ORNL/CP-102289

AUTOMATED DNA SEQUENCING SYSTEM*

R. L. Kress, G. A. Armstrong, and C. P. Ekkebus
Oak Ridge National Laboratory
Robotics and Process Systems Disision
P. O. Box 2008
Oak Ridge, Tennessee 37831-6426

R. J. Mural and L. J. Hauser
Oak Ridge National Laboratory
Life Science Division
P. O. Box 2008
Oak Ridge, Tennessee 37831

To be presented at the
American Nuclear Society Eighth International
Topical Meeting on Robotics & Remote Systems
Pittsburgh, Pennsylvania
April 1999

*Research sponsored by Oak Ridge National Laboratory, managed and operated by Lockheed Martin Energy Research Corp. for the U.S. Department of Energy under contract number DE-AC05-96OR22464.

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R. L. Kress, R. J. Mural, G.A. Armstrong, C.P. Ekkebus, L.J. Hauser (Oak Ridge National Laboratory)
P.O. Box 2008, Oak Ridge, TN 37831-6426
email: kressrl@ornl.gov
Tel:423-574-2468

ABSTRACT

Oak Ridge National Laboratory (ORNL) is developing a core DNA sequencing facility to support biological research endeavors at ORNL and to conduct basic sequencing automation research. This facility is novel because its development is based on existing standard biology laboratory equipment; thus, the development process is of interest to the many small laboratories trying to use automation to control costs and increase throughput. Before automation, biology laboratory personnel purified DNA, completed cycle sequencing, and prepared 96-well sample plates with commercially available hardware designed specifically for each step in the process. Following purification and thermal cycling, an automated sequencing machine was used for the sequencing. A technician handled all movement of the 96-well sample plates between machines. To automate the process, ORNL is adding a CRS Robotics A-465 arm, ABI 377 sequencing machine, automated centrifuge, automated refrigerator, and possibly an automated SpeedVac. The entire system will be integrated with one central controller that will direct each machine and the robot. The goal of this system is to completely automate the sequencing procedure from bacterial cell samples through ready-to-be-sequenced DNA and ultimately to completed sequence. The system will be flexible and will accommodate different chemistries than existing automated sequencing lines. The system will be expanded in the future to include colony picking and/or actual sequencing. This discrete event, DNA sequencing system will demonstrate that smaller sequencing labs can achieve cost-effective automation that can be expanded or changed as the needs of the laboratory grow.

Introduction

Automation of manufacturing occurs in specific stages of evolution. Difficult and tedious hand processes are automated first, creating a batch automated system in which intermediate steps and transitions depend on humans. Eventually, batch processes are combined, new ones are automated, and transitions between steps become the responsibility of a robot or fixed automated systems. Thus, a fully automated system is achieved. These stages occurred within the automotive industry decades ago, and today, a similar evolution of automation is occurring within the world of biology.

Many different areas of biology are experiencing a tremendous need for automation; the *Human Genome Project* (words and phrases italicized upon first use are defined in Appendix A), however, has generated a great deal of interest in high-throughput DNA *sequencing*. In the late 1980s, a goal was set to sequence all three billion base pairs (bp) of the *human genome*. After a few years of effort studies showed scientists almost as far from their goal as when they began, automation became the method of choice for achieving high throughput DNA sequencing. (Informative references for a simple background on sequencing are [1], [2], and [3].)

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The research community still needs a flexible, continuous-flow, automated DNA sequencing system. The system should be flexible in regards to the chemistries used and the hardware in place; if either needs to be altered, the change should be possible without major hassles in logistics, software, hardware, and cost. Oak Ridge National Laboratory (ORNL) is currently creating such a system. Government institutions and industry laboratories do not share academia's discounted price on labor. Because of this and other dilemmas, a great deal of research is devoted to moving DNA sequencing from labor-intensive, work-cell like automation with separate batch processes to a more fully automated approach. Eventually, it is expected that DNA sequencing will become like other chemical processes and move from batch operations to continuous-flow operations. However, a natural step in the change to a fully continuous flow process is the more complete automation of the batch process. This paper describes the approach taken at ORNL to automate the batch processes. Included are a brief discussion of other automated DNA sequencing centers, an overview of ORNL's current DNA sequencing approach and hardware, a section on the added automation expected for ORNL's laboratory, simulation results, and conclusions. Definitions for terms generally unfamiliar to robotics and automation researchers are included in Appendix A, and some biochemical processing robots are compared in Appendix B.

Background on Automated DNA Sequencing Centers

Hawkins et al. at MIT's Whithead Institute and the Center for Genome Research have developed the Sequatron series of automated DNA sequencing systems as referenced in [4]. Their integrated system emulates human manipulations performed in a standard laboratory environment. They have three production Sequatron systems to do the following: (1) DNA purification from M13 phage and *polymerase chain reaction (PCR)* products; (2) DNA sequencing reactions, using dye-primer and dye-terminator chemistries; and (3) finishing, which involves performing custom PCR amplifications and sequencing reactions on selected templates to close open gaps in an incomplete sequence. The sequencing Sequatron is based on a CRS Robotics A-465 arm and can perform approximately 2000 sequencing operations in three hours. Although this is an integrated automated DNA sequencing system, it is not flawless. Critical interface steps lack automation, and the complete system lacks flexibility. For example, it depends on a purification chemistry (solid phase reversible immobilization, or SPRI) that uses magnets and multiple washings to avoid centrifugation and solvent extractions as referenced in [4].

Washington University in St. Louis currently supports the Genome Sequencing Center (GSC), which produces approximately 25 *Mbp* of finished DNA sequence per year. The GSC is one of the world's premier centers because of its high throughput. Multiple (more than 60) lines of batch automation achieve this production, but a high degree of technician interaction is still required. Batch automation continues to be viable at the GSC because of low labor costs.

Excellent examples of automated DNA sequencing can also be found at the following institutions web site:

1) The McDermott Center at The University of Texas Southwest – http://gestec.swmed.edu/gas.htm
2) Lawrence Livermore National Laboratory – http://www.llnl.gov/automation-robotics/automation.html
3) Stanford – http://www-sequence.stanford.edu/group/techdev/auto.html
4) The SANGER Centre – http://www.sanger.ac.uk/Teams/Engineering/
5) European Molecular Biology Laboratory – http://www.embl-heidelberg.de/
6) The University of Washington – http://isdl.ee.washington.edu/GNL/acapella

This list is not intended to be all-inclusive and should merely serve as a starting point for the interested researcher.

Semiautomated DNA Sequencing Hardware Currently used at ORNL

DNA sequencing requires numerous operations by highly trained laboratory technicians and biologists. Operations include colony picking, pipetting, transferring reagents, centrifugation, vacuuming, filtering, mixing reagents, and transferring plates. These operations can be grouped into six broad steps:

1) Bacteria colony growth;
2) Sample colony extraction and preparation, including amplification of bacteria;
3) DNA removal and purification;
4) DNA amplification through the PCR process;
5) Sequencing preparation; and
6) Sequencing.

Table 1 summarizes these steps, the length of time required for these steps for the ORNL laboratory, and the specialized "machines" that can be used for these procedures if desired.

Table 1. DNA Sequencing Steps and Associated Hardware

Procedure	Approximate Time for ORNL lab (min)	Time requiring human interaction (%)	Specialized Machine(s)
Bacteria Growth	1440	<1	None
Sample Extraction and Preparation	45	100	Colony Picker Auto Centrifuge
DNA Purification	200	10	Processing Robot Automated Pipettor
DNA Amplification	210	16	PCR Machine
DNA Sequencing – Preparation	45	100	Automated Pipettor Auto. Centrifuge Auto. Vacuum
DNA Sequencing - Sequencing	360	<1	Sequencing Machine

In addition to the hardware indicated in Table 1, hand-held multichannel pipettors are also used for transferring reagents. Also note that standard biochemical processing "pallets" are referred to as microtiter plates and are further identified by the number of wells they contain. Common formats are 96-well microtiter plates arranged in an 8-by-12 well format and 384-well microtiter plates arranged like

the 96-well plates, with each well divided with a crosslike element forming 4 separate wells in each of the 96-wells. The last five steps in Table 1 are also illustrated in the process flow diagram in Figure 1, with inputs, outputs, and "pallets" identified. (The bacteria growth step is *not* shown in Figure 1.)

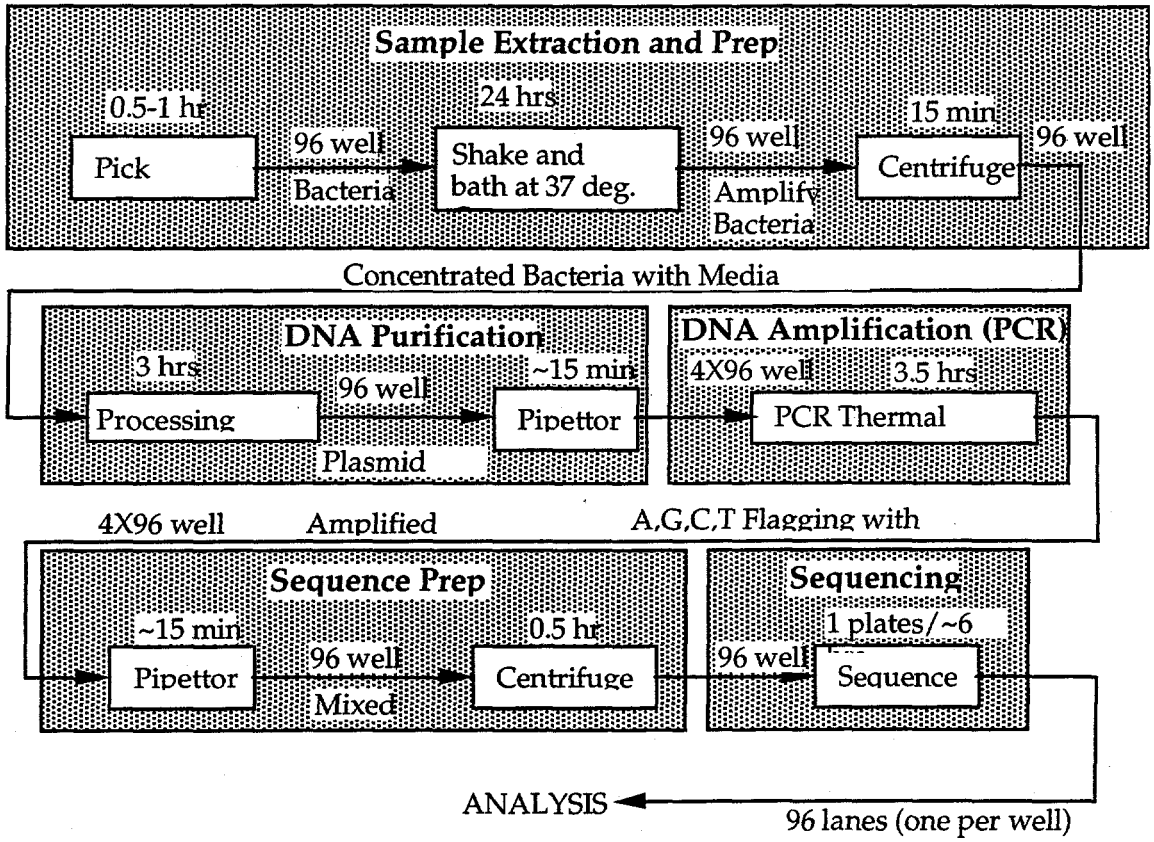

Fig. 1. ORNL's sequencing process flow following bacteria growth.

In ORNL's laboratory, bacteria colony picking is currently done by hand using standard laboratory techniques. Automated colony picking machines are commercially available, and there are plans to add one in the future. Refer to reference [1] for some examples and discussion of colony-picking machines.

A QIAGEN BioRobot 9600, shown in Figure 2, does DNA purification.

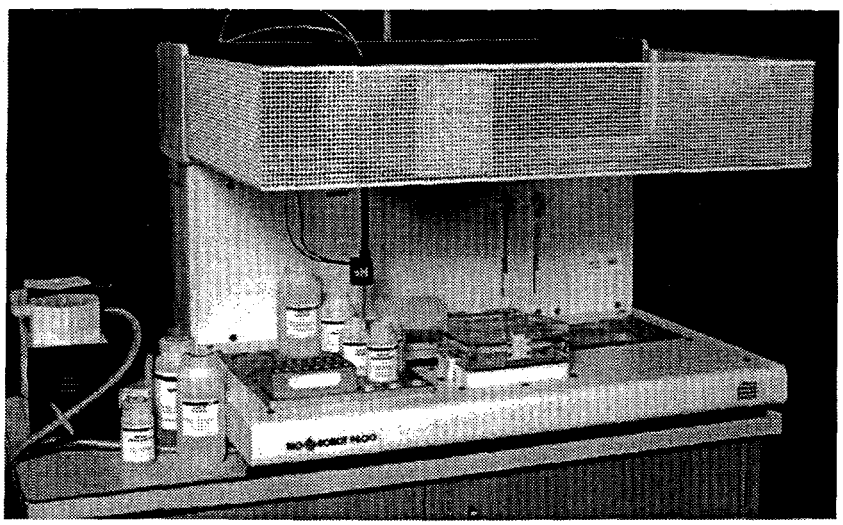

Fig. 2. QIAGEN BioRobot 9600.

This robot provides researchers flexibility in choosing purification chemistry; it uses a single pipettor, a shake rack, and a vacuum manifold to purify DNA. Protocols are ready to use on the connecting PC, however, scientists can program individualized chemistries which the robot will execute as well. A standard purification procedure using double stranded *plasmids* takes three hours and fifteen minutes. Other purification procedures can take longer. Technician interactions for ORNL's double stranded plasmid DNA purification include the following steps:

1) setup the robot (i.e., place reagents and bacteria pellets);
2) discard a filter, move a filter, place a tray;
3) blot/rap all ethanol out of filter;
4) remove waste tray and move microtiter plate; and
5) remove microtiter plate with purified DNA.

All five steps require technicians to open and close the BioRobot's vacuum manifold, a task that is difficult to automate and will require special modifications in the future. Similar purification and biochemical processing robots are available from other companies. A chart comparing a few of these machines with the BioRobot 9600 is provided in Appendix B.

The MJ Research Thermal Cycler (Tetrad), shown in Figure 3, automates the DNA amplification step. This thermal cycler uses *Peltier Blocks* to rapidly cycle and equilibrate temperature. It has motorized lids and a serial port, making it an ideal component in an automated system. Also, multiple programs containing temperatures and cycle times can be stored and recalled. In two hours, four 96-well plates containing suitable reaction mixtures and purified DNA can be cycled 60 times. Usually, cycle temperatures are 95, 50-55, and 70° C. The cycler can also be fitted to accommodate 384-well microtiter plates. In addition, it has a serial communication port to enable integration into the automated sequencing line and enable operation from a central controller.

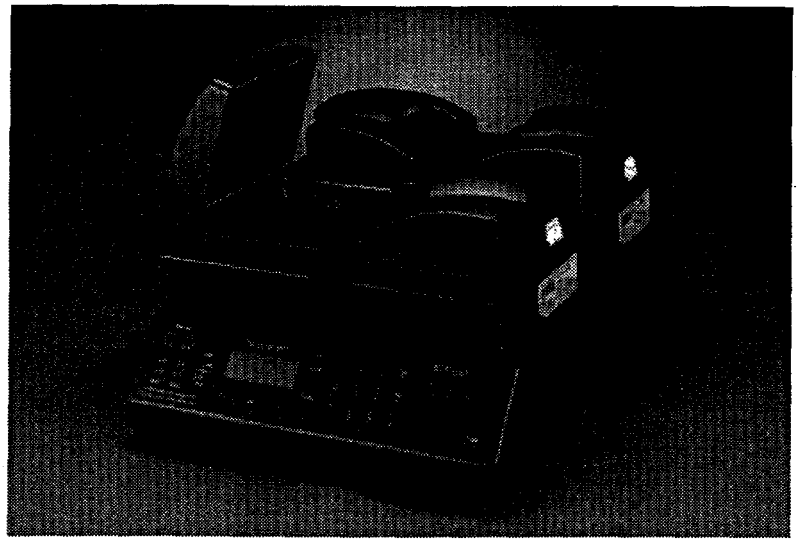

Fig. 3. MJ Research Thermal Cycler used for the PCR Step.

The Robbins Hydra 96 pipettor, shown in Figure 4, automates pipetting into 96-well microtiter plates. The Robbins pipettor can also handle 384-well plates.

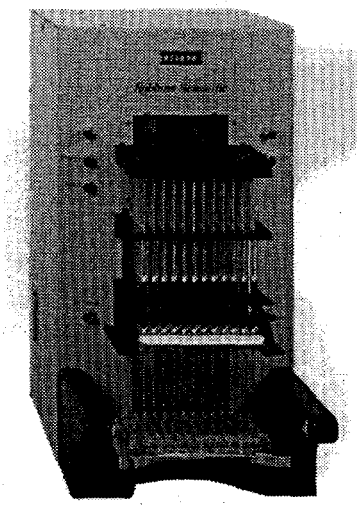

Fig. 4. Robbins Scientific Hydra 96, 96-Well automated pipettor.

Standing alone, the Robbins pipettor can hold 16 programs in memory. Programming the machine requires precise setting of the syringe depth, which generally requires hands-on testing with transparent trays. The Robbins is used for distributing the purified DNA template from a single 96-well plate into separate plates, one for each *nucleotide (A,G,C,T),* and depending on the sequencing approach, possibly a plate in the forward and reverse reaction directions for each nucleotide as well. The Robbins pipettor is also used to pool the separate nucleotide plates into a single plate during the sequencing preparation step after the amplification stage. The Robbins has a serial port, allowing it to be incorporated into an automated line and enable operation from a central controller.

Once purified and pooled, the DNA is then sequenced by an automated sequencing machine. Currently, ORNL's laboratory uses an ABI Model 373 and an ABI Model 377 automated sequencing machine. The ABI 373 and 377 machines are shown in Figure 5.

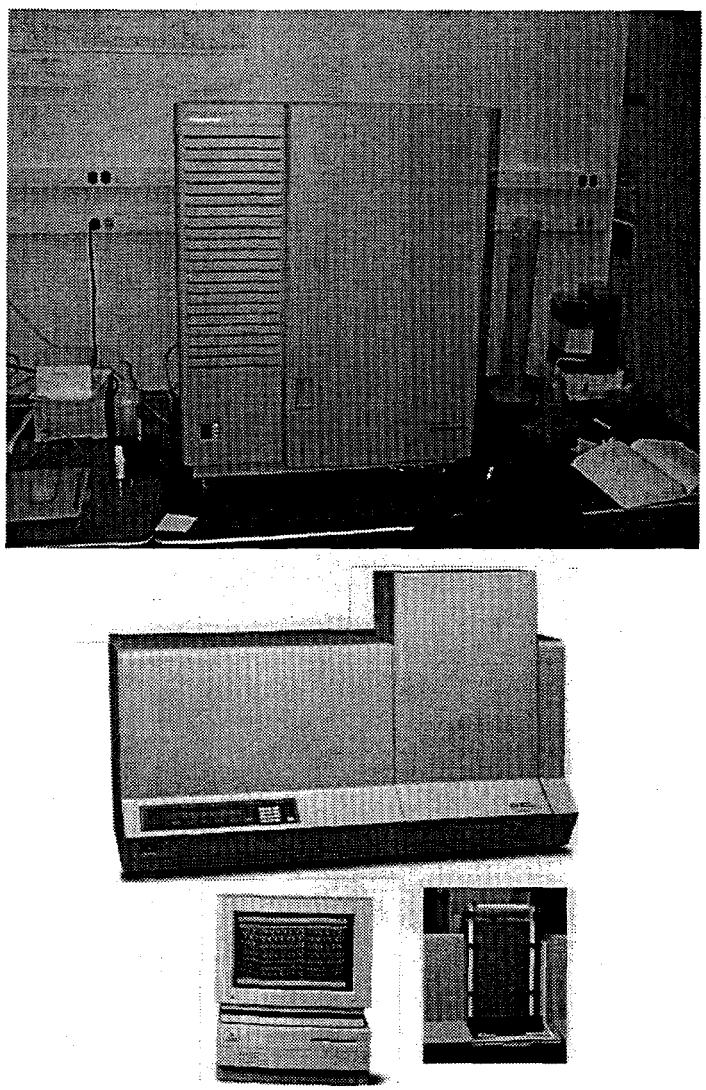

Fig. 5. ABI 373 and 377 automated sequencing machines.

The ABI machines sequence DNA by starting with the contents of each of the 96-well microtiter plate wells into an acrylamide *gel* and separating the DNA fragments by size using *electrophoresis*. This process separates the DNA fragments by length. A laser reads for each base (each fluorescing a different color) separately and repeatedly; thus, the base sequence of the complete DNA segment is determined. For more information on this topic refer to reference [1]. The data from the laser is transmitted by the ABI to an adjoining computer (Macintosh-based), where programs exist to sort the data and fit each sequence fragment into the appropriate place along the complete DNA segment. Senior researchers are then able to check the data and make judgment calls about questionable bases. The ABI is also equipped to do *genotyping*. Separate programs exist for this work, and different gels and plates must also be used.

All of the automated devices, described previously, have a maximum rate and a realistic rate at which they can operate. These rates are summarized for each of the machines in Table 2. (This Table assumes a five-day workweek and 600-bp read length per lane in the sequencing step.) Table 2 also includes the rate for the newest ABI sequencing machine, the Model 3700, which is planned for introduction into the ORNL's laboratory in the near future.

Table 2. Rates of various automated devices in ORNL DNA sequencing laboratory

Automated Machine	Production Rate in Various Units				
	Max or Real*	96-Well Plates/Day	Lanes/Day	Lanes/Week	10^6 Base Pair/Week
Qiagen BioRobot	Max	24	2304	11520	6.9
	Real	16	1536	7680	4.6
MJ Research	Max	48	4608	23040	13.8
	Real	32	3072	15360	9.2
Robbins Hydra	Max	48	4608	23040	13.8
	Real	32	3072	15360	9.2
ABI 373	Max	2	192	960	0.576
	Real	1.5	144	720	0.432
ABI 377	Max	3	288	1440	0.864
	Real	2	192	960	0.576
ABI 3700	Max	12	1152	5760	3.5
	Real	8	768	3840	2.3

* "Max" represents the maximum rate possible and "Real" represents a more realistic rate including some processing and down time.

Clearly the sequencing machines are the bottleneck in the process, even if the new machine were acquired. Because sequencing machines are expensive, easily costing $250,000 or more, in the future ready-to-be-sequenced template may be stored, transported, and sequenced at other organizations to more fully utilize the capability of ORNL's sequencing line. The DNA preparation robot is the second bottleneck in ORNL's automated line, and preparation techniques which do not require purification are being investigated.

Additional DNA Sequencing Hardware Being Integrated for Full Automation

To fully automate ORNL's sequencing center, the series of unconnected individually automated devices described in the previous section must be integrated into a hands-off, completely automated processing line. It is easiest to implement this conversion in two distinct phases. The first phase involves the automation of all steps after the DNA purification (QIAGEN BioRobot 9600) through the plate pooling step of the Robbins 96-well pipettor (the sequencing preparation step that immediately precedes the loading of the centrifuge in Figure 1). The second, more advanced automation phase will complete the smooth integration of all steps from bacteria colony picking through the running of the ABI Sequencing machine, thus enabling full hands-off operation of the entire process.

A key component of a more fully automated DNA Sequencing system is a robot handling system designed to transfer each plate from machine to machine. This is equivalent to using a human operator in a manufacturing cell. A rail-mounted robot was chosen over a stationary robot because it provides a larger table space for hardware to be arranged around the robot. The robot selected is a CRS Robotics A-465 track-mounted arm, shown in Figure 6.

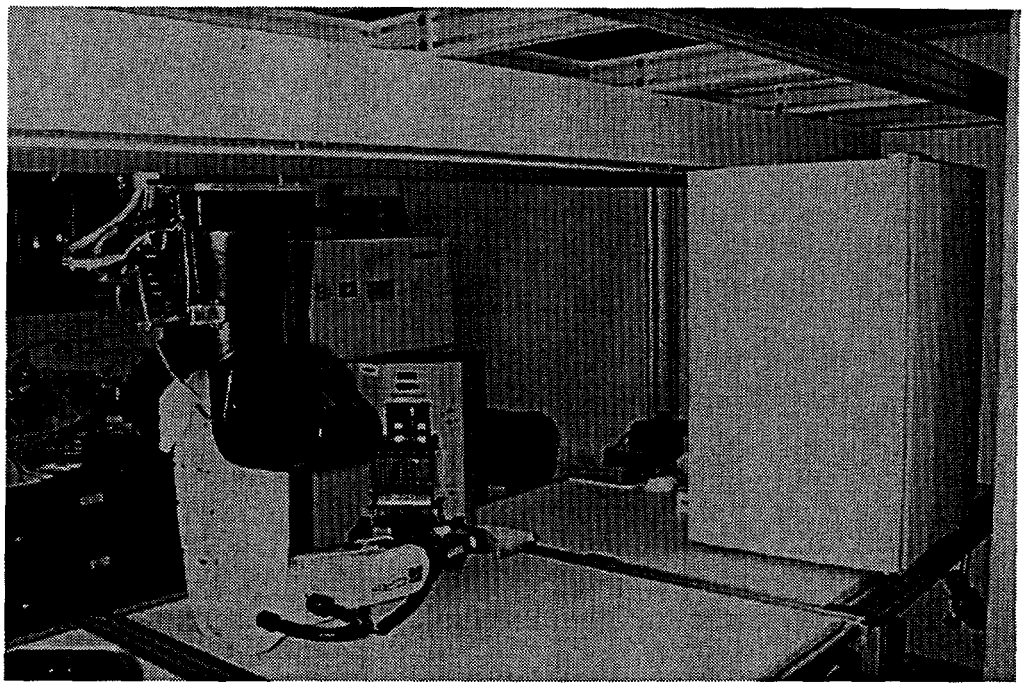

Fig. 6. The CRS A-465 robot arm mounted on a 4-meter rail.

The CRS robot has 6 degrees-of-freedom plus the track linear motion. The robot has a 3-kg payload, a 0.05-mm repeatability, a 711-mm reach, and a maximum speed of 1.02 m/s. It is supplied with the "C500" controller and RAPL III robot programming language from CRS. Along with the CRS arm, ORNL obtained the POLARA™ software for use as a supervisory controller.

Because of the nature of the samples being handled it is necessary to have a refrigerator as part of the automated line. A number of different stages necessitate cooled storage, therefore, the reaction mixtures for the PCR thermal cycler have to be chilled in darkness until they are used and mixed with template. After mixing, the four reaction plates taken to the PCR thermal cycler must be stored in the refrigerator if not used immediately. After the thermal cycling and plate-pooling steps, forward and reverse reaction plates must be stored in the refrigerator until an ABI sequencer is available. The refrigerator door is automated so that the robot does not have to open it, which might require additional steps to place and retrieve plates in the robot's grasp.

Another addition to the automated line is a plate holder, which sits between the Robbins pipettor and the thermal cycler. This plate holder is necessary to store plates during the distribution of template to the reaction mixtures. Used plates can also be placed on this rack for later disposal by a technician.

Several other pieces of equipment are being considered for the automated sequencing line. A bar code reader is being considered to monitor and control the information associated with a higher-throughput automated process. Before the robot performs an action with any plate, its bar code could be read to ensure the robot is carrying the correct plate. This is especially important if sequencing machines in other organizations are used, which is a likely scenario since the sequencing machines are the major bottleneck in the automated process (refer to Table 2).

Results

Simulations of the system were conducted with two different software packages. The first package used was Visualizer from CRS Robotics, which facilitated the layout and placement of equipment around the rail robot. Visualizer also helped with path planning in collision avoidance and singularity avoidance. The second software package used was Simscript, which models the use of automated equipment on a simulated process line with human interaction time. It can be used to determine system throughput, evaluate bottlenecks, and look at process line optimization. A typical result from a Simscript simulation is shown in Figure 7, in which utilization of the QIAGEN BioRobot is plotted versus system throughput.

Figure 7 assumes that there is infinite sequencing capability available and shows that as throughput is increased, the Qiagen becomes 100% utilized; that is, it does indeed become the bottleneck *if* sequencing capacity is available at off-site locations. Other conclusions from the Simscript simulations are as follows: (1) adding a second Qiagen BioRobot without adding a second MJ Research PCR machine does not significantly increase the maximum system throughput, (2) running the system continuously (i.e., 24 hours) versus "stacking" plates at the end of a regular day and running them until the refrigerator is filled only slightly increases the system throughput and is probably not warranted because of the added cost and complexity, and (3) system lockup can occur if careful attention is not given to the order in which machines are requested in the supervisory control software. This last conclusion required further investigation because of its implication on system reliability. Some simple Petri Net models were developed to help address these issues as referenced in [1] and [5]. These models are not within the scope of this paper but they are summarized in reference [6].

All of the hardware discussed in the previous section has been incorporated into a semiautomated system. The first phase of automation connects the output of the processing robot (QIAGEN BioRobot 9600) purification to the end of the plate-pooling operation, which is done by the automated pipettor in the sequence preparation step. This step includes the integration of the CRS A-465 rail robot, the QIAGEN biochemical processing robot, the Robbins pipettor, the MJ Research PCR thermal cycler, and the refrigerator.

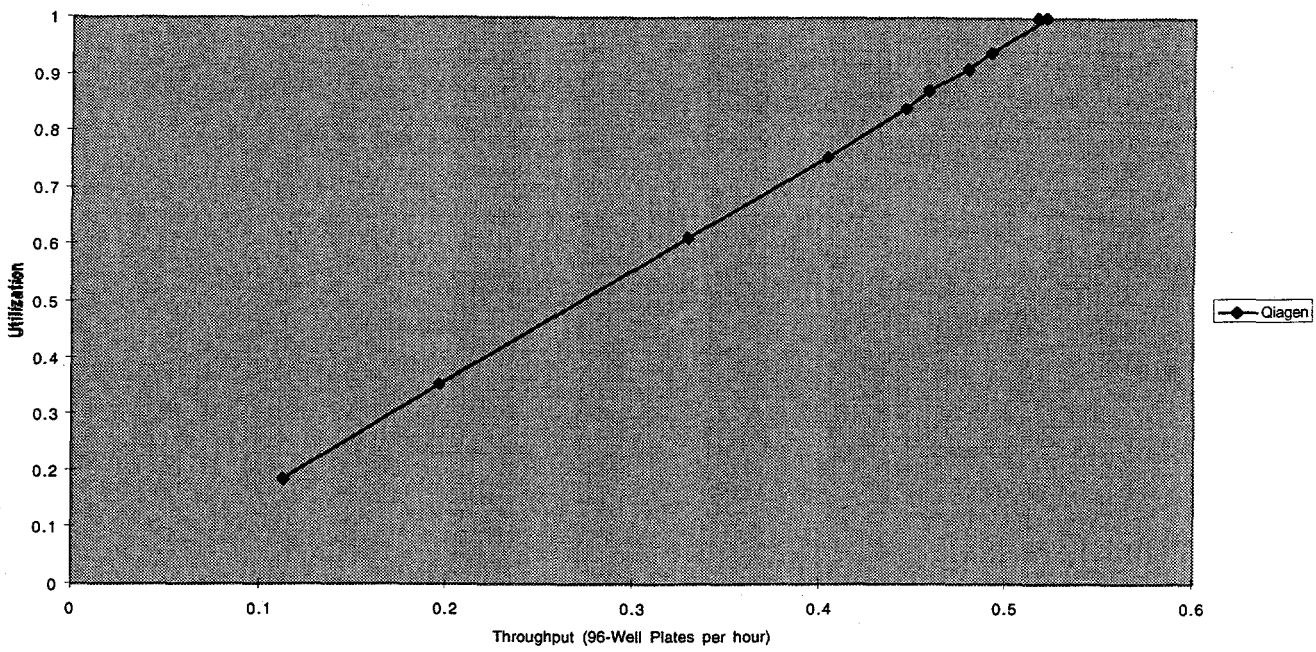

Fig. 7. Qiagen BioRobot utilization (1 = 100% utilized) versus system throughout.

Conclusions

ORNL is developing a core sequencing facility to support biological research endeavors at ORNL and to conduct basic sequencing automation research. This facility is novel because its development is based on existing standard biology laboratory equipment such as automated pipettors, PCR machines, biochemical processing robot, and off-the-shelf automated sequencing machines. A CRS Robotics A-465 arm moves microtiter plates between the automated machines. The system will be expanded in the future to include colony picking and/or the integration of the sequencing machines. This discrete event DNA sequencing system demonstrates that smaller sequencing labs can achieve cost-effective automation that can be expanded or changed as the needs of the laboratory grow.

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APPENDIXES

Appendix A: Small Glossary of DNA Sequencing Automation Terms

Note: Many of the following definitions are taken from reference [7].

A,G,C,T = Nitrogenous bases adenine (A), guanine (G), cytosine (C), and thymine (T). The bases (making up the "rungs" of the DNA ladder) are joined by hydrogen bonds.

bp = Base Pair; Purines always pair with Pyrimidines. In DNA: Adenine pairs with Thymine (2 Hydrogen bonds); Guanine pairs with Cytosine (3 Hydrogen bonds). In RNA, Adenine bonds with Uracil instead.

Complementary Base Pair = Bases are complimentary; for DNA, A is opposite T, and C is opposite G.

dNTP (deoxynucleotide triphosphates) = Precursor molecules that are used in the enzymatic synthesis of DNA.

DNA (deoxyribonucleic acid) = DNA is the universal code for amino acid sequences. Resembling a twisted ladder, each side has a sugar-phosphate backbone supporting nitrogenous bases (adenine (A), thymine (T), cytosine (C), and guanine (G)). The bases (making up the "rungs" of the ladder) are joined by hydrogen bonds. The strands run anti-parallel (in opposite directions) and are twisted into a double helix which turns every 3.4 nm. 5' and 3' refer the ends of the strands; they get their names from the carbons at those ends.

Electrophoresis = Technique used to separate DNA by size. Electricity is run through an agarose gel where the DNA has been placed in wells. Smaller segments of DNA run across the gel at a faster rate than larger segments. Uses of this technique include criminal identification and DNA sequencing. It can also be used to study proteins.

Gel = Used in electrophoresis. Often made of agarose, the viscosity of the gel can vary depending on the size of the DNA segments under study.

Gene = Sequence of base pairs that produces some physical meaning; 100,000 in a human.

Genome = "Gene" + "Chromosome"—refers to the entirety of our genetic information. Our entire genome is contained within each cell, although the portions of it, which are expressed (translated into proteins) depend on the function of the cell.

Genotyping = Determining the genetic makeup of a trait. [i.e., TT (homozygous dominant), Tt (heterozygous), or tt(homozygous recessive)]. This information is valuable in genetic counseling when attempting to discover the probability that parents carry or that their offspring will have the gene for an heritable disease.

Human Genome = 23 pairs of chromosomes [22 pairs of autosomes or somatic chromosomes and 1 pair of sex chromosomes: (XX for female, XY for male)] 46 chromosomes total. 3.5 billion base pairs long.

Human Genome Project: Project the goal of which is to "map" the exact location of every gene on human chromosomes.

Mbp = Mega base pair, that is, 10^6 bp.

Nucleotides = Monomers that make up DNA and RNA. They consist of (1) a 5-carbon sugar, either deoxyribose or ribose; (2) a phosphate group; and (3) a nitrogenous base or nucleoside.

PCR = Polymerase chain reaction. A method to amplify specific regions of DNA using oligonucleotide primers, dNTPs, and heat-stable DNA polymerase. Kary Mullins invented PCR in 1983.

Peltier Bock = Simple heater employing the Peltier effect to quickly and efficiently heat reaction mixtures to amplify DNA in the PCR process.

Plasmid = Extra chromosomal loops of DNA found in prokaryotes, containing genes that may benefit bacteria at some point (i.e., resistance to antibiotics and toxin production). Often used as vectors to add genes to bacteria's genome for experimental purposes.

Replication = Use of DNA as a template to make more DNA. This occurs when a cell is going to divide (mitosis).

Sequencing = Given a DNA string, identification of the order of the A, G, C, T molecules.

Vector = Carrier of desired DNA fragment or sequence, either a plasmid, lambda-phage, or bacterium.

Appendix B: Comparison of Some Commercial Biochemical Processing Robots

	AutoGen 740	Biomek 2000	Vistra 625	BioRobot 9600
Application (Enzymatic Reactions)	No	Yes	No	Yes
Application (M13)	Yes	No	Yes	Yes
Application (Plasmid)	Yes	No	Yes	Yes
Application (Sequencing Reactions)	No	Yes	Yes	Yes
Hardware (Liquid Detection)	Yes	No	No	Yes
Hardware (Pager System)	No	No	No	Yes
Hardware (Shaker Unit)	No	No	No	Yes
Hardware (Vacuum Pump & Manifold)	No	No	No	Yes
Reagent Kits (M13)	Yes	No	Yes	Yes
Reagent Kits (Plasmid)	Yes	No	Yes	Yes
Sample Set (M13)	20 to 160	No	1 to 32	1 to 96
Sample Set (Plasmid)	20 to 160	No	1 to 16	1 to 96
Software (Tested Protocols)	Yes	No	Yes	Yes
Software (User Programmable)	Partially	Yes	No	Yes
Software (Windows)	No	Yes	Yes	Yes
Throughput 96 Samples (M13)	9.5 Hours	No	3 Hours	2 Hours
Throughput 96 Samples (Plasmid)	9.5 Hours	N/A	5 Hours	3 Hours
Training On-Site	No	No	No	Yes
Warranty (Hardware)	1 Year	1 Year	1 Year	1 Year
Warranty (Software)	90 Days	90 Days	90 Days	1 Year