tools. Both tables show the complementary nature of each class of protocol and represent a set that encompasses venues of relevance to biologists, clinicians, forensics specialists, first responders and others whose main interest is in the collection and purification of bacterial cells and nucleic acids. While our emphasis has been sponsor driven and based on environmental samples and bio-agent targets, these protocols and devices are equally amenable to use in a clinical setting, for food testing, or for forensics. They fill a vital and often neglected need in the sample-analysis process.

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