IS-T 1851

Automation and Integration of Multiplexed On-Line Sample Preparation with Capillary Electrophoresis for DNA Sequencing

by

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PHD Thesis submitted to Iowa State University

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Date Transmitted: March 31, 1999

PREPARED FOR THE U.S. DEPARTMENT OF ENERGY

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To my lovely wife

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ABSTRACT

The purpose of this research is to develop a multiplexed sample processing system in conjunction with multiplexed capillary electrophoresis for high-throughput DNA sequencing. The concept from DNA template to called bases was first demonstrated with a manually operated single capillary system. Later, an automated microfluidic system with 8 channels based on the same principle was successfully constructed. The instrument automatically processes 8 templates through reaction, purification, denaturation, pre-concentration, injection, separation and detection in a parallel fashion. A multiplexed freeze/thaw switching principle and a distribution network were implemented to manage flow direction and sample transportation. Dye-labeled terminator cycle-sequencing reactions are performed in an 8-capillary array in a hot air thermal cycler. Subsequently, the sequencing ladders are directly loaded into a corresponding size-exclusion chromatographic column operated at ~60 °C for purification. On-line denaturation and stacking injection for capillary electrophoresis is simultaneously accomplished at a cross assembly set at ~70 °C. Not only the separation capillary array but also the reaction capillary array and purification columns can be regenerated after every run. DNA sequencing data from this system allow base calling up to 460 bases with accuracy of 98%. Further optimization of the operational conditions and software should extend the read length. The system is scaleable to a 96-capillary array and will benefit not only high-speed, high-throughput DNA sequencing but also in genetic typing, population screening and drug discovery.

The influence of three classes of fluorescence labels including dipyrrometheneboron difluoride (BODIPY), energy transfer (ET) and conventional fluorescein and rhodamine (ABI) on DNA sequencing have been examined with laser-induced fluorescence detection and poly(ethylene oxide)-filled capillary electrophoresis. DNA sequencing fragments were generated by

dye-labeled primer cycle-sequencing reactions in a hot air thermal cycler. A parameter, relatively-induced shift, was introduced to quantify the uniformity of electrophoretic mobilities of these fragments. BODIPY was found to introduce the smallest, but non-zero, effect for the dye-induced non-uniformity. Although ET dyes provided the highest sensitivity due to their unique spectroscopic property, they were found photodegradation easier than BODIPY and ABI dyes. Characterization also brings out some important tips to select the suitable dye set for the two-channel ratio-based DNA base-calling method.

CHAPTER 1. GENERAL INTRODUCTION

Thesis Organization

This dissertation consists of six chapters, beginning with a chapter of general introduction and ending with a general conclusion. In the introduction, a brief literature review has been conducted to summarize the related technologies in the dissertation and identify our approach to achieve the cost-effective, high-speed and high-throughput DNA sequencing. In chapter 2, 3 and 5, our approach in on-line integrating sample preparation into capillary array electrophoresis have been described step by step. Fluorescence dye chemistry in DNA sequencing also has been discussed in Chapter 4. Chapter 2, 3, and 4 are manuscripts published in a scientific journal. The dissertation then concludes with the significance of this research and the prospective for future research. The appendices include the information of hardware and software in detail.

Introduction

Life science has been envisioned as the science of the 21st century. Toward this direction, the genetic information for all organisms should be achieved, stored and understood. DNA sequencing is an essential technology used to identify genes and mutations, to confirm successful site-directed mutagenesis, and to analyze phage-display libraries. From its inception two decades ago, it has rapidly revolutionized biology, agriculture and medicine. Decoding the genetic information not only provides a better understanding of biology but also empowers us with the capability to manipulate and recreate a balanced environment for all living things.

DNA consists of a very long, threadlike polymer made from four monomeric components called nucleotides: deoxyadenosine (dA, abbrievated as A), deoxycytidine (dC or C), deoxyguanosine (dG or G), and deoxythymidine (dT or T). The nucleotides consist of three parts, a base that varies for the four

nucleotides, the sugar deoxyribose, and a phosphate group. The phosphate is attached to the 5' end of the sugar and a single hydroxyl group is attached to the 3' sugar position. The monomeric units are joined together by phosphodiester bonds between the 5' phosphate and the 3' hydroxyl group of adjacent sugars. As a result, a DNA strand has orientation: there is a 5' end with a phosphate group and a 3' end with a hydroxyl group. In most organisms, DNA is in a double helix. The two strands are complementary to each other; A is always paired with T and C is always paired with G. If the sequence of one strand is known, the sequence of the complementary strand is also known. It is the sequence of nucleotides in the DNA molecule that carries the genetic information. A gene is an ordered sequence of nucleotides representing the fundamental physical and functional unit of heredity. The complete set of genes from an organism is called a genome; in humans, it contains about three billion base pairs (bps) of DNA organized into 23 pairs of chromosomes. Research and related technology development aimed at mapping and sequencing the entire genome of a particular organism is called a genome project.

The U.S. Human Genome Project (HGP) is a joint effort of the Department of Energy and the National Institutes of Health formally initiated in 1990. Its stated goal is "... to characterize all the human genetic material-the genome-by improving existing human genetic maps, constructing physical maps of entire chromosomes, and ultimately determining the complete sequence ... to discover all of the more than 50,000 human genes and render them accessible for further biological study." The original 5-year plan was updated and modified in 1993[1] due to rapid progress. In the updated version of the five-year research goals of HGP (FY 1994 through FY 1998), one of major goals under "DNA sequencing" category is "...to develop technology for high-throughput sequencing, focusing on systems integration of all steps from template preparation to data analysis[2]." The program of study in this dissertation is a direct response to the call of the Human Genome Project.

Automation and Integration for DNA Sequencing

Automation of DNA sequencing began in 1986-1987 when Smith and co-workers in Hood's laboratory[3], workers in Ansorge's group[4], and Prober and co-workers at DuPont[5] replaced the radioactive labels with fluorescent labels and replaced autoradiography with laser-induced fluorescence detection. Laser-induced fluorescence allows the direct reading of the electropherogram into a computer for automated sequence determination so that it opens the door to integration of separation, detection and data analysis. This also makes the Human Genome Project possible. In Smith's method, dye-terminated primer labeling scheme was coupled with four-color four-channel fluorescence detection. In Prober's method, dye-labeled termination was combined with four-color two-channel detection. In Ansorge's method, only one-color one-channel detection but four separate lanes were used. Smith's, Prober's and Ansorge's fluorescence sequencers were commercialized by Applied Biosystems (ABI), DuPont, and Pharmacia, respectively. The DuPont sequencing technology was eventually sold to ABI. ABI now enjoys almost 90% of market share in DNA sequencing. Typically, the ABI model 377A has 36 lanes running simultaneously on a slab gel to produce sequencing rates of 200 bases/h/lane or 7200 bases/h/ slab.

Although an improvement over manual sequencing, current automated sequencers have some limitations. In the traditional slab gel electrophoresis with relatively thick gels, Joule heating degrades the separation performance and limits the separation speed that obtained at relatively lower voltage. Furthermore, preparation of a slab gel is tedious and awkward, and sample loading onto the wells of a slab gel requires significant manual dexterity. Both preparation and loading of slab gels are very difficult to automated. Also, it is difficult to generate larger number of channels on a slab gel because most current slab-gel sequencers employ on a scanning detection system so that the duty cycle of which decreases with an increase in the number of channels. The signal-to-noise ratio of detection degrades with the decrease of the duty cycle.

Fortunately, in a capillary-based system, all the problems listed above are minimized. The thermal properties of capillaries allow the use of higher electric field, which generate faster separation and higher resolution. The application of replaceable sieving matrix to capillary permits reloading of the fresh matrix automatically with a simple syringe pump. In the injection, it has smaller i.d. and only requires smaller amount of samples; and its flexibility helps in an interface. Highly dense packing of capillaries allows more channels to be used so that it will greatly increase the throughput. Therefore, multiplexed capillary electrophoresis has significant advantage over slab gel electrophoresis.

Multiplexed Capillary Electrophoresis

With the throughput pressure in sequencing human genome, technologies to automate and integrate multiplexed capillary electrophoresis with laser-induced fluorescence detection are under very rapid development. Recently, sequencing runs in a set of 96 capillaries have been demonstrated. Technical challenges are being explored intensively.

Excitation and Detection. Laser excitation and detection schemes for multiplexed capillary electrophoresis fall into two categories: those that use optical scanning techniques or those that use optical imaging. A scanner equipped with a confocal fluorescence detection system sweep across an array of capillaries back and forth during electrophoresis[6-7]. Several color-coding schemes have been tested with adequate good sensitivity. These confocal scanners use a small local illumination and detection volume requiring only modest excitation powers for optimal signal-to-noise. A simple sweep of this local volume ensures elimination of cross talk between adjacent capillaries. High repetition rate scan systems with sensitive closed feedback loops are required and therefore limit the number of channels that may be scanned. Recent development in this line is using a rotating objective instead of horizontal sweeping the capillary array that may lead to up to 1000 capillaries[8]. However, this is not a true parallel detection due to scanning optical system and data acquisition.

Optical imaging methods for DNA sequencing using multiple capillaries can be divided into two classes based on the location of illumination: on-column and end-column. The end-column methods advanced by both Dovichi's[9] and Kambara's groups[10], employed sheath-flows with multiple capillary systems that demonstrated very sensitive operation. Two laser beams are combined to cross the flow streams in a line for excitation, and a CCD is used for simultaneous detection perpendicular to the excitation beam. Two-dimensional packing of capillary array has been acclaimed[11-13]. However, it is quite difficult to flush the sheath flow chamber between runs.

Variations of on-column illumination include axial-beam, side-entrance and expended laser beam methods, advanced by Yeung's group[14-18]. In axial-beam illumination mode, optical fibers are inserted into the ends of capillary array and fluorescence is collected with CCD camera at the perpendicular direction. The difficulty with this scheme is the potential to alternation of mobility of fragments and affect separation. In the side-entrance illumination mode, a laser beam perpendicularly focuses on the capillary array on the same plane of the array. A wave-guide was used to achieve uniform illumination. In expanded laser beam mode, a laser expander lengthens the focused laser line to cut across a large number of capillaries. Alignment is trivial and rugged. A wide-angle lens that provides a low magnification allows close focusing and thus a better effective f-number without additional image distortion. Crosstalk can be minimized by adjusting the capillary-to-pixel ratio for imaging. The only drawback here is to require high laser power for excitation in order to achieve adequate signal-to-noise. It is preferable to have a highly sensitive CCD camera to collect fluorescence image. In general, the high read-out rate of CCD cameras for simultaneous detection of fluorescence from capillary arrays provides flexibility for higher sampling requirements as the capillary separation rates improve. Since imaging methods do not require moving optical elements, the only limitation to the number of capillaries used in a particular design is the

density of capillaries that may be packed into image field and the size of the image field.

Sieving Matrixes. One of the key focus in developing multiplexed DNA sequencing system is the development of sieving matrixes with high separation speed, long read length, and replaceable operation. The sieving matrix must have very low viscosity to flush in and out capillaries easily. In the early works, crosslinked polyacrylamides[19-28] were used as matrices to separate DNA. However, due to the instability over time, irreproducibility in the polymerization and cross-linking processes, and the fragile nature of the medium, crosslinked PAA in CE have not been reported to last for more than a few runs. The call for low to moderate viscosity entangled polymers for CE have initiated a matrix-hunting champion in recent years. Starting from linear polyacrylamide (LPA)[29-31], methylcellulose[32-33], hydroxyalkyl cellulose[34-35], polyhydroxy-polyethylene glycol-methacrylate[36], polyvinylalcohol[37-38], polyethylene oxide[39-40], fluorocarbon end-capped ployethleneglycol[41-42], polyvinylpyrrolidone[43] and poly N-substituted acrylamides[44-46] have been employed for DNA separations. In terms of separation and read length, LPA is still on the top of the list, which has been achieved up to 1000 bp. In terms of viscosity, polyvinylpyrrolidone (PVP, 4.5% with 27 cP) and polydimethylacrylamide (PDMA, 6% with 75 cP) seem to reach the low end with reasonable resolution up to 600 bp.

Coatings. Another important issue in DNA separation is the capillary inner surface modification to suppress both electroosmotic flow and capillary wall and DNA interaction. Most research groups applied covalently bonded polymers into the capillary inner surface. Hjeten's polyacrylamide[47-48] coating is widely used for DNA sequencing. Other coatings like DB-10[41-42] from J&W Scientific and polyvinylalcohol[49] were also applied in DNA separation. The temperature stability and lifetime of these coatings are less reported, but the degradation[50] of the coatings have been noticed. Although covalently coated capillaries are widely used, the coating methods increase their cost by requiring

in situ synthesis and often give rise to problems such as capillary fouling and coating inhomogeneity. Not only does this increase the cost for sequencing but also it decreases the reliability. Other approach is to employ non-covalent bonding. Fung and Yeung[40] first report the use of this method to suppress electroosmotic flow (EOF) with polyethylene oxide. Recently, ploydimethylacrylamide and polyvinylpyrrolidone also have been reported for suppression of EOF in DNA sequencing. The advantage of non-covalent coating is that they are more rugged, cost effective and renewable for each run.

New technologies. Several non-electrophoretic technologies such as single molecule sequencing, sequencing by hybridization, and mass spectrometric methods are under active development, but none of them seems to be able to replace the current electrophoresis separations in DNA sequencing. The reasons and comparisons are not given in detail here. Just briefly, single molecule sequencing is an elegant approach but it needs for a better labeling scheme and a faster exonucleose cleavage reaction. Sequencing by hybridization requires a good eye to determine a perfect match and a slight mismatch and also locate a repeat sequence into the different region in the assembling process. Mass spectroscopic methods progress very rapidly but it is still difficult to get a representative ion bundle of DNA fragments. Even though the ion bundle can be generated, the inherent isotope broadening and sodium and potassium ions attachment will still be a problem. So, with today's technology, multiple capillary electrophoresis is the most maturated format for multiplexed CE runs.

Automation of Sequencing Reaction

The "plus and minus" technique[51] developed by Sanger may be considered as the inception to the modern gel-based DNA sequencing technology. This technique not only was an inception to the present common dideoxynucleotide chain termination method[52-53] but also was the first report in which it was shown that single-stranded DNA fragments differing in size by only one nucleotide can be resolved electrophoretically on denaturing polyacrylamide gels. Since that

time, fractionation according to the relative molecular mass of the fragments has been a central principle of all widely applied new DNA sequencing techniques.

The preparation of so called "nested sets", that is the collections of DNA fragments from which the DNA sequence can be derived, is at the heart of current DNA sequencing reactions. In the "plus and minus" method and its more practical versions with terminating analogs such as dideoxynucleoside triphosphates, the nested sets are prepared enzymatically by copying a DNA strand with a DNA polymerase. This was first developed by Sanger and is the basis of modern enzymatical DNA sequencing protocol. Alternatively, a DNA fragment can be treated with base specific chemical reagents under conditions that generate partial degradation products forming a nested set. Maxam and Gilbert[54] used this concept to develop their chemical degradation sequencing method. Sanger's method has gained a great of popularity and occupied almost the whole of market share due to intense improvement of polymerases and commercial availability of high quality kits.

An important extension to Sanger's sequencing reaction is the introduction of cycle sequencing reaction[55-56], in which Sanger's method is thermally cycled when replaced normal polymerase with a thermally stable polymerase such as Taq polymerase. The main advantages of this modification is the use of much less template due to linear amplification, simple and fast reactions, and generation of practically equivalent amounts of high-quality sequence from both single- and double- stranded templates. The key component in the cycle sequencing reaction, thermostable polymerases[57-58], has been widely studied in recent years. The cycle sequencing reaction, based on labeling scheme, is divided into dye-labeled primer or dye-labeled terminator cycle sequencing reaction. The fluorescence dye chemistry[59-61] also advances rapidly.

The thermal cycler is used to perform cycle sequencing reactions or polymerase chain reactions. Automated thermal cycler, based on the heating properties, can be divided into three categories: heating block, water bath, and

hot air. The heating block thermal cyclers such as PE 9600, PE 2400 and MJ Researches can easily fit into microtube or microtitier plates, but normally have slow temperature ramp rate. The heating bath thermal cyclers can have faster temperature ramp rate but it relies on the robotic arm to move the reaction trays around different temperature bathes, which cause the problem on reliability in the long run. The hot air thermal cyclers use a high-speed fan blowing hot air to the reaction vessels at a fixed position. It not only increases the ramp rate due to this massive flow but also easily adapts into a capillary reactor. The disadvantage of moving the reaction into capillary is the surface effect to the reaction.

Therefore, the advancement in biochemistry, chemistry and instrumental design does enable the automation of DNA cycle sequencing reactions.

Challenge and Solutions toward Total Integration

The process of DNA sequencing as practiced today involves a series of steps. The first step is to isolate DNA from the biological sample and break them into fragments with convenient sizes because the chromosomes are too large to be sequenced directly. The second step is to amplify the smaller pieces of DNA biochemically through cloning or polymerase chain reaction in order to obtain enough amount of pure template for sequencing reaction. After mini-preparation of DNA template, the short piece is then subjected to the sequencing reaction in order to introduce a label for detection while generating a nested set of ordered fragments. The nested set of labeled DNA fragments, after purifying, pre-concentrating, denaturing and lyophilizing, is separated by slab gel electrophoresis and detected by laser-induced fluorescence. The sequencing raw data based on the fragment sizes and label colors are finally used to identify the nucleotide sequence and reassemble the short sequencing data in a complete sequence.

When considering the integration and automation starting from template, the processes after sequencing reaction such as purification, preconcentration,

denaturation and injection need to be integrated with separation and detection. Once the degree of multiplexing in separation and detection goes up to 1000 capillaries, how to provide such amount of sequencing samples in a compatible fashion magnifies the challenge to integration and automation. This is one of so-called front-end problems.

Ideally, the purification of sequencing reaction should remove fluorescently labeled primer or dideoxyribonucleotide terminators, salt contents, DNA template, and all ingredients and enzymes for reaction. However, practically, no single purification process removes all the above components. If absolutely necessary, multiple purification operations[62-64] have to be conducted. This is expensive and was not so necessary for slab gel electrophoresis. A potential problem is loss of material during this multiple procedure. Ultrafiltration[65], spin-column gel filtration[64], acetate-ethanol precipitation[64] and phenol-chloroform extraction[64, 66] are centrifugation-based methods. Solid phase sequencing[67-68] normally has solid phase support for either template[69] or primer[70]. Properly implementing a selective binding of DNA sequencing products is very important for this type of purification. Tuning the binding affinity is not so trivial that one can control the bonding and releasing of target DNA fragments. So, optimization of proper purification scheme is really dependent on the overall strategy.

Attempts to integrate automated thermal cycler for dideoxynucleotide DNA sequencing reaction with automated DNA sequencer based on slab gel electrophoresis have been made in several groups [65,71-75]. The first system is to use the same manual preparation protocol combined with an X-Y-Z robotic, a centrifuge, a thermal cycler and a DNA sequencer. This method relies on the centrifuge to purify and concentrate the sequencing products. The system is relative large and unreliable due to the difficulty in interfacing with centrifuge. The second system avoids using the centrifuge, but the X-Y-Z robot still is at the heart of integration and automation. Vacuum force under gel filtration cartridge

has been used to purify the reaction products instead of centrifugation[69]. A Solid-phase magnetic beads have also been utilized to bind the cycle-sequencing products.[70] Solid support manifold has been employed to bond with the template to facilitate the purification and isolation processes. Except solid support manifold for template, the multiplexed sample loading into slab gel electrophoresis is problematic. Although a capillary loader for slab gel has been developed, a DNA fragment is difficult to maintain in its denatured state after transfer. Immobilization of the DNA template in a solid support manifold requires more information about template before handling, which is not a common practice. Therefore, the limitations of robotics have never led us truly and totally into a full integration with slab gel electrophoresis.

When moving into the multiplexed capillary electrophoresis, we still have to face the same problems as those in slab gel. Basically, there are two philosophies regarding the integration and automation in the genomic community. One is to emulate the human manipulations performed in the laboratory with an articulated robotic arm and liquid handing robots. The other is to modify the standard operational procedure to fit into an on-line microfluidics. Although the first strategy with a combination of robotics, solid phase purification, multiplexed capillary electrophoresis is highly possible, the disadvantages with this strategy are expensive instrumentation, large reagent consumption due to the minimum volume capable to handle with robotics, and duty cycle time limited by slow kinetics of binding or releasing. However, the on-line integration approach has not been explored extensively. This is simply because of the difficulty in implementation of such for slab gel electrophoresis. The capillary format does provide us a new opportunity to interface with all-column sample preparation system. The potential gain in this strategy is not only in full integration and automation but also in reagent savings through miniaturization.

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CHAPTER 5. INSTRUMENTAL DESIGN FROM MULTIPLE SAMPLES TO MULTIPLE CAPILALRIES WITH AN INTEGRITY OF ON-LINE MANIPULATION

Introduction

In this chapter, more details of the instrument used in Chapter 3 will be described. Figure 5.1 shows the whole structure of this multiplexed instrument. The multiplexed microfluidics has been described. Both mechanically and electronically controlling hardware and software drivers will be discussed here. The failure in developing this dedicated and multiplexed on-line system is also revealed. The purpose of this instrumental design is to demonstrate the concept of the multiplexed on-line integration from DNA template to data analysis with the automatable capability. It is not a direct effort of commercialization. Therefore, most of components used here directly come from a commercial source. The software drivers are also developed based on a need rather a systemic and universal driver for each control unit.

Hardware

Overview

Figure 5.2 shows the flowchart of control units for automation. The host computer communicates with sensors and actuators through both RS232 protocol and Data Acquisition board with ISA standard. It provides the flexibility and adaptability to other application such as polymerase chain reaction. RS232 port 0 and 1 are the two original components with the host computer. AT232/2 adds two more ports to the host computer bus, configured into port 4 and 5. AT-MIO-16DE-10 is a multi-function data acquisition device,

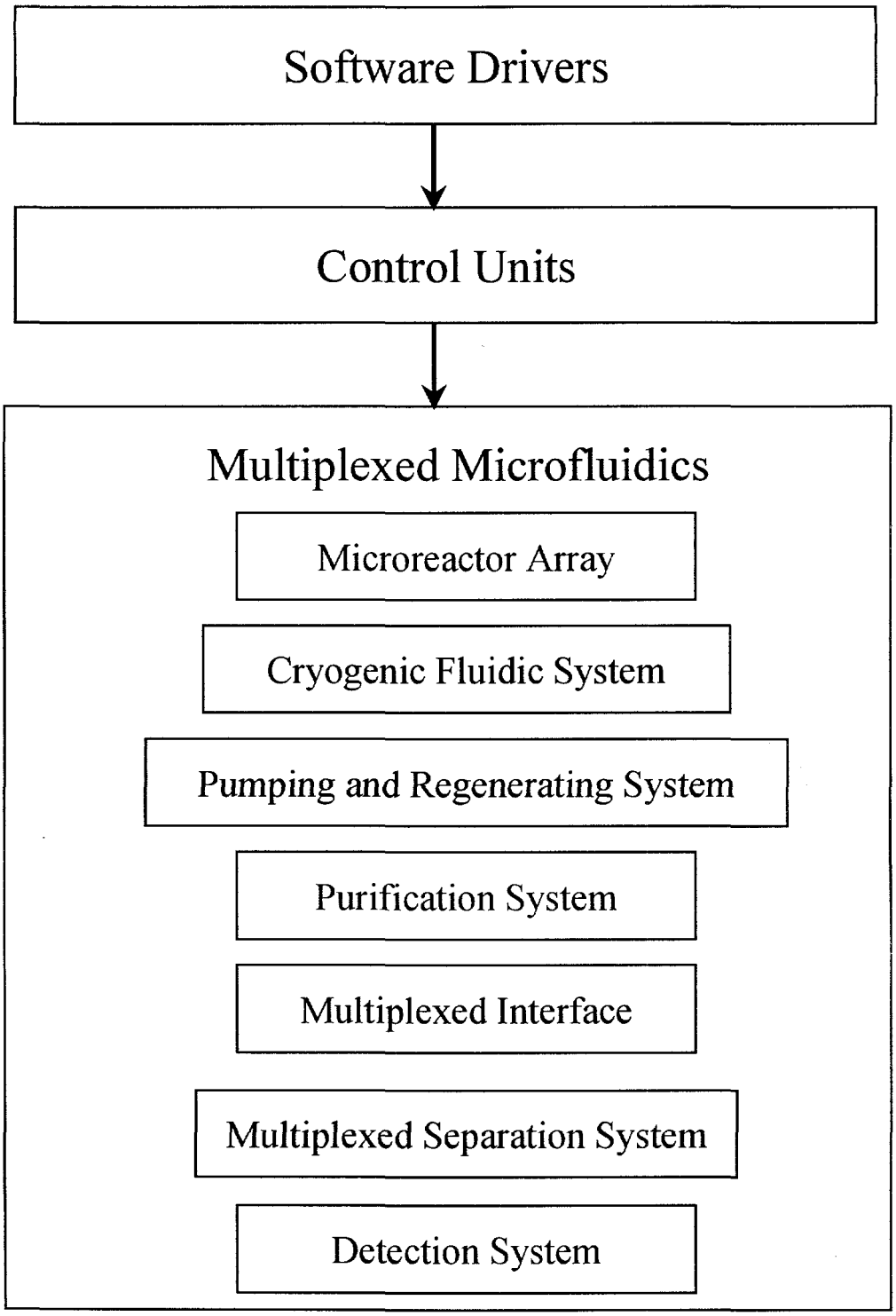

Figure 5.1 Block diagram of multiplexed template-to-sequence CE-based instrumentation.

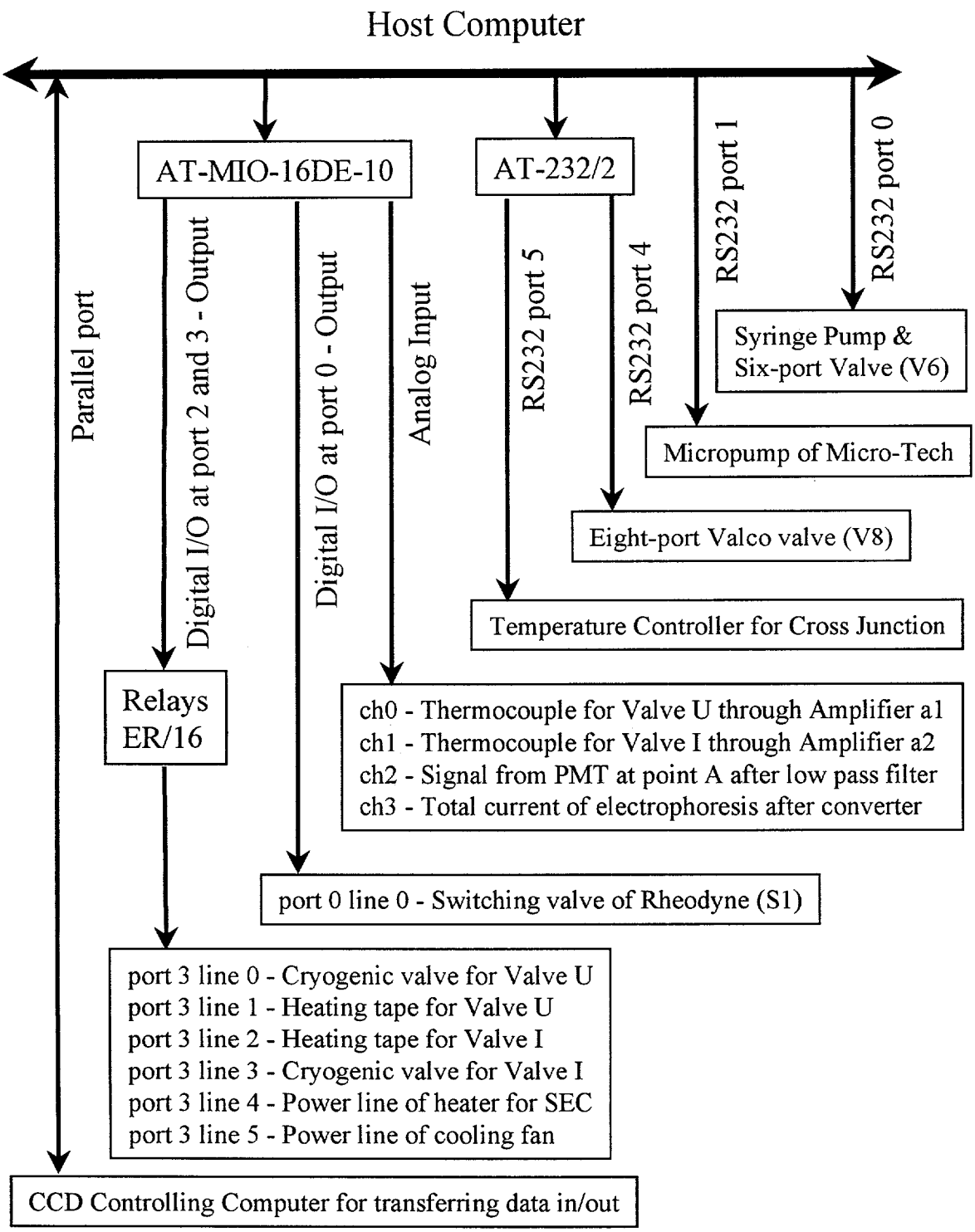

Figure 5.2 Flowchart of control unit for automation of multiplexed template-to-sequence CE-based instrumentation.

which provides 16 analog inputs, 2 analog outputs, 32 digital input/output, and 2 counters. 16 analog inputs were configured into 8 channels of differential inputs to reject the common noise. The digital lines are divided into four ports. Port 0 directly provides TTL signals to a control unit. Port 2 and 3 sends TTL signals to the relay ER/16 in order to control a power box where Bit 1 always energizes the corresponding actuator such as heater, valve or fan while Bit 0 de-energizes it.

Syringe pump & valve. The Kloehn model 50300 syringe pump is a programmable precision liquid metering instrument with non-volatile user program memory and system expansion input/output. It has a resolution of 48,000 increments for a full syringe stroke. It was equipped with a six-way distribution valve connecting into 1 mL syringe. Two-way communications with the host computer are via RS-232 protocol at port 0 at the baud rate of 9600. The base address of syringe driven module is set for 1. Figure A.1 in Appendix A shows schematic of setting parameters and connections of the syringe pump and its distributing valve. Before operating it, the syringe always requires to be initialized[1]. The "home" position of the syringe is only initialized once when the syringe pump is first installed while the "soft limit" needs to be initialized whenever it powers up.

Micropump. The micropump from Micro-Tech Scientifics is Ultra-plus MicroLC System which includes a pump controller and a pump unit. This pump is a dedicated instrument for microbore HPLC which is overqualified for the application here. However, it does provide the computer interface for RS-232 communication. It is necessary to purge the pump head and tubes before using it[2]. Figure A.2 in Appendix A shows the connection panel in the experiment.

Valco Valve. The eight-position distribution valve is controlled by a micro-electric multiposition valve actuator[3] from Valco. It consists of the control module, the stepper motor/gearbox assembly, a manual controller, a 110/220VAC to 24VDC power supply, and the interconnecting cables. Figure A.3 in Appendix

A illustrates the cconnections of 8-position Valco valve and its multi-position actuator.

Temperature Controllers. There are four types of temperature controlling subsystems involved in the instrument due to the availability and convenience of the instruments. First, Controlling the multiplexed freeze/thaw valves is through controlling the temperature of the solution plugs inside capillaries. The signal from thermocouple is amplified, calibrated, and then sent to a PID controller (software module from Labview). After compared with the setting, either cryogenic valve or heating tape is turned on to maintain at the setting point. The control loop is formed by one analog input and two digital outputs to drive the corresponding actions. Second, the temperature of the cross assembly is controlled by a CN77000 controller[4] from Omega, which communicates with the host computer through RS-232 protocol. Its software driver only allows changing the setting temperature. Due to the requirement of rapid cooling when switching to run electrophoresis, a cooling fan is added and can be controlled by a digital line. Third, the temperature of purification columns is managed by a self-regulated controller and a heating bath. When turned on, it rises to the pre-set temperature. In other wards, the temperature of these purification columns is not programmable. Finally, the temperature of multiplexed microreactors is controlled by an air thermal cycler. Interfacing to the host computer has not been done. Capillary microreactors must be placed carefully and firmly inside the thermal cycler, otherwise they will be broken by the inner fan. Figure A.4 in Appendix A shows the connections and setting for the first two temperature control subsystems.

Switching Valve. 24VAC-output power supply drives the two-position, six-port motorized valve[5] through a 5-pin DIN connector. A jumper cable is connected to pins 8 and 12 on the back panel of the terminal block. Pin 1 is linked to the ground end of the TTL signal from the data acquisition board while pin 11 is connected to its Hi/Lo digital line. When the TTL signal is set as Hi/5.0

volts, the valve turns to A position/Load and the "pathway" in software is point to "to syringe". While the signal is low, the system is driven by the Micropump on B position/Injection. Figure A.5 in Appendix A illustrates the pass selection using this valve. Note that the purification of sample starts only when this valve is turned to B position or the Micropump.

Technical Remarks

Sample loading. Sample loading from a microtitier plate is a very important step to place all 8 reaction plugs right inside the air thermal cycler. It is obvious that the length, the inner diameter, and the connection of capillaries in this micromicrofluidic system are crucial as described in Chapter 3. Besides that, some technical aspects are also needed to be aware. First, due to the different surface tension of solutions used to prepare cycle sequencing reaction, specially surfactant or carrying proteins added, bubbles are easily formed in the wells of microtitier plate. Elimination of these bubbles was found very helpful to increase the loading accuracy. Second, how to position the tips of loading capillary in the sample wells also affected the loading accuracy and uniformity of sample plugs. Capillary should be placed in the center bottom of the sample well and tilted a little bit rather than perpendicular to the well. Third, it should be avoid using high aspiration rate of syringe pump to drive the sample plug into the capillary microreactors. For example, the speed of syringe pump can be set at 500 for the commend parameter of "V". Forth, exercise need to be taken until the whole loading process can be done within 2-3 min.

Cycle Sequencing Reaction. Fresh enzyme and BSA are both important to the success of these reactions. Although the cycling time is a little bit long here, the improvement of reaction speed can be done by miniaturization.

Connection. There are more than 120 connecting points in order to make this multiplexed microfluidic system function. Extreme care must be given to make these connections. Both connectors with and without sleeves were used. For coupling without a sleeve, rules for standard HPLC should be followed as

long as the tube, ferrule, and nut are matched. For connecting capillaries, the sleeves are normally used. The inner diameter of the Teflon sleeves must fit with the outer diameter of capillaries exactly. The only luck we had is 0.015" i.d. and 1/16" o.d. Teflon tubing from Valco Instruments. The sleeve ends should be cut as flat as possible. Also, the length of sleeves is important because it is too long to be inserted with capillary or too short to allow the capillary stands the higher tension when bending and twisting. Two types of configuration for capillary and its sleeve, with (a) and without (b) extending 1-2 mm of the capillary out of the sleeve end, were employed. Except that the linkage at transferring capillary and separation capillary to the cross assembly in Figure 3.2 were used the type (b) configuration, all others with capillary were used the type (a) connection.

In the current instrumental setting, most troubleshooting involves making the connection right, specially putting separation capillaries into the cross assembly after gel filling. It is really helpful to pump a little bit of 1x TBE buffer into the cross region before coupling with the gel-filled capillary. To avoid bubbles formed at the capillary tips and get a sharp interface between sieving gel matrix and buffer is the key to success. Gel matrix can be poured in after connected only if the gating flow rate is high enough.

Column Packing. Packing a set of uniform size exclusion columns is an art rather than science. The trick is to get a set of uniform empty columns with very low flow resistance. Except the length and diameter of columns, one should pay a great deal of attention to choose frits so that the backpressure for the empty columns is lower than 10 psi. Many frits and filters have been tried, but the frits mentioned in Chapter 2 and 3 are the best so far. The longer the column the better the resolution even with the same capacity. Also the procedure used to pack each column should be identical, especially one should pay attention to the time used in settling the wetted resins (Sephadex) in packing reservoir.

Cross Assembly. The orientation of the cross assembly or Tee assembly is important to avoid the gravity effect on uniform flow rates among the parallel

channels. Due to the low flow resistance (estimated 2 psi) at the waste outlet of the cross assembly, the gating flow was found to be better from bottom up. The horizontal arrangement of these parallel channels is also found helpful. The residue salt in the channel may affect flow uniformity. The temperature variation may also exist, especially when the cleanup steps cause the heating tape partially wet. Figure A.6 in Appendix A shows the schematic of the cross assembly.

Alignments. In the side-entrance excitation scheme for multiplexed capillary, there are three critical alignments necessary to be achieved. They are the alignment of each capillary into the same plane, the alignment of laser beam in this co-planar array, and the alignment of the capillary array into the focus plane of CCD camera.

It is not a simple technique to pack capillaries into the same plane because few micrometer out of plane will results in tremendous loss of signal-to-noise and multiple focusing capability. Many factors could affect the quality of the alignment. The quality of a reference plane and the way to hold capillaries against this reference plane play an important role. Figure 5.3 shows the most successful capillary array holder. Four adjustable nuts and the flatness of glassy plastic surface really helped. How to open and clean the detection window is another consideration. For 360-μm-o.d. capillary packing, it is essential to blade out blurs on both sides after burning a 1-cm window. Packing small dusts into the window often kills the focusing of laser. One of additional problems of packing an array for online operation is the torsion introduced by the current connection scheme. Torque was produced along the capillary when screwing it into the cross with a sleeve. To eliminate the torsion affecting laser alignment, three sets of high-adhesive double-sticky tapes were placed right before the window region to restrict the propagation of torque force.

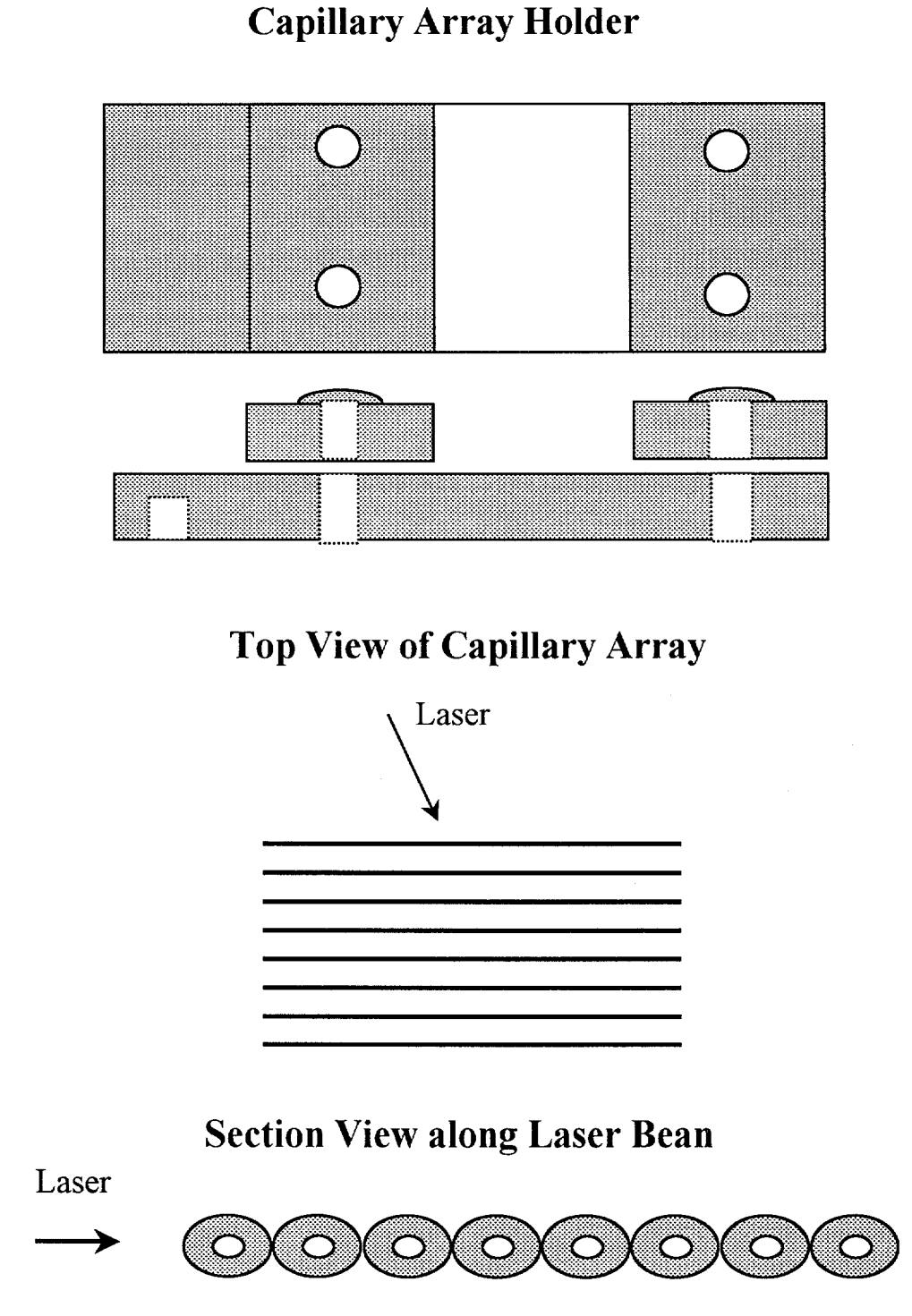

Figure 5.3 Capillary array packing and laser alignment for side-entrance excitation of multiplexed capillary electrophoresis.

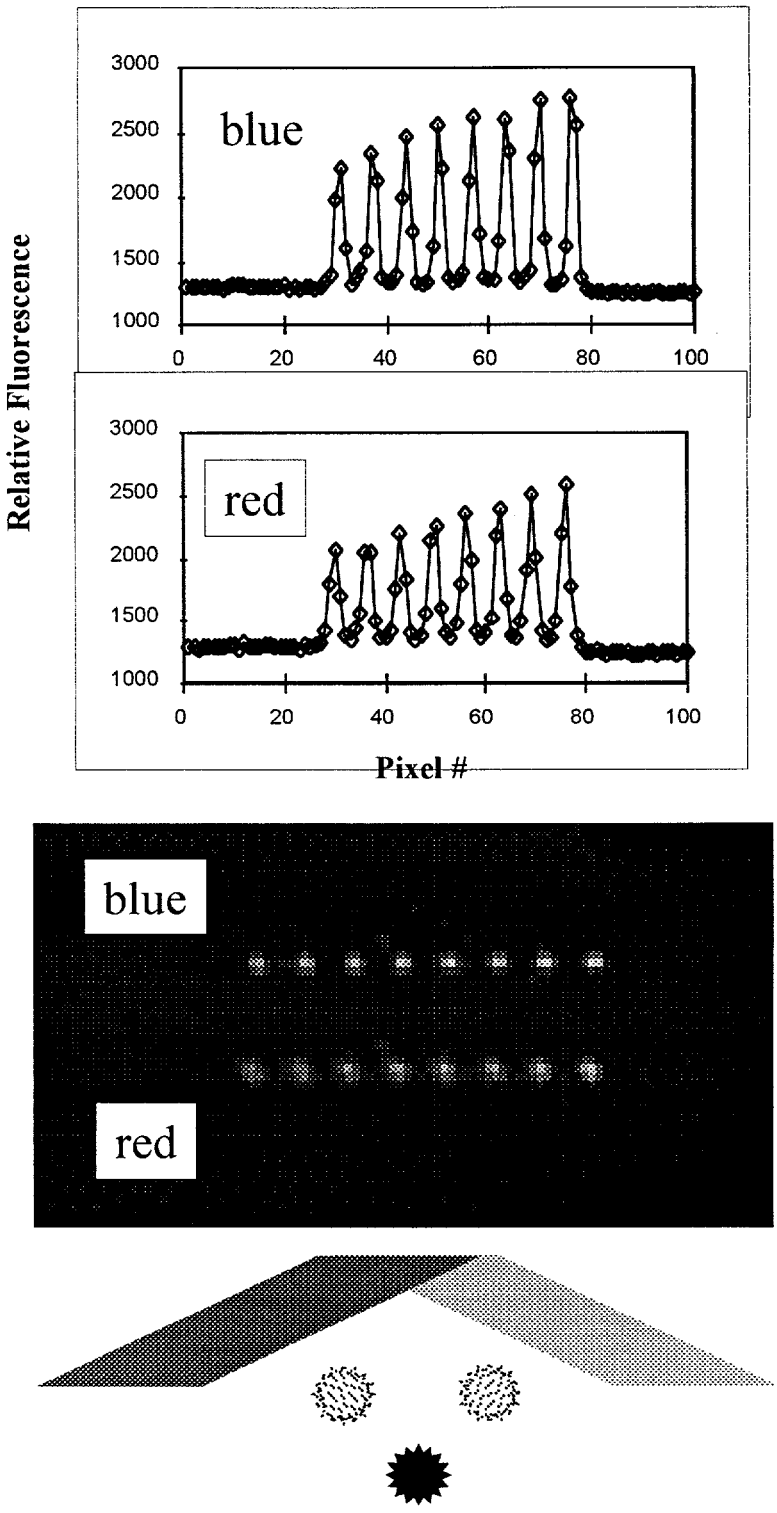

Figure 5.4 Focusing and image splitting of the background fluorescence of 8x capillary array.

It is obvious to align the laser beam into the capillary array plane and focus on the first capillary in order to get multiple focusing effect. However, we recently realized the importance of tilting the laser beam to the capillary array rather than normal to it. As shown in Figure 5.3, although the tilting laser beam changes the curvature of the capillary wall effect, it was found experimentally that it still maintains the multiple focusing property to certain tilting angle. The advantage of tilting is not focusing but eliminating the scattering background. We knew for a long time that the scatter lights mainly a scatter ring is the major background and source of noise in the electropherogram. Tilting the incident laser beam causes the parabolic shape of the scatter ring shifting away from the normal of the capillary array plane. By placing the CCD camera carefully, one can avoid collecting the scatter ring and reduce the background by a factor of at least 5.

Figure 5.4 shows the focusing and image splitting of the background fluorescence of 8x capillary array. Along the direction of laser illumination, the fluorescence background decreases to about 60% of the first capillary on the 8th capillary with the decrease of the laser power. The crosstalk between adjacent capillary, splitting ratio of Blue channel vs. Red channel and image size of one capillary have been discussed previously in our group[6-7].

Software

Control Software

The control software consists of two "while loop" structures and a termination module as shown in Figure B1 of Appendix B. All active functions from each instrumental driver are incorporated into this control software. The function of the first "while loop" is data acquisition from four channels including two thermal couples (0,1), elution monitoring after size exclusion column (2), and electrophoresis current (3). After conversion and calibration of the thermal

couples, information is passed to the second "while loop" through "local" variables. The second "while loop" primarily is two PID (proportional, integration, and differential) control loops of two freeze/thaw valves. Inside the loop, it also checks the status of the syringe pump, controls all the mechanical valves, aspirates and dispenses reagents and also manages the temperatures of the cross assembly and columns based on the given instruction. In the termination module, it clears all the memory for this control application and save the useful data through a user dialogue.

Base Calling Software

The base-calling scheme has been developed previously in our group. This is a user-friendly Labview version. It follows the same architecture, removing baselines of both blue and red channels, filtering high frequency noise, detecting all the peaks and synchronizing the peaks in both channels, inserting peaks based on a peak gap selector, and determining the bases with peak ratio histogram, and then displaying with colored codes. This program is more interactive and suitable for one to optimize the base-calling parameters. Figure B.2 in Appendix B lists the program in detail.

Conclusion

By choosing right hardware and software, on-line manipulation and automation from sample-to-answer is very attractive solution to improve the throughput in a parallel fashion. It demonstrated here all the individual elements for highly multiplexed operation can be automated. Multiplexed microfluidic control was achieved.

Refereences

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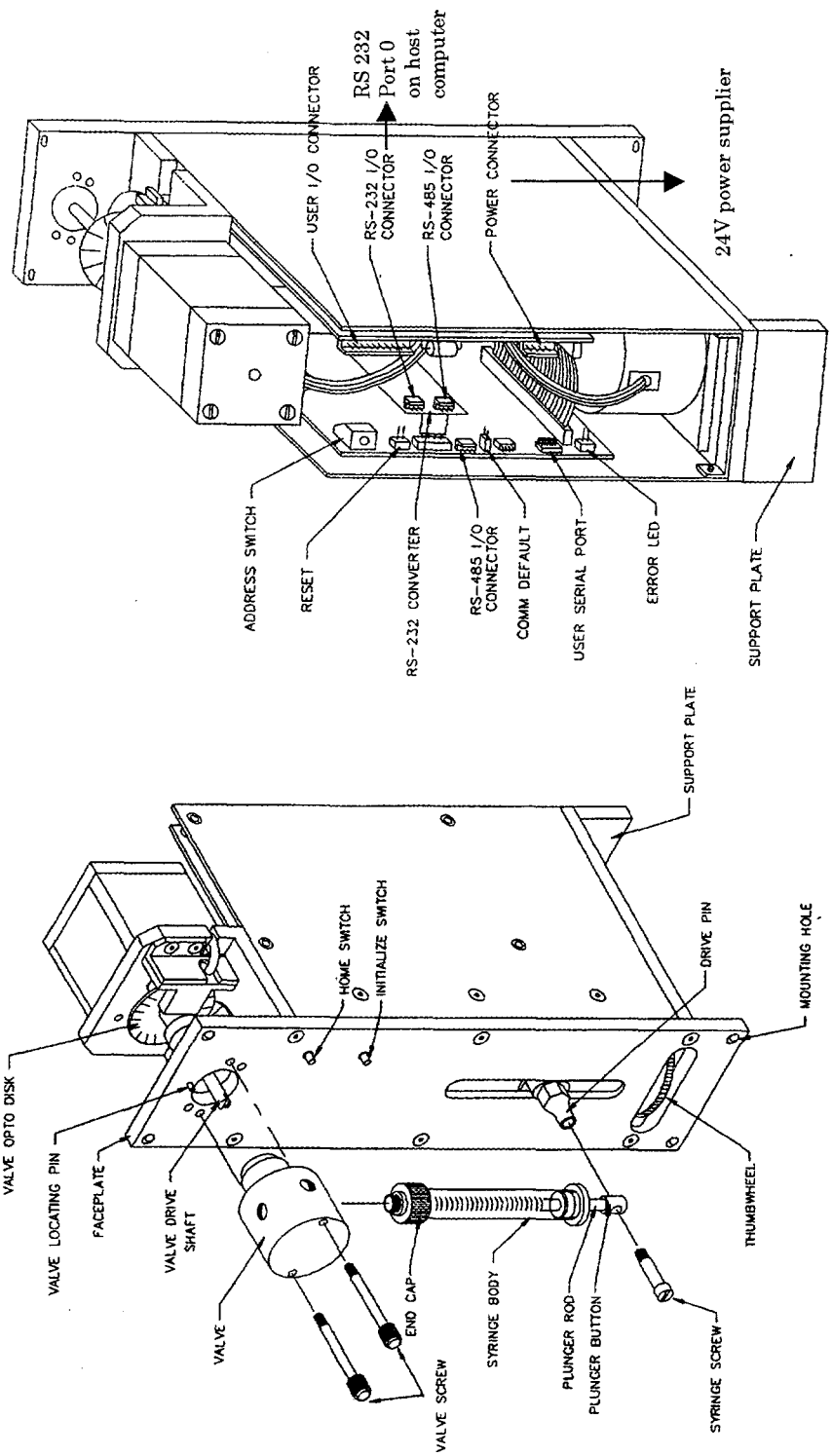
CHAPTER 6. GENERAL CONCLUSIONS

Automation and integration has been played an important role in the industrial revolution. It enhances up the productivity of mankind and liberates our mind and hands from many routine operations. Without doubt, automation and integration of all steps from DNA template to sequence will once again increase the speed and throughput of DNA sequencing.

This dissertation has described one of the avenues toward the total integration of DNA analysis based on multiplexed on-line microfluidics and multiplexed capillary electrophoresis. A protocol was developed to carry cycle sequencing reaction products through purification, denaturation, injection, separation and detection. The developed protocol from template to sequence has been validated in both single-channel and multiple channels. Scale-up of such a system is a mater of optimization and engineering. Compatibility of a set of technologies and principles is the key to the success of integration and automation. With microfabricated manifold Tees or Crosses, current instrument design should be ready to accommodate a 100 capillary system. Further extension of this work could focus on developing better biological reactions and miniaturizing some of the components used. The capability to provide information from raw sample in a highly parallel fashion makes it possible to obtain the genetic information in a timely manner, use it for disease diagnostics and even compare interspecies and intraspecies variations. The multiplexed framework based on this dissertation will be miniaturized once the other developments, such as separation on microchips, catches up. No matter what driving force will be used in the future, multiplexing, miniaturizing and integrating multiple components for high-throughput analysis is the general trend.

APPENDIX A. INSTRUMENTAL SETTINGS



Valve position:
A: switching valve position #2; B: waster bottle;
C: cross assembly manifold inlet; D: BSA solution;
E: de-ionized water; F: 1x TBE buffer.

Figure A.1 Front and rear panels of syringe pump and their connections

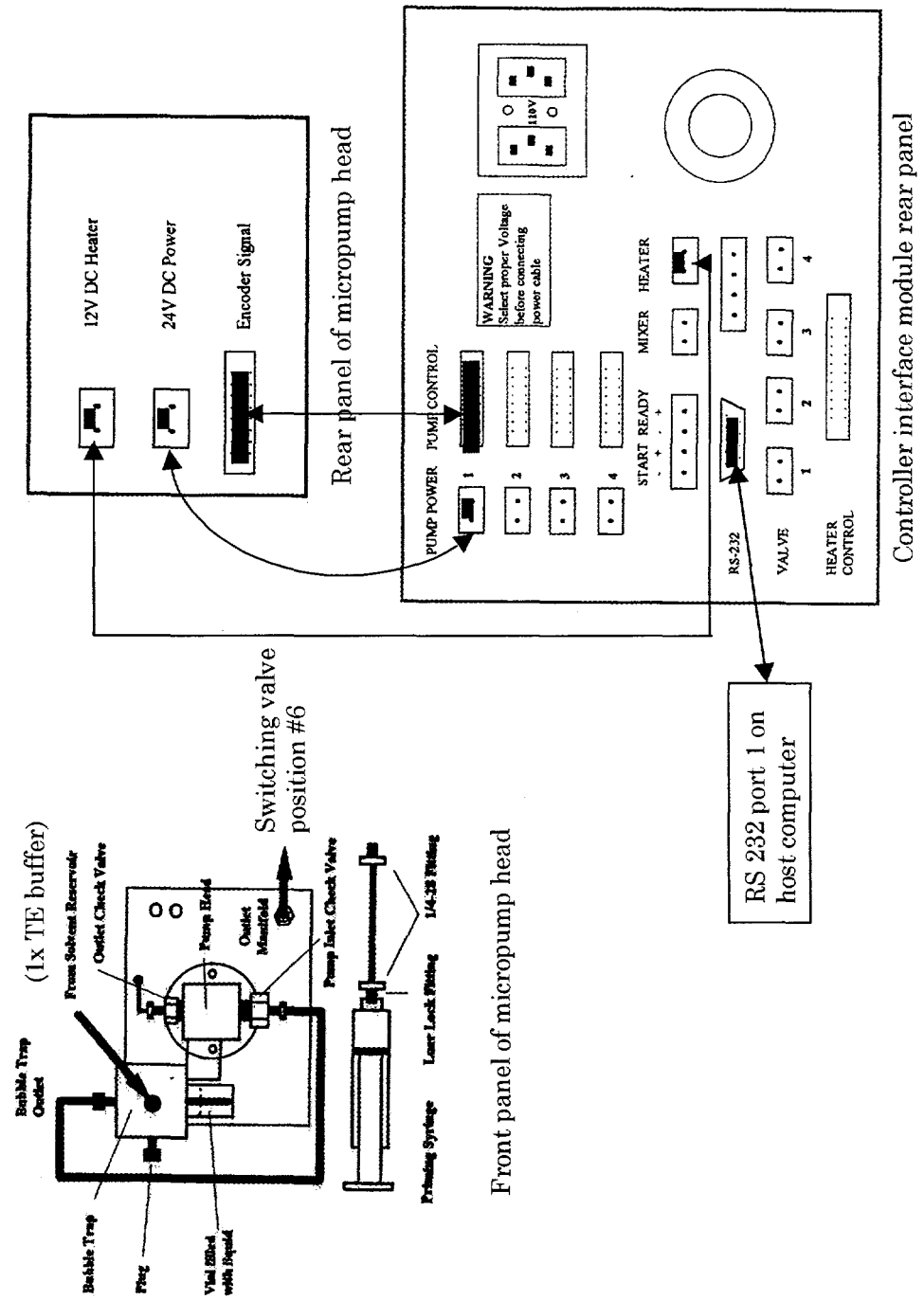
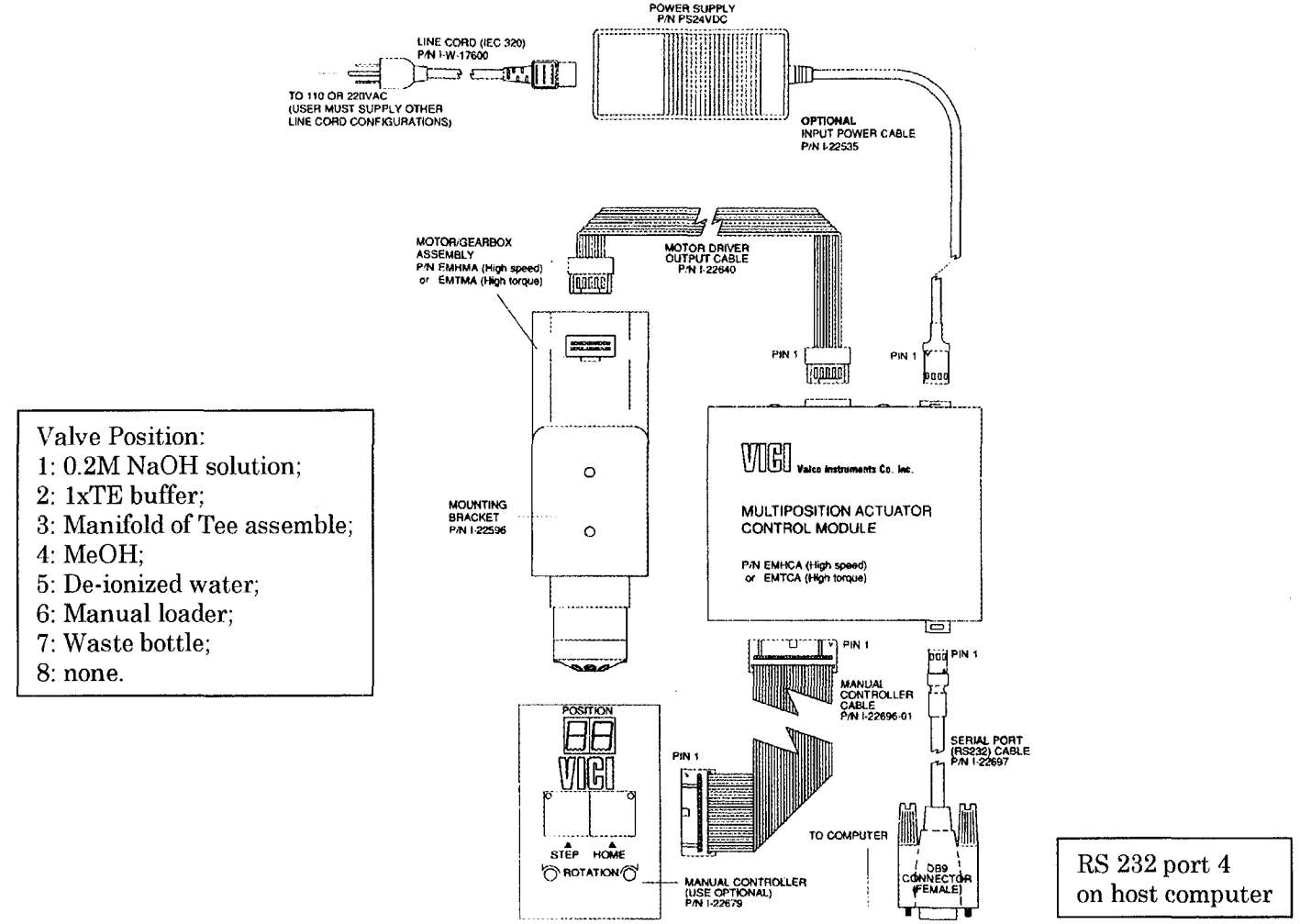


Figure A.2 Front and rear panels connections of micropump

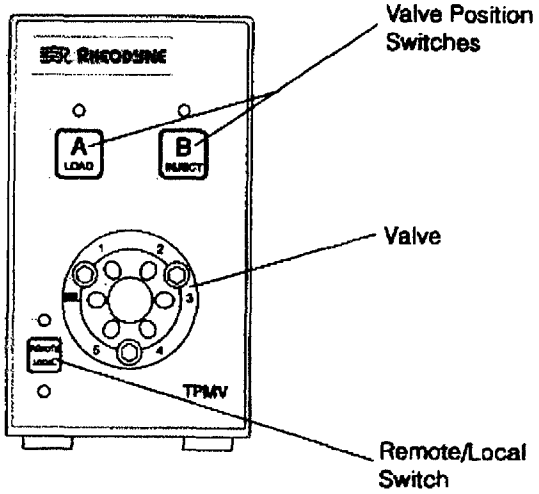


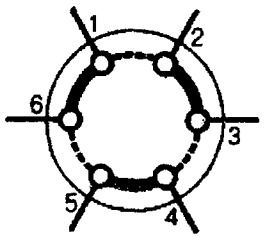
Valve Position:
1: 0.2M NaOH solution;
2: 1xTE buffer;
3: Manifold of Tee assemble;
4: MeOH;
5: De-ionized water;
6: Manual loader;
7: Waste bottle;
8: none.

RS 232 port 4
on host computer

Figure A.3 Connections for 8-position Valco valve and actuator

Front panel





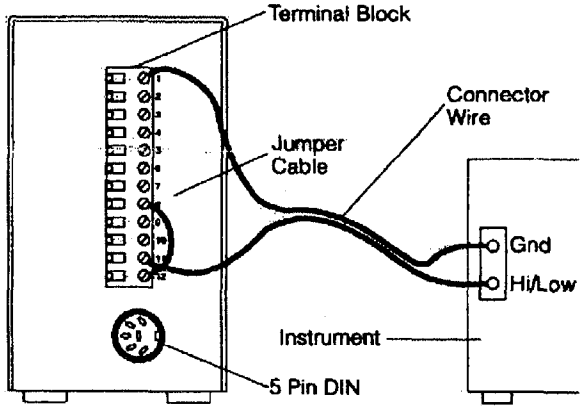
Switching Valve

POSITION A (ccw)

POSITION B (cw)

Connections:
1: center of 8-position valve;
2: A on syringe valve;
3: waste bottle;
4: not used;
5: not used;
6: outlet of micropump.

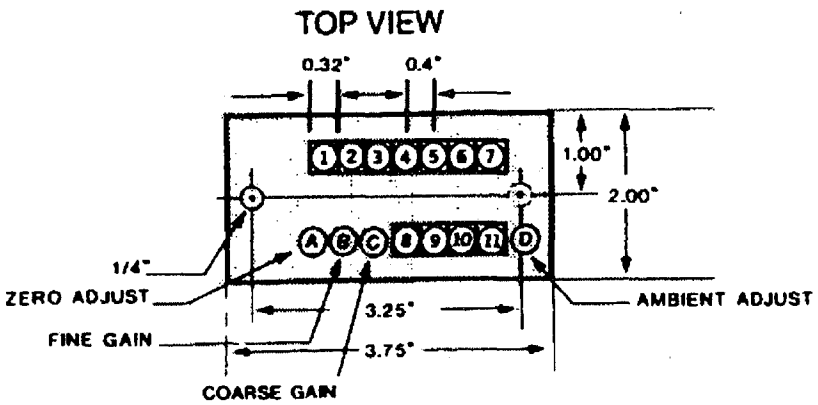
Rear Panel



5 pin DIN: power supper;
instrument: data acquisition board at digital port 0 line 0.

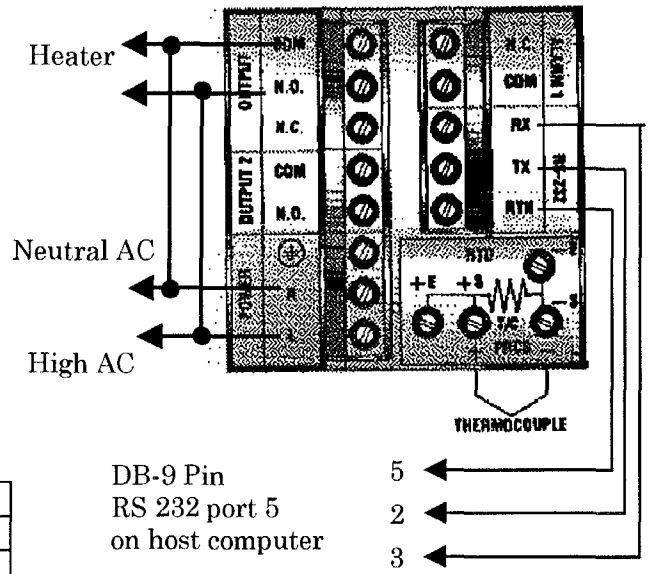
Figure A.5 Pass selection with Rheodyne 6-position valve

Omni-Amp IV Thermocouple Amplifier



PIN	FUNCTION	CONNECTION
1	Output	AT data board pin #3 or #5 for valve U or I
2	CMN	AT data board pin #4 or #6 for valve U or I
3	Ambient out	
4	not used	
5	not used	
6	AC	AC power line
7	AC	AC power line
8	+Input	Yellow for K type thermocouple
9	"-Input"	Red for K type thermocouple
10	CMN	
11	CMN	

Omega CN77000 series Controller



Communication Parameters:
9600 baud; no parity; 7 data bit; 1 stop bit.
Bus Format:
no check sums; no line feed; no echo; 232C;
command mode; cartridge return for separation.

Figure A.4 Connections and settings for temperature control

APPENDIX B. SOFTWARE

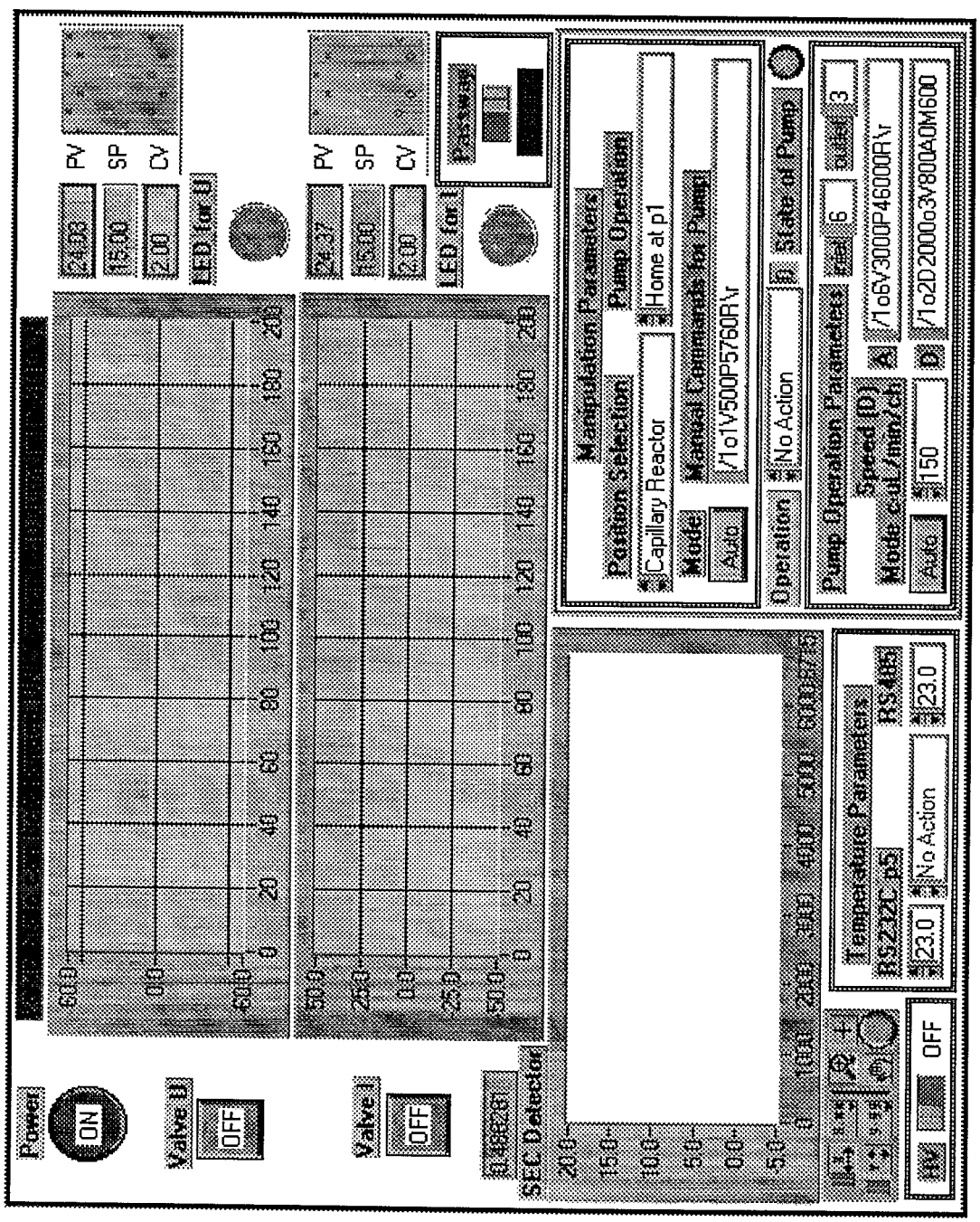


Figure B.1 Front panel of instrumental control software

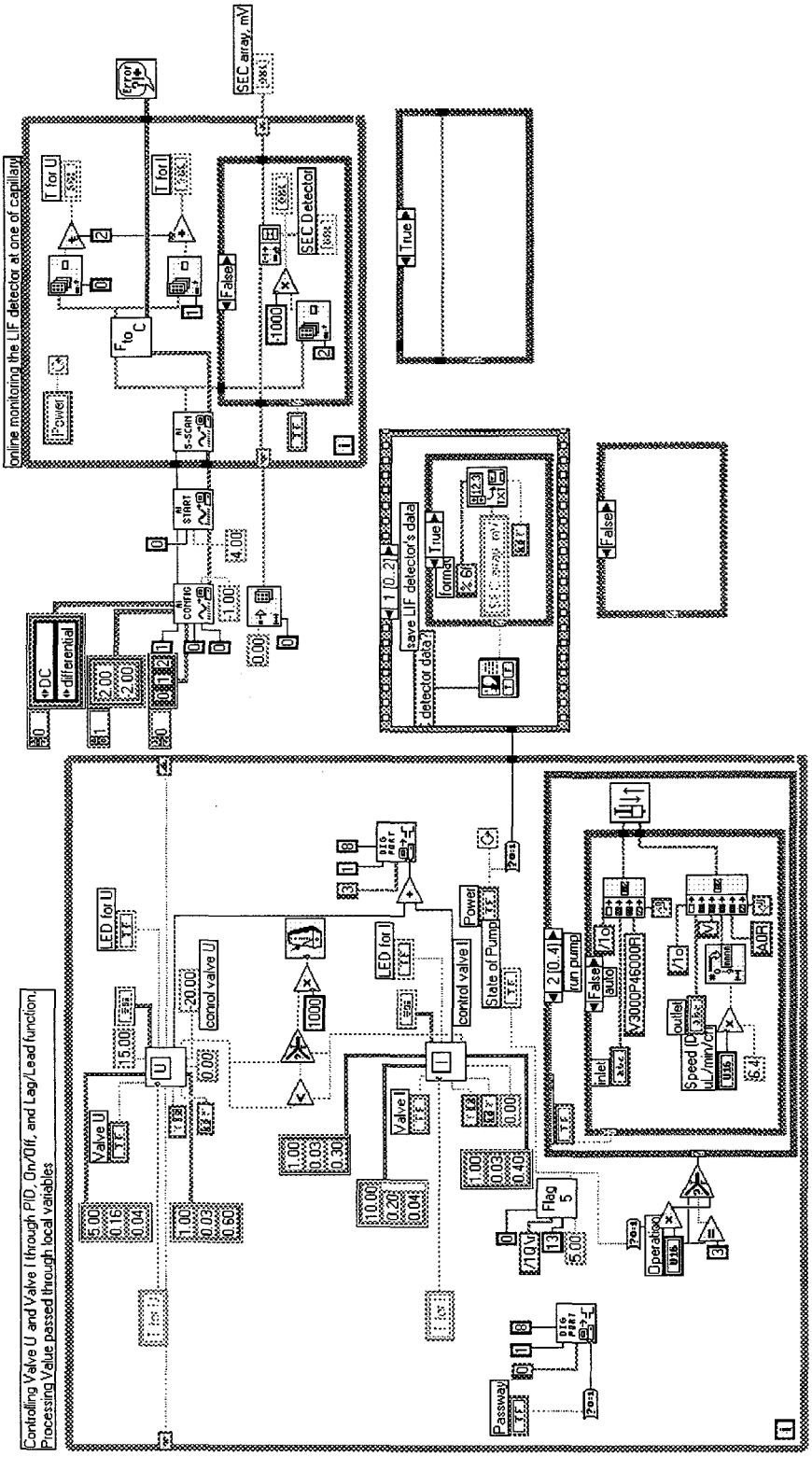


Figure B.1 Block diagram of instrumental control software

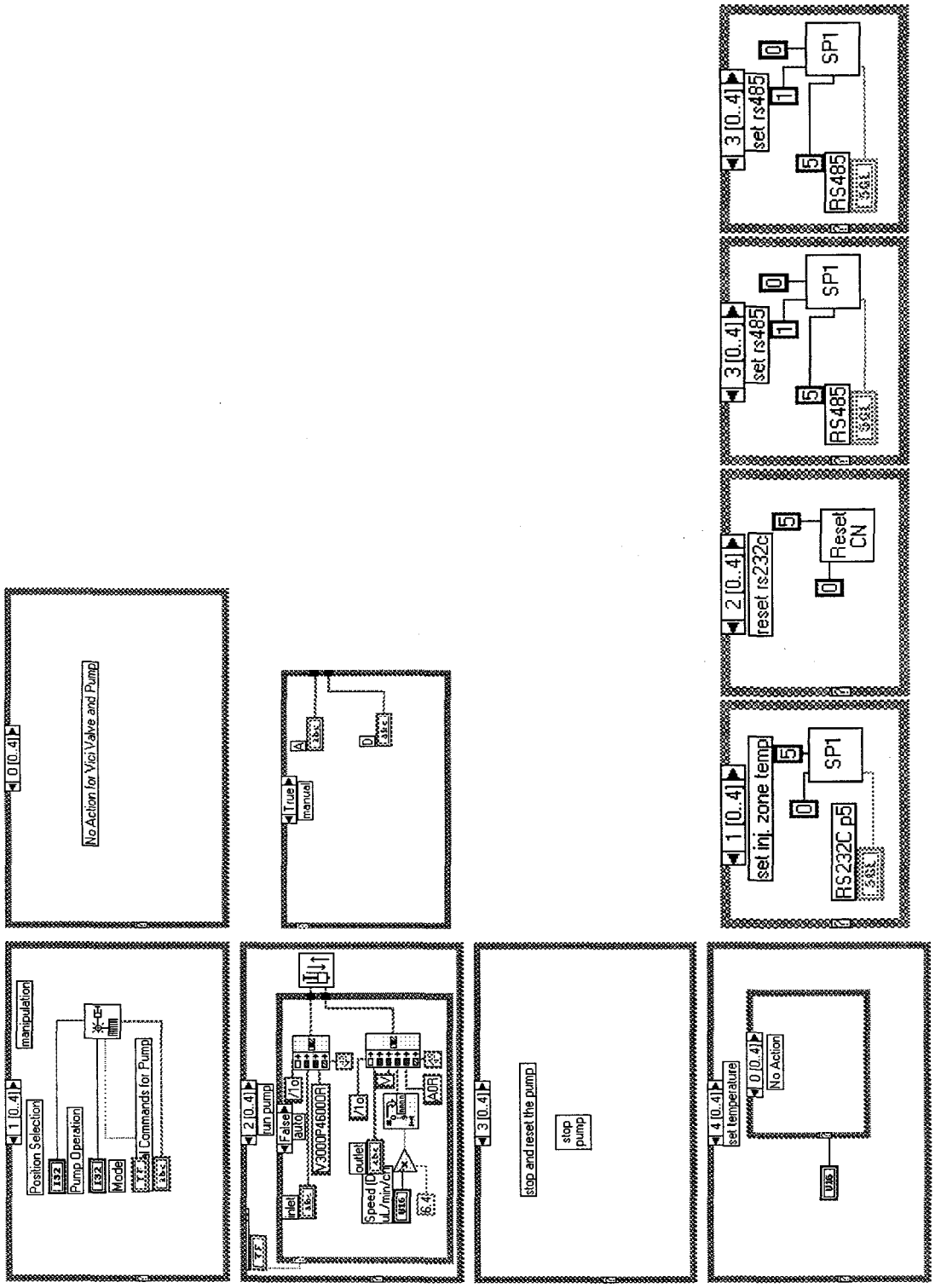


Figure B.1 Block diagram of instrumental control software (cont.)

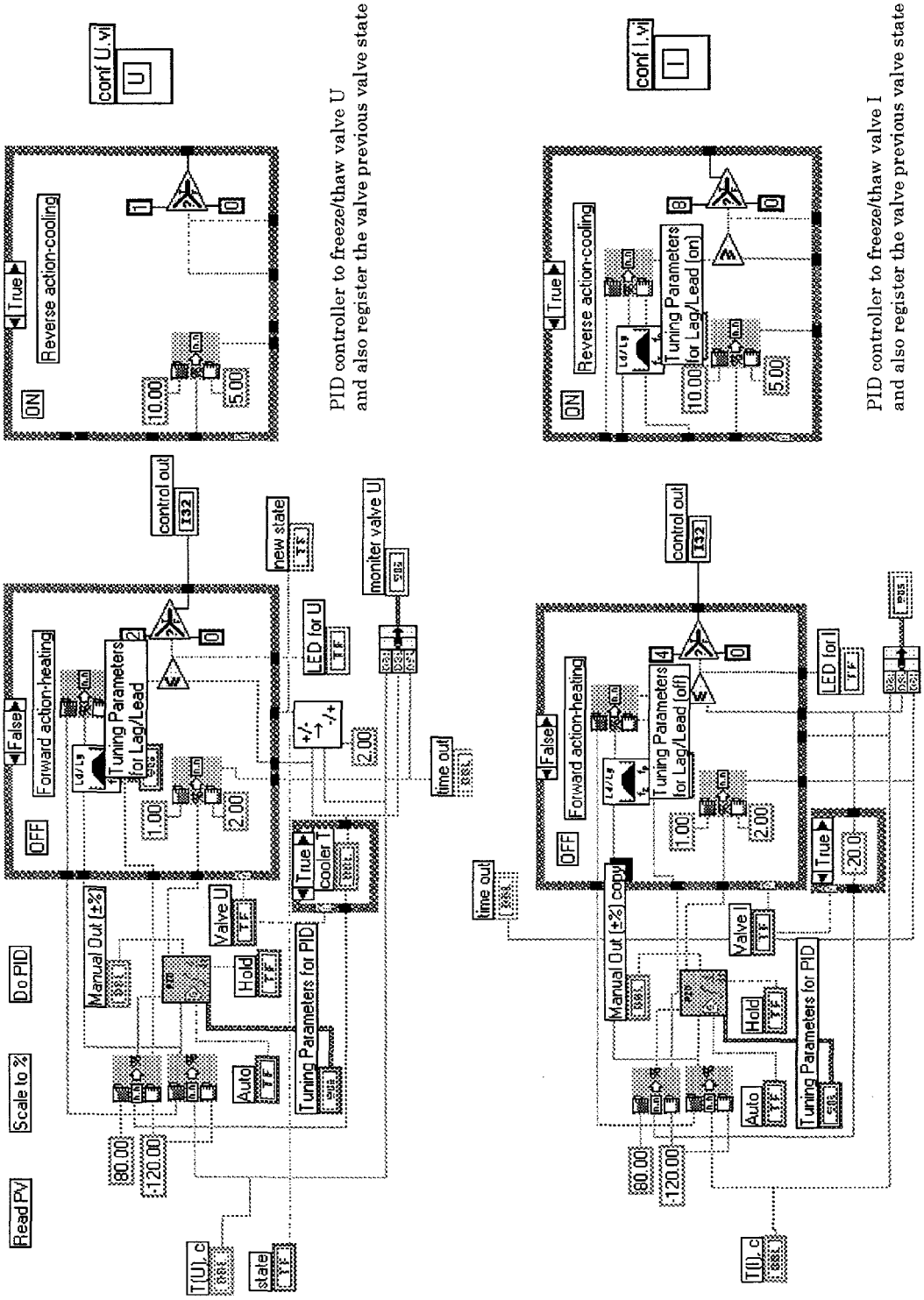


Figure B.1 Block diagram of instrumental control software (cont.)

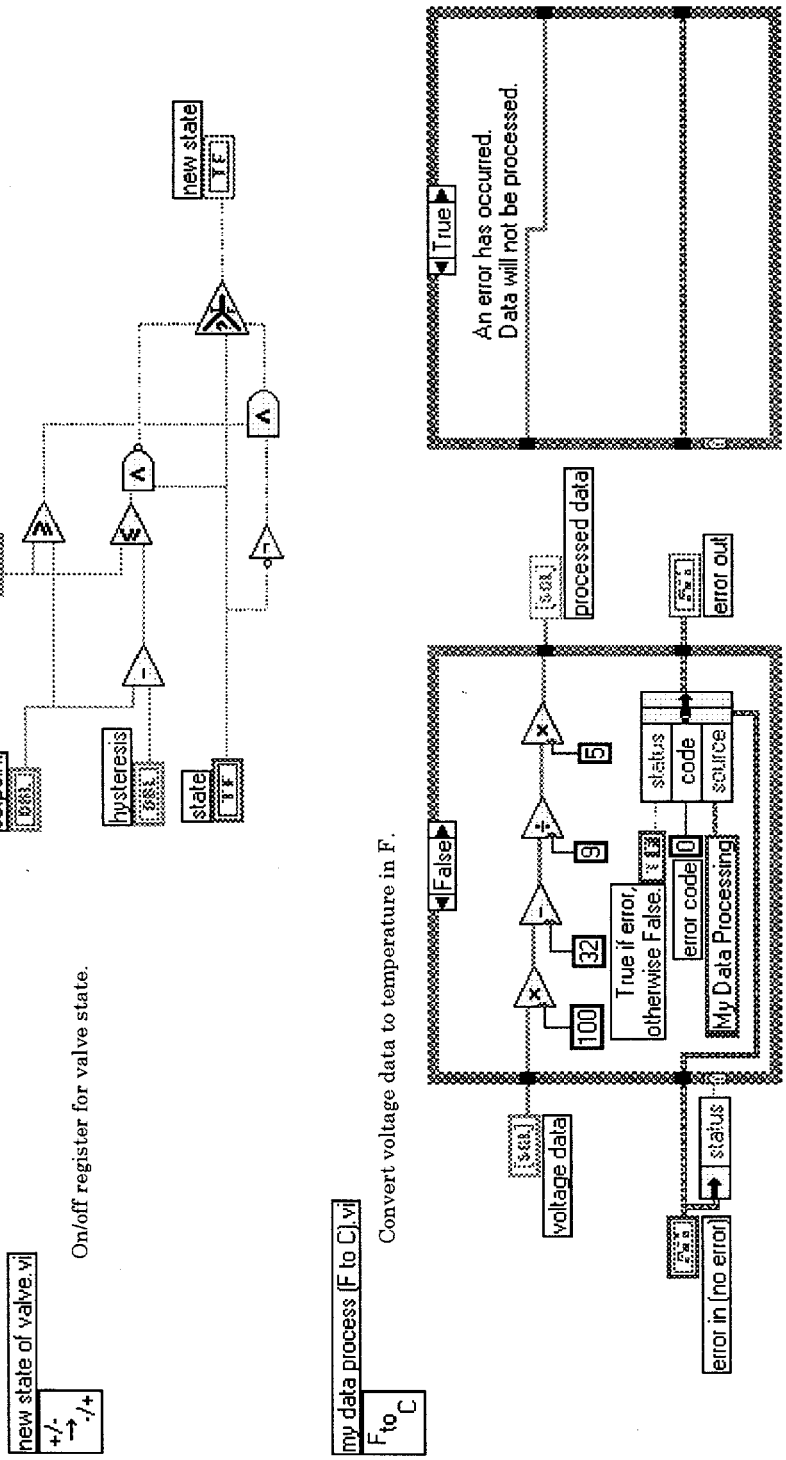


Figure B.1 Block diagram of instrumental control software (cont.)

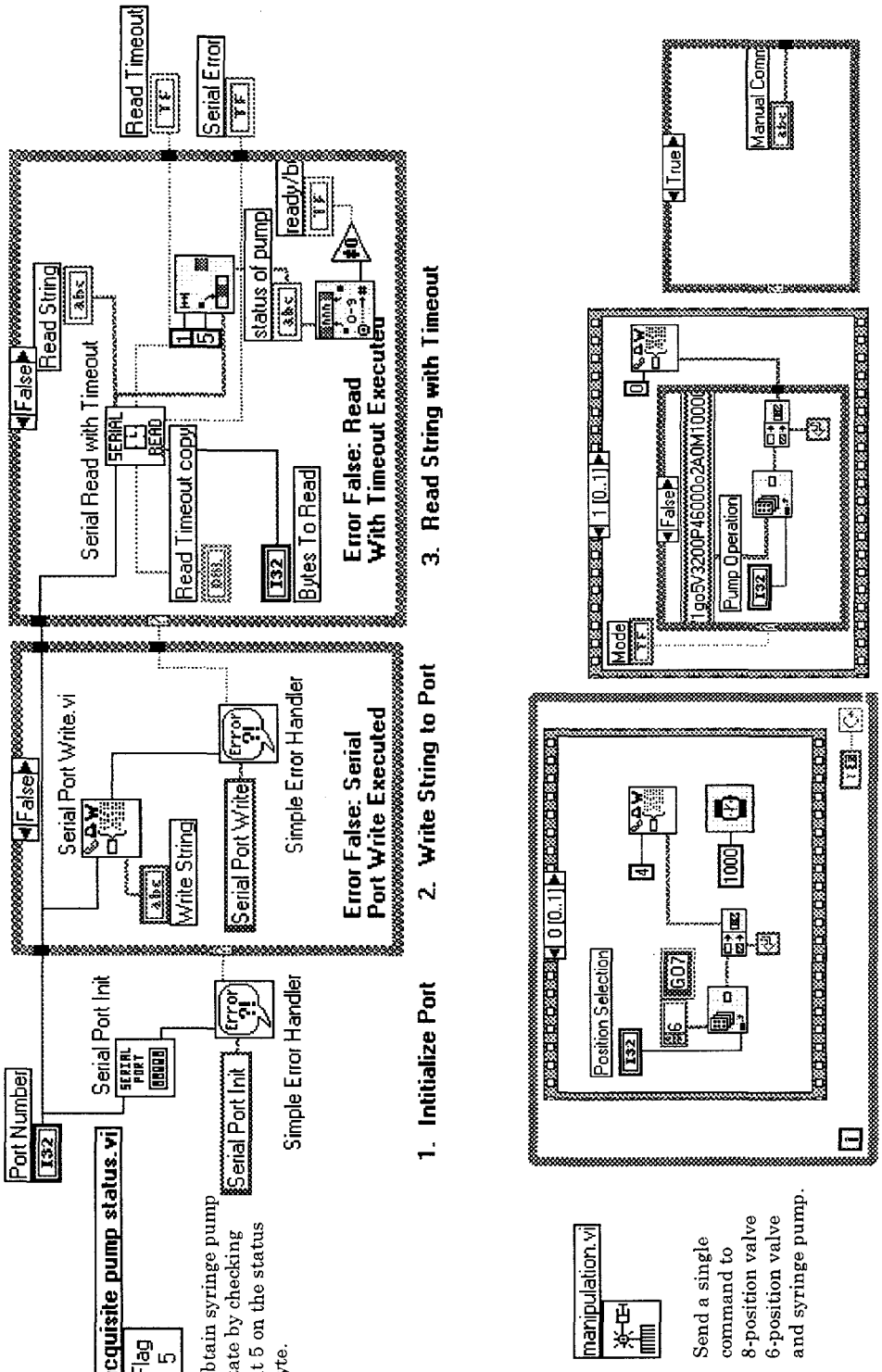


Figure B.1 Block diagram of instrumental control software (cont.)

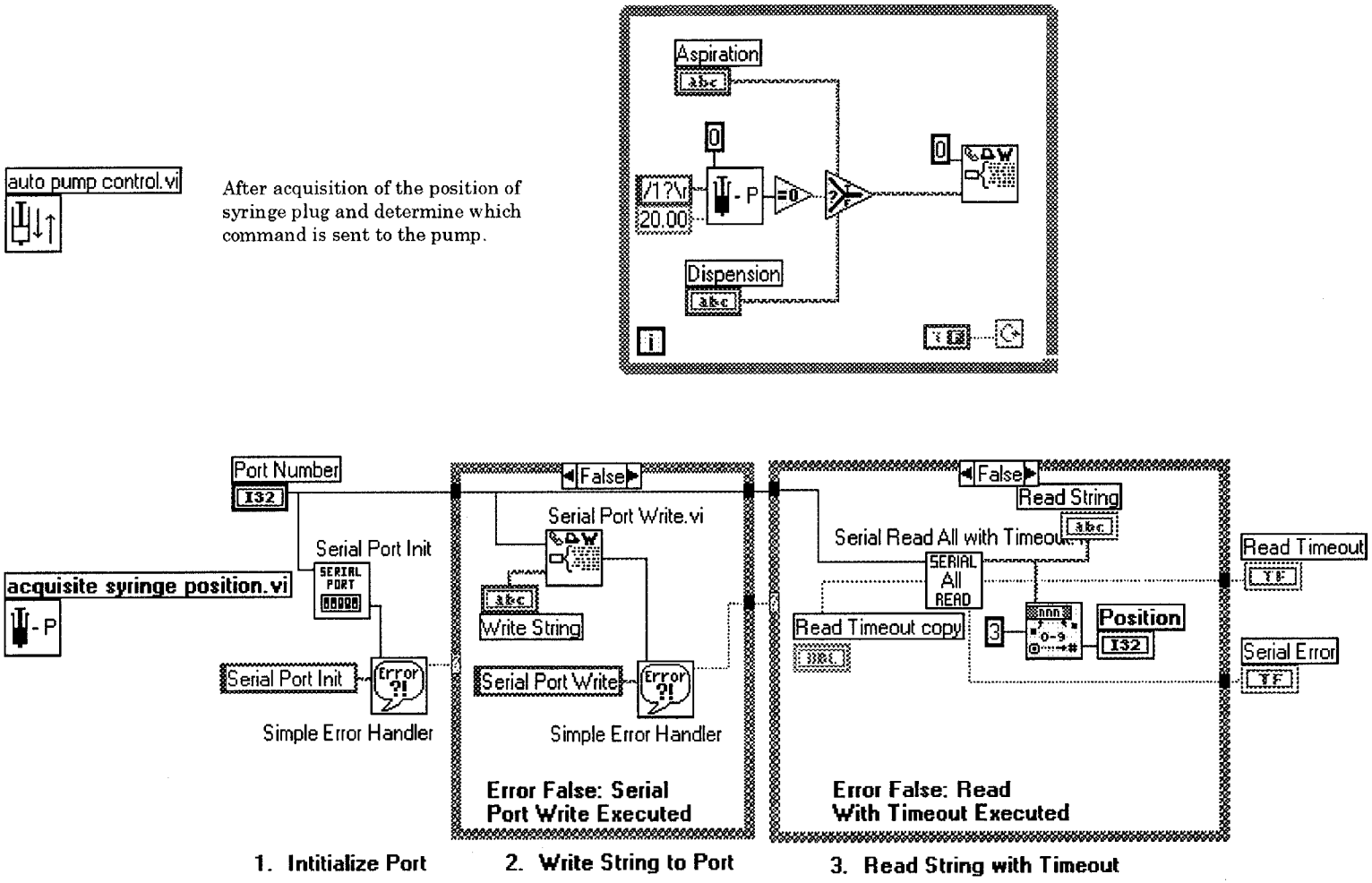


Figure B.1 Block diagram of instrumental control software (cont.)

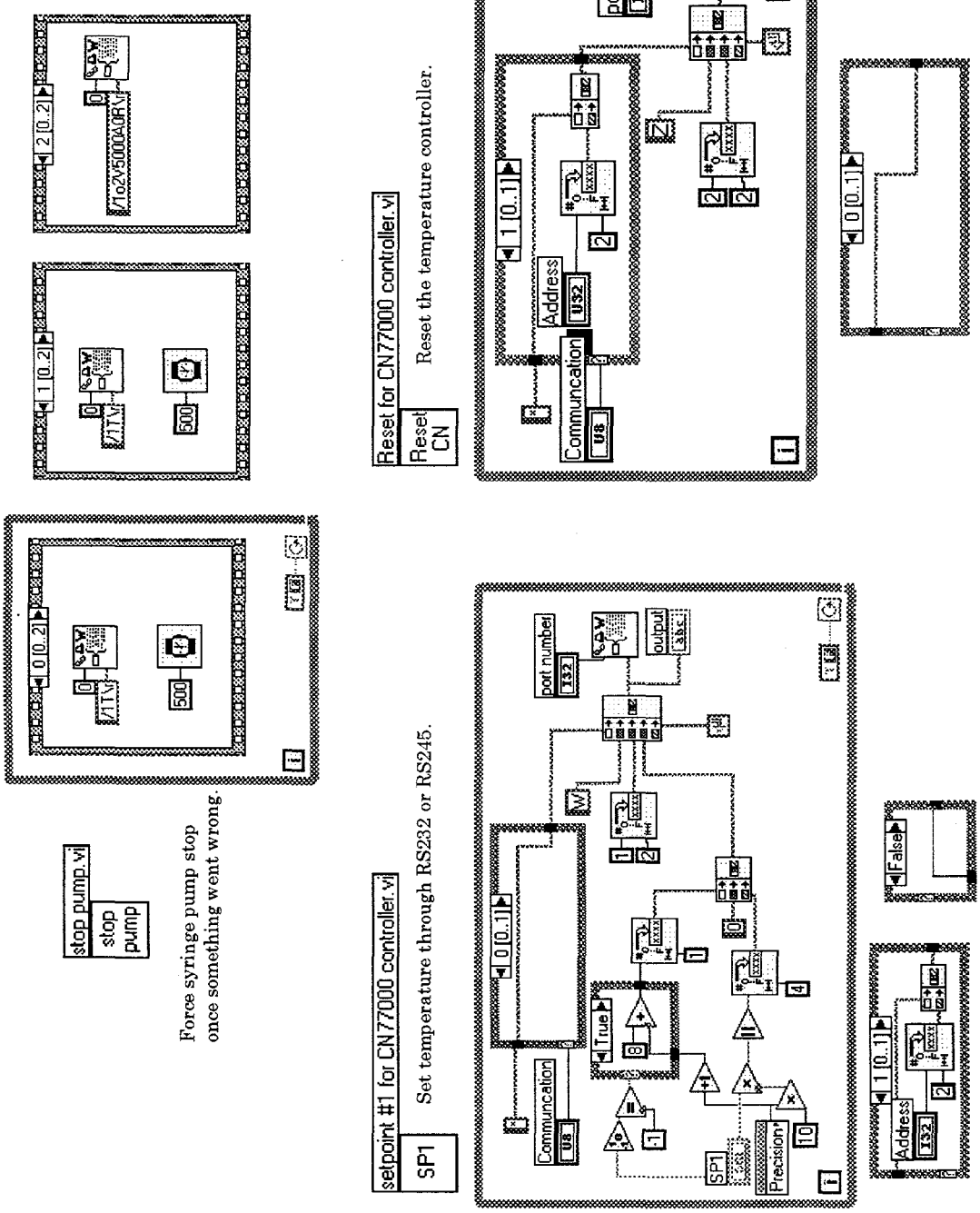


Figure B.1 Block diagram of instrumental control software (cont.)

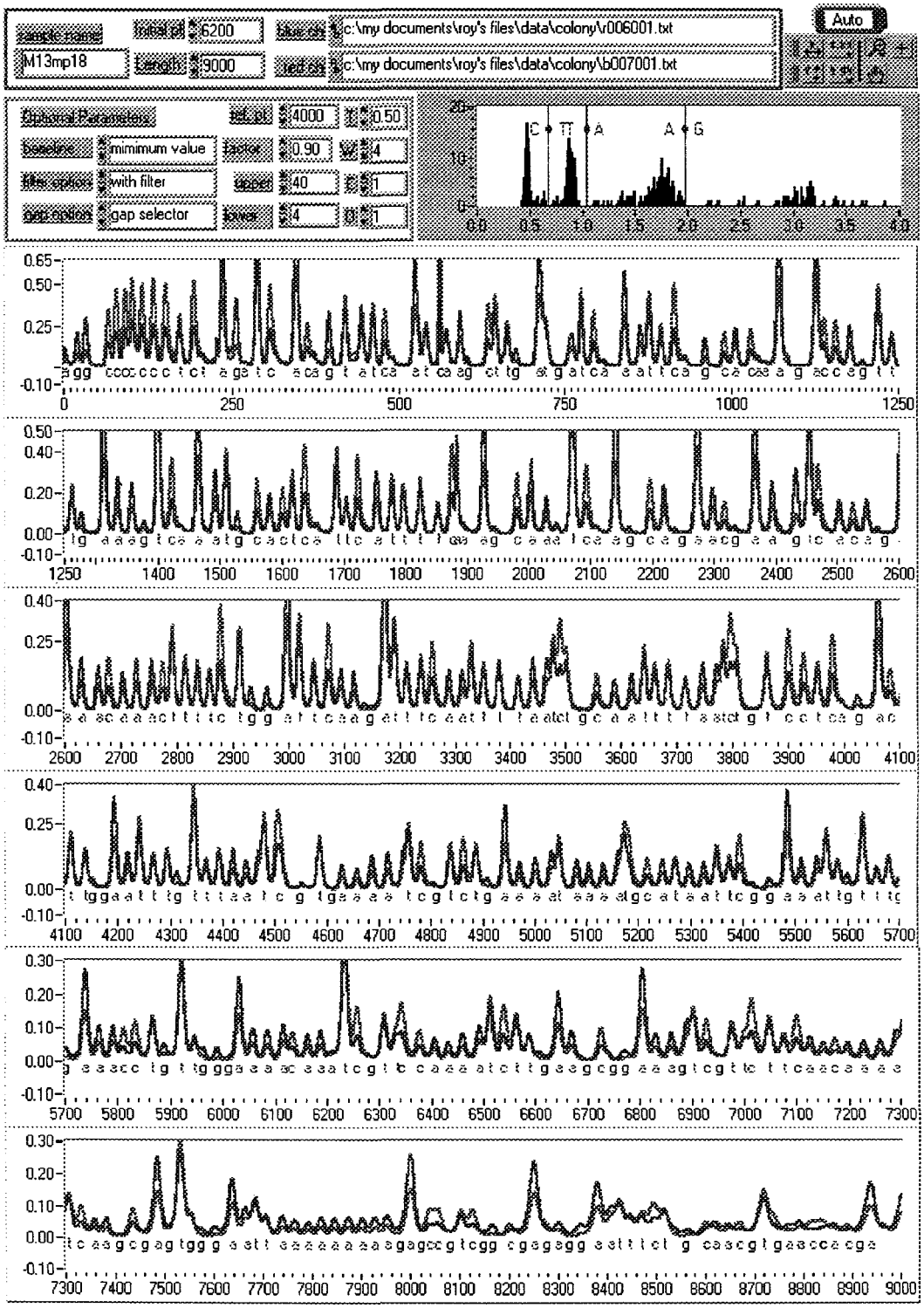


Figure B.2 Front panel of base-calling software

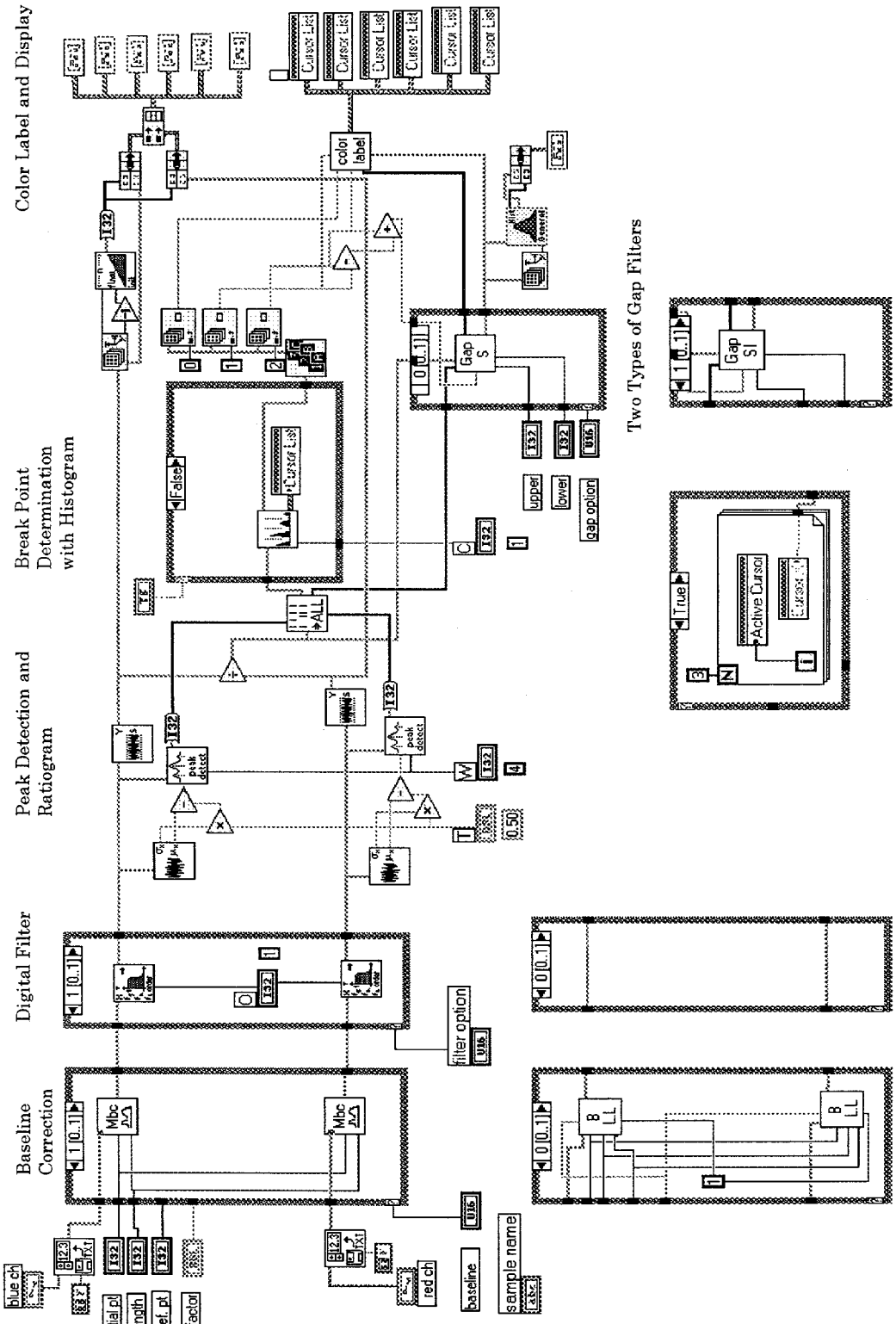


Figure B.2 Block diagram of base-calling software

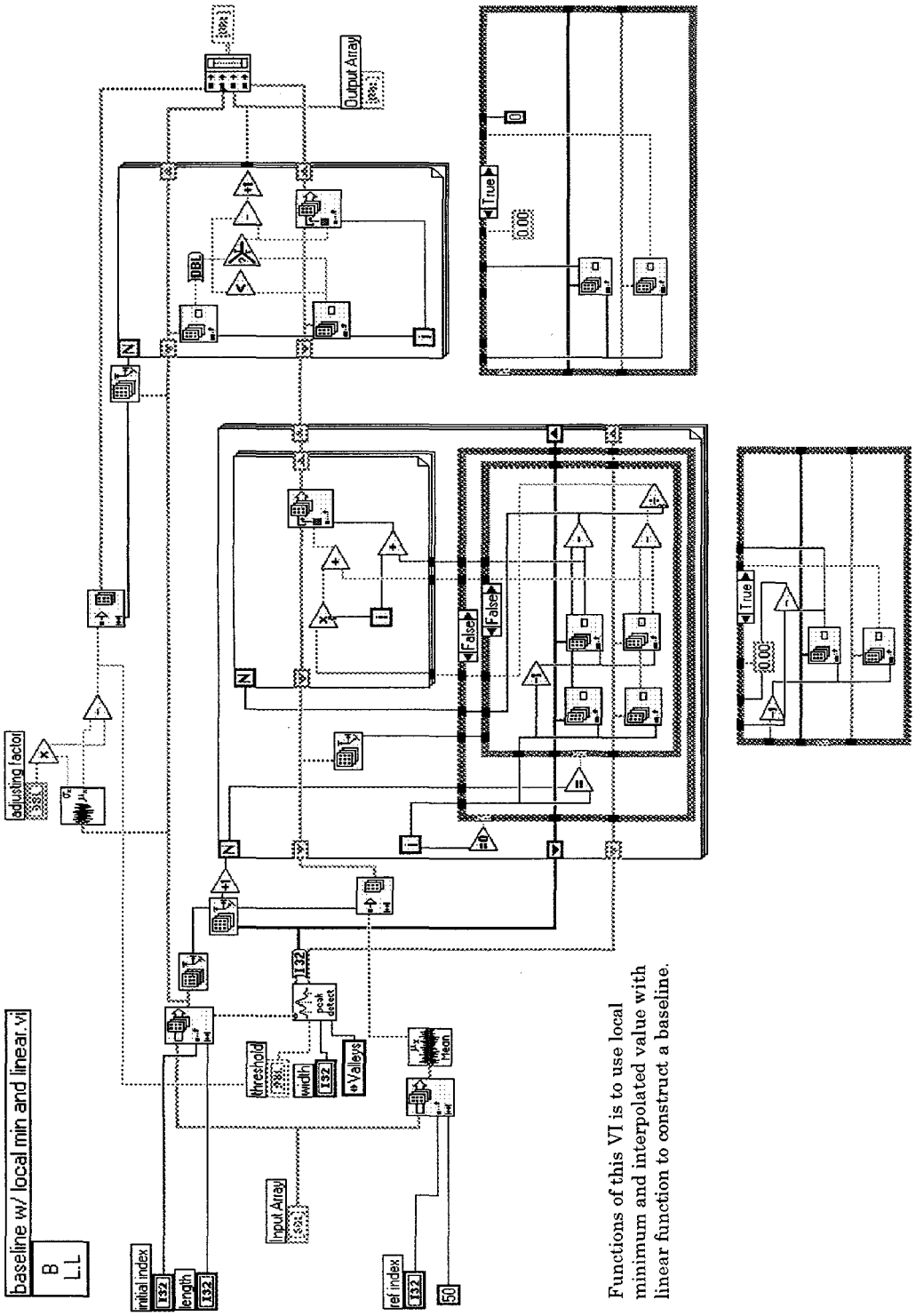


Figure B.2 Block diagram of base-calling software (cont.)

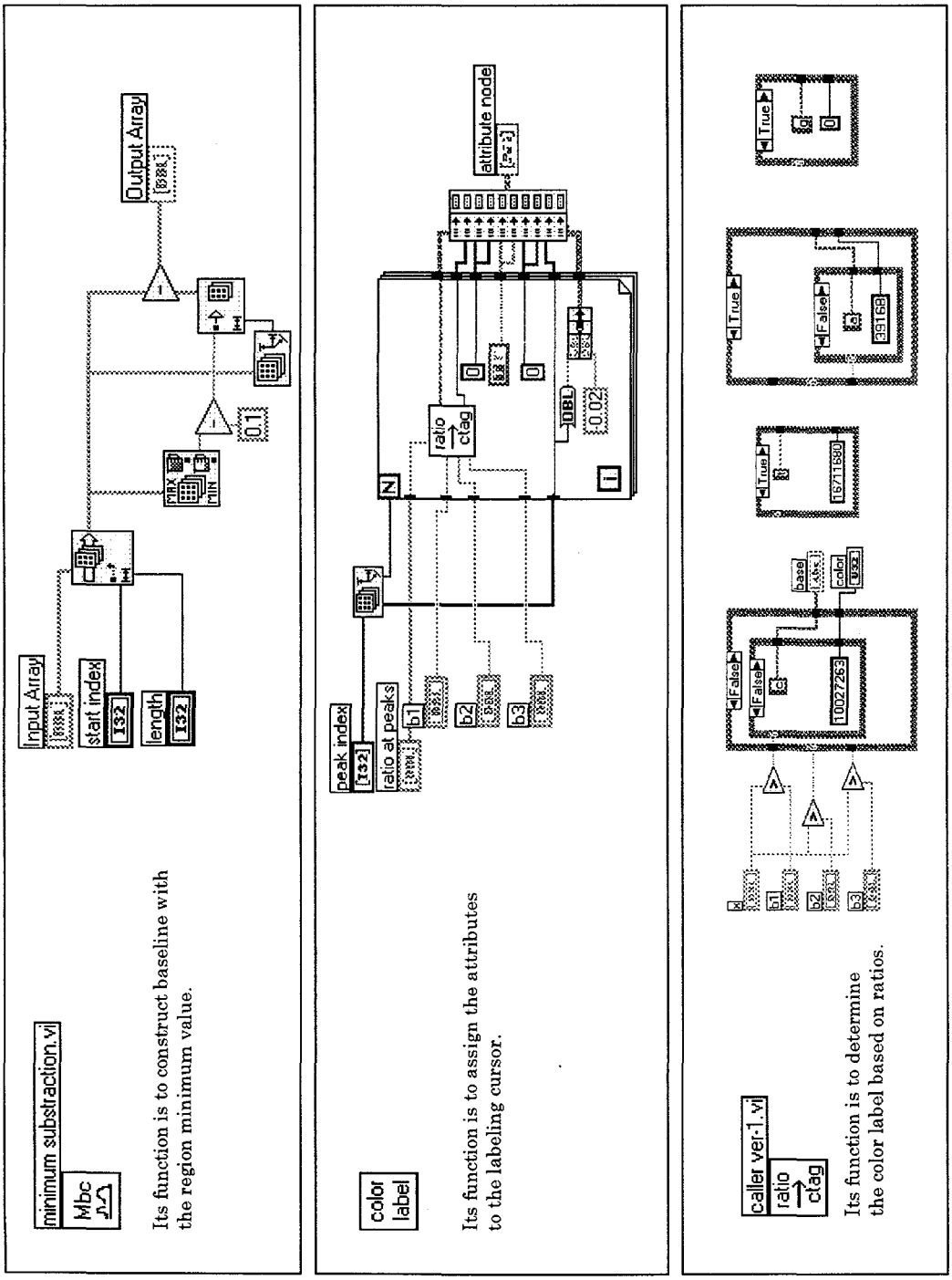
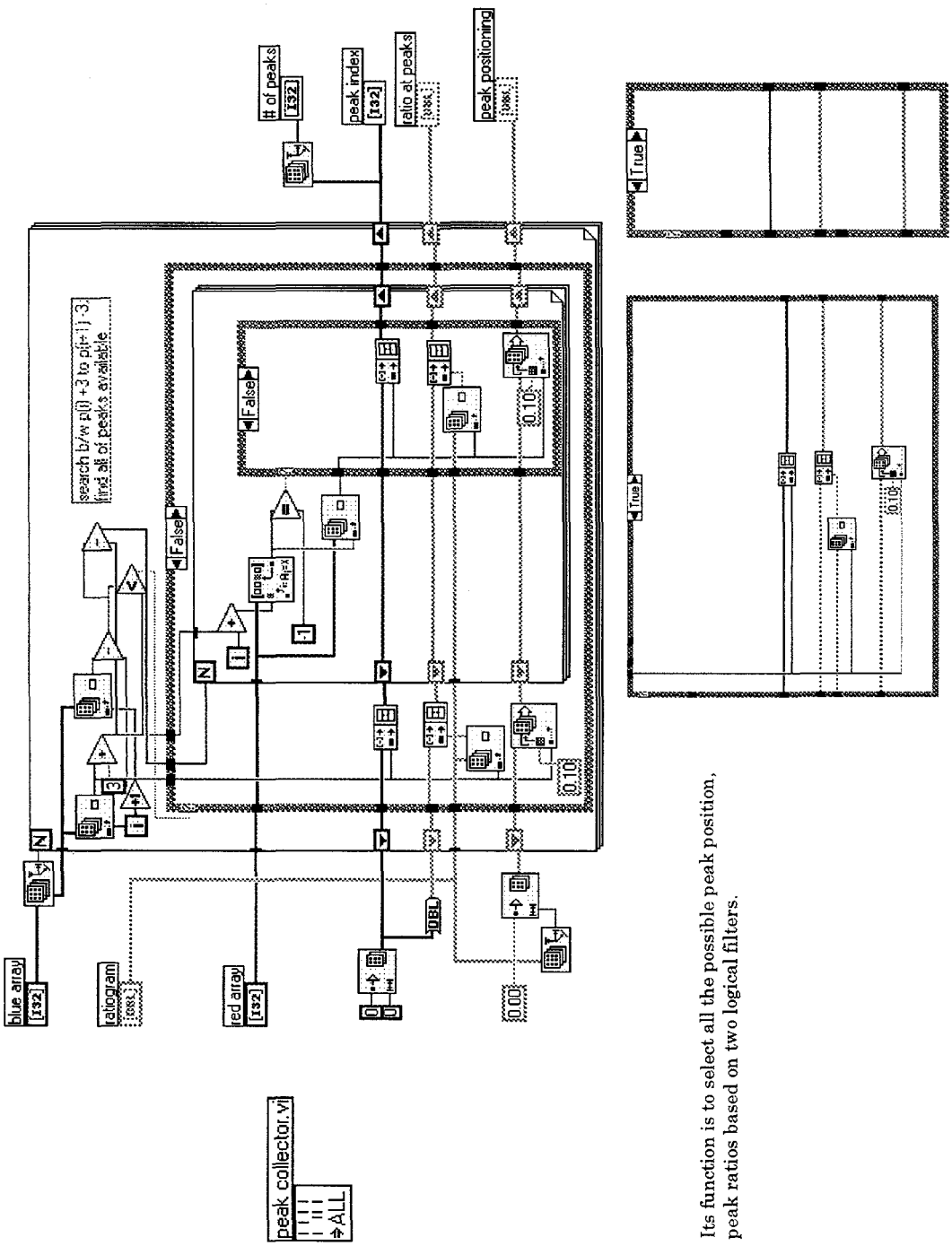


Figure B.2 Block diagram of base-calling software (cont.)



Its function is to select all the possible peak position, peak ratios based on two logical filters.

Figure B.2 Block diagram of base-calling software (cont.)

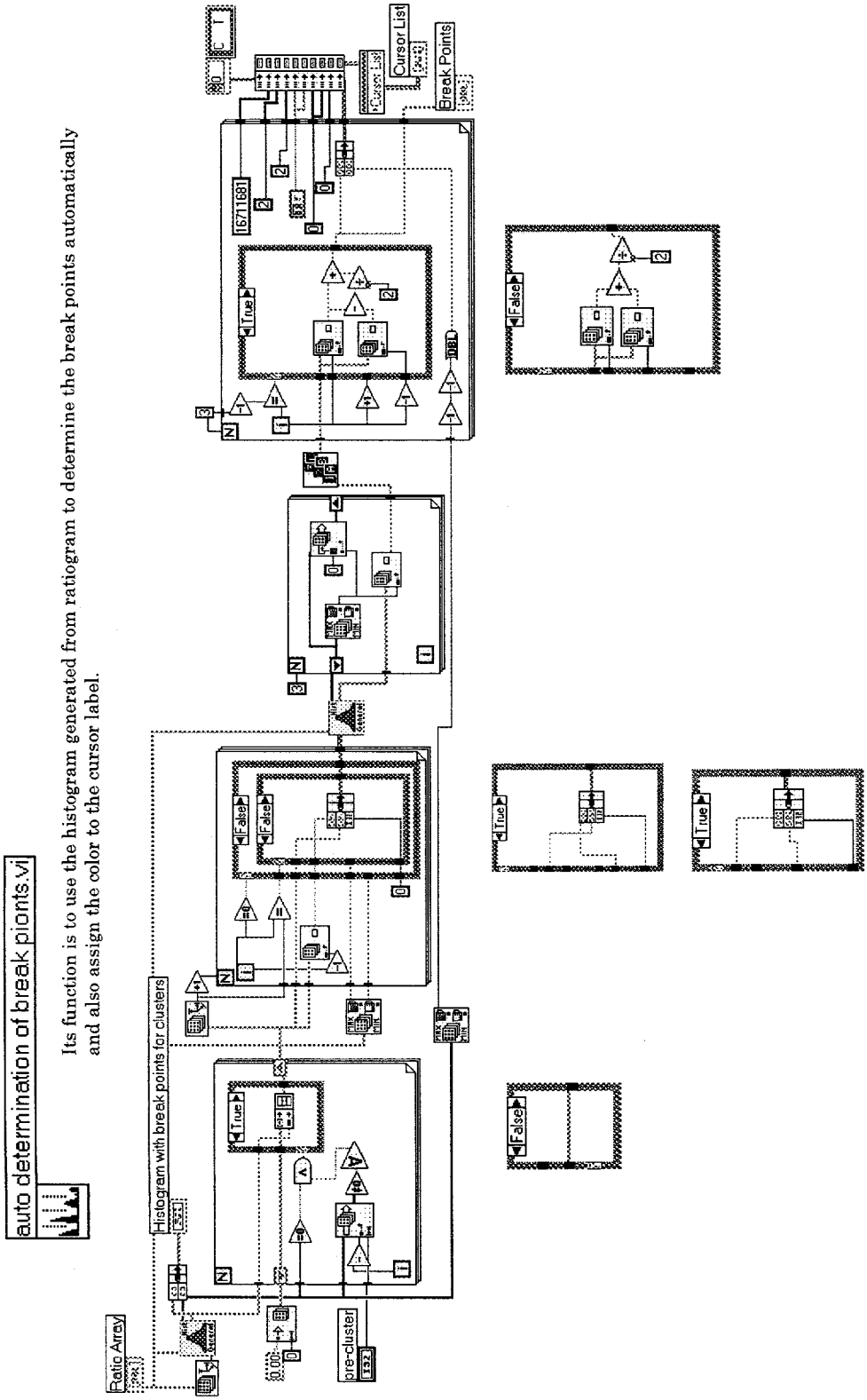


Figure B.2 Block diagram of base-calling software (cont.)

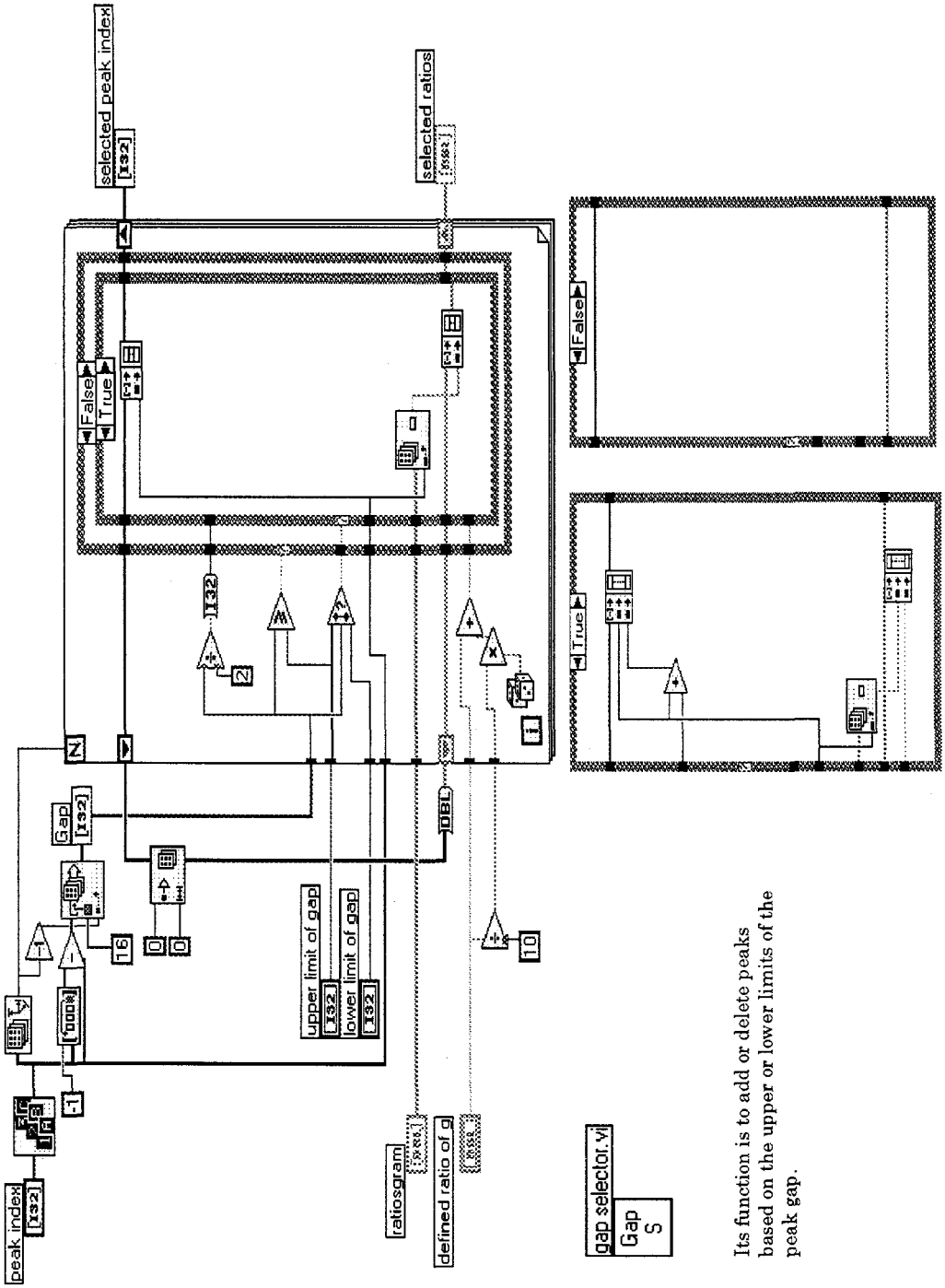


Figure B.2 Block diagram of base-calling software (cont.)

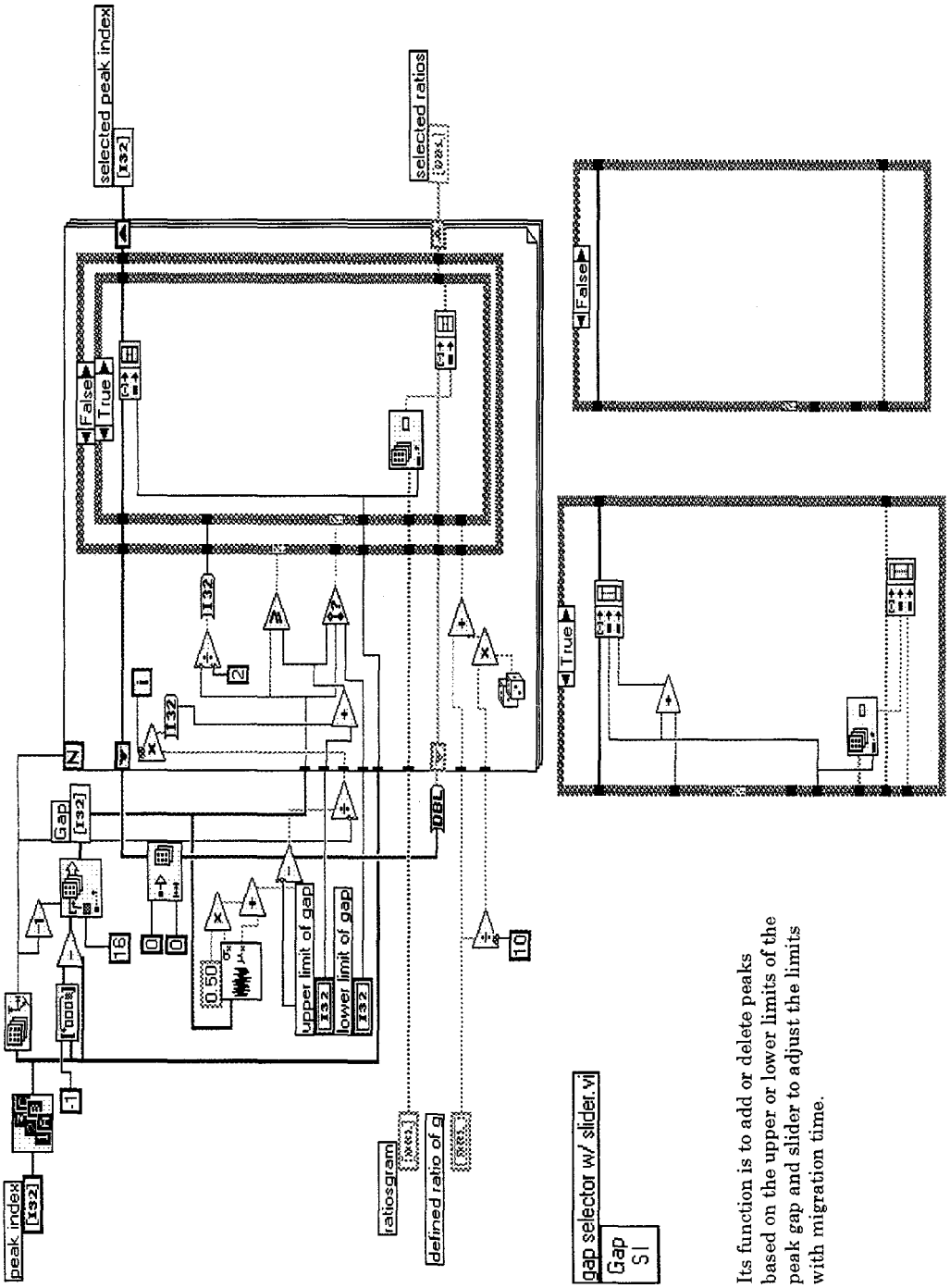


Figure B.2 Block diagram of base-calling software (cont.)

ACKNOWLEDGEMENTS

I am very grateful to Dr. Yeung, my major professor, for his guidance and encouragement, which made this work possible. I truly admire him for his curiosity, enthusiasm, diligence and dedication to research. Ever time when I discussed my research with him, no matter which is a good or bad result, his curious attitude, critical judgment or serious suggestion always motivates me to seek the answers and make research enjoyable. It is never enough how many thanks I want to say to him.

I am also very grateful to my committee members. Drs. R. S. Houk, D. C. Johnson, C. Y. Ng, R. Honzatko, and V. Sheares for their precious time and advice through the study program at Ames. I also would like to thank Drs. R. D. Foust and J. Espenson who introduce me this excellent education program at Iowa State University.

I would like to thank all the members in Yeung's group, both past and present, who share my best memory in my life, although I cannot mention all the names here. Thanks goes to Qingpo Li, Homing Pan, Yeongseng Kim, Michael Shortreed, and Xiaohong Xu for their sharpening my mind and teaching me tricks to do experiments. I want to think Nanyang Zhang, Ziqiang Wang, Qiufeng Gao, Xiaoyi Gong and Yonghua Zhang for sharing their upward spirits. I also would like to thank Ziqiang's wife, Qiufen's mother, Hui Su and Andrea Ho for their baby sitting my son Oliver when I was getting very busy in the lab.

Finally, I dedicate this dissertation to my wife and my father. My wife, Tianshu, who has been sharing every achievement of mine since 1985 we first met, I thank her for her sacrifices and support over the years when I struggled toward my goal. I am sorry about so much spare time I had spent in the lab.

This work was performed at Ames Laboratory under contract no. W-7405-Eng-82 with the U. S. Department of Energy. The United States government has assigned the DOE Report number IS-T1851 to this thesis.