ANL/BIO/01-1

Automated Cloning Methods

Biosciences Division
Argonne National Laboratory

Operated by The University of Chicago,
under Contract W-31-109-Eng-38, for the

United States Department of Energy

Available electronically at http://www.doe.gov/bridge

Available for a processing fee to U.S. Department of Energy and its contractors, in paper, from:

U.S. Department of Energy
Office of Scientific and Technical Information
P.O. Box 62
Oak Ridge, TN 37831-0062
phone: (865) 576-8401
fax: (865) 576-5728
email: reports@adonis.osti.gov

ANL/BIO/01-1

Automated Cloning Methods

by Frank Collart

Biosciences Division
Argonne National Laboratory, 9700 South Cass Avenue, Argonne, Illinois 60439

February 2001

This report is printed on recycled paper.

CONTENTS

CONTENTS (Cont.)

TABLES

FIGURES

FIGURES (Cont.)

FIGURES (Cont.)

PREFACE

This document describes work in progress. The information in this document is intended to aid researchers by describing a series of protocols and experiments designed to help produce bacterial expression clones in an automated format. Developing an automated system for gene cloning and expression requires an inherently different approach than that used in classical methods for expression cloning. Achieving the high-throughput capability of an automated system comes at the expense of system flexibility. Manual cloning methods can be altered by purchasing a new cloning kit. However, substantial modification of an automation protocol can be expensive and time-consuming in terms of rewriting and revalidating protocols. Establishing automated systems requires that researchers apply a more global approach in the evaluation and implementation of cloning and expression protocols. At the inception of a program to develop an automated system, protocols must be evaluated with respect to their compatibility with the protocols of other methods and for the feasibility of their implementation in an automated setting. Specific considerations for gene cloning and expression protocol selection and implementation in an automated system include the following:

- *Universal approach.* To achieve high throughput, automation protocols must be uniformly applicable to all input and output resources. In the context of structural genomics, a universal handle approach is required to permit cloning and expression of targets from many sources. Although several universal handle cloning systems are presently available, an array of compatible vector systems needs to be designed to provide for flexible expression in different host systems.

- *Integration of method requirements.* Minimizing manual intervention between the various components of the system optimizes the efficiency of an automated process. For automated expression cloning, the universal cloning sequences must be integrated into the primer design and vector design components. Similarly, the fusion tags incorporated during the cloning method must be compatible with downstream analytical methods to assess protein expression by immunological techniques and batch purification techniques for large-scale protein production.

- *Standardized components.* The economical implementation of an automated system requires the use of commercially available high-throughput hardware and components using a standard microplate format.

- *Robust methodology.* With the high throughput of many automated processes, individual sample control is often lost. Including methods

with increased latitude for sample stability and time flexibility enhances overall process efficiency.

Argonne National Laboratory's Biosciences Division has acquired an Automation Core System as a first step in the development of robotic capabilities to enable the application of a high-throughput approach to future divisional molecular biology programs. This capability is essential in terms of capitalizing on the explosion of data and resulting opportunities generated by the Human Genome Project. The automation system is presently used for the automated generation of proteins for application in the field of structural genomics. The experience gained by implementing these initial protocols will provide a platform for extending the system's capabilities for application in other growth areas of high-throughput molecular biology, including site-specific mutagenesis and protein interaction studies. With the Automation Core System, Argonne is uniquely positioned as a leader in the creation of new high-throughput capability in molecular and cellular biology; this capability is fundamentally important to future progress in biomedical and biotechnology research.

AUTOMATED CLONING METHODS

by

Frank Collart

1 INTRODUCTION

Argonne has developed a series of automated protocols to generate bacterial expression clones by using a robotic system designed to be used in procedures associated with molecular biology. The system provides plate storage, temperature control from 4° to 37°C at various locations, and Biomek and Multimek pipetting stations. The automated system consists of a robot that transports sources from the active station on the automation system. Protocols for the automated generation of bacterial expression clones can be grouped into three categories (Figure 1). *Fragment generation* protocols are initiated on day one of the expression cloning procedure and encompass those protocols involved in generating purified coding region (PCR)

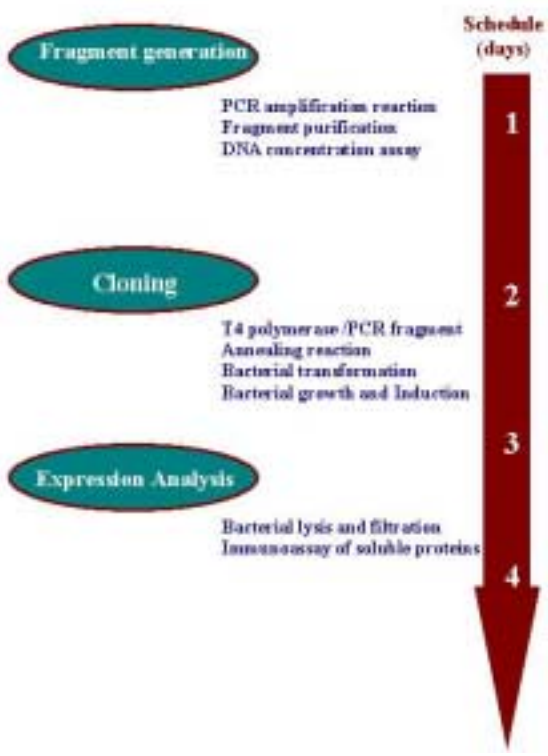


FIGURE 1 Schedule for Automated Cloning Protocol

fragments from target gene coding regions. *Cloning* protocols are initiated on day two and involve cloning the PCR fragment into bacterial expression vectors(s), transforming competent bacteria, and growing the transformed strains. Day three of cloning *(expression analysis* protocols*)* involves inducting bacterial expression, harvesting and lysing the induced bacteria, and preparing immunoassay plates from the soluble fractions. On day four, immunoassay plates are screened, and the successful expression constructs are identified.

2 OVERVIEW OF AUTOMATED CLONING PROTOCOLS

2.1 FRAGMENT GENERATION PROTOCOLS

The fragment generation protocols comprise two robotic methods and a program for preparing a thermocycler reaction plate. The PCR reactions are prepared via an automated method. The vendor supplies the forward and reverse primers in a 96-well format at a concentration of 100 μM. The primers are diluted and dispersed into 96-well PCR plates by using the Multimek pipetting station. The Biomek workstation is used to prepare the reaction mix and dispense the mix to the PCR plates. Table 1 provides schedule times for the generation of various numbers of PCR plates. The plates are currently transferred manually to Perkin-Elmer 9600 thermocyclers for DNA amplification. Argonne's Biosciences Division has three Perkin-Elmer 9600 thermocyclers and several other thermocyclers from other vendors. Upon completion of the amplification program, the PCR fragments are purified on the Biomek workstation by using Qiagen 96-well filtration plates. The purified fragments are collected in 96-well v-bottom plates, and the DNA concentration is assessed by using a picogreen fluorescent assay.

2.2 CLONING PROTOCOLS

The cloning protocols generate expression constructs used to transform competent bacterial cells. The PCR fragments derived from the fragment generation protocols are treated with T4 DNA polymerase to generate handles compatible with those on the bacterial expression vector. After the vector and cloned fragments are annealed, an aliquot of the annealing solution is used to transform a rare codon variant of the BL21 bacterial strain (Stratagene RIL rare codon strain). The transformed bacteria are cultured in LB for a brief period to allow the recovery of antibiotic resistance.

At present, this group of cloning protocols encompasses two automated methods. The LICR×1 automation method uses the Multimek workstation to array the purified PCR

TABLE 1 Comparison of Schedule Times for Automated Methods

	Time (min)		
Protocol	1 plate	2 plates	4 plates
Thermocycler plate preparation	16	24	42
Fragment purification	37	74	148
Picogreen DNA assay	9	14	25
LIC reaction	53	62	85
Annealing/transformation	60	70	107

fragments in a 96-well reaction plate. The plate is then transferred to the Biomek workstation to prepare the LIC reaction mix and set up the reaction plate. In the final segment of the LICR×1 method, the plate is incubated for 20 min at 75°C to inactivate the T4 polymerase. The Anneal1 automation method uses the Biomek workstation to combine the fragment and vector. The resulting expression constructs are used to transform chemically competent bacteria by a heat shock technique. The transformed bacteria are then mixed with bacterial growth media to recover antibiotic resistance and allow overnight growth of expression culture. Schedule times for the current cloning methods are provided in Table 1.

A current limitation of the cloning protocols is the need to manually adjust the concentration of the purified PCR fragments before initiating the cloning methods. A planned upgrade of the method includes the development of an automated Biomek method that uses the DNA concentration data derived from the picogreen fluorescent assay to reformat the fragment plates to a uniform DNA concentration. Other revisions of the protocol include merging the present two automated methods into a single automation protocol.

2.3 EXPRESSION ANALYSIS PROTOCOLS

The expression analysis protocols encompass two groups of methods that are based on their function. One group provides for optimal bacterial growth, lysis, and collection of a soluble protein fraction. The other group uses a series of immunological techniques to characterize the soluble protein fraction. These methods provide the basis for the selection of expression clones for progression to the protein purification component of the High-Throughput Protein Crystallography Project.

The method for the production of soluble protein is a SAMI method (BAC1) for promoting bacterial growth in deepwell plates. This method runs overnight; IPTG is added so that the induced expression cultures can be harvested the following morning. The Lysate1 SAMI method coordinates the collection and lysis of bacteria on a filtration plate. The soluble proteins are collected in a 96-well plate by the application of a vacuum filtration method.

The analysis methods involve preparing serial dilutions of the soluble protein fractions for subsequent immunological analysis. The analysis methods, performed on the Biomek workstation, provide for plate blocking, addition of antibodies, and washing of the immunology plates. The plates are manually read on a Victor 1420 fluorescence instrument.

2.4 SUMMARY

All of the steps required to generate expression clones have been demonstrated successfully. These procedures are now being integrated into what is virtually a total roboticized

process capable of handling more than 1,000 genes simultaneously. In the early phases of the project, all systems will be tested repeatedly to establish baseline performance.

3 MOLECULAR BIOLOGY ROBOT

The Argonne Molecular Biology Robot System (Figure 2) includes a number of stations (e.g., pipetting workstations, a heatblock, and incubators) that perform the equivalent of standard laboratory manipulations during an automation procedure. To allow for flexible programming of the system, the stations are treated in a modular manner. Thus, the activities of the stations are independent, and samples are transported between stations in such a way that the samples entering a station can arrive from and leave for any other station. This feature maximizes the flexibility of the method and allows many programs to run concurrently. Planned system upgrades compatible with the present design include a plate shaker, plate reader, and incubator.

3.1 SYSTEM COMPONENTS

Biomek 2000 workstation. The Biomek 2000 Laboratory Automation Workstation performs accurate and precise liquid-handling tasks within and between plates on the Core System. This workstation has interchangeable tools to meet a variety of research requirements. Some features incorporated in the system include the following:

1. Pipetting tools that accurately and precisely transfer 0.5–200-µL volumes.

2. A multichannel tool for dispensing aspirating liquids in volumes of 0.05–10 mL.

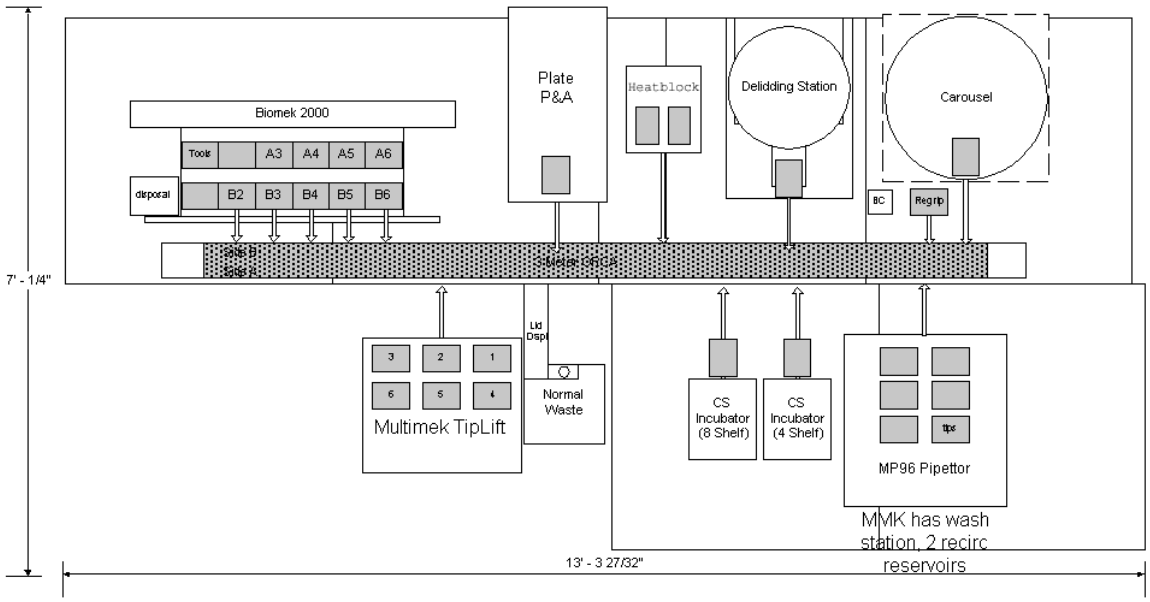


FIGURE 2 Schematic of Molecular Biology Robot System

3. A gripper device to allow for movement of labware.

4. Two thermal reservoirs to allow for heating/cooling of microwell plates.

5. A vacuum filtration system to handle 96-well (or equivalent footprint) filtration plates.

Multimek workstation. The Beckman Multimek is a 96-channel robotic pipetting station. Six predefined positions within the work envelope accommodate standard microplate or reservoir labware. The Multimek system is configured with two 96-channel disposable dispense head options that allow pipetting of 1–200 μL-volumes.

Microplate lidding station. The lidding station integrates automated microplate lid removal with run lid storage and replacement for up to six standard microplate lids.

Tip lift and disposal unit. The tip lift unit automatically indexes six columns of tip boxes in a configuration specifically designed to enable the automated laboratory system to continually deliver tips to the deck of the Biomek or the Multimek workstations.

Storage carousel. The storage carousel provides plate and tip storage and ambient incubation for transports on an automated system. The carousel allows random access for up to 10 hotels containing as many as 18 standard microplates. The carousel provides storage for automation supplies, including tips, deep-well plates, filtration plates, and standard 96/384-well plates.

CS incubators. The CS incubators provide automated storage of standard and deep-well microplates. The incubator effectively maintains a temperature from 4°C to 37°C and features automatic doors to create a closed system for reducing contamination.

Microplate print and apply. The microplate bar-code print and apply tracks sample identification and provides sample confirmation during automated handling and subsequent processing steps. The print-and-apply assembly has been teamed with a bar-code scanner to verify bar-code readability and allow tracking of experimental manipulations.

Heatblock. The robot system contains a variable-temperature heatblock designed to accommodate 96-well thin-walled thermocycler plates. The device permits independent incubation of up to two 96-well plates, allowing for increased throughput.

3.2 SOFTWARE

The software platform (SAMI-NT) resides on a Microsoft Windows NT system. The user is able to modify system parameters, analyze the status of tests in progress, and check the results of completed tests. SAMI-NT software has three main modules: Method Editor, Scheduler, and Run-Time. Methods are built in the *Method Editor* by using a flowchart diagram, with each icon in the cart representing a station in the robotic system. Methods can be created, edited, saved to disk, and executed. The *Scheduler Module* generates the code and timing to execute a selected method on numerous samples. Multiple assays can be scheduled to run simultaneously. The *Run Time Module* software is the interface presented to the user while the system is running the methods defined by the SAMI Method Editor. The software also performs the following:

- Error checking and recovery,
- Sample tracking and sample history generation,
- Instrument interfacing,
- Data management and presentation, and
- Scheduling optimization.

4 PRELIMINARY DATA

4.1 FRAGMENT GENERATION PROTOCOLS

Potential expression cloning methods were evaluated to assess the suitability of converting them to an automated system. The Ligation Independent Cloning (LIC) method [1–2] was selected for further evaluation on the basis of several attributes:

- Universal cloning handles can be incorporated at the PCR fragment design stage, enabling a general cloning approach to most of the selected targets.
- Cloning is directional.
- LIC vectors are available that incorporate at least two fusion tags.
- No restriction enzyme protocols are required.
- LIC is highly efficient for generating expression clones.

Fragment generation data were obtained for two independent 96-well plate experiments. Each experiment produced a single PCR plate for amplification, fragment purification, and DNA analysis. These experiments used two sets of forward/reverse primers arrayed in 96-well plates. Amplification products and purified DNA fragments were electrophoresed through agarose gels to provide a comparison with fluorescent assay data (Figure 3). This gel analysis validated the successful performance of the amplification and purification protocols. The results of these preliminary studies can be summarized as follows:

- PCR amplification rate averaged 65%, which is consistent with the rate observed for manual preparation of PCR amplification reactions.
- Gel patterns before and after purification were similar, thus validating the success of the automated purification protocol.

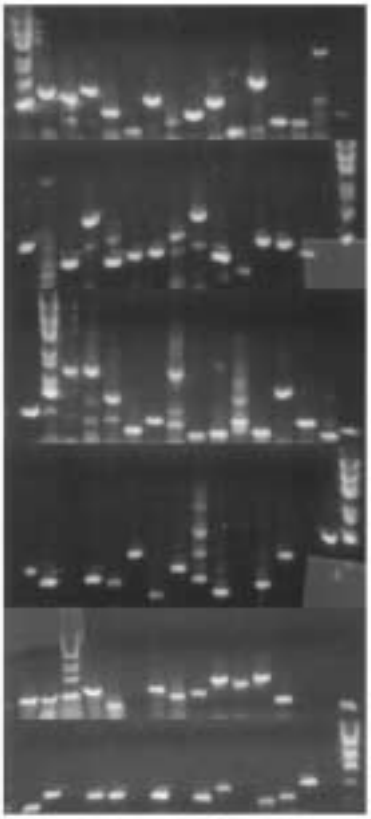


FIGURE 3 Gel Analysis of Purified PCR Fragments

- Relative fluorescence levels correlated with the amount of DNA observed in the DNA gel electrophoretic patterns.

4.2 CLONING PROTOCOLS

Implementing the cloning component of the automated cloning method on the present robotic system required the modification of the standard laboratory methods. The laboratory approach to expression cloning was to use LIC cloning kits available from Novagen. However, the multiple-vector strategy needed to implement a high-throughput structural genomics project requires the development of a set of unique cloning tools not currently available from commercial vendors. Because of this constraint, the automated cloning method must allow the use of off-the-shelf reagents in the cloning protocols. Using off-the-shelf reagents significantly reduces cost, because the highest cost element of the cloning protocols is the vector cloning kit. To determine the feasibility of using off-the-shelf reagents, several cloning reactions were prepared by using reagents prepared in the laboratory and T4 DNA polymerase from Promega. Analysis showed that the number of colonies obtained by using off-the-shelf components was comparable with that obtained by using the standard cloning kit (Figure 4).

Current laboratory cloning protocols use an annealing reaction with a total volume of 1.5 μL. This volume is achieved by mixing 1 μL of T4 DNA polymerase-treated PCR fragment with 0.5 μL of the pET30XaLIC vector. Although these volumes are within the pipetting capability of the Biomek workstation, increasing the volume for an annealing reaction would help in the implementation of an automated cloning method.

As data presented in Figure 5 illustrate, a ten-fold increase in the total reaction volume of the annealing does not compromise the efficiency of the reaction. This increased volume for the annealing reaction simplifies the setup of the annealing reaction on the automated system and facilitates the downstream protocol for T4 DNA polymerase heat inactivation.

The cloning protocol uses a heat-shock procedure to transform chemically competent *E. coli* bacteria. In the laboratory, this protocol is accomplished by transferring 13 × 100-mm test tubes containing the bacteria-expression construct mixture to a 42°C heat block for 15–20 s. In the automated cloning method, the heat-shock protocol requires transfer of a 96-well PCR plate from CS Incubator 2 at 4°C to a specially designed heat block. Various heat block temperatures and incubation times were

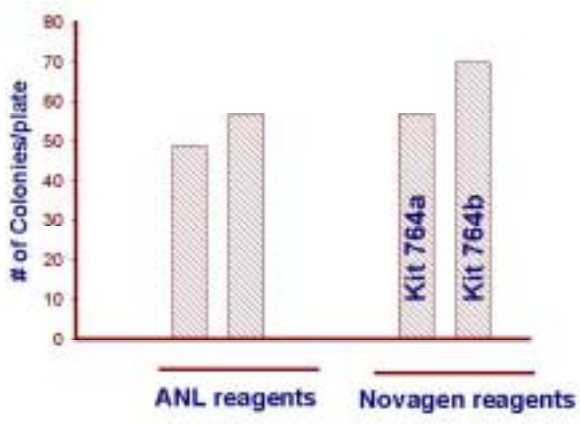


FIGURE 4 Comparison of cloning efficiencies obtained with cloning kits and Argonne-prepared reagents

examined (Figure 6) to evaluate the effectiveness of the automated heat shock protocol. The data validate the effectiveness of the automated heat shock protocol and suggest that a heat block temperature of 45°C and an incubation time of 40 s are optimal conditions.

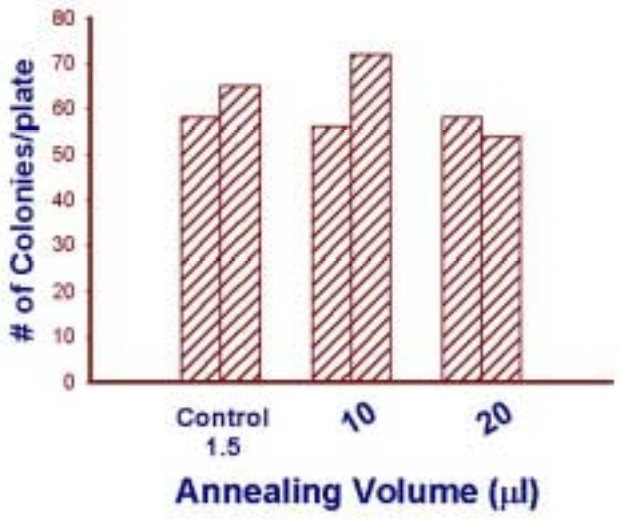


FIGURE 5 Effect of Total Annealing Reaction Volume on Cloning Efficiency

4.3 EXPRESSION ANALYSIS PROTOCOLS

4.3.1 Expression Analysis in Bulk Bacterial Cultures

The automated cloning method requires the elimination of plate cultures for expression cloning. One method to circumvent the initial plate culture is to perform the solubility analysis on bulk cultures obtained by overnight growth of the transformed bacteria. The LIC cloning approach is well suited for this type of analysis since this procedure yields a high percentage of expression constructs. Using SDS-PAGE to analyze bulk cultures of transformed bacterial cells (Figure 7) allows the evaluation of protein expression.

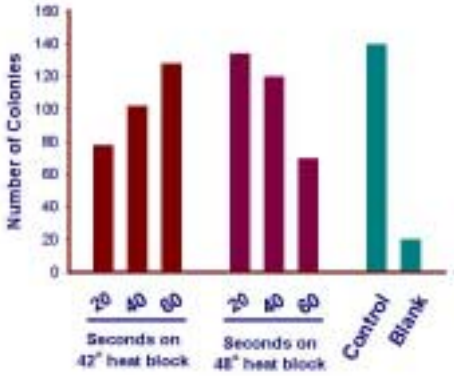


FIGURE 6 Evaluation of Various Heat Block Temperatures and Incubation Time on Transformation Efficiency

The level of protein expression observed in bulk cultures is comparable with that observed by analysis of individual clones. The data show that bulk cultures can be successfully used for the expression analysis component of the automated cloning method.

4.3.2 Filter Evaluation

The automated protein solubility protocols provide for the collection of induced bacterial cultures on a filter membrane, bacterial lysis, and collection of a soluble protein fraction in a v-bottom microwell plate. The essential component of this process is the filter plate, which must enable the collection of bacteria and have low protein binding. The filter should also be able to

contain 600 µL/well of filtrate since this capacity would eliminate the need for multiple filtration steps and thereby increase efficiency. Several filters were evaluated (Table 2) for their utility in the soluble protein protocols. Although filter plates suitable for bacterial collection or protein filtration were identified, no plates had the combined characteristics of bacterial collection and low protein binding.

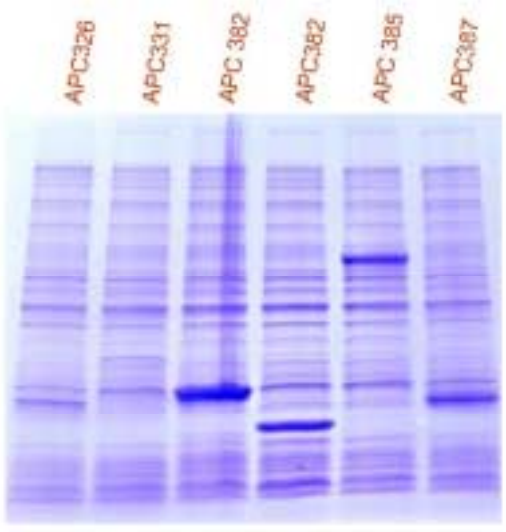


FIGURE 7 Protein Expression in Bulk Bacterial Cultures

To evaluate the protein expression analysis using filter plates, a Whatman Unifilter 350 with a hydrophobic PVDF was used for filtration of lysed bacteria. SDS-PAGE gel electrophoretic analysis of soluble protein fractions prepared by filtration on the Biomek system shows the recovery of soluble proteins (Figure 8). For many samples, the level of soluble protein recovered by filtration is comparable with that observed in the total protein samples. Several protein constructs (APC326 and APC331) do not produce soluble protein expression products.

4.4 MICROWELL PLATE ASSAY FOR DETECTION OF PROTEIN EXPRESSION

The automated cloning method employs expression vectors incorporating at least fusion tags. These fusion tags are the basis of the strategy used to detect soluble proteins. Soluble proteins are serially diluted in hydrophobic microwell plates (Immulon 4HB, high protein binding), and unbound sites are blocked by incubation with BSA. Tag proteins are detected by using incubation with an affinity ligand (e.g., nickel-coated peroxidase is used to detect His-tagged proteins) and an appropriate detection reagent. Figure 9 displays the results obtained from a serial dilution assay of soluble fractions from several expression constructs. The yellow in sample wells of the soluble protein fractions indicates the presence of His-tagged protein.

TABLE 2 Descriptions of Filter Plates Evaluated for Soluble Protein Protocol

Vendor	Plate Description	Membrane	Pore size (µm)	Bacterial Collection	Protein Filtration
Whatman	Unifilter 350	PVDF	0.45	No	Yes
Whatman	Unifilter 800	GF/D	6.0	No	—
Nalge Nunc	Silent Screen	Nylon	0.45	Yes	No

To evaluate the characteristics of the immunoassay method, soluble protein fractions were analyzed with the immunoassay method and SDS-PAGE gel electrophoresis. The results obtained by using these two methods are provided in Table 3. Comparison of the results in Table 3 shows that a false positive result was obtained for sample APC332, and two putative false-negative results were observed for APC 323 and 386. The false-negative results obtained via the immunoassay method may be a consequence of a His-tag component that is not accessible to the detection ligand. An inaccessible ligand would be scored as a negative expression system since the protein would not be suitable for subsequent protein purification protocols that use affinity resins.

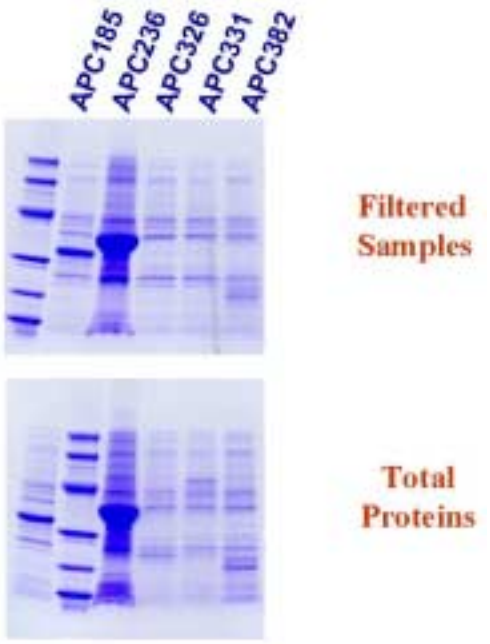


FIGURE 8 Denaturing Gel Electrophoretic Analysis of Soluble Proteins by Filtration Assay (Lanes 1 and 2 in total protein gel were reversed to allow for unambiguous gel identification)

4.5 EXPRESSION SUMMARY

To evaluate LIC for automated expression cloning, we used it to generate expression constructs manually by employing protocols similar to those envisioned for use in an automated system. Targets with the potential for novel folds were selected from the Protomap database. DNA sequence databases for multiple bacterial organisms were scanned to obtain as many representatives as possible for each fold. Primers were developed for individual targets, and the amplified coding regions were cloned into several LIC vectors containing various fusion tags. This process generated more than 100 confirmed expression constructs with representatives from five different organisms (Figure 10). The results validate the LIC approach as a candidate for implementation on an automated system. Furthermore, the clones generated are a valuable resource for assessing protein purification strategies and undertaking crystallization studies.

FIGURE 9 Immunodetection of Soluble His-Tag Proteins

TABLE 3 Evaluation of Soluble Protein Production by Immunoassay Methods

APC ID	Fusion MW	Solubility	His-tag
APC322	20423	Neg	+
APC323	107528	1	-
APC325	41980	Neg	-
APC326	26314	3	+
APC327	42052	1	+
APC330	81668	Neg	-
APC331	50056	2	+
APC332	28691	Neg	+
APC381	27242	1	+
APC382	16911	3	+
APC284	29442	1	+
APC285	22192	2	++
APC386	22297	3	-
APC387	31137	3	++
APC388	20539	1	++
APC389	28583	1	+

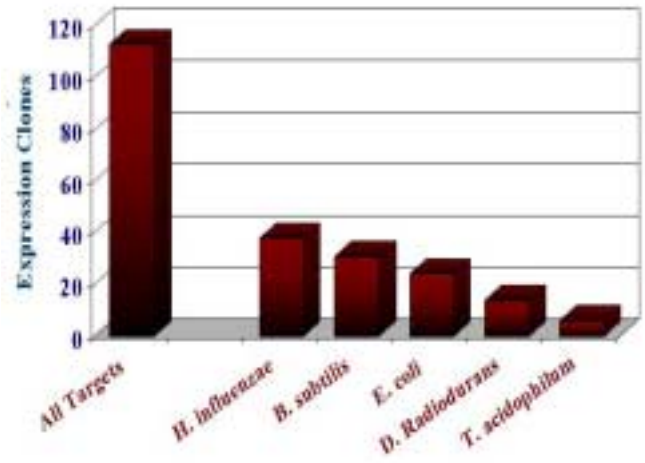


FIGURE 10 Expression Clones Generated by Using the LIC Cloning Approach

The number of clones generated in the manual evaluation of the LIC cloning method indicates our anticipated outcome for various stages of the automated cloning process. On the basis of data obtained from several hundred sequences submitted to our in-house oligo design program, an input sequence of average GC content currently has a 50% chance to yield acceptable primers for subsequent PCR-based cloning. Of targets that enter the cloning process, approximately 70–80% yield amplification products and can be cloned into a bacterial expression vector (Figure 11). On the basis of our experience with the expression of bacterial proteins, approximately 50% of targets entering the cloning protocol can be expressed, with approximately one-half of these expression clones yielding soluble protein.

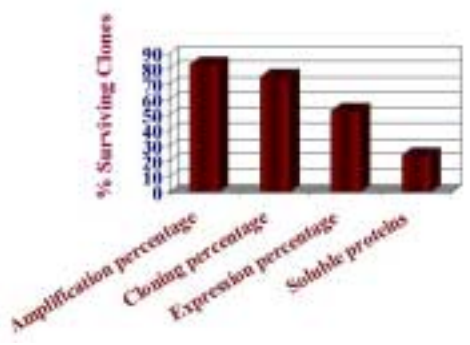


FIGURE 11 Summary of Anticipated Outcomes for Various Stages of the Cloning Protocol

Protocols have been developed that enable automated expression cloning. These protocols are currently being implemented on the Molecular Biology Robot System for eventual high-throughput expression cloning. Preliminary information that supports the validity of this automated approach includes the following:

- An automated method was established for the preparation of samples arrayed in 96-well plates for PCR amplification. The process uses forward and reverse primers arrayed in 96-deep-well plates. The rate of amplification success is comparable with that obtained in previous laboratory studies. These fragments have been successfully purified on the robot by using 96-well Qiagen filtration plates.

- A microplate picogreen fluorescent assay has been validated for determining the concentration of DNA fragments.

- Protocols have been developed for cloning of PCR fragments and using the annealed vector-insert for the transformation of chemically competent bacteria. An essential element of these protocols is verification of the utility of an automated method in which 96-well plates are used for bacterial transformation.

- The bulk culture analysis approach has been verified. The high cloning efficiency of the LIC method results in efficient protein expression in bulk cultures and eliminates the need for plating to select individual expression clones at the analysis stage.

- The utility of filter plates for bacterial collection and soluble protein analysis has been demonstrated. This capability eliminates the need to centrifuge bacterial cultures and lysates and potentially can replace the low-throughput protein gel electrophoresis method for verifying protein expression.

- A tag assay method for detection of soluble proteins has been established. The cloning protocol enables the construction of expressed protein with at least two dual-purpose fusion tags. These tags are used to detect expressed soluble protein and to facilitate subsequent purification.

5 RECOMMENDED METHOD REVISIONS AND ACTION ITEMS

On the basis of the experience gained during the development and implementation of the cloning protocols on the Argonne Molecular Biology Robot System, recommendations and actions have been assembled. These items will be implemented in subsequent revisions to the protocols and upgrades of the robotic system.

5.1 FRAGMENT GENERATION PROTOCOLS

The recommended fragment operation protocols are described in Sections 5.1.1–5.1.2.

5.1.1 PCR1 SAMI Method

1. Instruct the vendor to apply the oligo barcode to the "A" side of the oligo plate to allow reading and tracking within the SAMI method.

2. Revise the method so that the barcode is read before the PCR plate is set up.

3. Modify the microfuge holder so that it can incorporate a baseplate and filler to provide better temperature control for reagents.

4. Develop a strategy to increase throughput from the current limitation of three PCR plates. Limitation is the amount of reagent that can be stored in a microcentrifuge tube. Using reservoirs would alleviate this limitation, but the storage properties would have to be investigated.

5.1.2 Biomek PCRFrag1 and Picogreen SAMI Method

1. Merge protocols into a coherent SAMI protocol:

   - Translate plate pipetting operations to Multimek;

   - Validate transport properties for Qiagen filter and carrier. This task may include fitting a base on the current Qiafilter holder and obtaining more reservoir holders; and

- Incorporate DNA fragment transfer from square-well to shallow 96-well plate as a component of the fragment purification protocol.

2. Develop appropriate DNA controls for fluorescent assay. Prepare new picogreen assay kits that include DNA controls. Develop a strategy to incorporate these controls as part of the automated sample-preparation process.

3. Establish the relative fluorescence cutoff and DNA standard curve to allow for automated adjustment of DNA sample concentration.

5.2 CLONING PROTOCOLS

The recommended cloning protocols are described below.

1. Develop an automated fragment array program to eliminate the need to manually adjust the purified PCR fragment to achieve uniform fragment concentrations for the cloning reactions.

2. Eliminate manual reconfiguration of the deck between the Anneal2a and Transform Biomek methods. The process will require an evaluation of the transport and use characteristic of the reservoirs used to maintain stock cloning reagents.

3. Evaluate the Zymo-competent cell product that allows transformation of *E. coli* at 4°C. Incorporating this capability would eliminate heat shock transformation and associated use of the heat block.

4. Revise the SAMI method to use Multimek for whole-plate pipetting operations.

5. Develop ampicillin-based cloning vectors to increase transformation efficiency.

5.3 EXPRESSION ANALYSIS PROTOCOLS

The recommended expression analysis protocol is to continue screening filtration plates to identify a device that is capable of collecting bacteria and keeping protein binding low.

6 COST ESTIMATES FOR AUTOMATED GENERATION OF EXPRESSION CLONES

On the basis of the estimates provided in Sections 6.1-6.3, the cost for each validated expression construct will be approximately $45.

The estimated cost of fragment generation protocols is presented in Table 4, the estimated cost of cloning protocols is presented in Table 5, and the estimated cost of expression analysis protocols is presented in Table 6.

TABLE 4 Fragment Generation Protocols and Cost Estimates per 96-Well Plates

Component(s)	Cost ($)[a]
Pfx DNA polymerase	200
Qiagen filtration plate	150
Miscellaneous	100
Oligonucleotides	3,200
Total	3,650

[a] Cost estimates are based on catalogue price. Oligonucleotide value is based on an average primer length of 43 bases and a cost of $0.39/base for 96-well arrayed plates.

TABLE 5 Cloning Protocols and Cost Estimates per 96-Well Plates

Component(s)	Cost ($)
LIC cloning components	300
Consumables	25
Bacteriological supplies	10
Total	335

TABLE 6 Expression Analysis Protocols and Cost Estimates per 96-Well Plates

Component(s)	Cost ($)
Soluble Protein Production Protocols	
Bacteriological supplies	10
Consumables	50
Lysis reagent	10
Analysis Protocols	
Chemicals	40
Immunological supplies	20
Consumables	50
Total	180

7 REFERENCES

Aslanidis, C., and P.J. de Jong, 1990, “Ligation-Independent Cloning of PCR Products (LIC-PCR),” *Nucleic Acids Res.* 18:6069–6074.

Haun, R.S., I.M. Servanti, and J. Moss, 1992, “Rapid, Reliable Ligation-Independent Cloning of PCR Products Using Modified Plasmid Vectors,” *BioTechniques* 13:515–518.

APPENDIX A:

DETAILED FRAGMENT GENERATION PROTOCOLS

A.1 SETUP OF AMPLIFICATION REACTIONS

The PCR reaction plates are set up by using a SAMI method (PCR1) that uses the Multimek and Biomek workstations. An illustration of the PCR1 method is shown in Figure A.1.

Stations utilized: Carousel, Print and Apply, Multimek, Biomek, CS Incubator 2 at 4°C.

Program summary: Oligonucleotide and PCR plates are stored on Carousel 1. The forward/reverse primer plates and a 96-well oligo dilution plate are transported to the Multimek. The PCR plate is transported to the Print-and-Apply Station for barcoding with a PCR incremental suffix. The barcoded PCR plate is moved to the Multimek, and the Multimek program PCR2 is initiated.

The Multimek program PCR2 accomplishes the following (see Figure A.2):

- Prepares 10-fold dilution mixture of forward and reverse oligos,
- Transfers primer mix to PCR plate, and
- Utilizes the Wash Station to restrict tip usage to a single box.

The oligo and dilution plates are sent back to the carousel while the PCR plate is transported to the Biomek. Biomek runs program PCR prep1 to prepare reaction mixes and dispense components into the PCR plate.

The Biomek program PCR prep1 accomplishes the following (Figure A.3):

- Transfers individual reaction components into 8 wells in column 1 of a microwell plate and
- Mixes components and transfers them to the PCR plate.

The PCR plate is sent to the barcode reader for barcode tracking and is stored in the CS Incubator 2 until the SAMI program is completed. Lids are applied to the PCR plates before initiation of the DNA amplification program on the thermocycler.

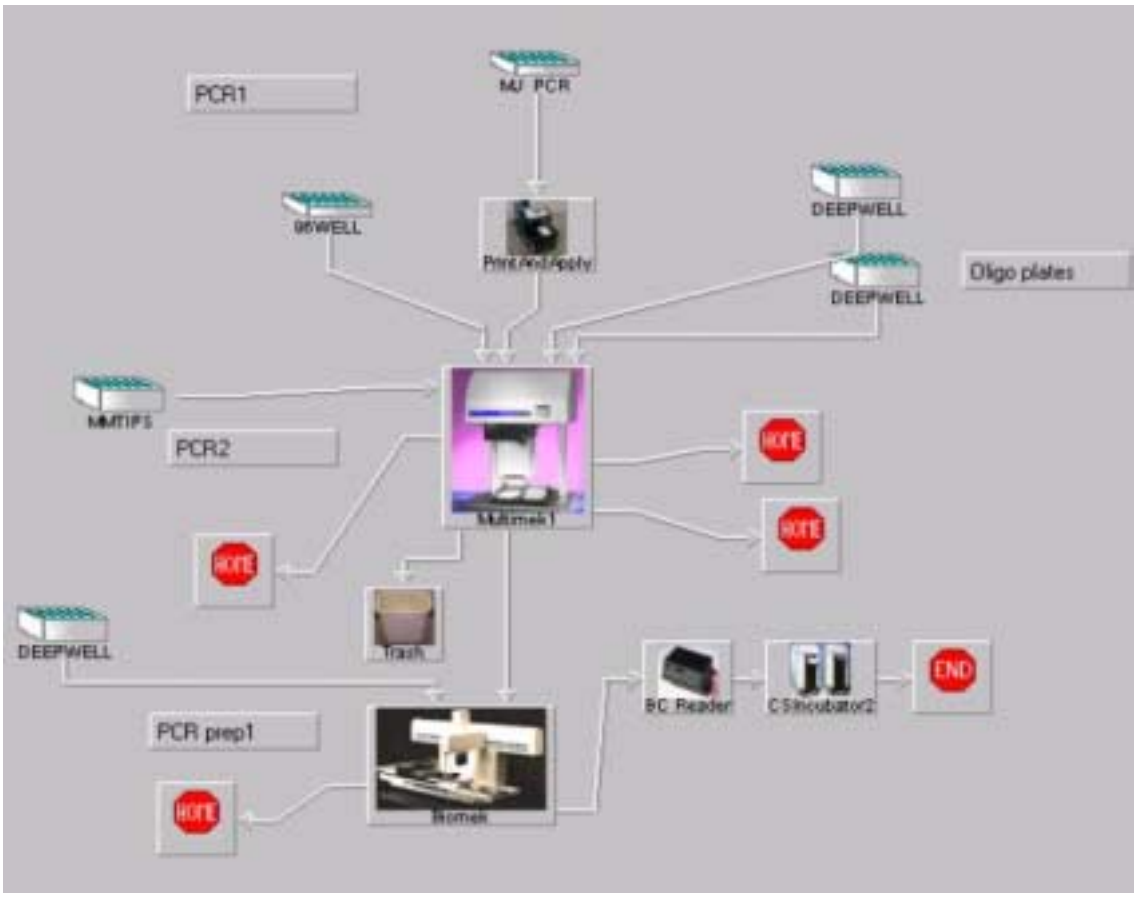


FIGURE A.1 Illustration of SAMI Method PCR1 for Automated Setup of PCR Reactions

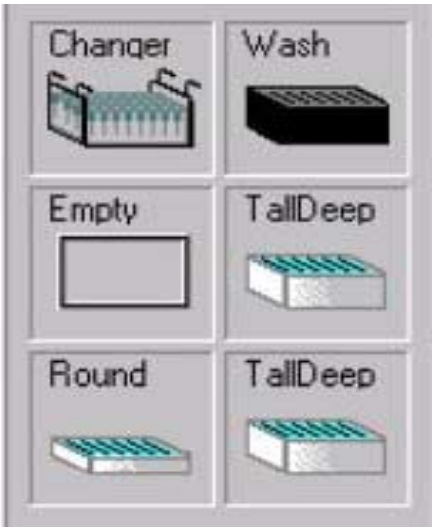
A.2 PURIFICATION OF AMPLIFIED PCR FRAGMENTS

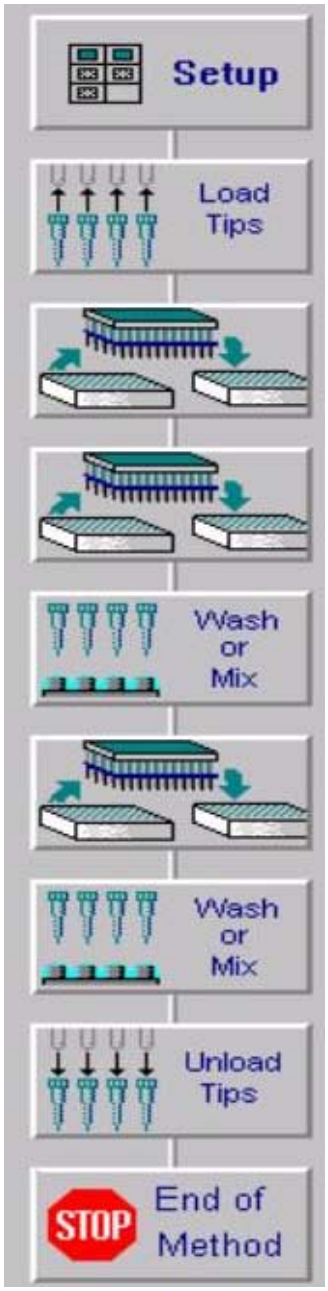
Upon completion of the thermocycler program, PCR plates are manually placed on the Biomek worksurface for fragment purification by using the PCR Frag1 program.

The Biomek PCR Frag1 program accomplishes the following (Figure A.4):

- Bulk dispenses Qiagen PB solution into the filter and PCR plate,
- Transfers contents of PCR plate to Qiagen filter plate,
- Applies vacuum and washes with Qiagen PE solution, and
- Adds elution buffer and collects purified fragments in square deepwell plate.

Multimek Deck Configuration





Transfer 17 µL from the wash station to PCR plate at position 1

Transfer 4 µL from the Talldeep plate at position 4 to the PCR plate at position 1

Transfer 4 µL from the Talldeep plate at position 2 to the PCR plate at position 1

FIGURE A.2 Multimek Method PCR2

```
Setup Configuration
Tool Rack at A2
Manually Move Tip Rack Holder to A6
Manually Move Tip Rack Holder to A5
Manually Move Tip Rack Holder to A4
Manually Move Thermal Ex to A3
Manually Move Microfuge 24 to A3
Manually Move 96-well deep to B3
Manually Move 96-well v-bottom to B5
Tool Rack at A1
Manually Move P250 Tip Holder to A5
Initial Configuration
// Check configuration
// Distiller water supply through Port 1
// Empty Wash Tool Reservoir
// Turn on Thermal reservoir chiller 30 minutes prior to method start
// Stock reagents placed in 3rd row (from back of Biomek) of Microfuge 24 holder
// Order stock reagents (right to left).
// Genomic DNA (minimum volume =100 uL, max = 1000 uL)
// 10x Pfx buffer (minimum volume = 600 uL, max = 1200 uL)
// dNTPs (20 mM) (minimum volume = 200 uL, max = 1000 uL)
// Magnesium Sulfate (minimum volume = 200 uL, max = 1200 uL)
// Pfx polymerase (minimum volume = 100 uL, max = 500 uL)
Reset Tip Rack at A5 to A1 (0 tips used)
Purge Wash8 using 2.00 ml Port 1
Bulk Dispense 0.585 ml Port 1 to B3 using Wash8
Pipette 75.00 µL from A3 to B3 using P200L - P250
Pipette 8.00 µL from A3 to B3 using P200L - P250
Pipette 17.00 µL from A3 to B3 using P200L - P250
Pipette 5.00 µL from A3 to B3 using P200L - P250
Pipette 9.00 µL from A3 to B3 using P200L - P250
Mix 200.00 µL at B3 using MP200 blowout - P250
Pipette 60.00 µL from B3 to B5 using MP200 blowout - P250
END
```

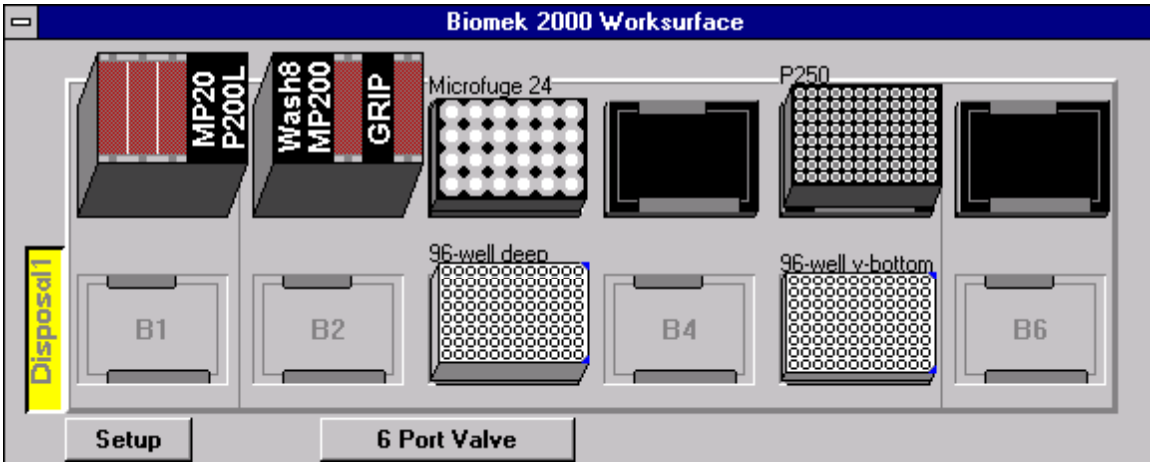


FIGURE A.3 Biomek Method PCR Prep1 for the Automated Preparation of PCR Amplification Reactions with Pfx DNA Polymerase

```
Setup Configuration
Tool Rack at A2
Manually Move Vacuum Manifold to A3
Manually Move QIAwell Holder to B5
Manually Move QIAprep to B5
Manually Move Labware Holder to A4
Manually Move Labware Holder to B6
Manually Move Labware Holder to B3
Manually Move 96-well square to B6
Manually Move Tip Rack Holder to A5
Manually Move P250 Tip Holder "B" to A5
Manually Move Collar Holder to B2
Manually Move Vacuum QIAcollar new to B2
Manually Move Labware Holder to B1
Manually Move PE9600 to B3
Manually Move Tip Rack Holder to A6
Manually Move P250 Tip Holder "A" to A6
Module Change at B4
Tool Rack at A1
Initial Configuration
// Place blotting paper in Labware Holders @ B1,B2
// Note: QIA holder in B5 is a modular reservoir holder.
// Reagent Volume Calculations (2mL Initial)
// EB volume = 10 mL
// PB use bottle
// PE use bottle
// Review port settings for proper buffer usage
Reset Tip Rack at A6 to A1 (0 tips used)
Reset Tip Rack at A5 to A1 (0 tips used)
Vacuum Control: Closed
// Make sure your vacuum pump is ON
//
//
// TRANSFER PCR PLATE TO QIA prep
Gripper Move B2, Vacuum QIAcollar new to A3
Gripper Move B5, QIAprep to A3
Purge Wash8 using 2.50 mL Port 5
Bulk Dispense 0.140 mL Port 5 to B3 using Wash8
Bulk Dispense 0.200 mL Port 5 to A3 using Wash8
Pipette 200.00 µl from B3 to A3 using MP200 blowout - B
//
// TRANSFER FROM QIAprep to WASTE
Vacuum Control: Open for 00:02:00
Svstem: Pause for = 00:00:15
```

FIGURE A.4 Biomek Method PCR Frag1 for the Automated Purification of PCR Fragments

```
// WASHING OF QIAprep PLATE WITH "PE"
Purge Wash8 using 2.50 mL Port 6
Bulk Dispense 0.750 ml Port 6 to A3 using Wash8
Vacuum Control: Open for 00:02:00
System: Pause for = 00:00:15
//
// BLOTTING THE PLATE
Gripper Move A3, QIAprep to B2
System: Pause for = 00:00:15
Gripper Move B2, QIAprep to A3
Vacuum Control: Open for 00:01:00
Gripper Move A3, QIAprep to B1
System: Pause for = 00:00:15
Gripper Move B1, QIAprep to A3
System: Pause for = 00:00:15
//
// POSITIONING OF THE ELUTION PLATE
Gripper Move A3, Vacuum QIAcollar new to B2
Gripper Move B6, 96-well square to A3
Gripper Move B2, Vacuum QIAcollar new to A3
Pipette 120.00 µL from B4 to A3 using MP200 blowout - A
System: Pause for = 00:01:00
Vacuum Control: Open for 00:03:00
Vacuum Control: Closed
//
// CLEANUP OF WASH8 TOOL
Purge Wash8 using 10.00 mL Port 1
Purge Wash8 using 10.00 mL Port 2
END
```

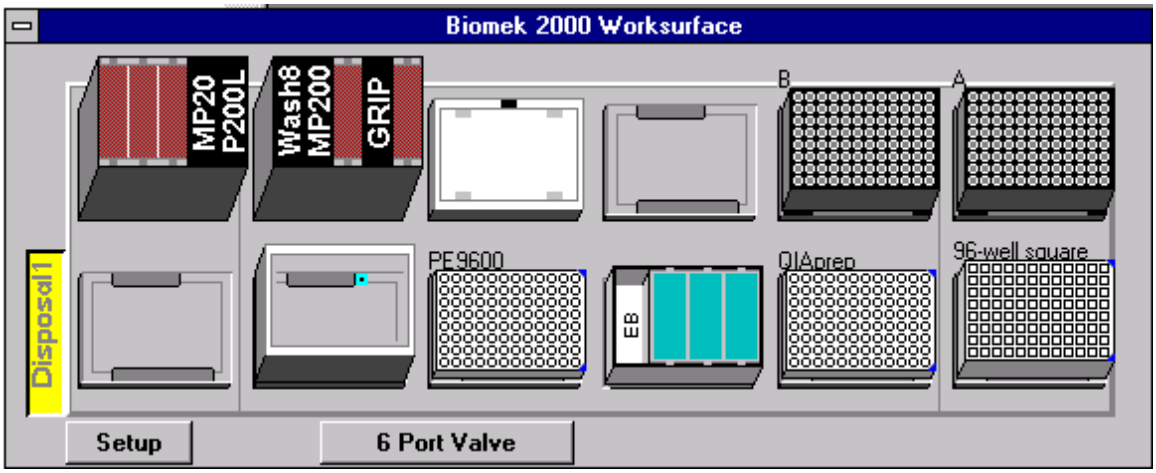


FIGURE A.4 (Cont.)

The DNA concentration of the microplate wells is determined by means of a fluorescent assay. This assay utilizes picogreen, which is a sensitive reagent for detecting dsDNA in solution. The assay conditions are designed to minimize the fluorescent contribution of RNA and single-stranded DNA. The DNA assay plates are prepared by using the Picogreen SAMI method, and the fluorescence is measured on a Wallac VICTOR fluorescence reader.

A.3 SAMI PICOGREEN METHOD

Stations utilized: Carousel, Print and Apply, Multimek, Biomek.

Method summary: Square deepwell and shallow 96-well plates are stacked in Carousel 1. Plates are transferred to the Biomek for implementation of the Biomek method by using picogreen (Figure A.5). During this method, the fragments are transferred to a shallow 96-well plate, and picogreen assay reagent is transferred from a reservoir into a fluorescent-compatible microwell plate. An aliquot of the purified amplification sample is transferred to the assay plate and mixed with the picogreen reagent. Plates are manually transported to the fluorescent reader.

```
Setup Configuration
Manually Move Tip Rack Holder to A6
Manually Move Tip Rack Holder to A5
Manually Move P250 Tip Holder to A5
Tool Rack at A2
Tool Rack at A1
Manually Move Lid to Off System
Module Change at B4
Manually Move 96-well flat to B6
Manually Move P20 Tip Holder to A4
Manually Move 96-well v-bottom to B5
Manually Move Thermal Ex to B2
Initial Configuration
Reset Tip Rack at A4 to A1 (0 tips used)
Reset Tip Rack at A5 to A1 (0 tips used)
Pipette 100.00 µL from B4 to B6 using MP200 - P250
Pipette 1.00 µL from B5 to B6 using MP20 deliver - P20
END
```

Figure A.5 Biomek Method Picogreen for the Automated Determination of DNA Concentration

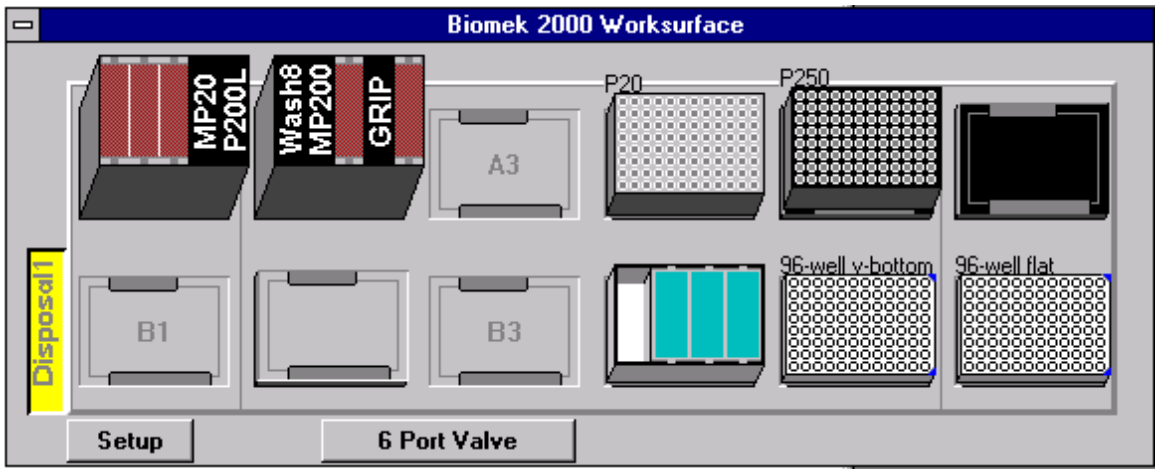


Figure A.5 (Cont.)

APPENDIX B:

DETAILED CLONING PROTOCOLS

B.1 PREPARATION OF FRAGMENTS FOR LIC CLONING METHOD

Stations utilized: Carousel, Print and Apply, Multimek, Biomek, CS2 incubator at 4°C, CS Incubator 1 at 37°C, Heatblock at 75°C.

Before the cloning protocols are initiated, the concentration of the purified PCR fragments is adjusted to allow for a uniform transfer of a fixed volume in the LIC reaction sequence. Replacing this manual intervention process with an automated method is a high-priority action item. The LIC reaction plates are set up by using a SAMI method (LICR×2) that utilizes the Multimek and Biomek workstations. An illustration of the LICR×2 method is shown in Figure B.1. PCR fragment and reaction plates are transported from the carousel to the Multimek that uses the LICR×1program to array the purified PCR fragments in a 96-well reaction plate.

The Multimek program LICR×1 accomplishes the following (Figure B.2):

- Arrays 10 μL of purified PCR fragments into an LIC reaction plate and
- Utilizes the Wash Station to provide an opportunity to reuse tip box.

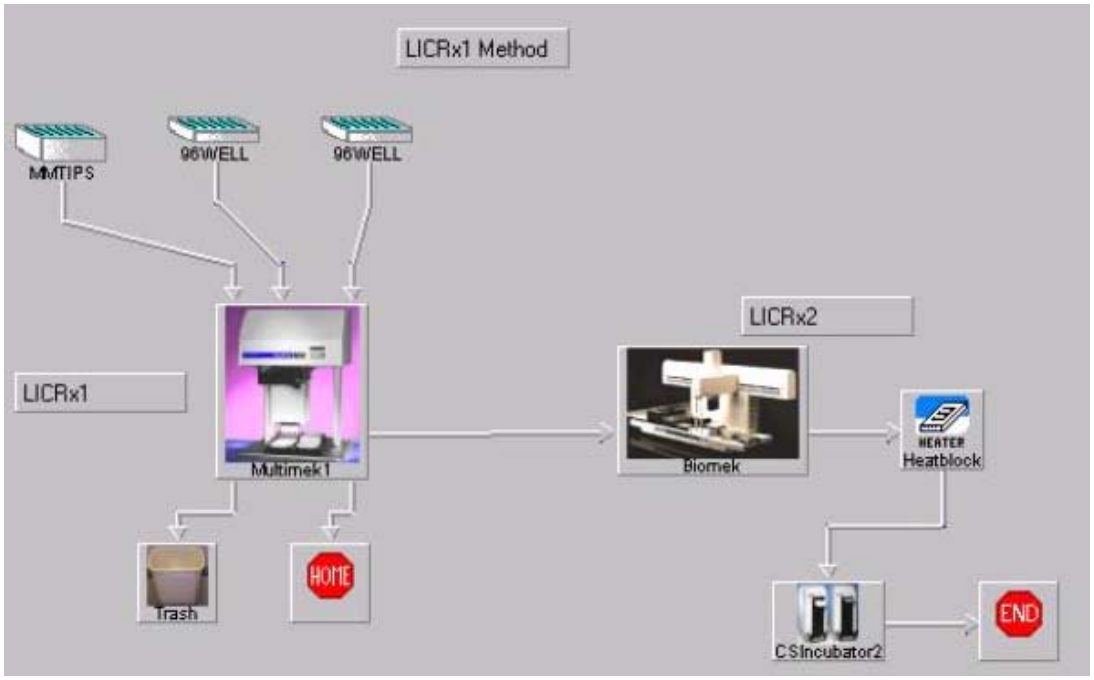
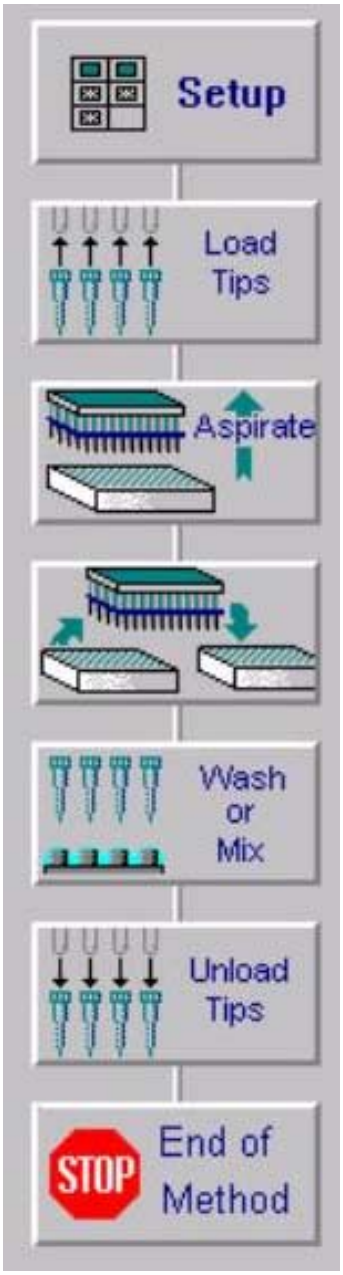


FIGURE B.1 Illustration of SAMI Method LICR×1 for Automated Setup of LIC Reactions

Multimek Deck Configuration



Aspirate 10 µL from the flat bottom plate at position 4

Aspirate 4 µL from the round bottom plate at position 2 to the round bottom plate at position 1

FIGURE B.2 Multimek Method LICR×1

The LIC reaction plate is transported to the Biomek workstation for completion of the LIC reaction. At present, the Biomek LICR×3a method is configured for a single column of 8 reactions. The present method is scalable to several 96-well plates but would require reconfiguration of the reagent stocks in the Microfuge 24 holder.

The Biomek program LICR×3a accomplishes the following (Figure B.3):

- Mixes individual reaction components in a reaction tube,
- Transfers the reaction mix into 8 wells in column 1 of a microwell plate, and
- Reconfigures the Biomek worksurface for subsequent methods.

Once the LIC reaction has been set up, the plate is transported to temporary storage at room temperature for 18–25 min. The flexible storage interval allows for completion of the enzymatic component of the LIC reaction and enhances scheduling efficiency for the SAMI method. When the enzymatic reaction component is complete, the plate is transported to the Biomek for initiation of the LICR×3b method.

The Biomek program LICR×3b overlays LIC reactions with 50 μL of mineral oil. The mineral oil prevents evaporation from the LIC reactions during subsequent methods. The LIC reaction plate is transported to the heatblock (manually set at 75°C) and incubated for 20 min to inactivate T4 DNA polymerase. In the final step of the method, the LIC reaction plate is transported to the CS2 incubator for storage at 4°C.

B.2 FRAGMENT CLONING AND BACTERIAL TRANSFORMATION USING THE SAMI ANNEAL1 METHOD

Stations utilized: Carousel, Print and Apply, Multimek, Biomek, CS Incubator 2 at 4°C, CS Incubator 1 at 37°C, Heatblock at 45°C.

The LIC annealing and bacterial transformation plates are set up by using a SAMI method (Anneal1) that utilizes the Multimek and Biomek workstations. An illustration of the Anneal1 method is shown in Figure B.4. The v-bottom 96-well and the LIC reaction plates from the LICR×2 method are transported to the Biomek workstation. Annealing reactions are prepared by implementing Biomek method Ttest3-1 The Biomek LICR×3a method is configured for a single column of 8 reactions. However, the method is scalable to several 96-well plates after reconfiguration of the reagent stocks in the Microfuge 24 holder.

Setup Configuration
Tool Rack at A2
Tool Rack at A1
Manually Move Thermal Ex to A3
Manually Move Thermal Ex to B2
Manually Move P20 Tip Holder to A5
Manually Move P250 Tip Holder to A6
Manually Move MJ Thermocycler plat to B2
Manually Move MJ Thermocycler plat to B3
Manually Move Microfuge 24 to A3
Module Change at B1
Initial Configuration
Reset Tip Rack at A6 to A1 (0 tips used)
Reset Tip Rack at A5 to A1 (0 tips used)
Pipette 200.00 µL from A3 to A3 using P200L - P250
Pipette 90.00 µL from A3 to A3 using P200L - P250
Pipette 90.00 µL from A3 to A3 using P200L - P250
Pipette 60.00 µL from A3 to A3 using P200L - P250
Pipette 18.00 µL from A3 to A3 using P200L - P250
Pipette 50.00 µL from A3 to B2 using P200L - P250
Pipette 10.00 µL from B2 to B3 using MP20 - P20
System: Pause for = 00:30:00, Message
Pipette 40.00 µL from B1 to B3 using MP200 blowout - P250
END

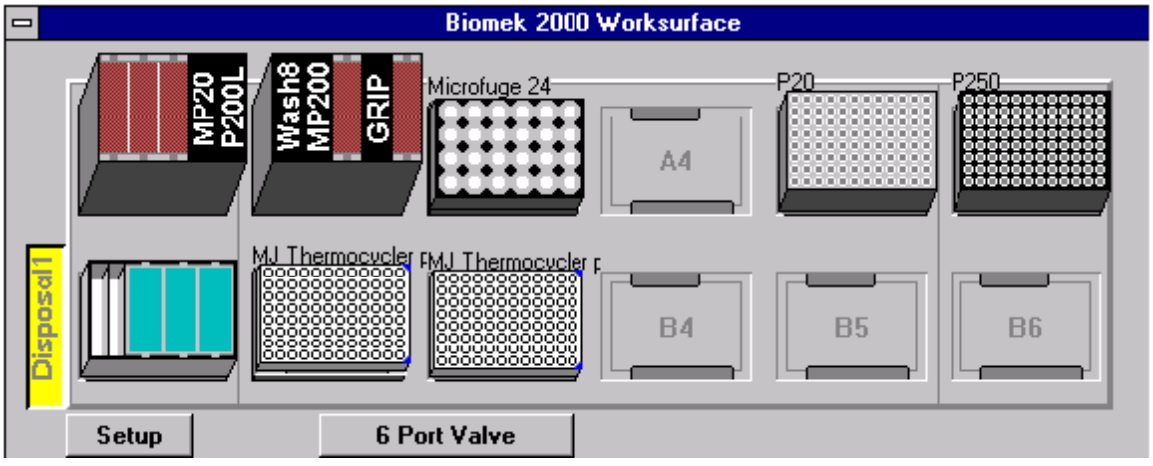


FIGURE B.3 Biomek Method LICR3a for the Automated Preparation of LIC Reactions

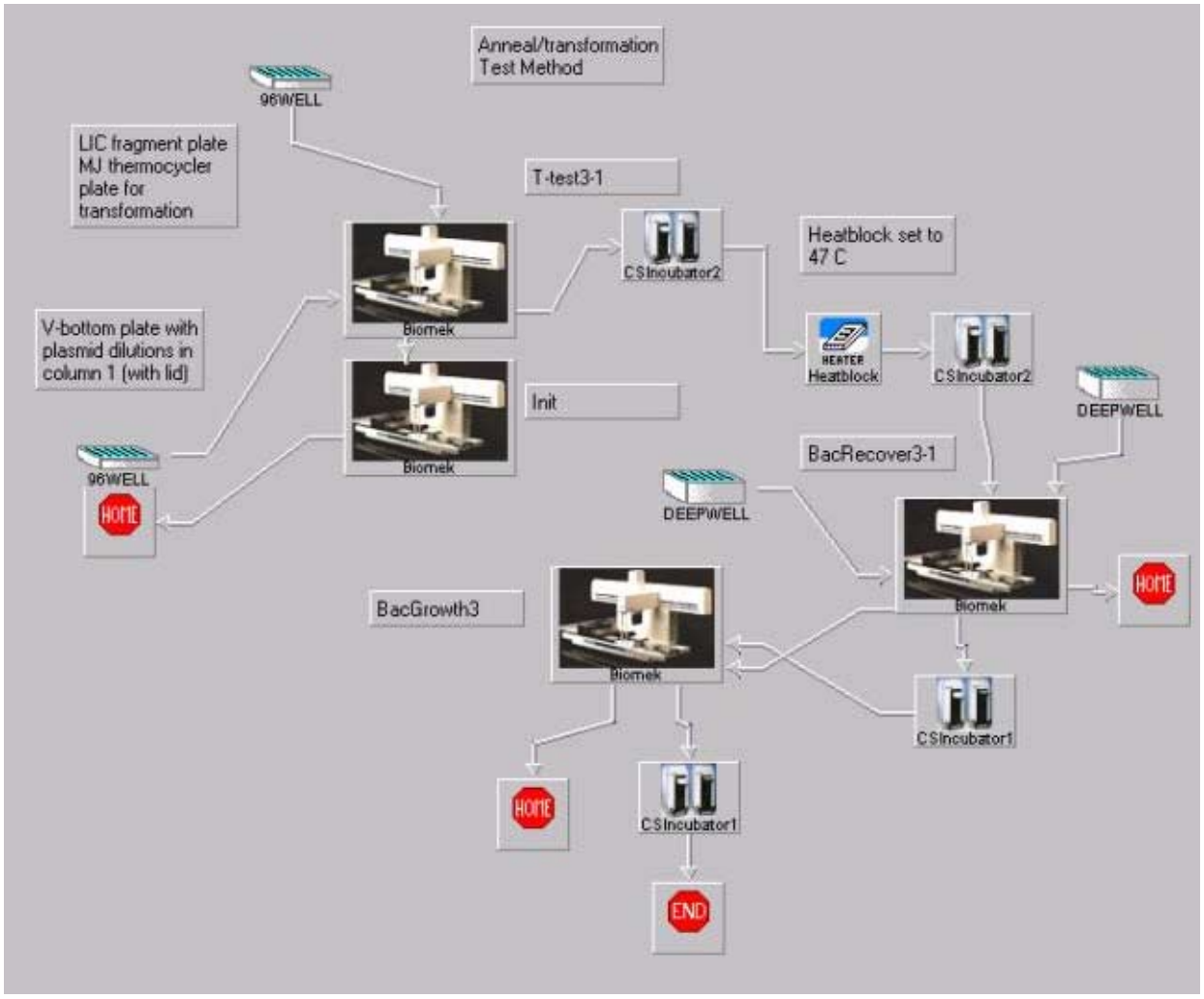


FIGURE B.4 Illustration of SAMI Method Anneal1 for Automated Setup of Annealing Reactions and Bacterial Transformation

In the Biomek program Ttest3-1 (Figure B.5),

- A prepared solution of the expression construct in the Microfuge 24 rack is distributed into 8 wells in column 1 of a v-bottom 96-well plate,

- 1.5 µL of the LIC reaction is transferred to the corresponding wells of the v-bottom plate and mixed with the vector solution,

Setup Configuration
Tool Rack at A2
Tool Rack at A1
Manually Move Tip Rack Holder to A6
Manually Move Tip Rack Holder to A5
Manually Move Thermal Ex to A3
Manually Move Thermal Ex to B2
Manually Move P20 Tip Holder to A5
Manually Move P250 Tip Holder to A6
Module Change at A3
Manually Move MJ Thermocycler plate to B4
Manually Move 96-well v-bottom to B3
Manually Move P250 Tip Holder "mix tips" to A4
Initial Configuration
Reset Tip Rack at A6 to A1 (0 tips used)
Reset Tip Rack at A4 to A1 (0 tips used)
Reset Tip Rack at A5 to A1 (0 tips used)
Gripper Move B4, MJ Thermocycler plate to B2
// COMPETENT CELLS
Pipette 60.00 µL from A3 to B2 using MP200 - P250
// Expression plasmid addition
Pipette 5.00 µL from B3 to B2 using MP20 - P20
Gripper Move B2, MJ Thermocycler plate to B4
END

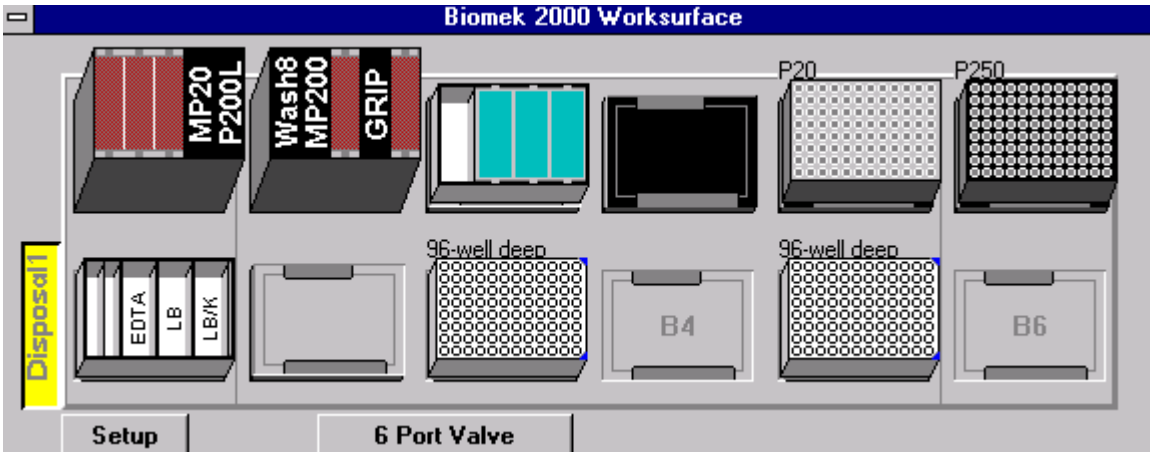


FIGURE B.5 Biomek Method Ttest3-1 for Automated Transformation of Chemically Competent Bacterial Cells with Heat-Inactivated LIC Cloning/Annealing Reactions

- The annealing plate is moved to temporary storage for 15–25 min. During this period, the worksurface of the Biomek is reconfigured for the subsequent transformation reactions. This reconfiguration involves placing a thermal reservoir containing chemically competent bacteria on the cold block at Biomek position A3. Upon completion of the annealing reactions, the annealing plate is transported to the Biomek.

- EDTA solution is distributed into 8 wells of an MJ thermocycler 96-well plate.

- 8 μL of the annealing reaction is transferred to the corresponding wells of the transformation plate and mixed with the EDTA solution.

- The Gripper tool moves the transformation plate to a chilled reservoir.

- 55 μL of chemically competent bacteria is added to 8 wells of the transformation plate.

The transformation plate is transported to the CS Incubator 2 for incubation at 4°C for 5 min. The bacteria are heat shocked by transport to the heatblock (set to 45°C) for a 40-s incubation. After heat shock, the transformation plate is returned to the CS Incubator 2 for 5 min. The transformed cells and a deepwell plate are transported to the Biomek workstation so that the bacterial recovery plate can be set up.

The Biomek program BacRecover1 accomplishes the following (Figure B.6):

- Fills three columns of a 96-deepwell plate with LB broth from the reservoir at B1 and

- Transfers appropriate amounts of transformed cells to LB broth in the deepwell plate.

The deepwell plate is transferred to the CS Incubator 1 for incubation at 37°C for 10–30 min. After recovery, a new deepwell plate and the transformed bacteria plate are transported to the Biomek for setup of the bacterial growth plate.

The Biomek program BacGrowth accomplishes the following (Figure B.7):

- Fills three columns of a 96-deepwell plate with LB broth from reservoir at B1 and

- Transfers appropriate amounts to LB broth in the deepwell plate.

The deepwell plate is transferred to the CS Incubator 1 for overnight growth at 37°C.

```
Setup Configuration
Tool Rack at A1
Tool Rack at A2
Manually Move Thermal Ex to A3
Module Change at A3
Manually Move Tip Rack Holder to A4
Manually Move Tip Rack Holder to A5
Manually Move P20 Tip Holder to A5
Manually Move Tip Rack Holder to A6
Manually Move P250 Tip Holder to A6
Module Change at B1
Manually Move Thermal Ex to B2
Manually Move MJ Thermocycler plat to B4
Manually Move 96-well deep to B3
Initial Configuration
// Add LB to position 3 in reservoir at Biomek location B1
// Add LB/Kan to position 4 in reservoir at Biomek location B1
Gripper Move B3, Lid to B6
// Use wash8 to add LB recovery broth
Reset Tip Rack at A6 to A2 (8 tips used)
Bulk Dispense 0.350 mL Port 4 to B3 using Wash8
Pipette 70.00 µL from B4 to B3 using MP200 - P250
Gripper Move B6, Lid to B3
END
```

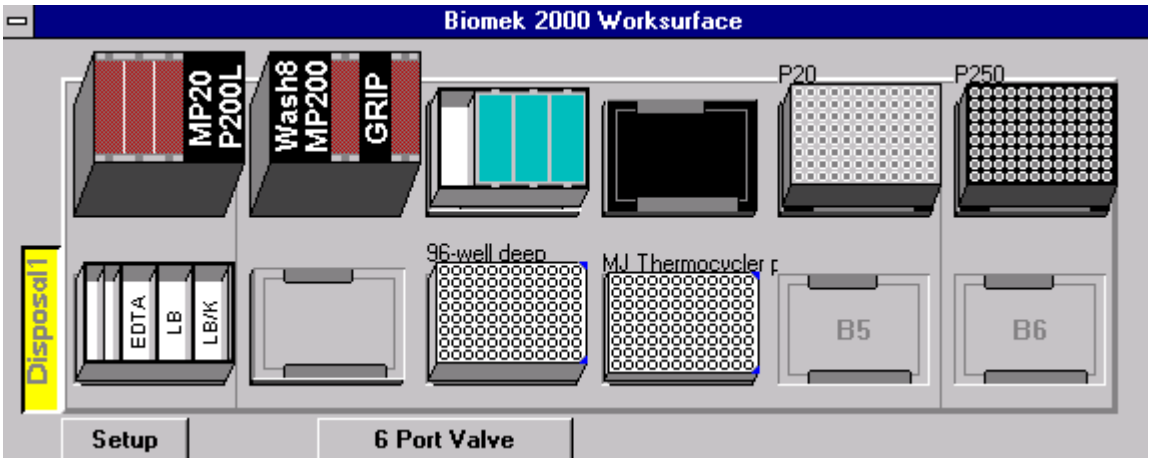


FIGURE B.6 Biomek Method Bacrecover1 for Automated Plating of Transformed Bacteria to Allow for the Recovery of Antibiotic Resistance

```
Setup Configuration
Tool Rack at A1
Tool Rack at A2
Manually Move Thermal Ex to A3
Module Change at A3
Manually Move Tip Rack Holder to A4
Manually Move Tip Rack Holder to A5
Manually Move P20 Tip Holder to A5
Manually Move Tip Rack Holder to A6
Manually Move P250 Tip Holder to A6
Module Change at B1
Manually Move Thermal Ex to B2
Manually Move 96-well deep to B3
Manually Move 96-well deep to B5
Initial Configuration
Gripper Move B3, Lid to B4
// LB/Kan dispense to col 3 and 5
Bulk Dispense 1.500 mL Port 5 to B5 using Wash8
Aspirate B5 for 1.00 s using Wash8
// Distribute bacteria from recovery plate to growth plate
Pipette 10.00 µL from B3 to B5 using MP200 - P250
Pipette 20.00 µL from B3 to B5 using MP200 - P250
Pipette 100.00 µL from B3 to B5 using MP200 - P250
Gripper Move B4, Lid to B5
```

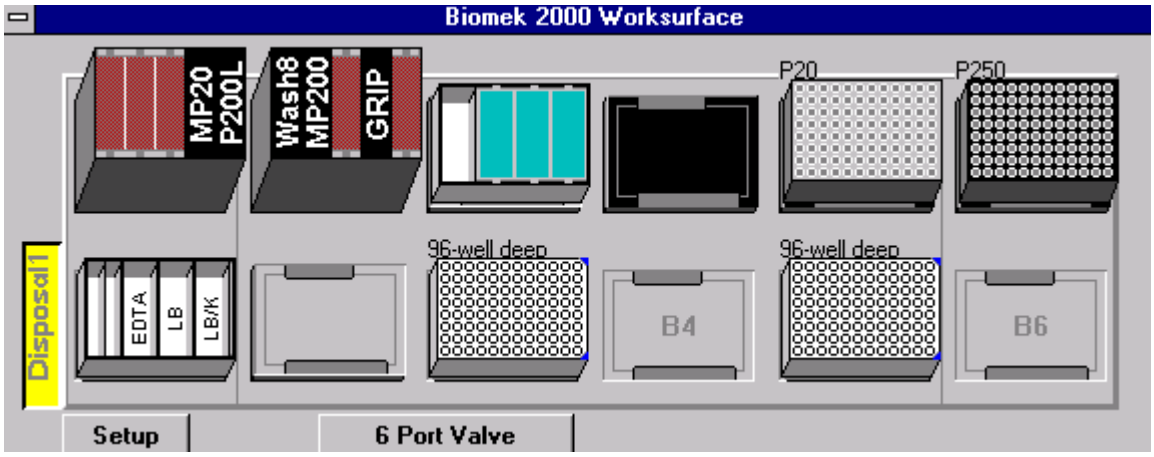


FIGURE B.7 Biomek Method Bacrecover1 for Automated Plating of Transformed Bacteria in Selective Medium

APPENDIX C:

DETAILED EXPRESSION ANALYSIS PROTOCOLS

C.1 BIOMEK BACLYSATE METHOD

The Biomek BacLysate method prepares a soluble protein fraction from the induced bacterial cultures. The method provides for the collection of the induced bacteria on a filtration membrane by using a series of pipette and vacuum filtration steps. The bacteria are lysed on the filtration membrane with a commercially available bacterial lysis reagent (BugBuster, Novagen, Inc.). The bacterial lysate is mixed for several minutes, and the soluble proteins are collected in a 96-well v-bottom plate by vacuum filtration. The schedule time for the 24-well method is 16 min. This method is currently implemented for 1–3 columns (8–24 wells) of a deepwell culture plate. To increase schedule efficiency, a 96-well plate scale method will be implemented on the Multimek.

C.2 IMMUNOASSAY FOR SOLUBLE EXPRESSED PROTEINS

C.2.1 Biomek Immuno2 Method

The Biomek Immuno2a method prepares serial dilutions of protein solutions for subsequent immunoassay procedures. The present procedure is designed as a stand-alone Biomek method for 2–4 protein solutions with a schedule time of 5 min. The primary purpose of this method is to evaluate the design methodology for the immunoassay plates. Future methods will use the Multimek workstation to prepare multiple dilution plates of the soluble protein plate.

C.2.2 Imm2a Method

The Imm2a SAMI method processes the serial dilution plates prepared from the soluble protein plates. A series of 2–3 dilution plates will be prepared for each soluble protein plate. The method is configured as a series of Biomek methods separated by incubation intervals. This type of arrangement allows maximum schedule efficiency for a large number of plates. As can be observed from the schedule times (Table C.1), increasing the number of plates by a factor of 9 results in a two-fold increase in the schedule time.

TABLE C.1 Schedule Times for Imm2a Method

Number of plates	Time (h:min)
1	2:54
2	3:15
3	3:37
6	4:41
9	6:09

The Biomek methods of the SAMI Imm2a are as follows:

- *ImmBlock* — Removes protein solution and adds BSA solution as a blocking agent.

- *ImmHisTag* — Removes BSA blocking solution and adds HisTag detection reagent.

- *ImmWash2* — Removes resident solution and washes plate.

After the last wash procedure, detection reagent is added and the absorbance is determined by manual intervention by using a Wallac 1420 VICTOR F fluorometer.