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TITLE: Biological Agent Sample Preparation for the Detection
and Identification of Multiple Agents by Nucleic
Acid-Based Analysis

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13. ABSTRACT (Maximum 200 Words) We have designed, developed and tested an instrument prototype for automating the "front end" of nucleic acid test procedures. The AutoLyser" instrument which we have developed, provides fully automated purification of viral, bacterial and human genomic DNA and RNA from clinical samples, cell culture and swabs in as little as 10 minutes, and is capable of purifying between 1 and 8 samples at a time. No manual steps are required during a purification run. Samples are rendered non-infectious and are processed inside a disposable plastic device which can be quickly attached or removed from the instrument by relatively unskilled technical personnel. The possibility of contact with an infectious sample is almost eliminated. Centrifugation is not required since reagents are moved within the sample cartridge by means of precision syringe pumps and reagent selection valves under software control. Purified nucleic acid template is eluted in water and is ready for use in PCR or other downstream operations.				
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Foreword

Phase I of the above contract was carried out by Instant Genetics, Inc. from Dec. 17, 1999 to June 16, 2000, during which time we demonstrated the feasibility of constructing a portable apparatus capable of purifying one DNA containing sample at a time and detecting a specific nucleic acid sequence of a target DNA.

The focus of work covered by this FINAL REPORT was to design and develop a DNA purification instrument which could purify multiple samples (up to 8 in number), with a goal of carrying this out within a 10 minute time frame.

Additional desirable features were to reduce the size and weight of each processing site and to provide for reagent storage within the instrument case. In order to keep the weight of individual modules to a transportable size it was decided to limit each instrument processing module to 4 sites and then to link multiple modules to a single computer via USB communication protocol. Each site would communicate randomly with the instrument, thereby preventing a malfunction in one site from interfering with the correct processing in other sites.

Background In July 1977 Instant Genetics signed a CRDA agreement with USAMRIID to facilitate the development of automated DNA purification instruments. Pursuant to this agreement, the Diagnostic Systems Division (DSD) of USAMRIID worked closely with Instant Genetics to provide testing of new instrument designs, evaluation of devices such as the IGene hand cartridge DNA purification system, and systematic evaluations of the yields of DNA from different instrument prototypes using different reagents and test protocols. DNA purification yield was evaluated by the DSD lab using quantitative and semi-quantitative nucleic acid (PCR) tests with instrumentation that was not available to Instant Genetics.

Shortly before the present work was funded, however, the US was attacked by airplanes and by anthrax, with a consequence that USAMRIID's mission was altered, and the PCR based lab services from USAMRIID were no longer available to Instant Genetics. This occurred at a time when the yields of DNA purified by a re-designed AutoLyser and by the IGene hand cartridges, which had been high, unexpectedly decreased.

Instant Genetics has recently obtained a Cepheid Smart Cycler system for its laboratory and work is currently in progress to restore the high DNA yields. We now look forward to the resumption of close cooperation between USAMRIID and IGene Diagnostics (Instant Genetics changed its name to IGene Diagnostics, Inc. in 2002) and to the refinement of an instrument system that can deliver high yields of DNA and RNA from blood, tissues and other biological samples, rapidly, safely and with minimum technical training.


Bob Fields, Ph.D., Principal Investigator

1 December 2002
Date

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Introduction

The detection of nucleic acid (NA) sequences using PCR, or other DNA or RNA amplification methods, requires first that the nucleic acid be isolated and purified from a starting material. For "real time" PCR this purified NA must then be combined with enzymes, buffers, salts, nucleoside triphosphates, DNA primers, and dyes or detection probes in a composition such that thermal cycling will exponentially increase the number of target NA sequences.

In the case of an RNA target, a reverse transcriptase (RT) enzyme must first be used to produce a cDNA to act as a template for PCR amplification, or a DNA polymerase enzyme having RT activity must be used. This RT step introduces a delay in the total time required to obtain a PCR result but it does not change the requirements for the thermal cycling and detection apparatus. It does increase the requirement for template purity and protection. If the target RNA template exists as a small number of molecules in the presence of a much larger amount of other nucleic acids, synthetic RNA, termed "carrier RNA", must be added early in the purification procedure to protect the target RNA from losses. If a separate RT enzyme is being employed, additional enzyme inhibitors must be guarded against, since the final yield of amplified DNA will be the product of two separate enzyme-mediated steps, each of which may have its own inhibitors.

The most effective purification strategy for each type of sample matrix depends on many factors, including the nature of the molecular structures surrounding the NA. Is the target NA sequence enveloped within large amounts RNA and DNA, or is there a surrounding protein capsid? How tightly bound are the matrix materials? Are there covalent bonds between the target NA and other molecules that must first be broken before purification is attempted? If so, which enzyme or reducing reagent should be used in a "pre-purification" digestion or for the reduction of disulfide bonds? Does the original material need physical disruption, such as liver or brain tissue, which needs to be dissolved using a glass/teflon homogenizer, or frozen bone or tooth material which must be fractured into a powder form before liquid extraction? Does sonication or boiling of the sample before purification increase the yield? If so, what is the optimum length of time for such treatment? Finally, will a special filtration step be required to remove enzyme inhibitors? Whole blood samples, for example, contain heme degradation products that inhibit TAQ enzyme, even at very low concentrations, if not removed.

The AutoLyser® instrument that has been constructed under this contract is controlled entirely by software. All sample handling steps are carried out automatically by software without a requirement for manual manipulations. The AutoLyser® automatically withdraws a liquid sample into a sealed extractor cartridge, provides agitation to liberate the nucleic acids, adsorbs the impure NA onto a membrane in the device and then washes the bound NA using one, two or three wash solutions, finally dispensing purified NA into a screw top vial.

Body

Note: the Figures referred to below are located in the Appendix at the end of this Report.

Figure 1, Overview of the AutoLyser® system, is a montage of 3 pictures which together illustrate the main features of the instrument which has been developed by Instant Genetics for USAMRIID under this contract.

A and B show two instrument modules, each of which contains 4 sample processing sites, numbered 1 - 8. It will be noted that each site has 2 Snap-on Cartridge Holders and 3 tube holders (black circles) associated with it. The Carrying Handles allow the modules to be moved and transported easily. The Luer Lock fittings connect to fittings on IGene Cartridges and allow reagents to enter these Cartridges when a NA purification is being carried out.

C shows a laptop computer which has the AutoLyser® software installed. This software runs under the Windows 98 operating system, and so may be installed on any benchtop as well as laptop Windows computer. The communication between the computer and the two modules is by means of 2 standard USB cables, which are not shown in the picture.

D is a hand-held Laser scanner similar to the type used in super markets which is used to scan barcodes on Sample tubes (if these are present), on the vial that is to receive the purified nucleic acids, and to scan the lot number of the Reagents and Cartridge extractors that are used by the instrument. All this data archived in a log file, for permanent documentation of each run.

E shows a picture of the IGene disposable sample processing Cartridge. These cartridges are supplied in sealed, tear-open Autoclave bags (see Fig. 4), and are attached to the instrument console by pushing them down onto the Snap-on Cartridge Holders, where they are locked into place. The cartridge has a vial attached which is to receive the purified nucleic acids. This vial has a barcode label with a unique barcode number and an additional cap attached to it, shown in red in the photograph. At the conclusion of a nucleic acid purification run, this vial is unscrewed from the cartridge and the attached cap is immediately screwed on, thereby minimizing the chance of receiving airborne NA contamination, or conversely, releasing any of its purified NA into the ambient air.

F shows a Cartridge held in place on an instrument console. The nucleic acid sample is brought to the instrument in a sealed Sample Input Tube and then is directly attached to the cartridge via a cap and screw threads on a siphon tip tube, as shown in F. In this way ambient NA contamination is reduced to a minimum when attaching a Sample Input Tube to the cartridge.

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Biological Agent Sample Preparation

Figure 2 shows the Startup Screen for the AutoLyser® when only one 4-Site Module is being used. Extractor cartridges are placed on the instrument, and the sample I.D. is entered next to the Site number that has been selected for the DNA purification Cartridge. The barcode on the Nucleic Acid vial attached to the Cartridge that will receive purified nucleic acids is then scanned in, next to the Sample I.D. In this way, the Sample ID is permanently locked with the barcoded identifier number on the nucleic acid vial before, even the purification run has begun. This information is permanently stored in a Log file. Above the Sample ID entry fields there is shown the name of the Extraction Program that is being used, the Run Number and the Date and time of run. After the Run program has been selected these 3 fields are updated automatically by the Instrument, so that only Sample ID and barcode need to be scanned in. Once this has been done, the "Begin Run" button is clicked and those Sites which have had information entered will be processed automatically with no further user interaction required. Sites with no Sample ID will be ignored by the software.

Figure 3 shows the Startup Screen for an 8-Site AutoLyser® instrument. Any of the 8 Sites may be used for purification, or any combination or all of them may be used for a run. In this version of the software, once a run has begun, new Sites may not be added for sample processing until after the first run has completed. However if the maximum number of sites are being used, and further samples are to be processed, information for the next run, including barcoded NA vial numbers, may be entered into the startup screen while the current run is in progress. At the conclusion of the run, the NA vials are removed, the used Cartridges lifted off the instrument and placed in the trash, and the new Cartridges are attached. One has only to click "Begin Run" to begin the processing of a further 8 samples. The setup time between consecutive runs is shortened to less than two minutes for 8 samples.

On each side of the main screen there are "clickable" buttons. These buttons allow convenient access to all instrument operations that may be required for purification of NA and for maintenance of the AutoLyser® instrument. The top left green button "Load New Program" allows the user to select and load particular software processing programs, the orange button below, "Purge Reagent Lines" automatically flushes out all reagent lines to remove air bubbles and replace old reagent with fresh reagent after changing a reagent reservoirs. "New Run" is self explanatory; "Abort Run", on the upper right hand side, halts all further actions in all Sites. "Examine LOG" brings up a window displaying a comprehensive log file in ASCII format, which may be saved as a file, printed or attached to an email. "Quit" is used when it is desired to turn the instrument off. The "Enter Setup" button brings up a new window in which the lot numbers of reagents may be scanned in, when changing reagents. The "Edit Software" button brings up the purification program, written as a text file, which can be edited and saved by the user. A message window below the main window gives prompts to the user for the software operation, as well as error messages.

Figure 4 shows an IGene Cartridge in its "tear open" Autoclave bag, as it is supplied to the user. The barcode on the purified NA vial may be seen, as well as the barcode giving the lot number of the Cartridge. The cartridge is locked onto the AutoLyser® console by pressing the Cartridge internal valve handles onto the two Snap-on Cartridge holders show in **Fig. 1-F**.

Figure 5 shows a Reagent Bottle with its barcoded label and a number of reagents inside the reagent compartment of an AutoLyser® 4-Site Module. The sealed reagent bottle is used as its own reservoir, and simply replaces an old reagent bottle in the instrument with no decanting or making up of reagents required. The drop-down Reagent door gives easy access to all reagents from the outside, and allows the liquid level of each reservoir to be observed through the window in the door.

AutoLyser® Engineering

It was required that we consolidate, as far as possible, the motorized elements of a single site AutoLyser® purification instrument in order to create an instrument that was capable of carrying out 8 NA purifications which would also be easily transportable. **Fig.6** shows a consolidated cartridge actuator module, in which we were able to bring two sample processing sites together into a single module. Two of these Dual Site Cartridge Actuators were positioned side by side in a highly compact Four Site AutoLyser® chassis, as shown in **Fig. 8**.

An additional design objective to facilitate service and maintenance operations and to make access to each instrument module direct without the necessity of removing other components. Each sample processing site of the AutoLyser® needs to have one digital syringe pump and one reagent selection valve. The Four Site AutoLyser®, therefore, requires 4 digital Syringe pumps and 4 reagent selector valves. These devices are interconnected by power and communication cables and also by fluid delivery lines. **Fig. 7** shows how these eight digital components have been mounted together on a tray which can be slid out from the interior of the instrument console. Each pump and valve component is rigidly held on the tray by means of two screws which enter from below the tray. Cables are attached to each component from a cable harness, by means of removable plugs at the back, and Teflon reagent tubing has been organized into bundles that allow the entire instrument tray to be removed without having to disconnect any of the elements. If it is required to change a single valve or syringe pump, the only fluid fittings that need to be unscrewed are those connected to the particular device. The replacement device then needs only be secured with 2 screws from below and connected to the fluid lines and electric push on edge connector plugs. The design shown in **Fig 7** allows this to be carried out in a short time.

In the same way, the Dual Site Cartridge Actuators described above are secured to the Instrument chassis with 4 thumb screws; power and communications are delivered to each unit by means of removable edge connectors. **Fig. 8** shows the accessibility of these modules after the instrument cover has been removed. Such modules can be quickly swapped out for new modules to keep the instrument running, and to allow examination and service of the old module to be carried out remotely. **Fig. 8** also shows the tops of the syringe pumps after the component tray has been returned to its normal position. The bundles of reagent lines may also be seen returning to the reagent reservoir section of the instrument.

Precision Fluid Handling

The AutoLyser® digital syringe pumps are capable of pumping precisely at flow rates calibrated in microliters per hour, since the syringe pistons in these pumps are advanced by stepper motors receiving instructions from a computer. In practice however, precise delivery of small amounts of liquids depends importantly on other factors, such as the internal geometry of valves and the fluid pathways through which the liquids must pass. Many refinements were made to the AutoLyser®'s fluid manipulation system, as reproducibility is a fundamental requirement for an analytical instrument that is to repetitively purify nucleic acids. **Fig. 9** shows one example of an engineering and manufacturing solution that was applied to the fluid coupling between the AutoLyser® and its disposable purification Cartridge. The picture on the upper left shows the "dead space" that is formed in a conventional luer fitting, and how this was eliminated by altering both the male and the female luer fitting. An illustration of the results of this refinement which shows the reliable dispensing of 15 microliter drops into a relatively large diameter tube of the extractor Cartridge, is shown at the bottom of **Fig. 9**.

Tests

During most of the period of this Contract, the DSD lab of USAMRIID was not available to IGene Diagnostics to carry out tests of specific purification protocols for the AutoLyser®, or to enable joint working together to optimize particular combinations of sample treatments for purification of genomic DNA. Nevertheless, we were able to investigate several parameters of the AutoLyser® system, and some of our results are shown in **Fig. 10**. The picture of the gel at **A** shows the results of DNA recovered over a range from 100ng to 30µg from an AutoLyser purification. The oligonucleotides range in size from 35pb to 21,000bp and were derived from an EcoR1 digest of lambda phage DNA obtained from Sigma. The Standard lane shows the fluorescence of 1 ug DNA applied directly to the gel, and the other lanes are the DNA recovered from an AutoLyser® NA purification Cartridge after processing the amounts shown.

The reproducibility of recoveries of purified DNA over 4 orders of magnitude by the AutoLyser® is shown in the Standard curve at **B**, in which triplicate AutoLyser® runs were carried out using samples of e.coli plasmid having copy numbers between 100 thousand and 100 million.

Each four-site AutoLyser® instrument module has a footprint of 15" x 24" and is 9" high. The total weight is 48 lbs., allowing it to be manipulated and operated by one person. **Fig. 11** shows the ease with which the AutoLyser® may be transported from on lab to another, or into the field for use in a mobile setting.

Conclusions

We have developed a nucleic acid purification instrument that can be used to purify single or multiple samples of DNA or RNA without needing to be located in a separate clean room. The NA purifications take place inside a closed Cartridge. There is minimum setup time required, the instrument is robust, portable and built up from highly reliable components which can be programmed to carry out Nucleic Acid purifications from many different types of samples.

This instrument system is now ready to enter the protocol refinement stage, in which various sample treatment protocols will be compared in order to select the best program for each sample matrix. The robotic system that has been engineered is precise and the disposable Cartridge design allows multiple treatment protocols to be introduced, including enzyme digestions, pH changes, solvent extractions, heat treatments and specific filtration steps for removal of contaminants. The syringe pumps are capable of forcing viscous solutions through membranes, as may be required when dealing with a wide variety of unknown input sample types.

References

There were no journal submissions or publications disclosing any part of the work supported by this award.

No scientific presentations were made regarding the instrument that was supported by this award.

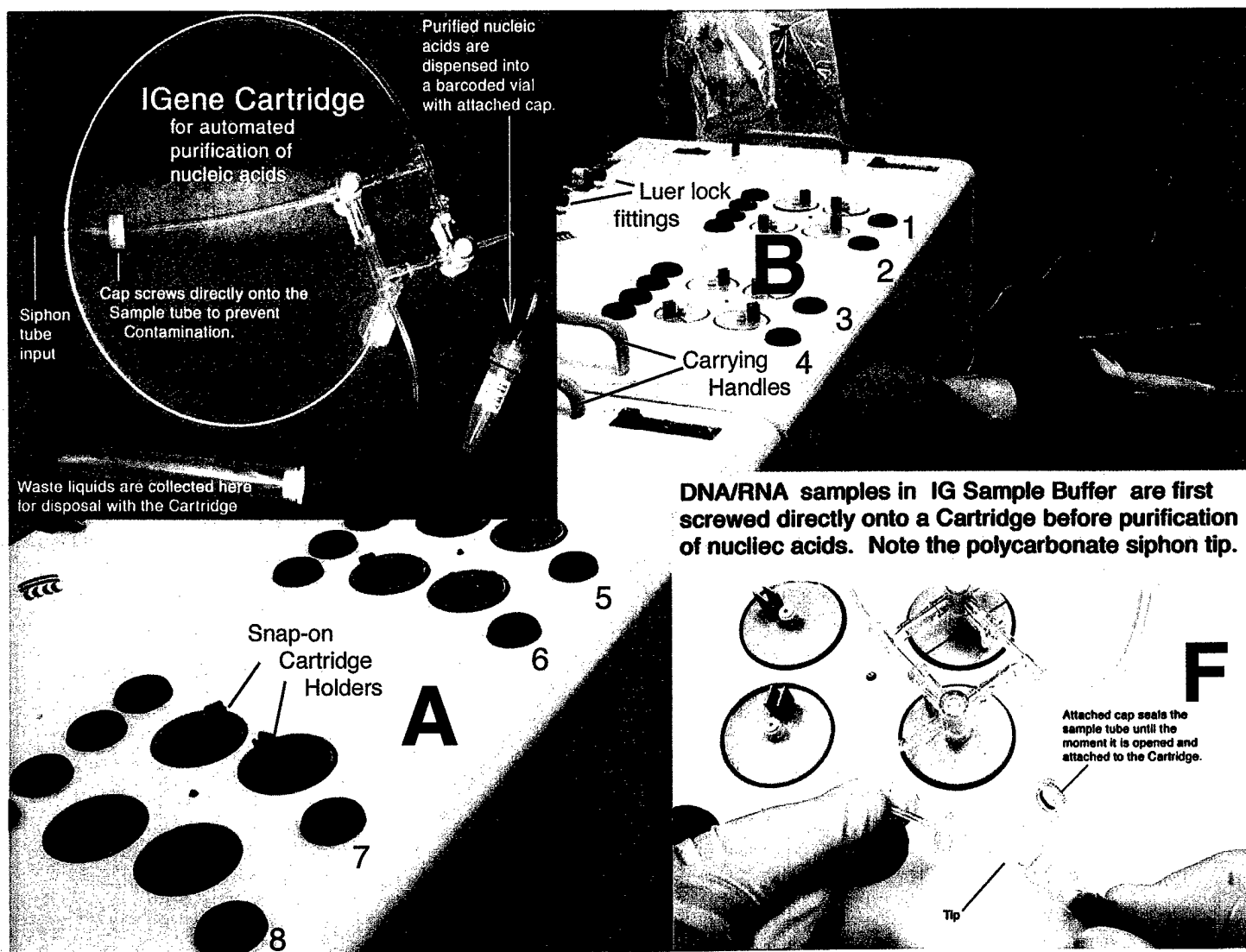
No person received pay or obtained a graduate degree from this award.

Appendices:

- Figure 1 Overview of the AutoLyser® system
- Figure 2 AutoLyser® Start up Screen for 4-Site
- Figure 3 AutoLyser® Start up Screen for 8-Site
- Figure 4 Cartridge in sealed packaging
- Figure 5 Reagent bottle
- Figure 6 Consolidation of Cartridge Actuator
- Figure 7 Consolidation of Syringe Pumps & Valves
- Figure 8 Front View of AutoLyser®
- Figure 9 Engineering of Fluid Delivery
- Figure 10 Test Results of AutoLyser®
- Figure 11 Transport of AutoLyser®
- Figure 12 Summary of AutoLyser® Features

Fig. 1

Overview of the AutoLyser® system



Please refer to the text on page 6 of the Final Report for detailed explanations of elements labelled A through F, above.

Fig. 2

AutoLyser® Start-up Screen for 4-Site (1 Module) configuration

The screenshot shows the IGene Diagnostics software interface. At the top, the title "IGene Diagnostics" is displayed in a stylized font. Below the title, there are three input fields: "Extraction Program" with the value "1-100ul", "Tube Number" with the value "1", and "Date" with the value "02/05/2003". To the right of these fields is an "ABOUT RUN" button. Below the input fields is a table with three columns: "Station", "Sample Volume (L)", and "Nucleic Acid Via No.". The table has four rows, each with a station number (1, 2, 3, 4) and empty input fields for the sample volume and nucleic acid via number. To the right of the table is a "Quit" button. At the bottom of the screen, there is a text box with the following instructions: "After new Extraction is attached, Enter Sample ID, NA Vol No. & Load New Program (if necessary). Then click [Begin Run]."

Station	Sample Volume (L)	Nucleic Acid Via No.
1		
2		
3		
4		

Fig. 3

AutoLyser® Start-up Screen for 8-Site (2 Module) configuration

IGene Diagnostics

Extractor Program: Run Number: Date: **2/5/03**

Station	Sample Name/ID	Nucleic Acid Vial No.
1	<input type="text"/>	<input type="text"/>
2	<input type="text"/>	<input type="text"/>
3	<input type="text"/>	<input type="text"/>
4	<input type="text"/>	<input type="text"/>
5	<input type="text"/>	<input type="text"/>
6	<input type="text"/>	<input type="text"/>
7	<input type="text"/>	<input type="text"/>
8	<input type="text"/>	<input type="text"/>

After new Extractor is attached,
Enter Sample ID, NA Vial No. & Load New Program
(if necessary). Then click [Begin Run].

Fig. 4

Cartridge in sealed packaging

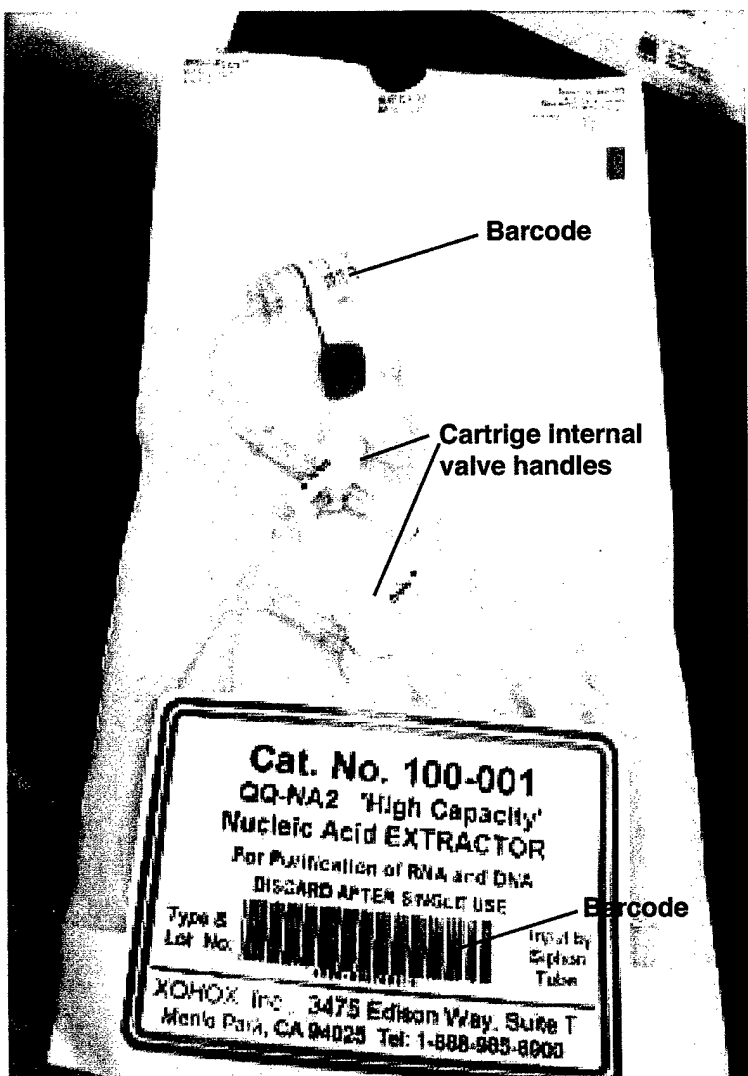


Fig. 5

Reagents are easily replenished.

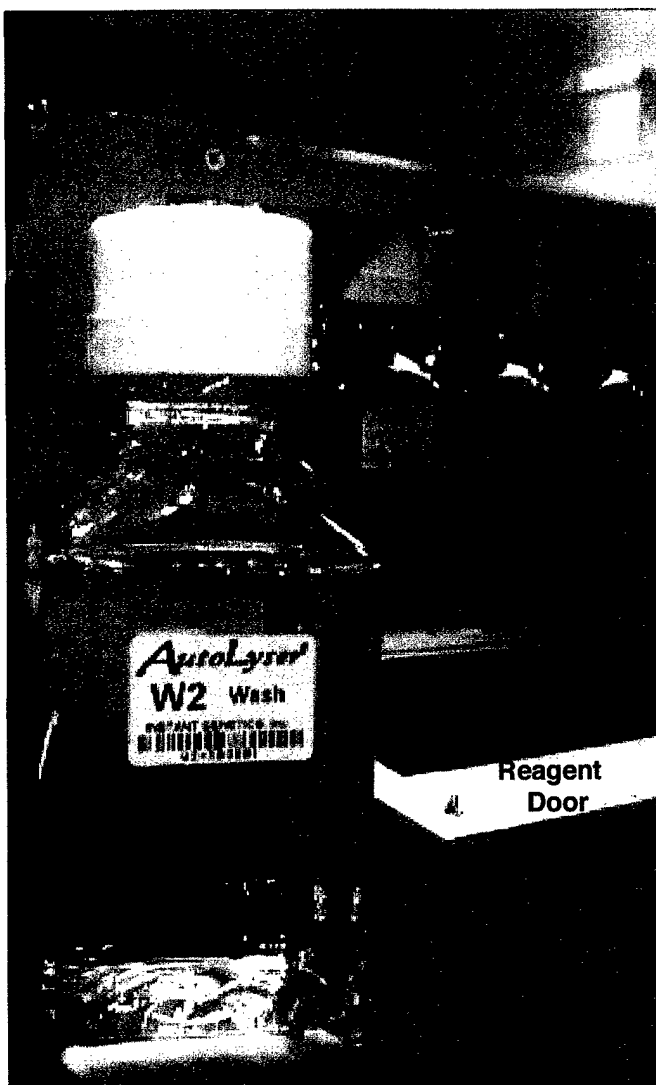


Fig. 6

Consolidation of Two Cartridge Actuator Modules into one Dual Site Module

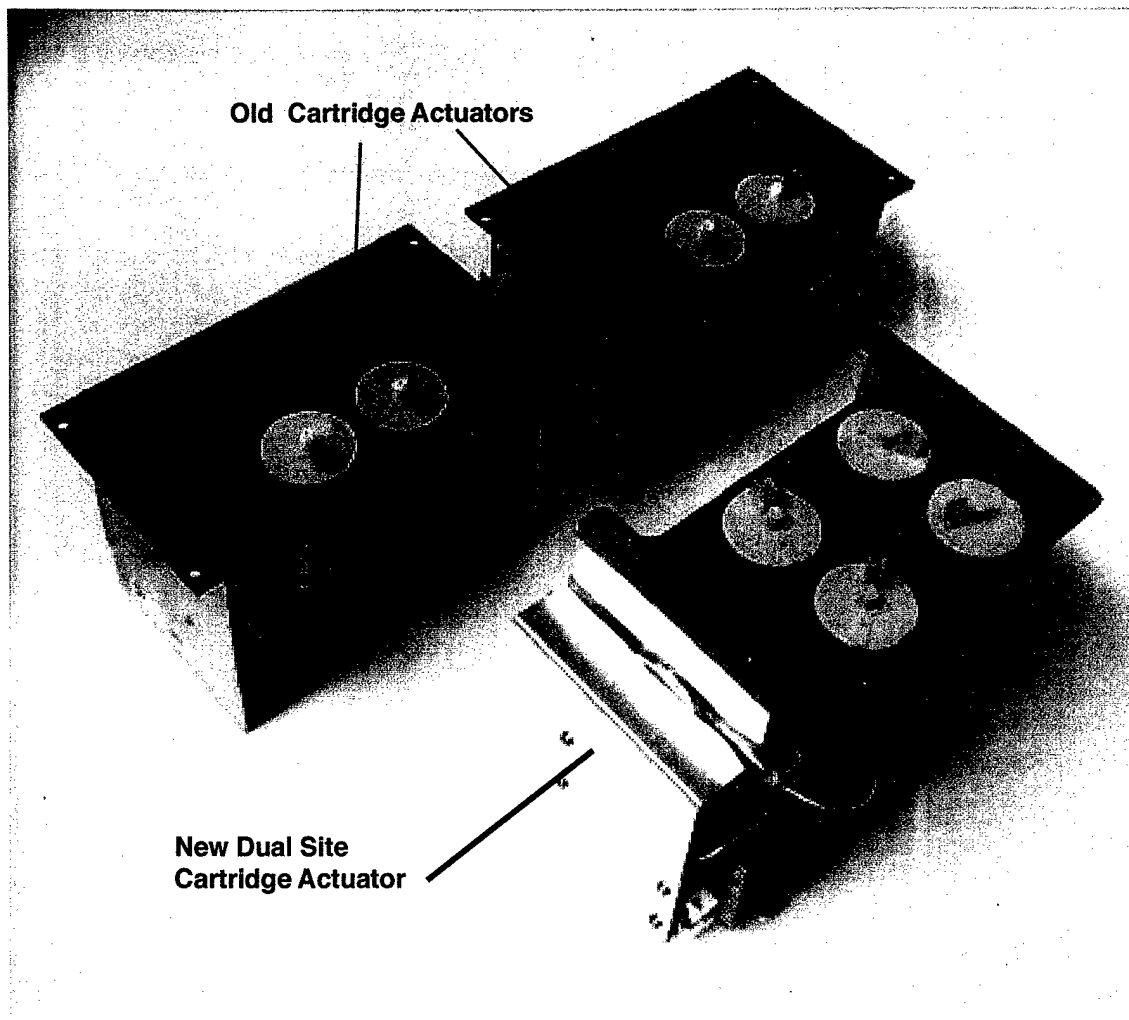


Fig. 7

Consolidation of Four Sets of Syringe Pumps and Reagent Selection Valves onto one Tray

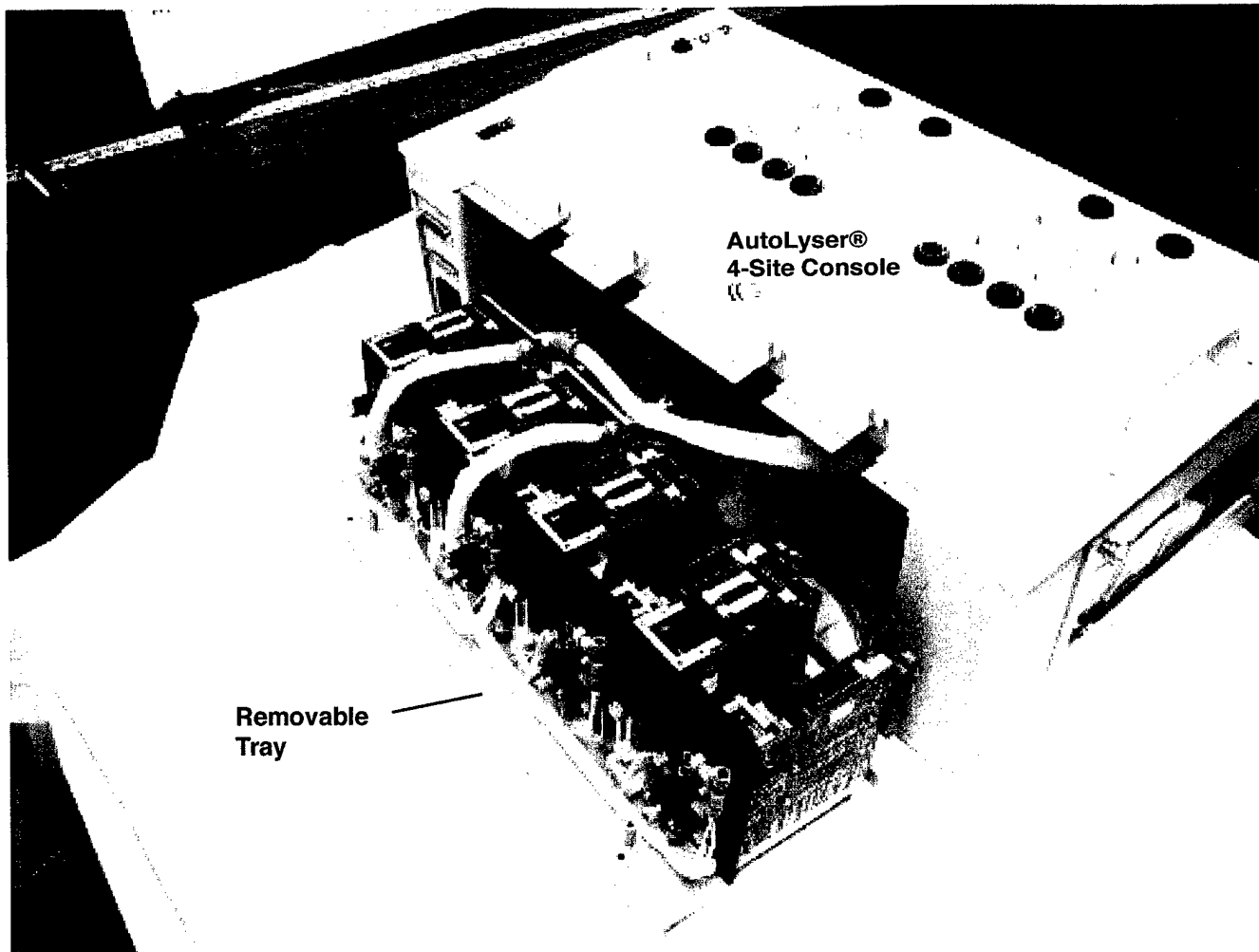


Fig. 8

Front View of AutoLyser® with Cover Removed

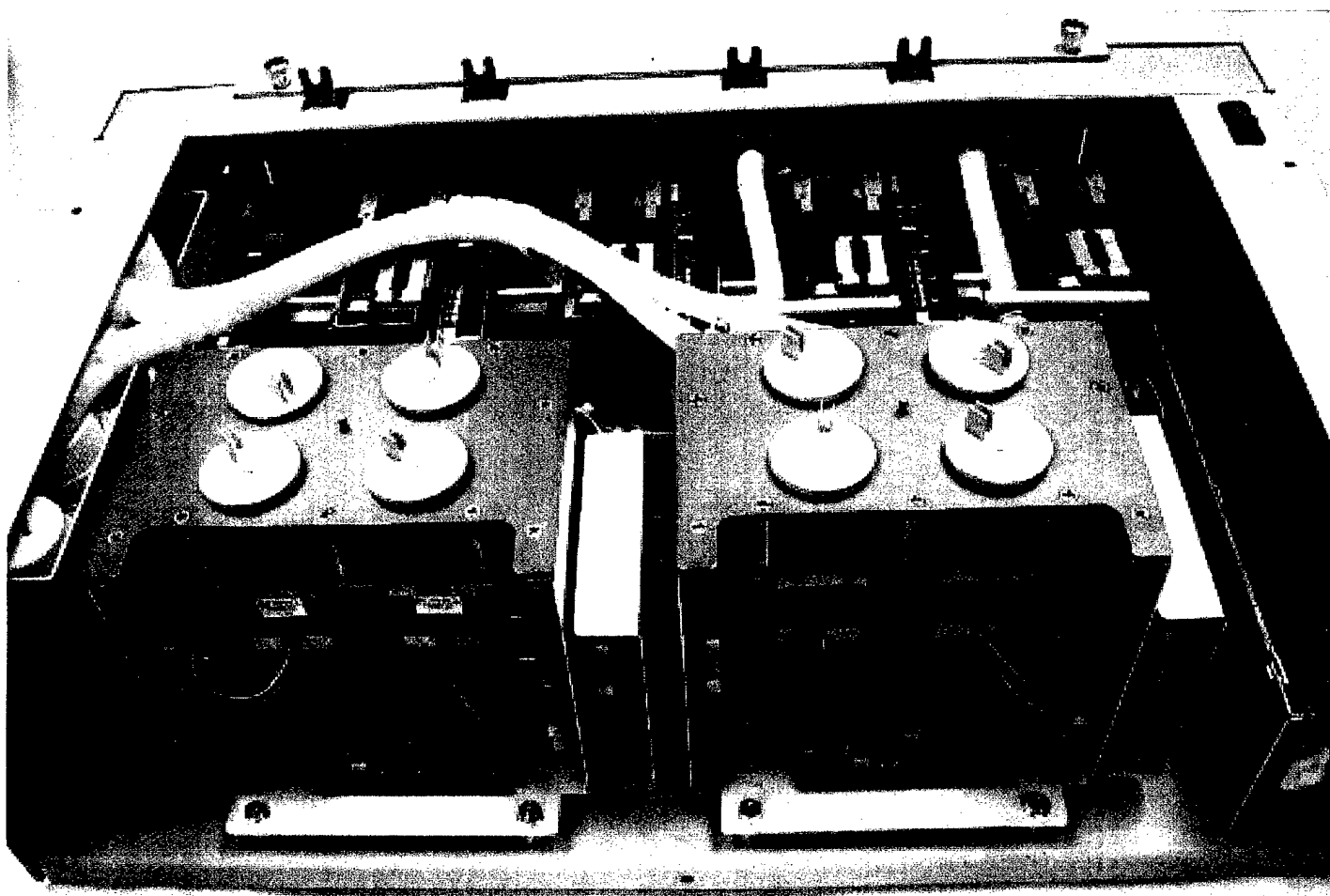
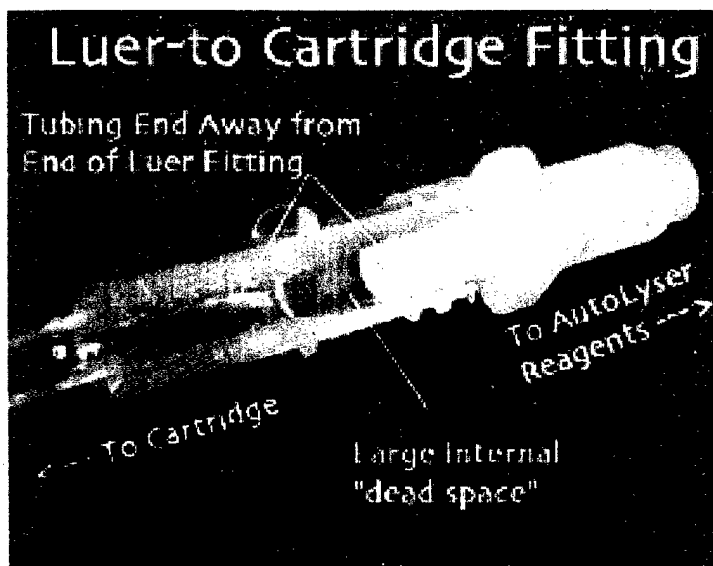


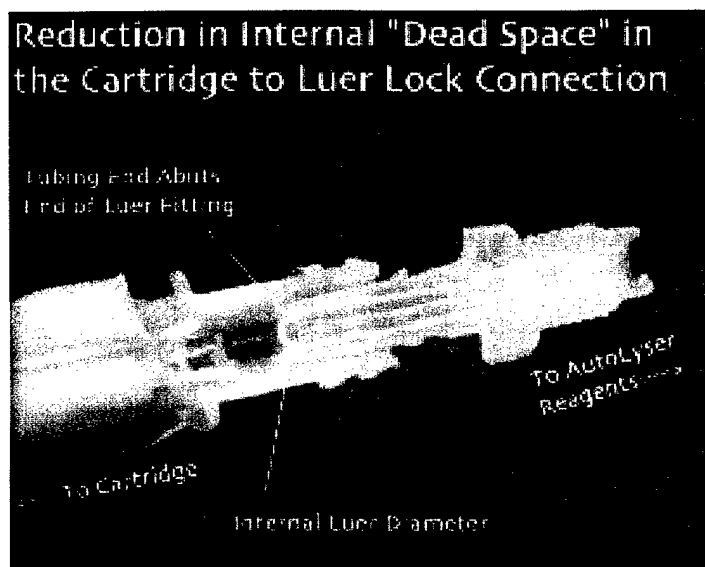
Fig. 9

Engineering the AutoLyser® Fluid Delivery System to Permit Control of Small Volumes

Old Cartridge Attachment



New Cartridge Attachment system



The Result: Precise control of small microliter volumes of reagent and Nucleic Acid solution

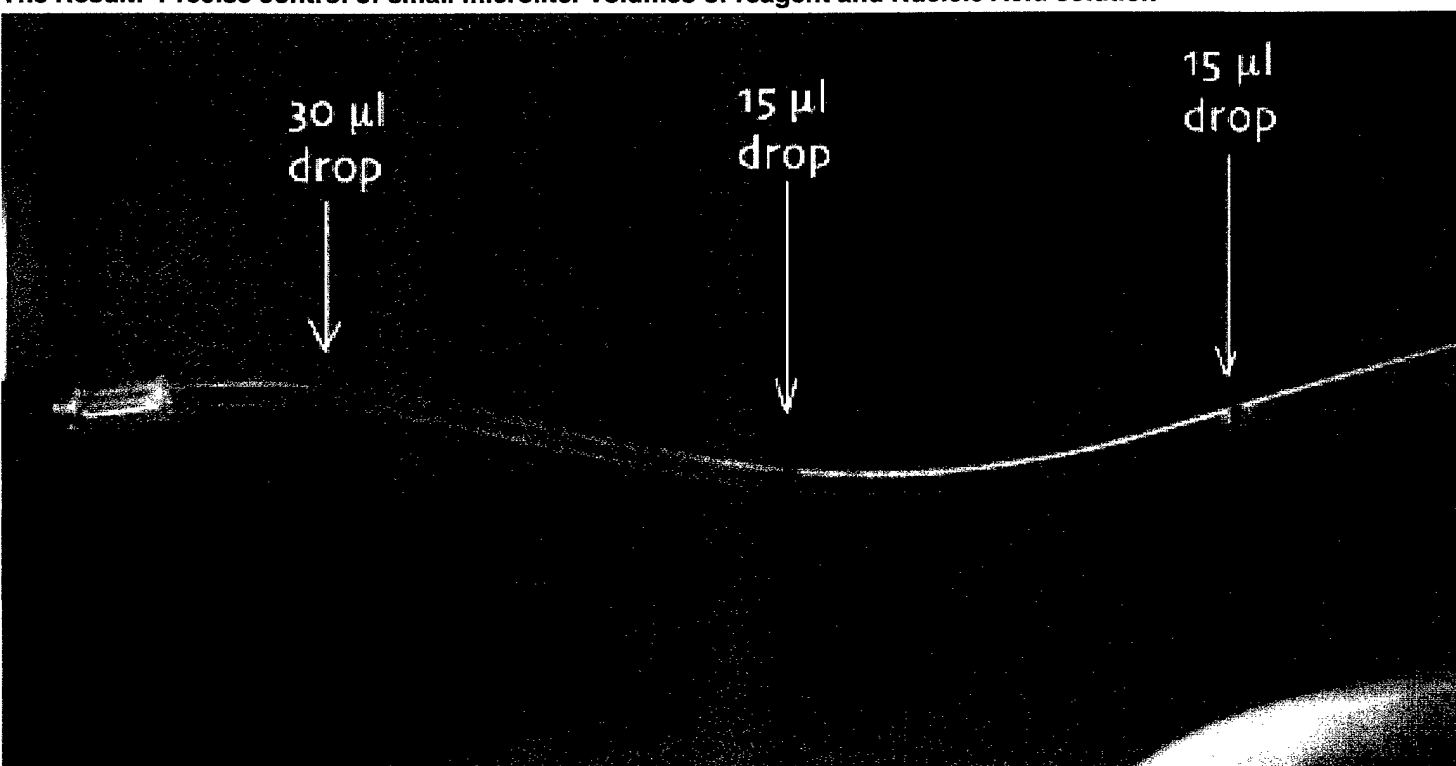
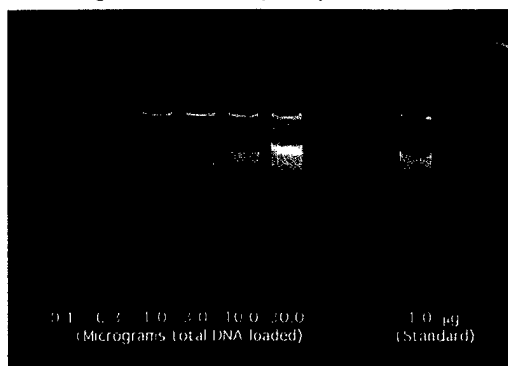


Fig. 10

Test Results For AutoLyser® DNA Extraction

A. Capacity of the IGene Cartridge for purifying DNA

Recovery of DNA from an EcoRI digest of lambda phage DNA using automated AutoLyser® purification.



Bands visualized on a 6% TBE gel using SYBR® Green I dye.

B. Reproducibility

Reproducibility of AutoLyser® purification of *e. coli* plasmid DNA over 4 orders of magnitude dilution

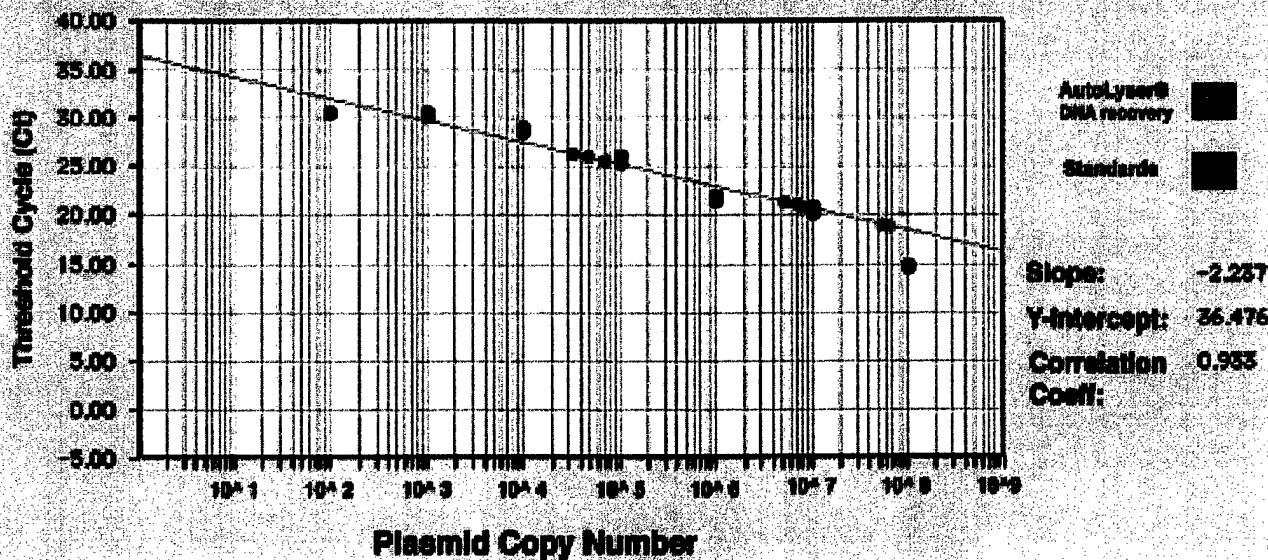


Fig. 11

Easy Transport and Quick Setup of AutoLyser® NA Purification

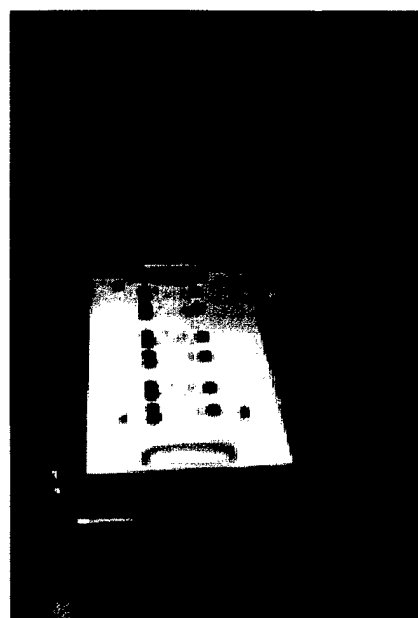
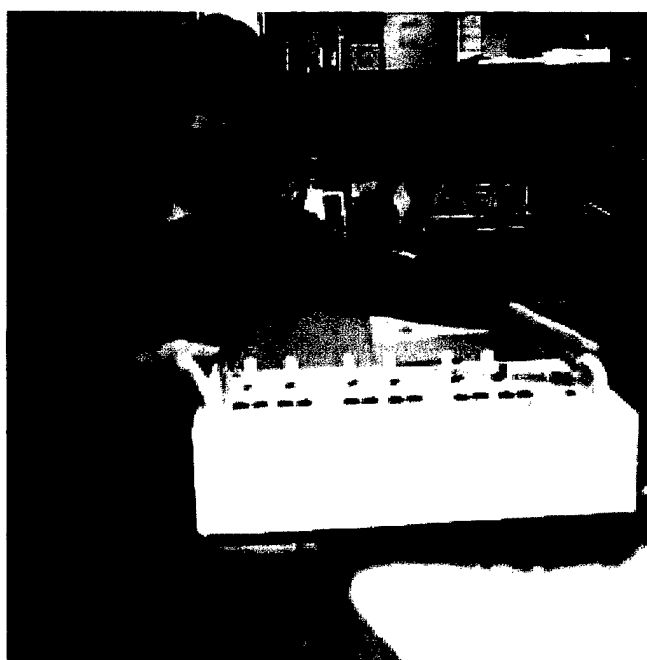


Fig. 12

Summary of AutoLyser® Features

● Rapid processing speed

DNA and RNA can be purified from cells, plasma or swabs in as little as 5 minutes, depending on the sample type. The template is eluted in water and is immediately ready for use in PCR or other downstream operations.

● Fully automated and reproducible

The AutoLyser® completely automates the "front end" of nucleic acid testing and makes it possible to quickly prepare one or more samples for PCR whenever test results are needed. Reproducibility is the end result of automation and is not dependent on the training or skill of a user.

● Processes up to 1.0 ml sample volumes

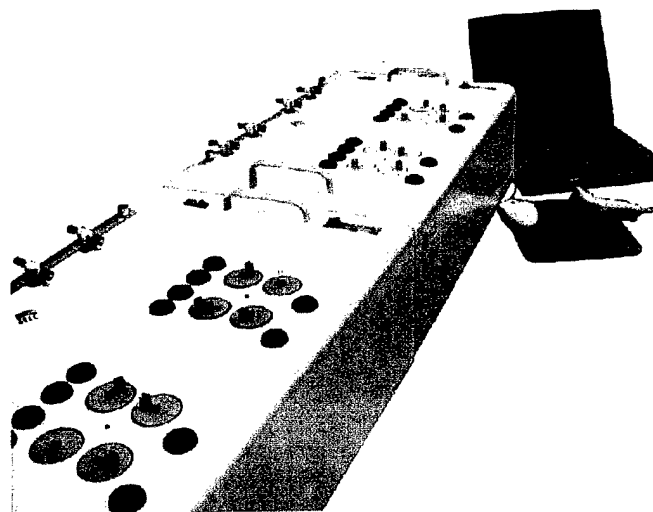
Large sample volumes of extracts or environmental liquids may be directly processed without the need for a separate sample concentration step.

● Low risk of worker infection

Samples are rapidly rendered non-infectious and are processed entirely within a closed cartridge. The possibility of contact with an infectious sample is almost eliminated.

● No technical skills required

The AutoLyser® instrument is simple to load and operate. There is very little instrument set up time, no manual steps are required during the run, and minimum maintenance.



● Low risk of cross contamination

Samples are processed and combined with PCR test components within a disposable sealed cartridge.

● No centrifugation

Reagents are moved within the sample cartridge by means of precision syringe pumps and reagent selection valves. Centrifugation is not required.

● Completely Transportable

The AutoLyser® is a self-contained, portable instrument, able to be taken into a remote environment for rapid, on-site purification of nucleic acids.

Comparison of AutoLyser® features with other systems:

System:	Setup Time	Number of Samples Run	Time to Purify RNA/DNA	Architecture	Mobility
Autolyser	3 minutes	8 samples	5 - 15 min.	sealed Cartridge	Portable
Roche MagNA pure	30 minutes	32 samples	70 minutes	open architecture	Not portable
Nuclisense	35 minutes	32 samples	65 minutes	open architecture	Not portable
BioRobot	30 minutes	96 samples	45 minutes	open architecture	Not portable