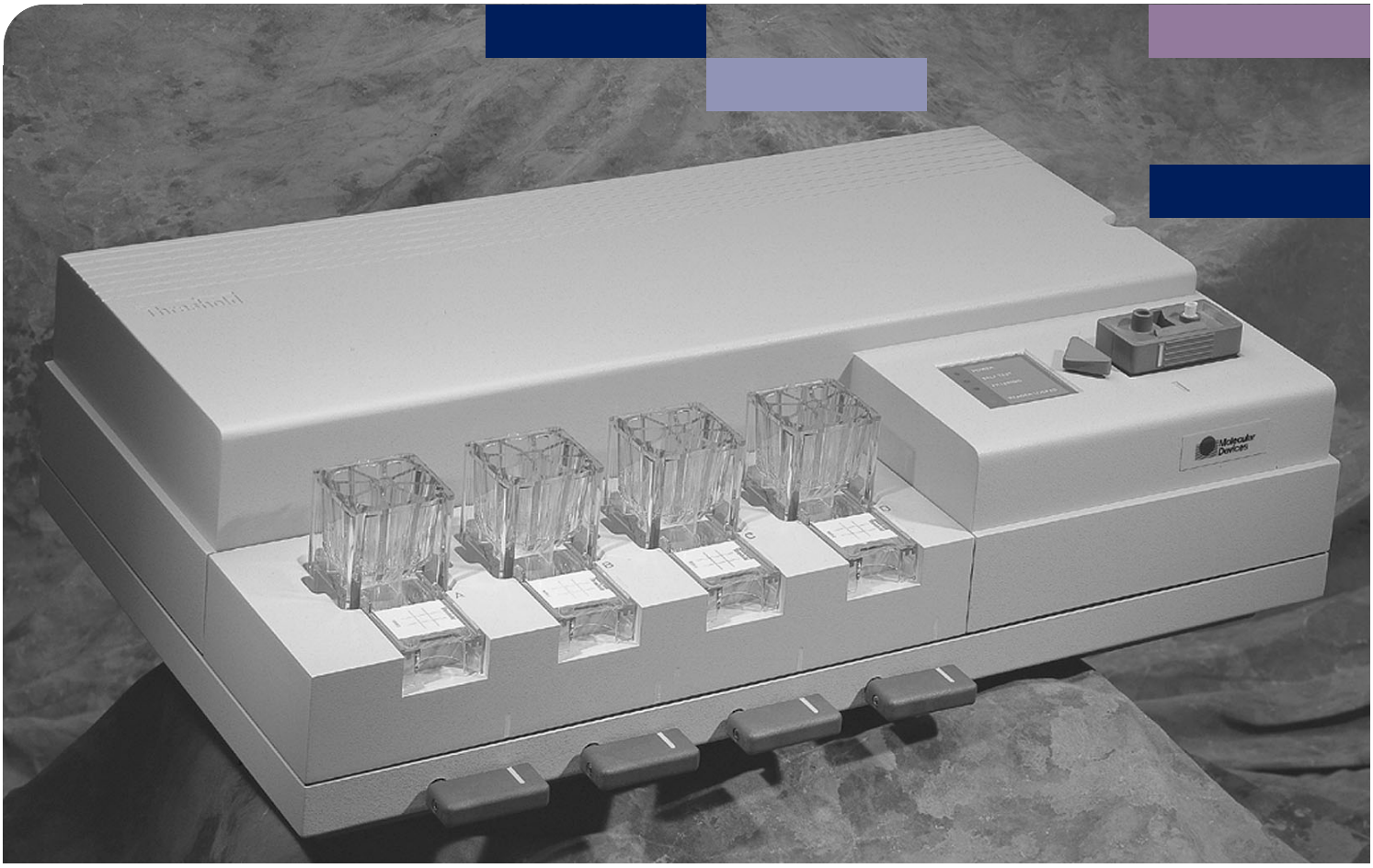
MOLECULAR DEVICES

Threshold® System User Guide

A BETTER WAY TO ANALYZE BIOMOLECULES



Product manufactured by Molecular Devices, LLC.
3860 N. First Street, San Jose, California, 95134, United States of America.
Molecular Devices, LLC is ISO 9001 registered.

Threshold System User Manual

Molecular Device LLC

Part #0012-0099

Rev. B

Preface – About This Manual

What's New for the Threshold System

Two versions of software for the Threshold System are now available: Threshold Enterprise and Threshold Software.

Threshold Software

Threshold Software includes all of the software features that have previously been available to Threshold System users. It runs on Windows NT, 2000, XP, 7, and 10.

Threshold Enterprise

Threshold Enterprise 1.0 for Windows (Threshold Enterprise) is designed for customers that require 21 CFR Part 11 compliance features. Threshold Enterprise includes password protection, audit trails, and electronic signatures and statements. Threshold Enterprise works together with a separate application, Enterprise Administrator.

Threshold Enterprise includes a new report, the Spike Recovery Report. The report extends the calculations on Threshold data to determine spike recovery and account for different pretreatment protocols. These calculations help validate the quantitation accuracy of the test method, used to determine low levels of analytes in samples.

The Extended Power Fit was added to the previously-available fitting routines to extend the range of negative quantitation with a power fit. With the Extended Power Fit, all sample values are included for quantitation. The Power Fit continues to include samples, with signals down to 10% below the background signal.

Table of Contents

Chapter 4– Threshold Software

Chapter 5– Threshold Enterprise

Chapter 6– Calibration and Quantitation

Chapter 7– System Troubleshooting

Chapter 8– Enterprise Administrator

Chapter 9– Immuno-Ligand Assay Introduction

Chapter 10– General ILA Procedure

Chapter 11– The ILA Checkout Assay

Chapter 12– ILA Labeling Procedure

Chapter 13– ILA Sandwich Format Optimization

Chapter 14– ILA Competitive Format Optimization

Chapter 15– ILA Troubleshooting

Chapter 16– Total DNA Assay Introduction

Chapter 17– Total DNA Assay Procedure

Chapter 18– Total DNA Checkout Assay

Chapter 19– Total DNA Sample Pretreatment

Chapter 20– Total DNA Assay Troubleshooting

Appendix A- Incorporation of Biotin

Appendix B- Incorporation of Fluorescein

Appendix C- ILA Worksheets

Appendix D- Total DNA Recommended Supplies

Appendix E- Software Installation

Appendix F- Threshold Enterprise and Threshold Software Reference

Chapter 1– The Threshold System

Threshold System

The Threshold System is the combination of an assay method and a means of detection. The system provides rapid and highly sensitive measurement of a variety of analytes at low concentrations. Applications include the Immuno-Ligand Assay for quantitative measurement of analytes, and the Total DNA Assay for measuring picogram levels of total DNA. Molecular Devices provides reagents for each application.

Each assay uses the same basic design (Figure 1-1):

- ***Reaction***: Formation of a reaction complex involving the analyte (substance being measured).
- ***Separation***: Capture of the complex onto a membrane by filtration on the Threshold Workstation.
- ***Detection***: Sensitive measurement of enzyme-catalyzed pH response with the Light Addressable Potentiometric Sensor (LAPS).
- ***Analysis***: Threshold Enterprise / Software facilitate data handling, computations for the standard curve, and statistics for measurements of analyte in replicate samples.

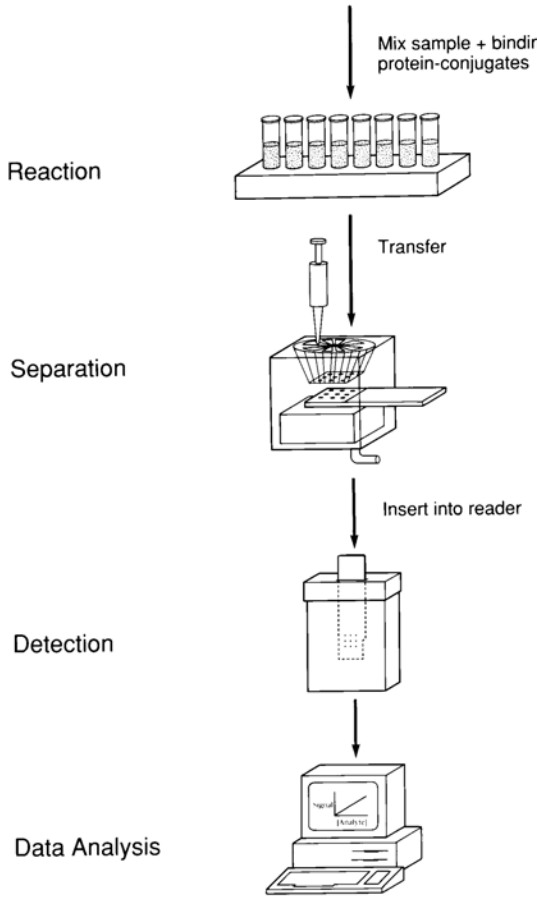
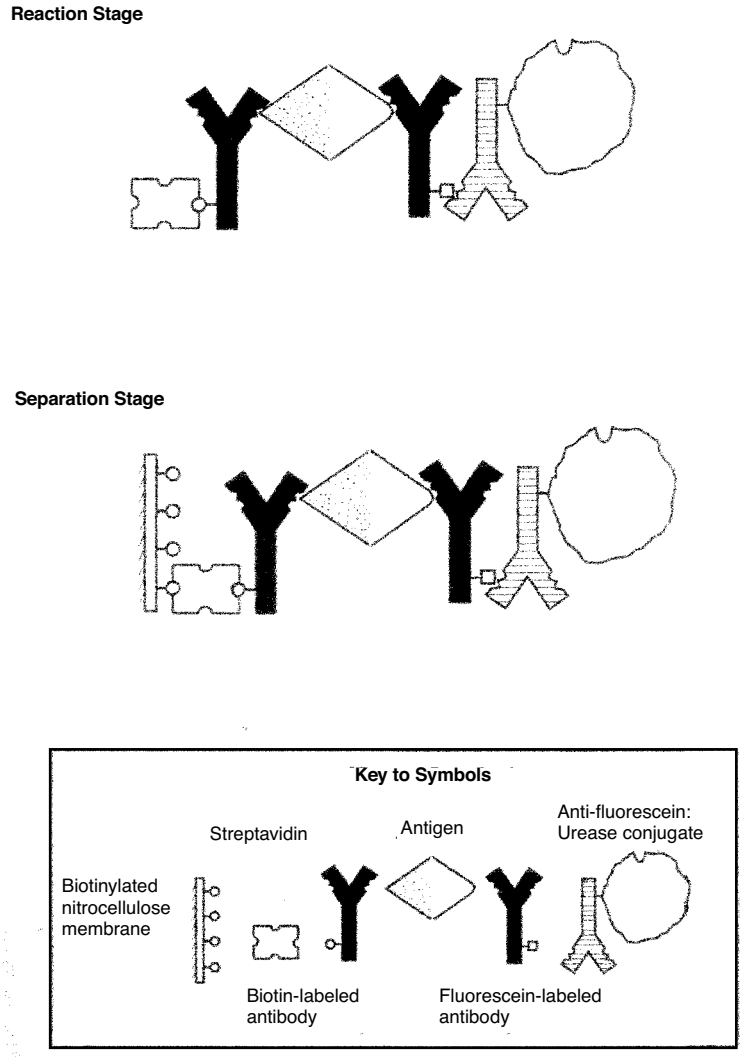


Figure 1-1: Assay Stages of the Threshold System

Phase 1: Formation of the Complex

The first phase of the Threshold Assay is the formation of a complex between binding components of high specificity and affinity. It may involve reactions of antibody and antigen, complementary strands of DNA, or any other specific binding pair.

Integral to the formation of the complex is the use of one or two haptens —biotin, or biotin and fluorescein. Molecules involved in the reaction complex are labeled with one of these haptens. The labeling configuration differs with each application. For example, Figure 1-2 shows the use of both haptens in the sandwich format of the Immuno-Ligand Assay.



Reaction Stage: streptavidin, b-Ab, Ag, f-Ab, and anti-fluorescein:urease bind to form this complex in solution.

Separation Stage: the complex is captured onto the biotinylated nitrocellulose membrane during filtration.

Figure 1-2: Formation of the Immuno-Ligand Complex

The Threshold Assay can accommodate other reaction schemes and combinations of indirect and direct labeling. In all cases, a complex comprised of the analyte, streptavidin, and urease conjugate is formed. Both sandwich and competitive assay formats can be used.

Streptavidin and Biotin

The streptavidin/biotin complex is useful in a wide variety of applications, including cytology, gene probes, affinity purification, histopathology, and other solid-phase binding assays. In the Threshold Assay, streptavidin binds to biotin in the reaction complex and to biotin on the nitrocellulose membrane, linking the complexes to the membrane (Reference 1 on page 1-12).

Streptavidin has an exceptionally high affinity for biotin (10^{-15} M) and one streptavidin molecule can bind four biotin molecules, forming a very stable complex. This provides a highly specific capture of the complex on the membrane. Streptavidin (molecular weight = 60,000 Daltons) is from the bacterium, *Streptomyces avidinii*.

Phase 2: Filtration on the Threshold Workstation

The second phase of the Threshold Assay is the separation and capture of the labeled complex.

- The Threshold Workstation filters the reaction solution, capturing the complexes onto the biotinylated nitrocellulose membrane of the Threshold stick.
- Filtration occurs in an eight-channel filter unit that houses a Threshold stick (Figure 1-3). Eight samples may be collected on different sites of one stick.
- The membrane on the stick is coated with BSA-biotin (bovine serum albumin-biotin), which limits nonspecific attachment of proteins. Streptavidin binds to the biotin in the reaction complex and to biotin on the nitrocellulose membrane.

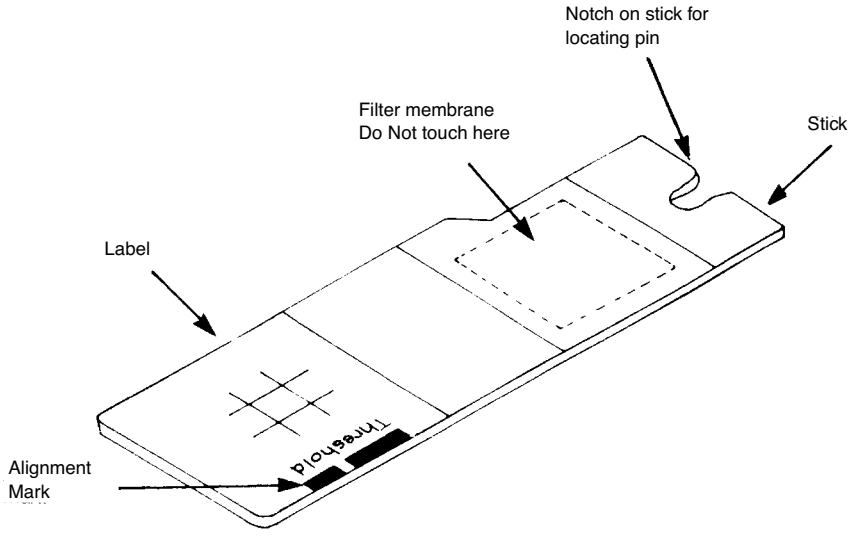


Figure 1-3: Threshold Stick with Biotinylated Nitrocellulose Membrane

- After filtration, the membrane is washed on the same filter unit. Each capture site of the membrane now contains the complexes. The complexes contain quantitative amounts of urease, in direct (sandwich format) or inverse (competitive format) proportion to the amount of analyte in a sample.

Threshold Stick with Capture Membrane

The Threshold stick contains a biotinylated nitrocellulose membrane. Common applications of nitrocellulose membranes are DNA and RNA hybridizations, protein transfers, and filtration of aqueous solutions. The membrane is 120 microns thick and has a pore size of about 0.45 microns — the pores occupy about 80% of the total membrane volume. Molecular Devices prepares the membrane by irreversibly immobilizing BSA-biotin onto the surface.

Phase 3: Detection and Analysis

The third phase of the Threshold Assay is the detection stage. Detection of analytes uses a proprietary silicon sensor.

- First, the stick is placed in the Threshold Reader which contains the enzyme substrate, urea. The Threshold Reader holds the stick membrane tightly against the silicon sensor. The sample sites on the membrane are in precise alignment and in close contact with the surface of the sensor. The actual detection volume is approximately 0.5 microliter of substrate solution, between a sample site on the membrane and the sensor.
- As the urease molecules of the reaction complex hydrolyze urea, a significant pH change occurs in this microvolume of substrate solution. The Threshold Workstation and computer monitor the change in surface potential on the sensor. This change is proportional to the amount of analyte present in the sandwich format, and inversely proportional in the competitive format.
- Threshold Enterprise / Software automatically quantitates data from test samples using a standard curve.The software allows adjustment of the standard curve for accurate quantitation.

Urease and Urea

Urease catalyzes the hydrolysis of urea to form one carbonate and two ammonium ions. Ammonium production changes the pH of the substrate solution, creating a change in the surface potential on the silicon sensor.

Urease (molecular weight = 540,000 Daltons) is a metalloenzyme with six subunits. The nickel ion, bound in the active site of urease, is essential for enzymatic activity (Reference 1 on page 1-12).

Urease is from the jack bean, *Canavalia ensiformis*. Urease conjugates are prepared using the heterobifunctional cross-linking reagent SMCC (succinimidyl 4-N-maleimidomethyl cyclohexane-1-carboxylate) (Reference 3 on page 1-12).

Instrument Components

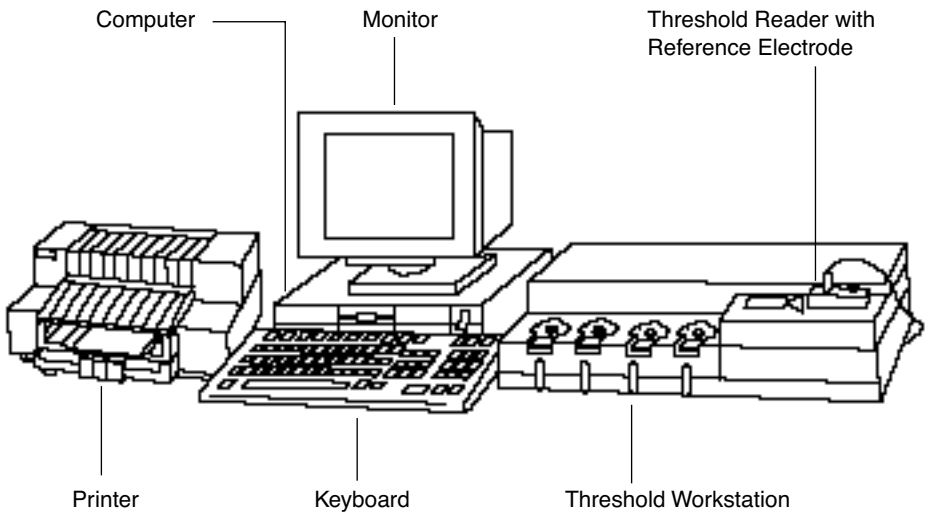


Figure 1-4: Threshold System

Threshold Workstation

The Threshold Workstation performs the separation and detection phases of the assay—it captures the reaction complex onto a stick, then reads the stick. This semi-automated workstation contains four independent filter units and a Reader which contains the silicon sensor (Figure 1-5).

Each filter unit consists of a filter base and an eight-channel filtration device called the filter block. The membrane on the stick is tightly fitted between the filter base and filter block. The components of the filter unit are manually locked together, using a locking lever on the workstation.

- When the test tube reactions are complete, the samples are transferred to the eight channels in the filter block. Quantitative capture of the complexes in the reaction mixture occurs during filtration through the membrane. Threshold Enterprise / Software allows selection of three filtration flow rates: LOW and SPECIAL flow rates for capture and a faster rate (HIGH) for washing away nonspecific enzyme.
- The Threshold stick is inserted into the Threshold Reader to measure the enzyme-labeled complexes. The Threshold Reader precisely aligns the eight capture sites on the membrane with the sensor's measurement sites. The Threshold Reader contains the highly sensitive silicon sensor.
- Threshold Enterprise / Software, running on a computer connected to the workstation, controls the Reader diagnostics, setup conditions, signal and data analysis, and recording of results.

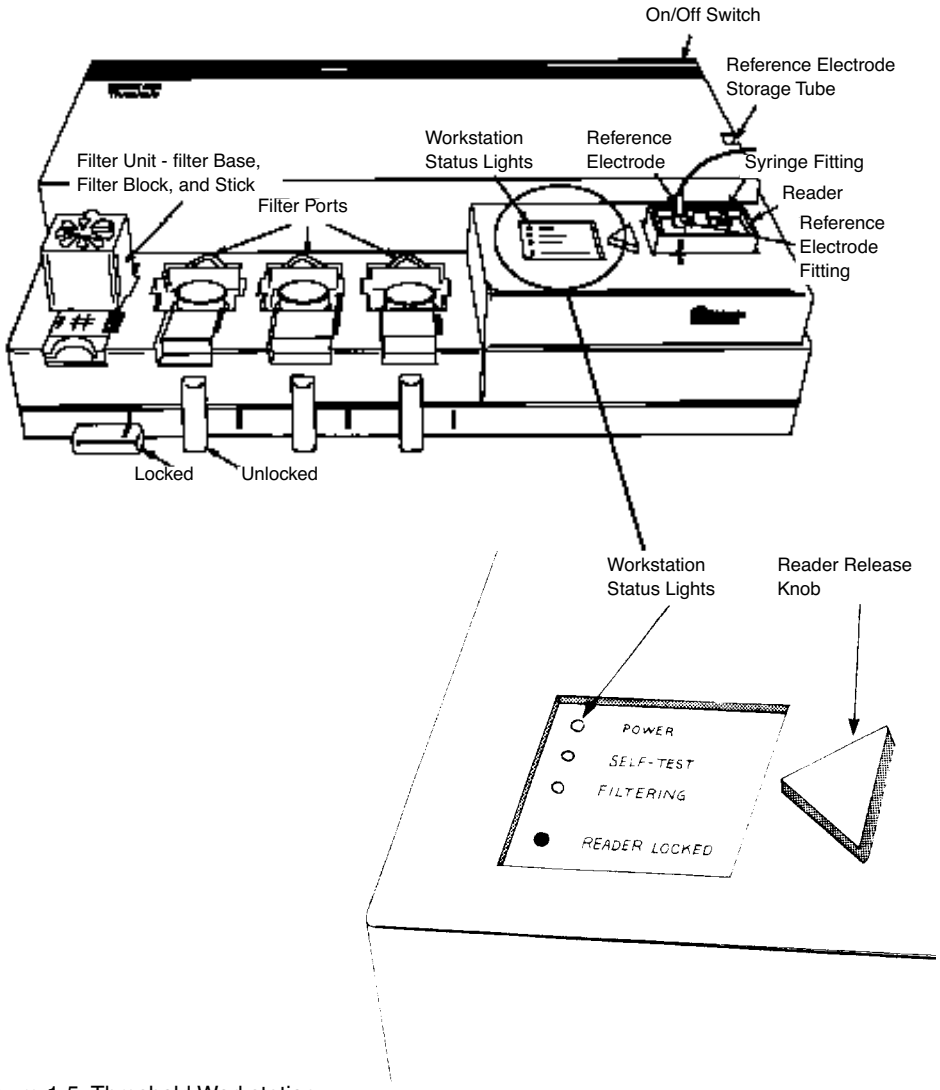


Figure 1-5: Threshold Workstation

Threshold Reader

The Threshold Reader houses a highly sensitive detector—the Light-Addressable Potentiometric Sensor (LAPS). When a Threshold stick is inserted into the Threshold Reader, containing Substrate Solution, the LAPS detects the change in pH, caused by the enzyme labeled complexes on the stick.

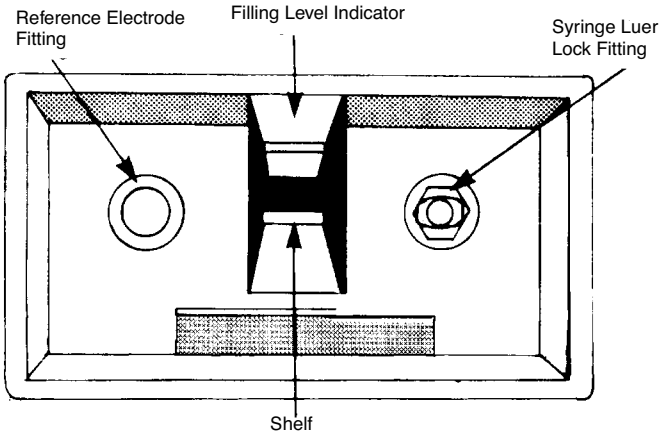


Figure 1-6: Threshold Reader

Reference Electrode

The Reference Electrode, located in the Threshold Reader, measures the potential of the Substrate Solution. This provides a reference for the change in surface potential of the sensor, caused by enzymatic hydrolysis of urea during the reading (detection) step. This electrode should be full at all times with 3 M KCl saturated with AgCl.

Computer and Software

Threshold Enterprise / Software controls, collects, and analyzes data from the workstation. A computer is supplied with each Threshold System.

Printer

A printer is supplied with each Threshold System.

Principles of Sensor Technology

The Light-Addressable Potentiometric Sensor (LAPS)

The light-addressable potentiometric sensor (LAPS) is the detector for the Threshold System. It is a solid-state semiconductor electrode, that detects changes in potential, due to chemical reactions (References 4, 5, 6, on page 15).

- The LAP sensor, in the Threshold Workstation, employs a pH-sensitive insulator surface to specifically measure pH changes. This pH-sensitive insulating layer, impervious to biological solutions, is deposited on a silicon chip. All electronic functions that provide specific measurements at multiple sites on the sensor are external to the sensor. Intensity-modulated, light emitting diodes (LED) adjacent to the sensor, but isolated from electrolytes, control the measurements at each site.
- The LAP sensor is equivalent to a capacitor, across which alternating current may flow. The components of the capacitor are the electrolyte, the insulator, and the silicon chip. To complete the electrical circuit, the LAP sensor uses these components: a counter-electrode and a Reference Electrode (to contact the electrolyte), an adjustable DC voltage source, an AC ammeter, and a gold wire contact to an uninsulated spot on the silicon (Figure 1-7).

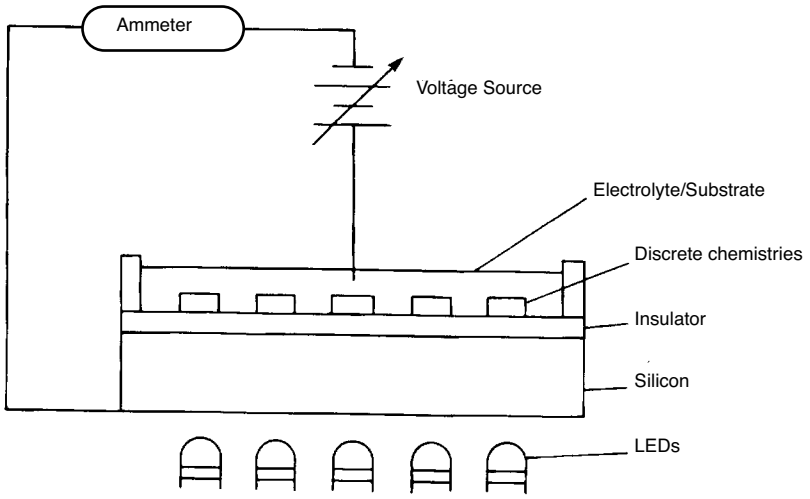


Figure 1-7: Structure of the LAP Sensor

- For a potentiometric measurement at a single site on the surface of the insulator, a LED illuminates the site on the back of the silicon chip. Measurements occur at eight sites. A ninth site is an internal reference.

- Light from a LED generates charge carriers in the silicon. As these charge carriers separate under the influence of an electrical field in the silicon, an electrical current flows in the circuit to which the chip is connected. The LED intensity is modulated (typically at 10 KHz), generating an alternating photocurrent. The magnitude of this current depends upon the electrical potential across the sensor. This electrical potential comes from two sources:

  a. The potential applied by the voltage source.

  b. The potential at the measurement site produced by the urease reaction. Urease hydrolyzes urea, causing an increase in pH.

pH-Dependent Surface Potential on the Sensor

The potential across the electrolyte-insulator interface is pH-dependent because of the affinity of the insulator surface for protons. Protons from the electrolyte bind to the insulator surface, changing the amount of charge at the surface. Because of this pH-dependent charge accumulation, the electrical field within the silicon is dependent upon the local pH. This pH-dependent electrical field influences the applied voltage-dependent amplitude of the alternating photocurrent in the LAP sensor circuit.

Enzyme-Catalyzed Potentiometric Response

The hydrolysis of urea by urease increases the pH at the insulator surface. (Recall that enzyme molecules are immobilized at discrete sites on a capture membrane.) The rate of change in pH is proportional to the number of enzyme molecules at a site. As the pH changes, the magnitude of the photocurrent changes because of the effect of pH on the sensor's surface potential. The Threshold System can compensate for this change by adjusting the voltage applied to the LAP sensor.

The change in applied voltage, required to return the photocurrent to its former value, is a direct measure of the change in pH at the insulator surface. Because the volume and buffer capacity of the electrolyte between the enzyme capture membrane and the LAP sensor's surface are fixed, the rate of change in voltage adjustment required to maintain the AC photocurrent is related directly to enzymatic activity. The direct conversion of enzymatic activity to an electrical response by the LAP sensor, combined with enzyme-linked assay technology, produces a highly sensitive detection of analyte.

The sensitivity of the Threshold Reader comes from the sensor's direct conversion of an enzymic reaction to an electrical response. About 3×10^7 urease molecules produce a measurable response in one minute, over the background drifts of the detector circuitry.

Stability, Reliability, and Performance

The LAP sensor from Molecular Devices is designed to overcome several of the practical difficulties facing semiconducting chemical sensor devices. The insulating layer is very stable, microscopically flat, and impervious to charged ions. LAP sensor chips have been stored in saline for over two years, and individual sensors have been used for thousands of determinations, with no deterioration in performance. This silicon-based chemical sensor provides fast, extremely sensitive, multiple site detection of a wide variety of analytes in a simple, stable, and reliable workstation.

References

1) Wilchek, M., and Bayer, E.A., The avidin-biotin complex in bioanalytical applications, *Analytical Biochemistry*, **171**, 1-32 (1988).

2) Blakeley, R.L. and Zerner, B., Jack bean urease: the first nickel enzyme, *Journal of Molecular Catalysis*, **23**, 263-292 (1984).

3) Ishikawa, E., Imagawa, M., Hashida, S., Yoshitake, S., Hamaguchi, Y., and Ueno, T., Enzyme-labeling of antibodies and their fragments for enzyme immunoassay and immunohistochemical staining, *Journal of Immunoassay*, **4(3)**, 209-327 (1983).

4) Briggs, J., Biosensors emerge from the laboratory, *Nature*, **329**, 565-566 (1987).

5) Hafeman, D.G., Parce, J.W. and McConnell, H.M., Light-addressable potentiometric sensor for biochemical systems, *Science*, **240**, 1182-1185 (1988).

6) Sigal, G.B., Hafeman, D.G., Parce, J.W., and McConnell, H.M., Electrical properties of phospholipid bilayer membranes measured with a light-addressable potentiometric sensor, In. Chemical Sensors and Microinstrumentation, Murray, R.W., Dessy, R.E., Heineman, W.R., Janata, J., and Seitz, W.R. (Eds.), American Chemical Society, Washington, D.C. (1989).

7) Bousse, L., Hafeman, D., and Train, N., Time-dependence of the Chemical Response of Silicon Nitride Surfaces, *Sensors and Actuators*, **B1**, 361-367 (1990).

8) Bousse, L., Owicki, J.C., and Parce, J.W., Biosensors with Microvolume Reaction Chambers, *Chemical Sensor Technology*, **4**, 145-166, (1992).

2

Chapter 2– Unpacking and Installation

Installing the Threshold System

Operating Requirements

Check that the following facilities are available.

Table 2-1: Required Facilities

Facilities	Details
Line Voltage	110 VAC at 60 Hz or 220 VAC at 50 Hz; 4 outlets
Operating Temperature	+15°C to + 40°C (+ 60°F to + 105°F)
Operating Humidity	0 to 85% (non-condensing)
Disposal Containers for Waste Reagents	
Bench Space for the Workstation	21.5 x 14.75 x 6.5 inch (55.5 x 37.5 x 16.5 cm)
Bench Space for the Computer	
Bench Space for the Keyboard	
Bench Space for the Printer	

System Setup

To set up the Threshold System:

1) Place the Workstation, computer, keyboard, monitor and printer on a work bench (Figure 2-1 and Figure 2-2).
2) Place the monitor on top of the computer.
3) If there is an Auxiliary Manifold, place it on top, or to one side, of the Workstation (Figure 2-5 and Figure 2-6).

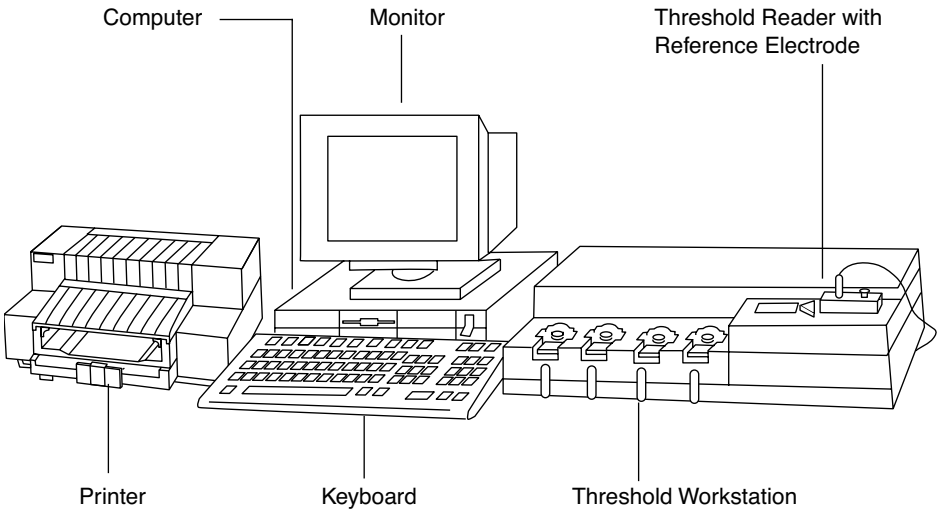


Figure 2-1: Front View of the Threshold System

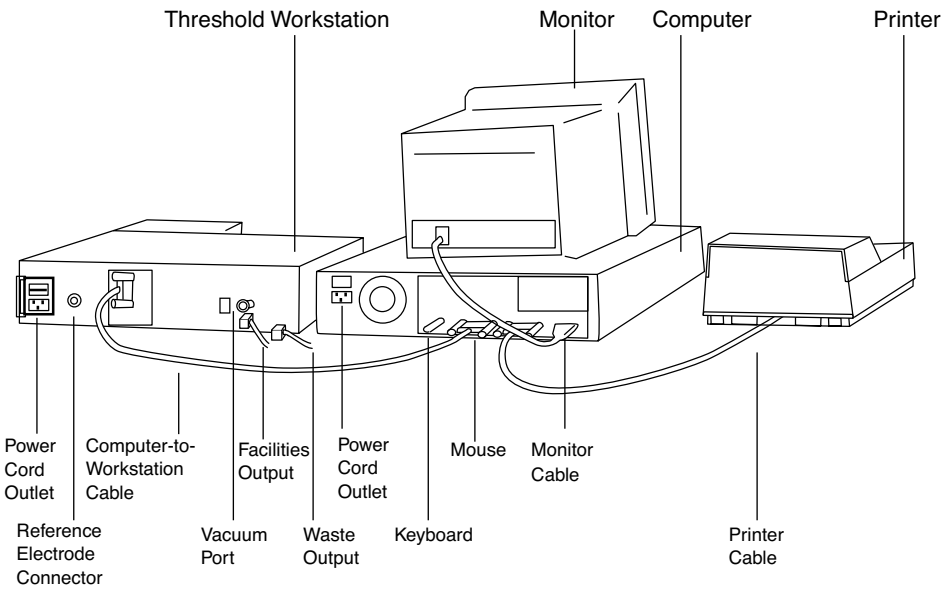


Figure 2-2: Rear View of the Threshold System

Attaching the Cables

Printer Cable

The printer cable has a metal Centronics male connector, at one end, and a 25-pin male connector, at the other end (Figure 2-3). To attach the printer cable:

1) Plug the blue metal Centronics male connector into the printer.
2) Plug the 25-pin male connector at the other end of the cable into the back of the computer (Figure 2-2).

Caution: *For the printer to function, firmly connect and clip on the Centronics connector.*

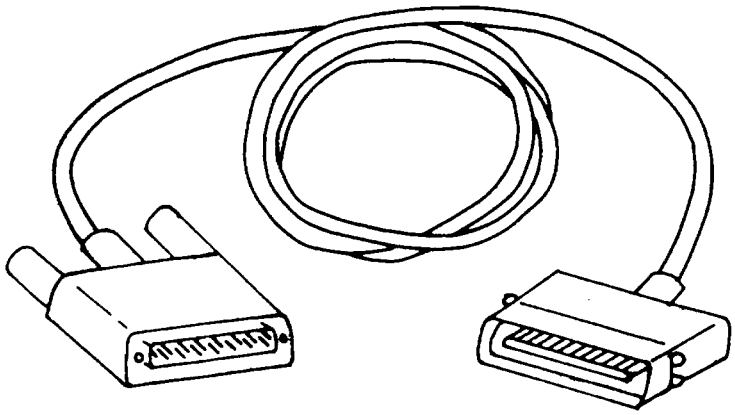


Figure 2-3: Printer Cable

Workstation Cable

The Workstation cable has a 9-pin female connector at one end and a 25-pin male connector at the other end (Figure 2-4). To attach the Workstation cable:

1) Plug the 25-pin male connector into the Workstation.
2) Plug the 9-pin female connector, at the other end of the cable, into the back of the computer (Figure 2-2).

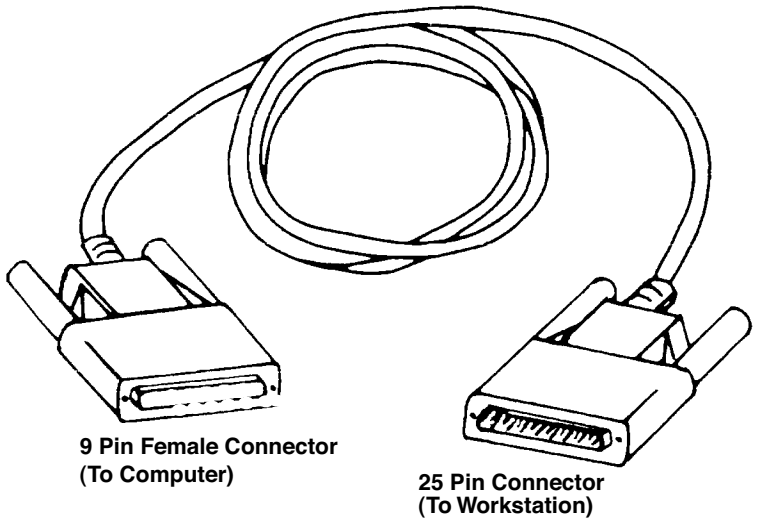


Figure 2-4: Workstation Cable

Computer Monitor Cable

1) Attach the computer monitor cable. The computer monitor cable is built into the monitor and has an 8-pin male connector at the free end.
2) Plug the 8-pin male connector into the back of the computer (Figure 2-2).

Keyboard and Mouse Cables

1) Plug the round end of the keyboard cable into the small port on the back of the computer labeled with the keyboard icon.
2) Plug the round end of the mouse cable into the small port on the back of the computer labeled with the mouse icon.

Attaching the Power Cords

To attach the power cords:

1) Plug the printer power cord into the printer and into a power outlet.
2) Plug the computer power cord into the computer and into a power outlet. The outlet on the computer is located next to the fan.
3) Plug the monitor power cord into the monitor and into a power outlet.
4) Plug the Workstation power cord into the Workstation and into a power outlet.

Connecting Waste and Overflow Tubing to the Workstation

To connect waste and overflow tubing to the Workstation:

1) Connect the waste tube to the connector block in the "Waste output" port on the back of the Workstation (Figure 2-2).

Caution: *When using biohazardous samples, submerge the exit end of the waste tubing into a collection trap containing disinfectant. Use enough concentrated disinfectant to be at its active strength when the trap is full of waste liquid. To avoid overflow, place the trap in easy view.*

2) Connect the overflow tube to the connector block in the "Facilities Output" port on the back of the Workstation.

Caution: *Do **NOT** plug the tubing into the Vacuum Port.*

Hooking Up the Auxiliary Manifold

To hook up the Auxiliary Manifold:

1) Place the Auxiliary Manifold on top of the Workstation or beside it.
2) To connect either 1 or 2 Auxiliary Manifolds to the Threshold Workstation, see instructions below and Figure 2-5 and Figure 2-6.

Connecting a Single Auxiliary Manifold

Control Cable

1) Plug the control cable into the control socket on the back of the Workstation.
2) Plug into either of the control IN/OUTsockets on the Auxiliary Manifold.

Tubing

1) Insert the tubing connector into the selected port, until it clicks.
2) If there is no click, remove the connector, push down on the port release tab on top of the port and try to insert the connector again.
3) To disconnect the tubing, push down on the port release tab and remove the connector.

Vacuum Tubing

The vacuum tubing is a clear tube with metal connectors.

1) Connect the tubing to the vacuum port on the Threshold Workstation
2) Plug into either vacuum IN/OUT port on the Auxiliary Manifold (Figure 2-5).

Caution: *Ensure that the vacuum tubing is connected to the vacuum port on both ends. If one end of the vacuum tubing is connected to a waste port, when the open end of the waste tube is in water, the water could be sucked into the system and cause serious damage.*

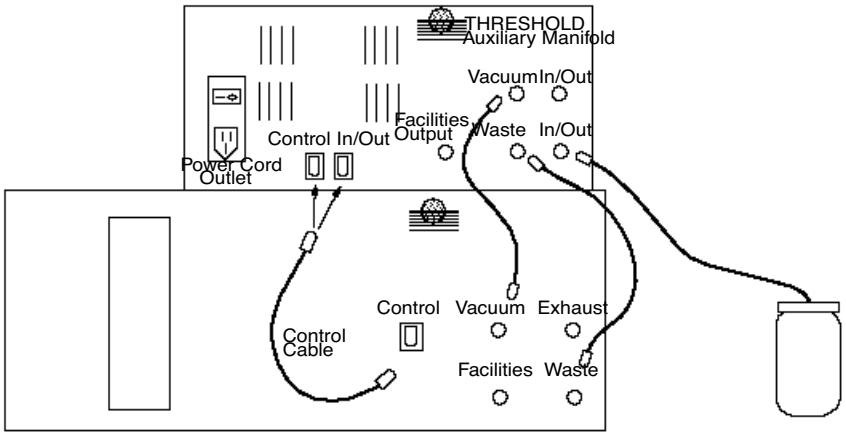


Figure 2-5: Auxiliary Manifold Connections

3) Connect the second clear tube, with metal connectors, to the waste port on the Workstation and either waste port on the Auxiliary Manifold.
4) Attach one end of the original waste tubing, from the Threshold Workstation, to the other waste port on the Auxiliary Manifold
5) Place the other end of the tubing into a sink or waste receptacle.

Caution: *If using biohazardous samples, submerge the exit end of the drain tube into a collection trap containing disinfectant. Use enough concentrated disinfectant so that it is at active strength when the trap is full of waste liquid.*

Connecting A Second Auxiliary Manifold

To hook up a second Auxiliary Manifold:

1) Plug the second Auxiliary Manifold's power cord into an electrical socket.
2) Use the control cable, vacuum tubing, and inter-unit waste tubing, accompanying the second Auxiliary Manifold, to connect it to the first Auxiliary Manifold.
3) Attach the waste tubing to a waste port on the second Auxiliary Manifold. This serves the entire system. The waste tubing is attached to a waste port on the second Auxiliary Manifold and serves the entire system (the Workstation and both Auxiliary Manifolds, Figure 2-6).

Note: *If placement of the manifold(s) requires longer waste or vacuum tubes, remove the connectors from the existing tubes and insert them into the supplied 30- inch clear tubes.*

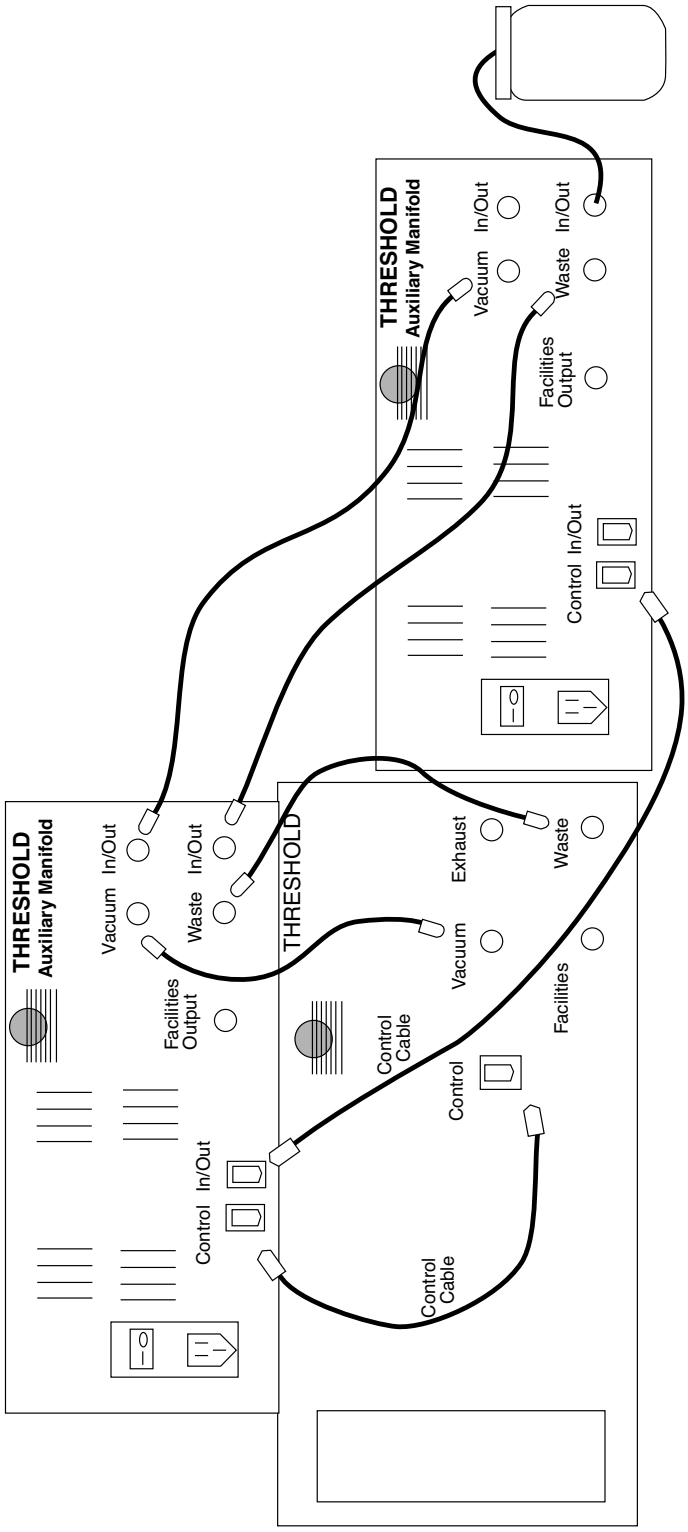


Figure 2-6: Connecting a Second Auxiliary Manifold

Turning on the Equipment

To turn on the equipment, set the power switches on the Workstation, Auxiliary Manifold, computer, monitor and printer to ON.

Examining the Status Lights

- The green power light on the Workstation should be ON (Figure 2-7).
- The Filter light on the Workstation should be OFF.
- If there is an Auxiliary Manifold, the green power light should be ON.

Caution: *If the green power light on the Auxiliary Manifold is **NOT** ON, it will not drain. This may cause damage to the instrument.*

1) Check that the printer status lights are ON, as shown in the printer owner's manual.
2) If the power or status lights are OFF, check that the power cords are connected and the power switches are ON.

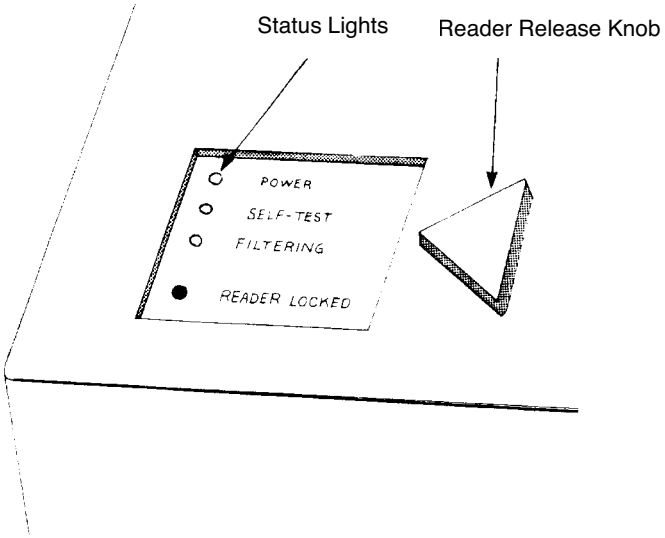


Figure 2-7: Workstation Status Lights

Hydrating and Inserting the Reader

For instructions on hydrating the Threshold Reader and inserting it in the Workstation, please refer to Chapter 3, "Operating Procedures and Maintenance."

Filling and Connecting a Reference Electrode

For instructions on filling the Reference Electrode, please refer to Chapter 3, "Operating Procedures and Maintenance."

1) Connect the Reference Electrode (microelectrode) cable to the connector, labeled "Reference Electrode," on the back of the Workstation (Figure 2-2).
2) Insert the Reference Electrode tip into the raised opening, on the left top of the Threshold Reader (not the white Luer Lock fitting) (Figure 3-7).

Testing the System

Power

1) Turn on the power for the Workstation, computer, monitor, and (if part of system) the Auxiliary Manifold(s).
2) Green power status lights should be lit on both the Workstation and Auxiliary Manifold(s).

Initial System Test

To perform an initial test of the system, using Threshold Enterprise / Software:

1) Select **Test** from the **Instrument** menu.
2) Threshold Enterprise / Software will then perform self-diagnostic tests and report "Pass" or "Fail" for each test. (See Figure 3-8 in Chapter 3, “Operating Procedures and Maintenance.”
3) For "Fail" results, consult the suggestions on the computer screen, or see Chapter 6, “Calibration and Quantitation.”

Threshold Workstation and Auxiliary Manifold(s) Setup Test

To test that the Threshold Workstation and Auxiliary Manifold(s) are set up correctly:

1) Select **Instrument**, **Filter**, and highlight **High** to test that the Threshold Workstation and Auxiliary Manifold(s) are set up correctly,
2) The vacuum pump should come on and the filter light on the Workstation should blink, and then stabilize, within 15 seconds.
3) Carefully push down and release one of the filter port check valves on the Workstation and each Auxiliary Manifold.
4) There will be a brief hissing sound as the valve is depressed.
5) The filter light on the Workstation should blink, and then stabilize, in approximately 15 seconds.
6) In Threshold Enterprise / Software, select **Instrument**, **Filter**, and highlight **Off**.
7) The drain pumps should come on, and the drain light on the Auxiliary Manifold(s) should be lit, for approximately 30 seconds.

Disassembly for Local Moves

To disassemble the Threshold System for local moves:

1) Remove the Reference Electrode from the Threshold Reader and put the electrode into its storage tube on the right side of the Workstation.
2) Remove the Threshold Reader from the Workstation and put on the Reader cap.
3) Drain the vacuum system of fluid:
   a. Select **Instrument**, **Filter**, and highlight **High.** This will turn the vacuum on.
   b. Select **Filter** again from the **Instrument** menu, and highlight **Off**.
   c. The drain pump will run for approximately 30 seconds.

d. While the drain pump is running, lift the right front corner of the Workstation (where the Threshold Reader is located) to help the fluid drain out.

e. Repeat this process (a. to d.) several times, until the fluid stops draining from the Workstation.

4) Wrap the ends of the overflow and waste tubes, and tape them to the top of the Workstation.

5) Turn off the computer, printer, and Workstation.

6) Disconnect the power cords and cables for the Workstation, computer, and printer.

7) Keep the equipment upright and level, while moving.

Disassembly for Shipping

To disassemble the Threshold System for shipping purposes:

1) Remove the Reference Electrode from the Threshold Reader and put the electrode into its storage tube on the right side of the Workstation.

2) Remove the Threshold Reader from the Workstation and put on the Reader cap.

3) Drain the vacuum system of fluid:

a. Select **Instrument**, **Filter**, and highlight **High.** This will turn the vacuum on.

b. Select **Filter** again from the **Instrument** menu, and highlight **Off**.

c. The drain pump will run for approximately 30 seconds.

d. While the drain pump is running, lift the right front corner of the Workstation (where the Threshold Reader is located) to help the fluid drain out.

e. Repeat this process (a. to d.) several times, until the fluid stops draining from the Workstation.

4) Disconnect the waste and overflow tubes, and clamp their ends.

5) Disconnect the Reference Electrode cable from the back of the Workstation.

6) Remove the Reference Electrode and its storage tube from the Workstation.

a. Discard the storage tube.

b. Rinse the PVC tube inside and out with deionized water, using the same technique as filling the Reference Electrode (see Chapter 3, "Operating Procedures and Maintenance.")

7) Put the Reference Electrode back into its shipping box.

8) Empty all liquid from the Threshold Reader, using a 30-ml syringe, and flush it twice with deionized water. Put on the Reader cap.

9) Put the Threshold Reader back into its shipping box.

10) Repack the equipment in the original shipping boxes.

Caution: *If the Threshold Reader and Reference Electrodes are ever dry for more than one hour, rehydrate them for* ***at least*** *one hour before use (see Chapter 3, "Operating Procedures and Maintenance.")*.

3

Chapter 3– Operating Procedures and Maintenance

Operating Procedures

This chapter describes operating and maintenance procedures for the Threshold System. These procedures will be used for both ILA and Total DNA assays. Some procedures will be used for each assay, whereas others will be used less often.

Starting Up the System

To start up the system:

1) Turn on the computer, monitor, printer and Threshold Workstation.
2) If an Auxiliary Manifold is connected to the system, turn on the manifold.
3) Check that the following are ON:
   a. The Workstation and Auxiliary Manifold(s) green power lights.
   b. The printers on-line light.
4) Launch Threshold Enterprise / Software. If starting up Threshold Enterprise, log on through the **Log On…** feature in the Security menu.
5) The Opening Screen opens. (Figure 3-1).

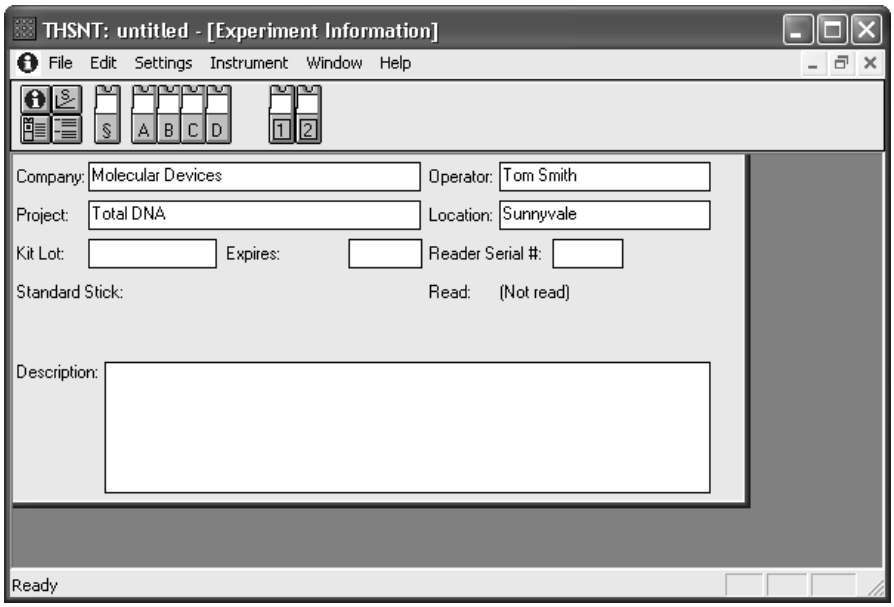


Figure 3-1: The Opening Screen

Note: *If any system component does not start up, refer to Chapter 7, "System Troubleshooting," the computer manual, or the printer manual. If the problem persists, call Molecular Devices Technical Support.*

Inserting a Reader in the Workstation

To insert a Reader in the Workstation:

1) Check that you are holding the Reader with the serial number facing you (Figure 3-2).
2) Insert the Reader into the cavity in the right-hand corner of the Workstation (Figure 3-3). *The serial number and the two gold contacts must be facing you. The Reader openings must be up.*

3) Gently press down on the Reader until it clicks into place.

- In this position, the Reader cannot move upward but it can move downward slightly, when pressed.

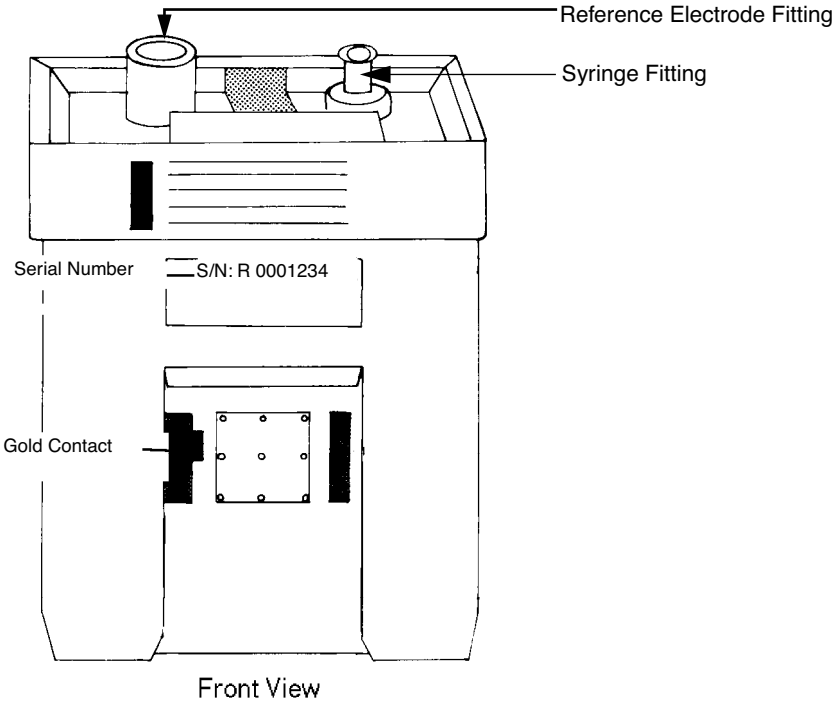


Figure 3-2: Front View of Reader

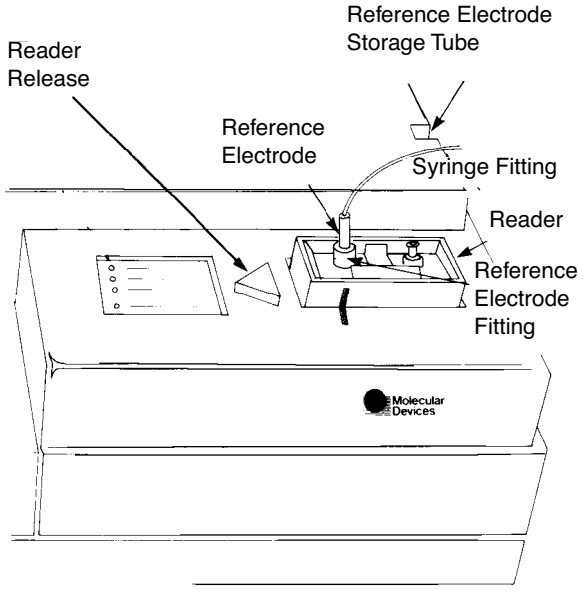


Figure 3-3: Reader Inserted in Workstation

Filling the Reference Electrode

The Threshold Workstation uses the MI-402 Micro-Reference Electrode made by Microelectrode Inc., Oak Hill Park, Londonderry, NH, 03063.

The Threshold System is provided with two Reference Electrode kits. Each electrode kit comes with the following components:

- One complete Reference Electrode, to be filled with the reference solution.
- One extra PVC tube, with a ceramic junction.
- One fine plastic transparent tubing (PE tubing).
- One bottle of electrode reference solution (3M KCl saturated with AgCl).

In addition, you need to provide:

- One 26-gauge hypodermic needle.
- One 1-ml syringe.

Procedure

The following procedure applies to filling a new Reference Electrode, as well as refilling an electrode already used in the Workstation. To fill the Reference Electrode:

1) Prepare a clean area on the bench to disassemble and fill the electrode.
2) Place the PE tubing onto the 26-gauge needle. *The tubing should go onto the needle, forming a leak-free seal.*
3) Fill the 1-ml syringe with the electrode solution, supplied with the Reference Electrode.
4) Place the needle and the attached plastic tubing on the syringe, making sure that you press the plunger until there are no air bubbles in the tubing and the syringe.
5) Remove the electrode to be filled from the box or the Workstation.
6) Hold the two portions of the white plastic electrode cap, and gently pull them apart (Figure 3-4). The clear PVC tube, with a ceramic end, cannot be pulled directly from the assembled electrode.

Caution: *Do not bend the fine electrode wire that remains attached to the larger half of the cap.*

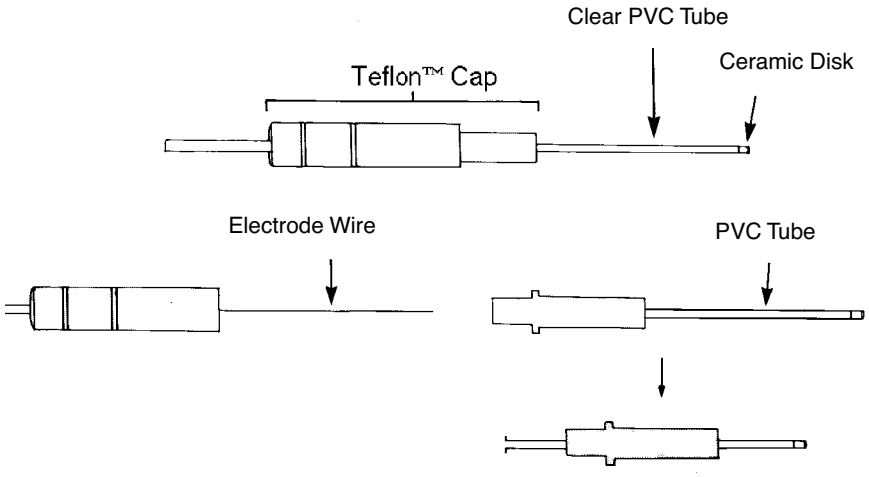


Figure 3-4: Reference Electrode

7) Slide the bottom portion of the cap away from the clear PVC tube and set them down on the workbench.
8) Gently insert the PE tubing into the PVC tube of the Reference Electrode, until it contacts the ceramic disk at the tip.
9) Hold the electrode's PVC tube horizontally and immobile.
10) Press on the syringe plunger to expel the electrode solution, while slowly withdrawing the PE tubing from the PVC tube. *This procedure should also be used when displacing the old solution with fresh electrode solution.*
11) Carefully insert the electrode wire into the open end of the PVC tube.
12) Gently draw the PVC tube back into its plastic cap (Figure 3-4).
13) Gently, but firmly, fit the two halves of the plastic cap together.
14) Keep the ceramic tip wet.
    - If the PVC tube has not been used before, the electrode should stand in the storage tube (See also: "Maintaining and Storing the Reference Electrode" on page 3-23) or Reader (Figure 3-5), for at least one hour.
    - If the electrode has been refilled, it should be returned to the storage tube, or Reader, and may be used immediately.
15) Test the Reference Electrode:
    a. Insert it into the raised opening on the left top of the Reader, not the white Luer Lock fitting, (Figure 3-5).
    b. Select the **Instrument** Menu in Threshold Enterprise / Software.
    c. Choose **Test**. (See "Performing Test-Diagnostics" on page 3-9.

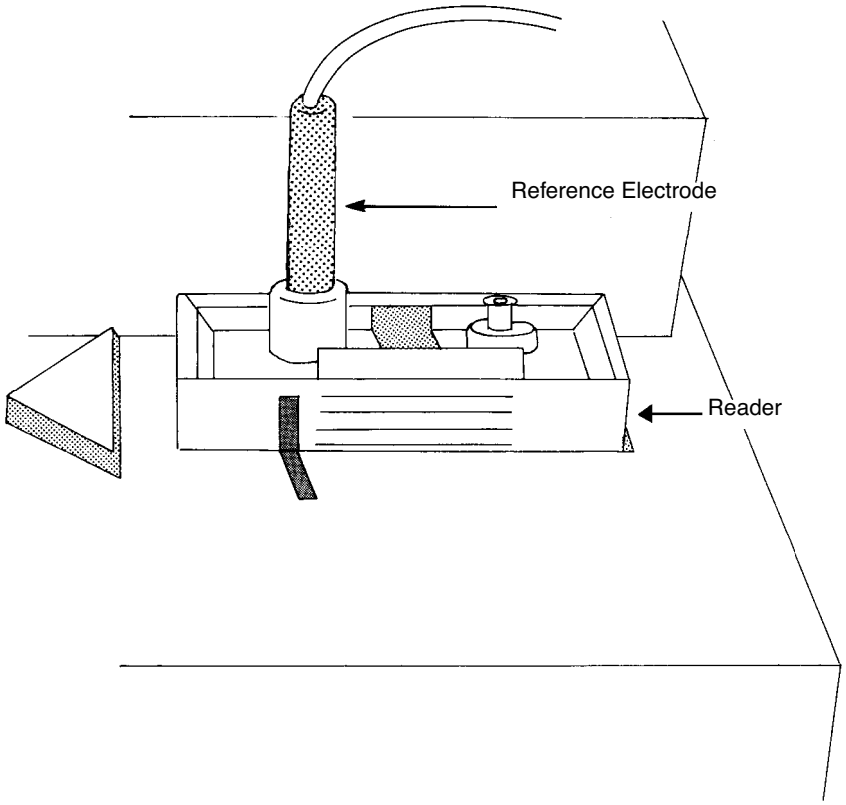


Figure 3-5: Reference Electrode Inserted Into the Reader

Note: *If the Reference Electrode is malfunctionin,g see Chapter 7, "System Troubleshooting."*

Removing and Adding Liquid To the Reader

Removing Liquid

To remove liquid from the Reader:

1) Attach an empty 30-ml syringe to the Luer Lock syringe fitting on the top right of the Reader (Figure 3-6).
2) Withdraw all the liquid from the Reader.

Caution: *Be careful not to spill liquid onto the Reader or Workstation. If a spill occurs, wipe it dry immediately.*

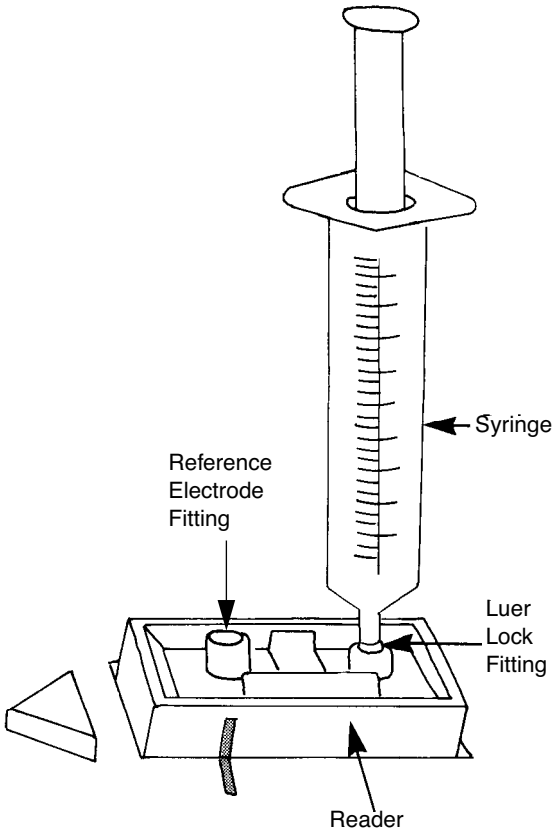


Figure 3-6: Syringe Attached to the Luer Lock Fitting on the Reader

Adding Liquid

To add liquid to the Reader:

1) Use a Luer Lock syringe body as a funnel to add 28-30 ml of Substrate or Storage Solution.
2) Place the syringe, without the plunger, on the Luer Lock fitting on the top right of the Reader.
3) Pour 28-30mL Substrate or Storage Solution into the syringe. The liquid will enter the Reader by gravity.
4) Check the level of liquid in the Reader by looking into the liquid level indicator on the top center of the Reader. The liquid should be just above the shelf in the indicator cavity (Figure 3-7).

Caution: *Be careful not to spill liquid onto the Reader or Workstation. If a spill occurs, wipe it dry immediately.*

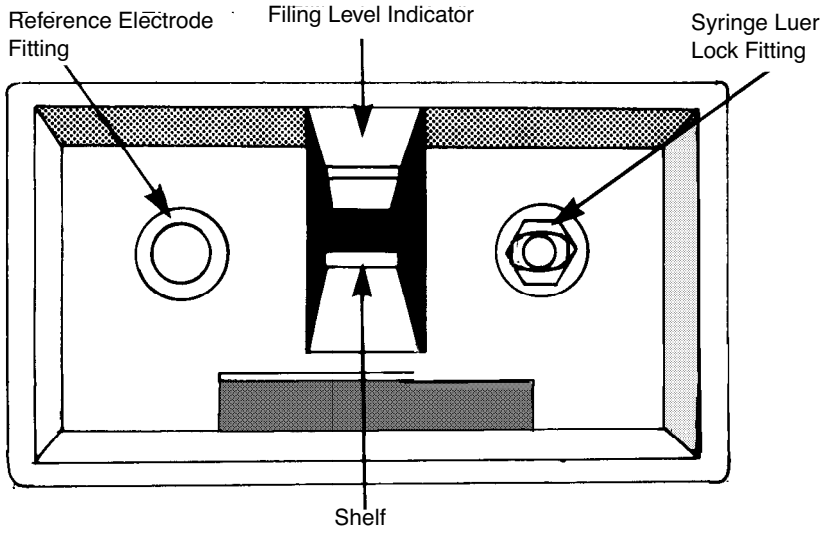


Figure 3-7: Liquid Level Indicator in the Reader

Performing Test-Diagnostics

To perform the system self-diagnostic test:

1) Select **Test** from the **Instrument** menu (CTRL+T or F7).
2) Threshold Enterprise / Software runs the system self-diagnostic tests and shows the results on the screen (Figure 3-8).
3) If a result is "Fail," follow the instructions on the screen or see Chapter 7, “System Troubleshooting.”. The Threshold System also performs the self-diagnostic tests prior to reading a stick. Sticks can not be read unless all tests are passed.

Figure 3-8: Testing Finished Dialog

4) When all the test results are "Pass," the Threshold System is ready to use. For a description of the tests, see Appendix F, “Threshold Enterprise and Threshold Software Reference.”
5) Click **Print** to print the results to the current printer, if required.
6) Click **OK** or press ENTER to return to the Opening Screen.

Preparing the Substrate Solution

To prepare the substrate solution:

1) Mix 0.6 ml of Substrate Concentrate with 30 ml of Wash Solution (equilibrated to room temperature) in a 50 ml disposable tube. Mix thoroughly.

- The Total DNA Assay kit provides IX solution for the Wash Solution.
- The ILA kit contains 10X Wash Concentrate, which should be diluted 1:10 in deionized water to make the Wash Solution.

Caution: *Substrate Solution must be at room temperature before adding it to the Reader. Cold substrate can alter the results of the assay.*

Hydrating a Blank Stick

To hydrate a blank stick:

1) Add approximately 45 ml of 1X Wash Solution to a 50-ml beaker. There should be enough solution to cover the membrane on the stick.

2) Open one Blank Stick package by tearing across at the notches, without twisting the stick. For the Total DNA 8 stick Assay kit, use a regular biotinylated stick as a Blank Stick.

   Caution: *Always hold the stick package by its edge to avoid damaging the membrane. Never touch or contaminate the membrane.*

3) Put the stick into the beaker of Wash Solution and fully immerse the membrane.
4) Hydrate the Blank Stick in the Wash Solution for at least 15 minutes.

Assembling a Filtration Unit

Attach the Filter Bases to the Workstation

To attach the filter bases to the Workstation:

1) Remove the filter base from its wrapper and place it on a filter port on the Workstation. The collar should sit directly on the port (Figures 3-9 and 3-10).

   Caution: *Do not touch or contaminate the areas on the filter base and filter block that contact the membrane on the stick.*

2) Press down on the filter base until it snaps in place.

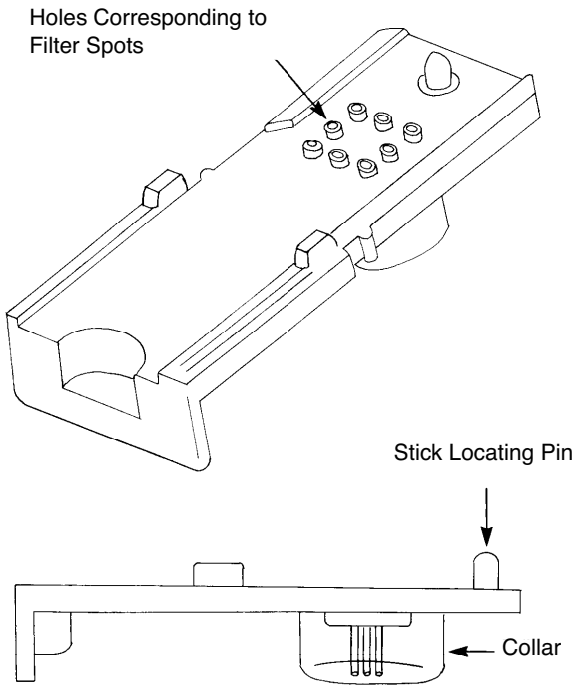


Figure 3-9: Filter Base (top and side views)

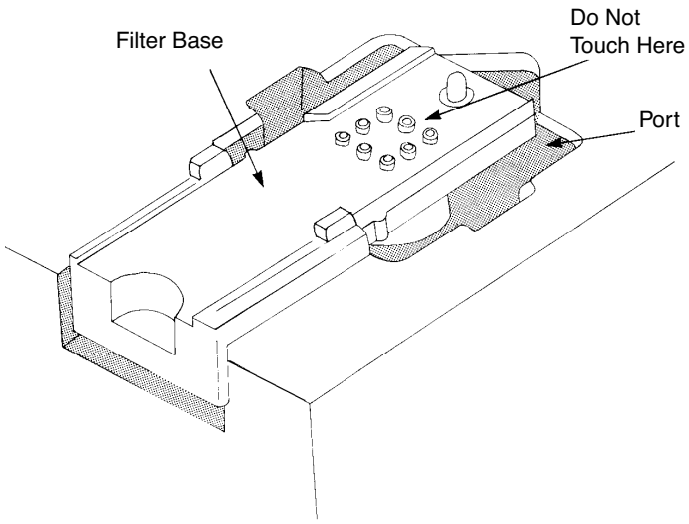


Figure 3-10: Filter Base in Filter Port on the Workstation

Put a Stick on the Filter Base

To put a stick on the filter base:

1) Open one stick package by tearing across at the notches, without twisting the stick. Hold the stick by the label only.
2) Place the stick flat on the filter base, label and membrane, facing up (Figure 3-11). Gently push the stick down and towards the locating pin, as far as it can go.
   - The notch on the stick should fit firmly against the locating pin.
   - The stick should lie evenly between the sides of the filter base.
   - The labeled end of the stick should be even with the filter base.

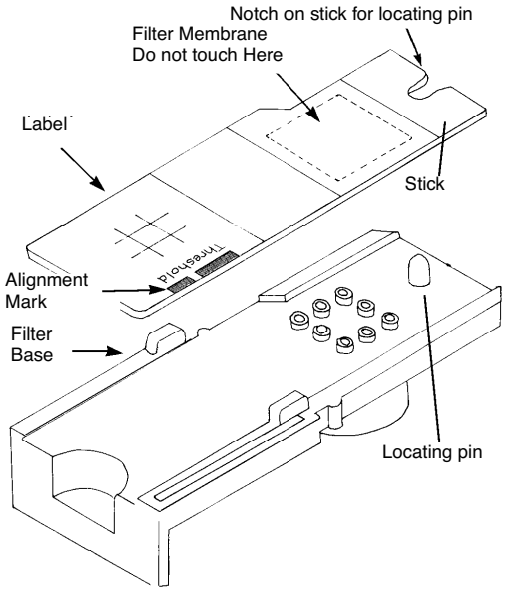


Figure 3-11: Stick on a Filter Base

Attach the Filter Block to the Filter Base

To attach the filter block to the filter base:

1) Put a filter block on the filter base so that the legs of the block straddle the filter base, and the pin indicator on the block aligns with the locating pin on the filter base (Figure 3-12).

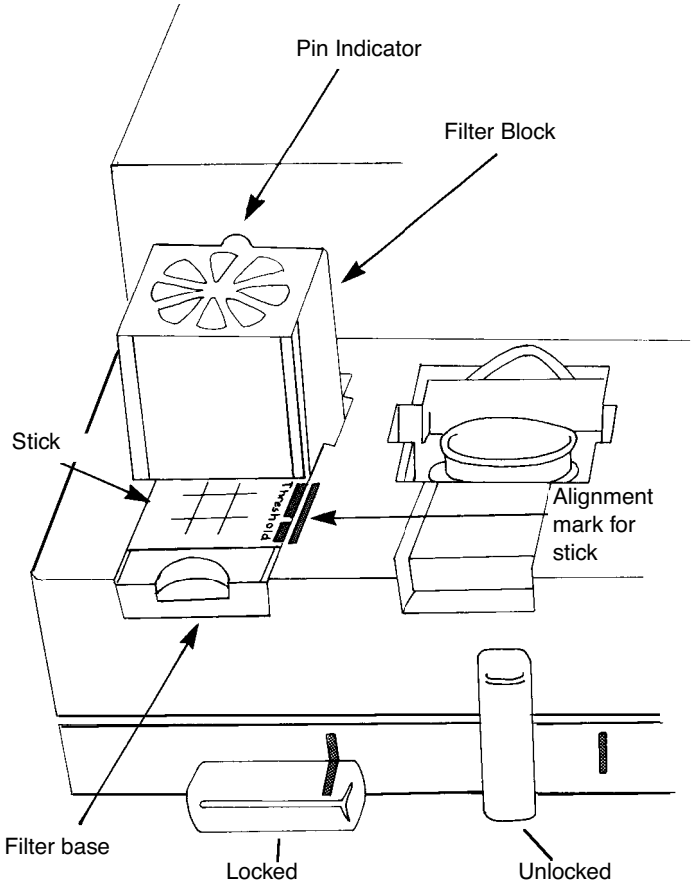


Figure 3-12: Filter Block Attached to Filter Base

2) While pressing down on the top of the filter block, lock the filter block to the filter base by turning the locking lever on the Workstation clockwise to the 3 o'clock position (Figure 3-12).

   If the filter block is properly locked, it cannot be removed from the filter base.

Disassembling and Removing a Stick from the Filtration Unit

To disassemble and remove a stick from the filtration unit:

1) Turn the locking lever on the Workstation one-quarter turn upward (counter-clockwise) to the unlocked, 12 o'clock position.
2) While holding the stick and filter base in place, remove the filter block.
3) Lift the labeled end of the stick to remove it from the filter base. Place the stick in a beaker containing enough Wash Solution to cover the membrane.
4) The filter base may be removed from the Workstation by pinching it at the semicircular indent and lifting it upward.
5) Do not re-use filtration units for Total DNA Assay or biohazardous samples. Filter blocks and bases which have not been used for biohazardous samples may be recycled.

They are made of polycarbonate.

Cleaning the Filter Bases and Filter Blocks

To clean the filter bases and filter blocks:

6) First soak the filter bases and filter blocks in 2% Liquinox (or similar laboratory glassware detergent) for at least 30 minutes.
7) Rinse them at least three times with tap water, and another three times with deionized water.
8) Shake the parts to remove excess water, and allow to dry.

- Avoid direct heat.
- Avoid hard abrasives or scouring.
- Do not machine wash.

Note: *Filter bases and filter blocks may be used up to three times each, after proper cleaning and drying.*

Reading a Stick

1) Select **Read** from the **Instrument** menu.

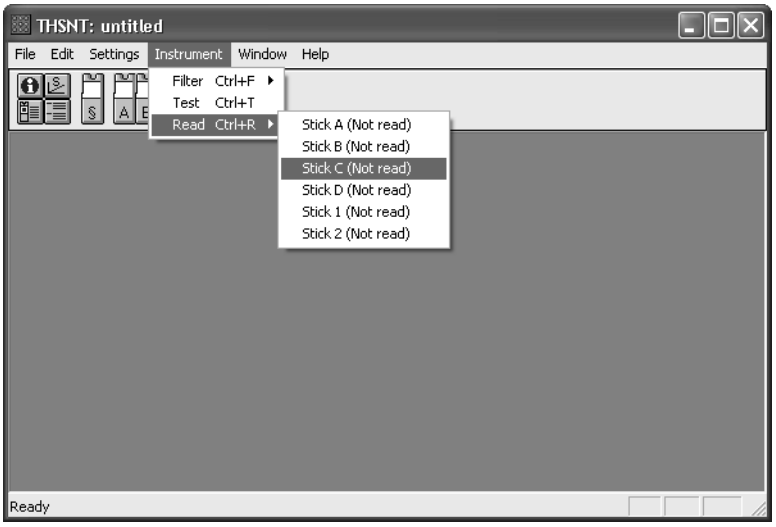


Figure 3-13: Stick List Menu

2) Use the mouse to select a stick to read in the stick list menu (Figure 3-13).
   The Threshold Enterprise / Software will perform the self-diagnostic tests.

   Caution: *Reading a new stick replaces the data for that stick.*

3) Wait for the "Put the stick in the Reader..." message (Figure 3-14).

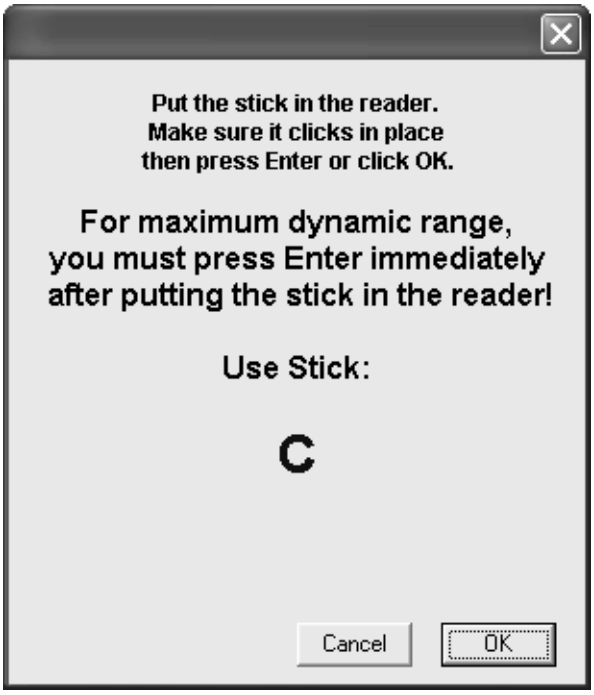


Figure 3-14: Put the stick in the Reader... Message

4) Hold the stick vertically by the labeled end, with the label facing you.
5) Gently blot the notched end of the stick to remove drops of excess Wash Solution.
6) Insert the stick in the Reader. The notched end of the stick should be down and the label facing you (Figure 3-15).
7) Press down lightly on the stick until it snaps softly into place.
8) Click **OK** or press ENTER immediately.

Caution: *Click **OK** immediately after inserting the stick into the Reader, otherwise the dynamic range of the assay can be reduced.*

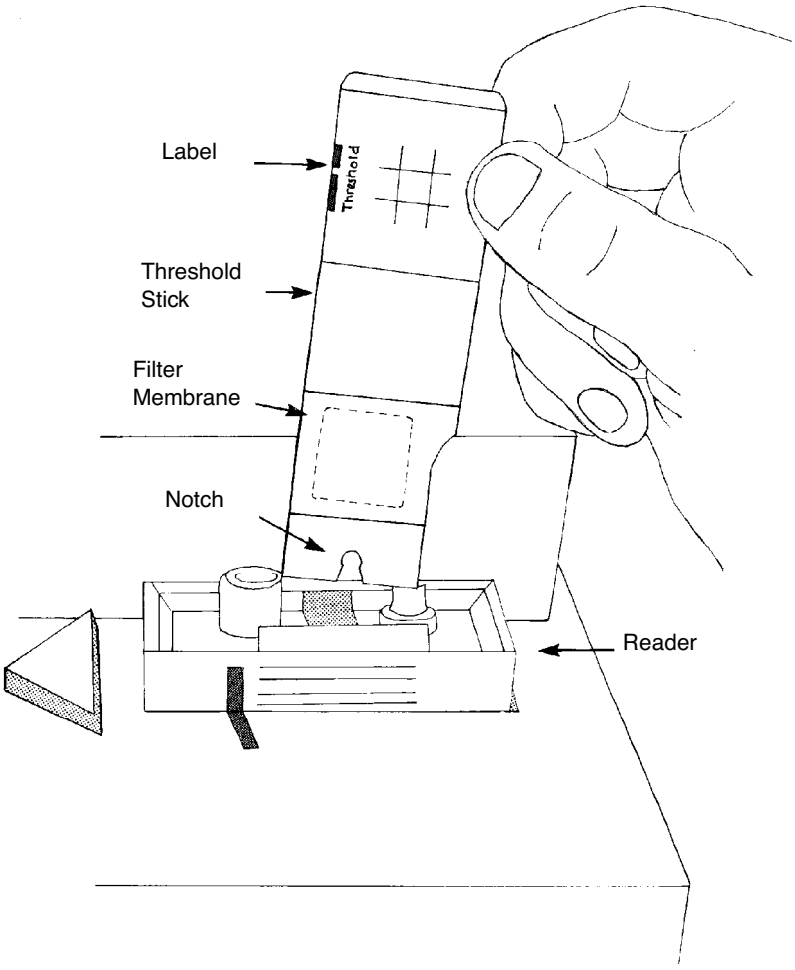


Figure 3-15: Inserting Stick Into Reader

9) The Stick Display window opens (Figure 3-16), showing kinetic plots of the signal measurement.

10) When the read is complete, slopes, signals and values appear on the screen. Values will only appear if a standard curve has been loaded.

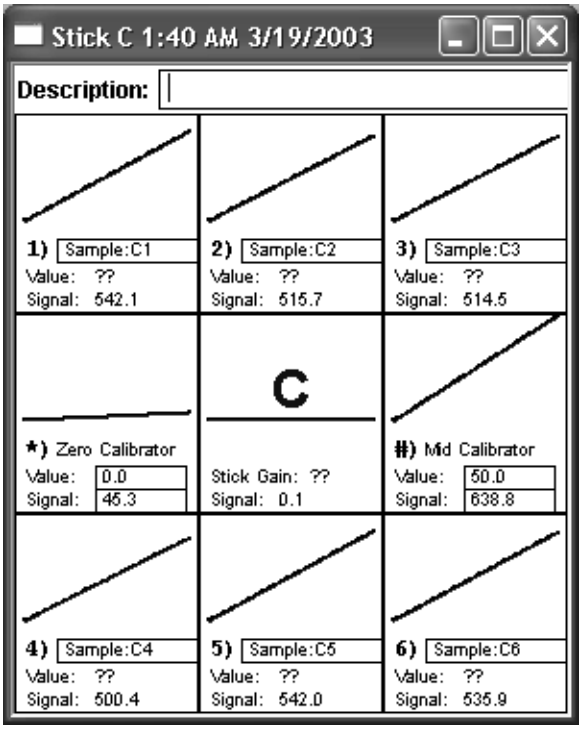


Figure 3-16: Stick Display Window Showing the Kinetic Plots

11) Remove the stick from the Reader only when the red indicator light, "Reader Locked", goes off.

Caution: *When removing a stick, be careful to prevent liquid from dripping into the Reader housing.*

12) At any time during the read, press ESC to cancel the read.

Removing a Reader From the Workstation

To remove a Reader from the Workstation:

1) Push the Reader release (triangular knob) to the left (Figure 3-17).
2) Lift the Reader up.

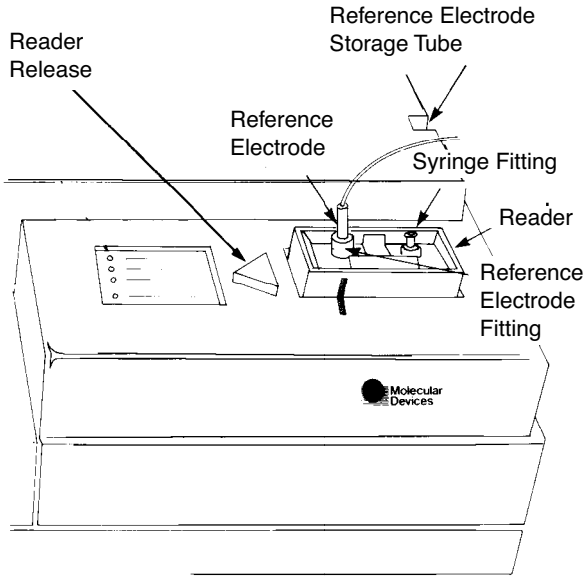


Figure 3-17: Reader Inserted in Workstation

Shutting Down the System

To shut down the system (after all the sticks are read, or at the end of the day):

1) Select **Shut Down** from the **Start** menu in Threshold Enterprise / Software. Click Yes.
2) When Threshold Enterprise / Software indicates that it is safe to do so, turn off the computer and all the other components of the system.

Maintenance

Storing the Reader

Daily Storage

Keep the Reader full (30 ml) of Substrate Solution (0.6 ml Substrate Concentrate added to 30 ml Wash Solution). Alternatively the Reader can be stored in Wash or diluted Wash Solution (1 part 1X Wash Solution: 4 parts deionized water). This will reduce the growth of Urea crystals on the outside edges of the gasket.

Longer Periods of Storage

1) Fill the Reader with Wash or diluted Wash Solution (1 part 1X Wash Solution: 4 parts deionized water); keep the cap on the Reader.
2) To avoid evaporation, put Parafilm around the Reader cap.

When a Reader has been stored for a long period of time, be aware that evaporation may have occurred. If the silicon chip has been partly dehydrated, the dry detection spots will give lower rates.

Note: *A rehydration for 24 hours with 30 ml of fresh Wash Solution is strongly recommended, prior to any use.*

Opening the Reader

Only Readers with serial number R000-6000 or higher, can be opened and cleaned. Older Readers cannot be opened without destroying the seal. The hex ball driver provided with the cleanable Reader is needed to open it.

Caution: *Vinyl or cotton gloves must be worn while handling the gasket, the silicon sensor, and the titanium surface of the plunger.*

To open the Reader:

1) Empty the Reader and rinse it with 30 ml of deionized water. Do not wet the gold contacts on the front of the Reader.
2) Partially remove all the screws, using the hex ball driver:
   a. Start with one-half of a turn on the middle screws on each side.
   a. Continue with each pair of diagonally-opposed screws.
3) Completely remove the 6 screws, using the same pattern as above.
4) Carefully pull apart the front and back Reader cases, taking care to avoid wetting the threaded inserts and the screws.
5) Use a clean laboratory wipe and remove excess liquid to keep the insert areas dry.
6) If the gasket becomes unseated from the ridge, press it back into place. *It is not necessary to remove the gasket unless it is damaged and needs to be replaced.*

   Caution: *Never scratch the surface of the silicon sensor or touch it with your fingers. Never touch the plunger with bare hands or latex gloves.*

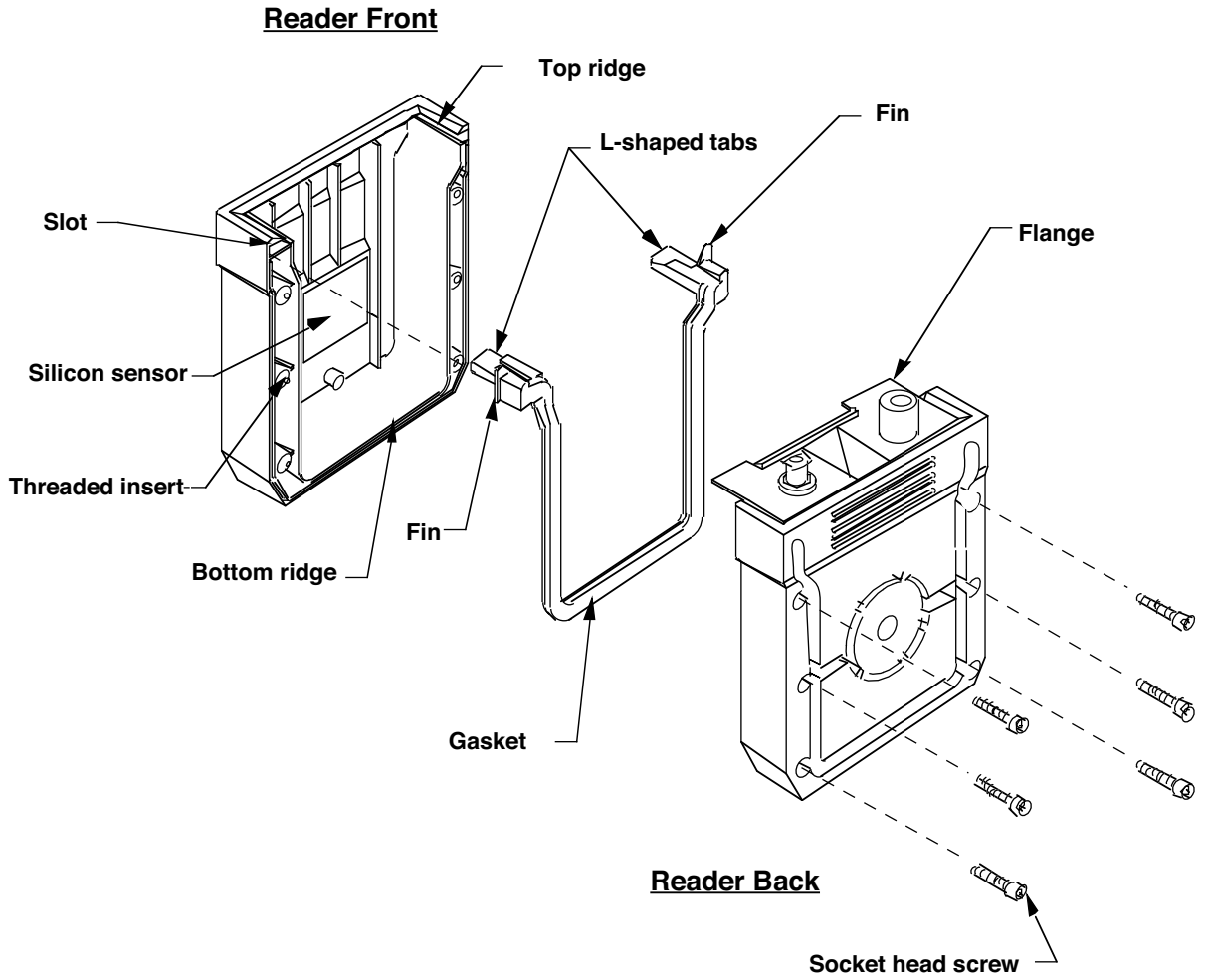


Fig 3-17b: Cleanable Reader

Cleaning the Silicon Sensor

To clean the silicon sensor:

1) With a clean swab dipped in Wash Solution, gently wipe the surface of the silicon sensor to remove any protein contamination. Be careful not to disturb the clear sealant at the edges of the silicon surface. Disturbing the sealant will disable the Reader, by causing a short circuit in the current path and will void the warranty.

   Caution: *Swabs with wooden handles can scratch the silicon sensor. This will void the sensor warranty. We recommend using foam swabs with plastic handles.*

2) Complete the cleaning by gently wiping the plunger surface. The plunger is the 16x16mm square metal structure, in the Reader back, which aligns directly opposite the silicon sensor, when the Reader is assembled.

3) Rinse the silicon sensor and the plunger surface by wiping gently with a swab soaked in deionized water.

Replacing the Gasket

Note: *The gasket needs to be replaced only if it is damaged or seated incorrectly.*

To replace the gasket:

1) Remove the old gasket and wipe dry the Reader case areas which hold the gasket.
2) Soak a clean swab in isopropyl alcohol and gently wet all surfaces of the L-shaped tabs of the gasket to lubricate it. Be sure to wet the cavities on the bottom of the gasket tabs.
3) Slide the L-shaped gasket tabs into position in the slots at the top of the front case until the thin fins are nearly touching the surface of the Reader.
4) If there is resistance, apply a little more isopropyl alcohol to the problem area and try again.
5) Press the remaining part of the gasket into position so that it seats properly over the rib of the case.
6) Close the Reader, following the instructions below.

Closing the Reader

To close the Reader:

1) Stand the front and back Reader cases on a flat surface with their mating edges facing each other.
2) Bring them together, guiding the top flanges of the back case into the ridge in the top of the front case. Make sure that the cases' edges are properly aligned. This may require a slight manipulation.
3) Place the screws into the screw holes.
   a. Using the hex driver, partially screw in the middle screws on both sides.
   b. Repeat with one pair of diagonally-opposed screws, then with the remaining two screws.
   c. Follow this pattern as you continue to tighten the screws, just until snug. Do not over-tighten the Reader screws.
4) Wipe the exterior of the case with clean, dry tissue paper.
5) Test the Reader for leaks by filling it with 30-ml Wash Solution or Substrate Solution.

   Caution: *Do not use more than 30 ml of solution in the Reader. Overfilling the Reader will make it leak.*
6) If leakage occurs, liquid will appear at the bottom of the Reader, or will be detected by the Threshold Enterprise / Software during the diagnostic tests.
7) If leakage is detected:
   a. Open and reassemble the Reader, ensuring proper alignment of the gasket.
   b. Tighten the screws further.

   For further assistance, contact Molecular Devices Technical Support.

Maintaining and Storing the Reference Electrode

To maintain and store the Reference Electrode:

1) Check that the clear plastic tube is filled with KCl/AgCl reference solution past the visible point (under the white plastic cap).

   Refer to "Filling the Reference Electrode" on page 3-5 of this chapter for filling the Reference Electrode.

2) Store the Reference Electrode in a 12 x 75 mm storage tube, in the holder on the right-side of the Workstation. It should contain diluted Wash Solution (1 part 1X Wash Solution: 4 parts deionized water).
3) Cover the tube with Parafilm to avoid evaporation.

Cleaning the Reference Electrode

The ceramic tip of the Reference Electrode should be white or off-white. If the ceramic tip is clogged by material, usually dark dots or crystals appear on the tip, then cleaning is required.

To clean the Reference Electrode:

1) Dip the Reference Electrode in warm deionized water for one hour.
2) If the dots or crystals do not disappear, the ceramic tip tube should be replaced by the spare one provided in the electrode kit.

Note: *The ceramic tip of the Reference Electrode has to be hydrated in the storage tube or Reader for at least 1 hour prior to use.*

Cleaning the Filter Ports, Flushing the Vacuum and Drain System, and Disinfection

Salts or contaminated liquids, (such as biohazardous samples), may accumulate over the four filter ports on the Workstation or on the Auxiliary Manifold. Before cleaning the ports, look at the rear panel of the instrument.

Facilities Output

Spills along the outer housing of the filter port drain into the bottom reservoir, connected to the "Facilities Output" port on the rear panel. Excess liquid flows from the tubing connected to the "Facilities Output" by gravity. Thus, the tubing and collection trap must be below the level of the "Facilities Output" port.

To clean the filter ports:

1) Use a plastic squirt bottle and gently squirt the filter ports with deionized water.
2) Keep the port free from granular debris.
3) Excess liquid will flow from the exit tubing. *The vacuum pump does not need to be turned on during this cleaning process.*

Waste Output

Liquid that enters through the filter port, during filtration, collects in the upper reser-

voir, which connects to the vacuum system and, through a separate draining pump, to the "Waste Output" port on the rear panel of the Workstation.

To prevent salt buildup in the tubing and drain pump, perform the following on a monthly to quarterly basis:

1) Flush the drainage system with deionized water.
2) Load a used filter base and filter block, without a stick, onto one of the vacuum ports.
3) Turn the vacuum on High and dispense approximately 500 ml of water into one of the wells of the filter block.
4) Turn the vacuum Off.
5) The liquid flows from the vacuum reservoir through the drain system tubing.

To disinfect the drain system:

1) Flush the drain system with disinfectant, (such as laboratory bleach, diluted 1:10 with deionized water).
2) Allow sufficient time for chemical disinfection, and flush with deionized water.

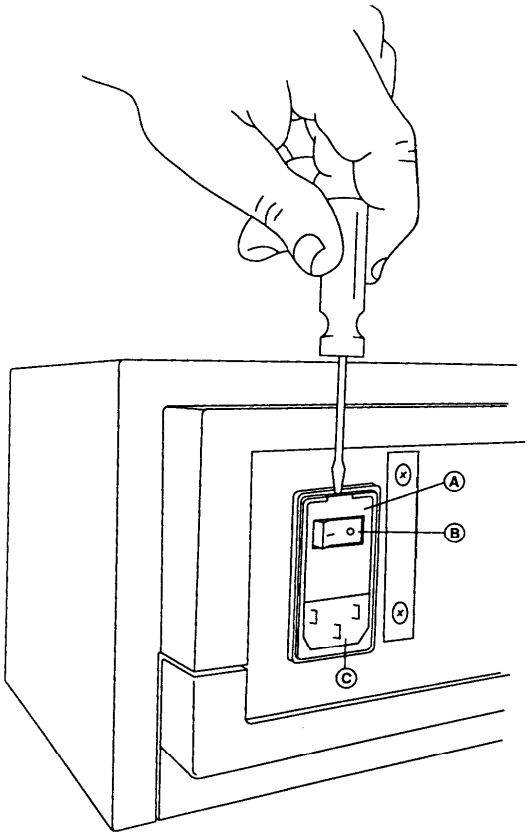
Caution: Do not connect any waste tubing to the "Vacuum Port" on the rear panel.

Changing the Fuse

A spare fuse comes with the Workstation. If you no longer have a spare fuse, obtain a new one from Molecular Devices.

To change the Workstation fuse:

1) Switch the instrument Off.
2) Remove the power cord from the power outlet and then from the Threshold Workstation.
3) Turn the instrument around for easy access to the rear panel.
   - On the left side of the rear panel (as viewed from the back) is the power plug connector (Figure 3-18).
   - The plastic cover of this assembly conceals the fuse box.



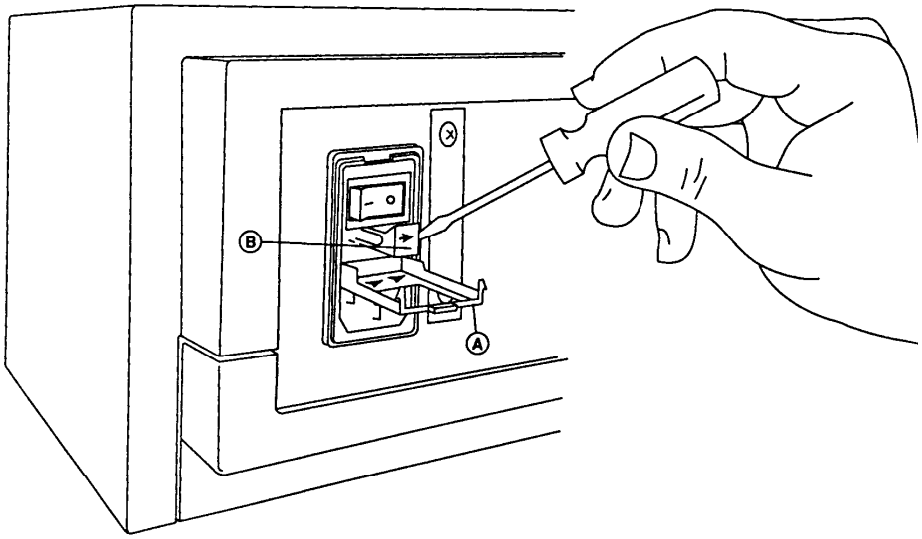
A. Fuse Box Cover

B. ON/OFF Switch

C. Power Plug Port

Figure 3-18: Opening the Fuse Box

4) Use a small flat-head screwdriver to gently pry open the fuse box cover. Insert the screwdriver, from above, into the slot behind the tongue at the top of the plastic cover. The cover will hinge down (Figure 3-19).
   - When open, the fuse box is visible in a light grey housing, below the ON/OFF switch, on the right side.
   - Note the white arrow pointing to the right, on the exposed end of the box, and the two identical white arrows on the inside of the opened portion of the cover. These will remind you to return the fuse box to the correct slot, when replacing it.
5) Use the same small flat-head screwdriver (Figure 3-19) to gently pry the fuse box forward and out.



A. Fuse Box Door Cover

B. Fuse Box

Figure 3-19: Removing the Fuse Holder

6) Once the fuse box is out, you can see the fuse. Remove the old fuse and replace it with the new Slowblow fuse. It does not matter which end of the fuse is forward.

Reverse the steps above to complete the procedure:

1) Insert the fuse box, containing tthe new fuse, into the right slot, with the white orientation arrow pointing to the right, as you face the back of the instrument.
2) Slide the fuse box all the way in.
3) Close the hinged drawer, making sure the two clips snap securely.

This completes the fuse removal and replacement procedure. To proceed to use the Workstation, attach the power cord and turn the instrument on.

Note: *There are orientation arrows on the fuse box and fuse box cover.*

Chapter 4– Threshold Software

4

Introduction

Threshold Software is a Microsoft® Windows™ application that controls, collects, and analyzes data from the workstation. This chapter presents the basic skills and concepts needed to start using Threshold Software and provides instructions for how to use the software to run a Threshold assay.

The instructions provided in this chapter are also applicable when using Threshold Enterprise. Threshold Enterprise includes all the features of Threshold Software and many additional features, particularly for maintaining data security (see Chapter 5, "Threshold Enterprise.")

Using Threshold Software

Threshold Software can be operated using the mouse or keyboard. In this chapter, instructions are given for using the mouse, with keyboard alternatives in parentheses. Menus can be selected with a single click of the left mouse button, or with the keyboard shortcut keys. Shortcut keys are underlined letters in the menus. To use a shortcut key, hold down the ALT key to activate the Menu Bar then press the underlined character. Text fields and menu drop-down lists may be selected with a single click of the left mouse button, *or* by using the keyboard. Press TAB to move forward and SHIFT+TAB to move backward between fields, and the up and down arrow keys to scroll through lists.

Further details of the menus and commands can be found in Appendix F, "Threshold Enterprise and Threshold Software Reference."

The Threshold Main Screen

The basic features of the Threshold Software main screen are shown in Figure 4-1. The main screen consists of a Menu Bar, Toolbar, main window area, and Status Bar.

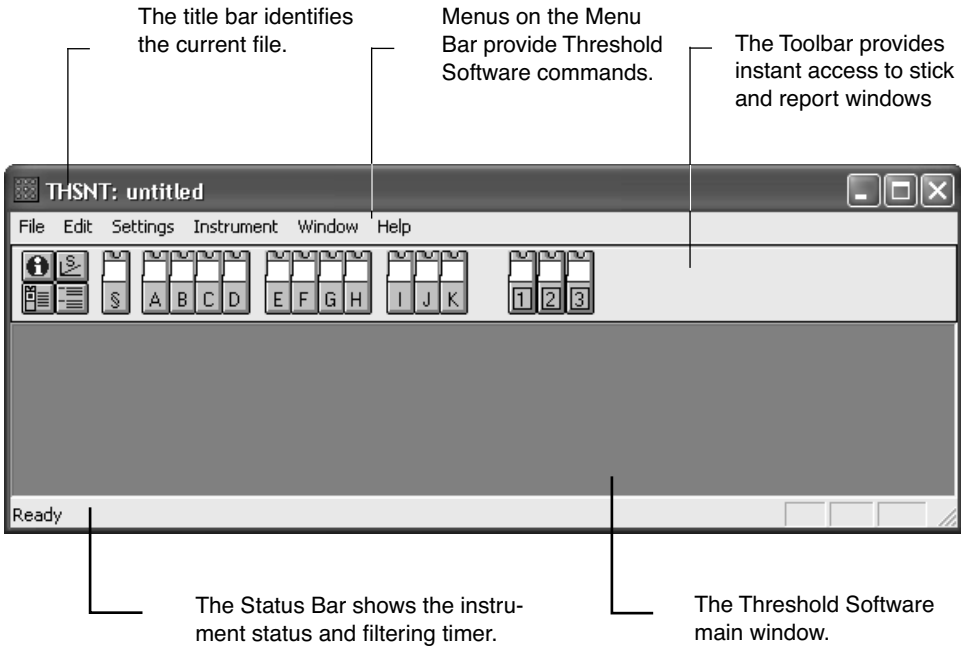


Figure 4-1: The Threshold Software Screen

The Toolbar Buttons

The Toolbar is shown in Figure 4-2. The Toolbar consists of buttons that provide instant access to display the different Threshold Software windows.

The Stick displays consist of:

- Kinetic plots
- Quantitation for each data and blank stick

The Report displays consist of the:

- Experiment Information window.
- Stick Report (signal and quantitation for each of the samples on each stick).
- Group Report (signal and quantitation for replicates of each sample).
- Standard Curve.

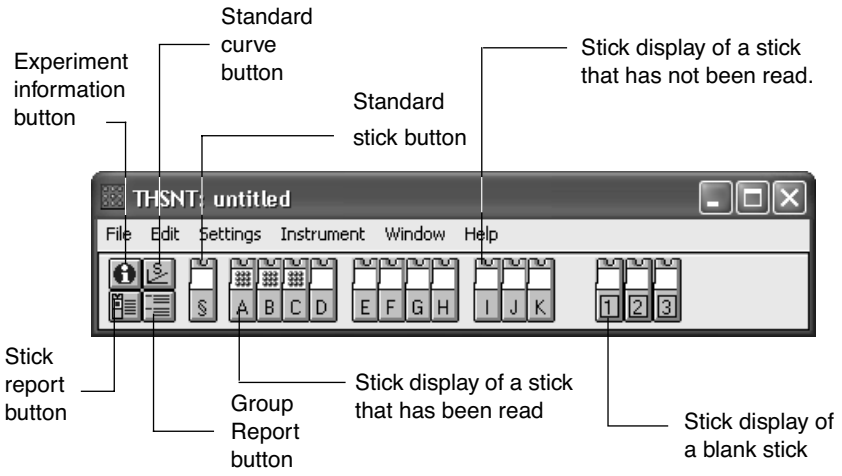


Figure 4-2: The Toolbar

Function Key Shortcuts

The twelve function keys, F1-F12, all have assigned shortcut functions in Threshold Software. These are shown in Table 4-1, "Function Key Shortcuts."

Table 4-1: Function Key Shortcuts

Function Key	Function
F1	Starts Threshold Software Help
F2	Opens the Save As dialog
F3	Opens Vacuum Setting dialog
F4	Opens Read Stick dialog
F5	Displays Standard Curve graph
F6	Opens Stick Display dialog
F7	Starts instrument diagnostic test
F8	Opens Print Reports dialog
F9	Opens Clear Stick Data dialog
F10	Quits software, warning is given if data not already saved
F11	Opens software registration screen
F12	Displays Threshold Software Title screen

Keystroke Shortcuts

There are a number of keystroke combinations that give direct access to software functions, without having to open the corresponding menus. These are shown in Table 4-2, "Direct Keystroke Shortcuts."

Table 4-2: Direct Keystroke Shortcuts

Keystroke	Function
CTRL+N	Creates a new experiment
CTRL+O	Opens an existing experiment
CTRL+S	Saves the current experiment
CTRL+P	Prints the current window
CTRL+Z	Undoes the most recent text edit
CTRL+X	Cuts the highlighted text, saves to Clipboard
CTRL+C	Copies the highlighted text to the Clipboard
CTRL+V	Pastes the Clipboard contents to the current field
CTRL+SHIFT+S	Opens the Copy Stick to Standard dialog
CTRL+SHIFT+C	Opens the Copy Stick dialog
CTRL+SHIFT+V	Opens the Paste Stick dialog
CTRL+SHIFT+X	Opens the Clear Stick Data dialog
CTRL+SHIFT+R	Opens the Reset Stick dialog
CTRL+F	Opens the Vacuum Setting dialog
CTRL+T	Starts instrument diagnostic test
CTRL+R	Opens Read Stick dialog

Using the Software for a Threshold Assay

This section covers the operations required when using the Threshold Software to run a Threshold assay. This section details how to perform the following operations:

- Start Threshold Software and access menu items
- Create a new file and open an existing file
- Use Threshold self-diagnostics
- Collect and analyze data
- Save assay results in a file for future use
- Print assay results and analyzed data
- Export data
- Exit the Threshold Software

Step 1- Starting the Software

Software Registration

To start Threshold Software:

1) Launch the application by double-clicking the desktop shortcut or by clicking the **Start** button, selecting **Programs** and selecting **Threshold Software** or **Threshold Enterprise**.
2) If not completed yet, the Software Registration dialog opens.
3) Enter the appropriate information for each field:

Table 4-3: Software Registration

Field	Enter Information
Registered User:	Your name
Company Name:	Your company name
Instrument Serial Number:	Instrument serial number (S/N) – located on the back of the Threshold Workstation
Software Serial Number (Threshold Enterprise only):	Software Serial Number – provided with your copy of Threshold Enterprise.

Click **OK** (ENTER).

Note: *Until the user completes all fields, the* ***Software Registration*** *dialog will continue to reappear at startup.*

4) The Threshold Software Title Screen opens, showing the registration information and the software version number.

Click **OK** (ENTER).

Note: *To access the Threshold Software main screen at any time, press* F12.

Opening Screen

The Opening Screen consists of the Menu Bar, the Toolbar, and the Experiment Information window (Figure 4-3). The Experiment Information window displays the company name and system user name from the Software Registration dialog. If desired, these defaults may be changed in Preferences (see “Preferences” on page 4-10).

Step 2 - Experiment Setup

Once registered, the user may create or open an experiment. However, for Threshold Enterprise, the user must be logged on AND have the appropriate permission set to be able to change experiment setup information (see Chapter 8, “Enterprise Administrator.”).

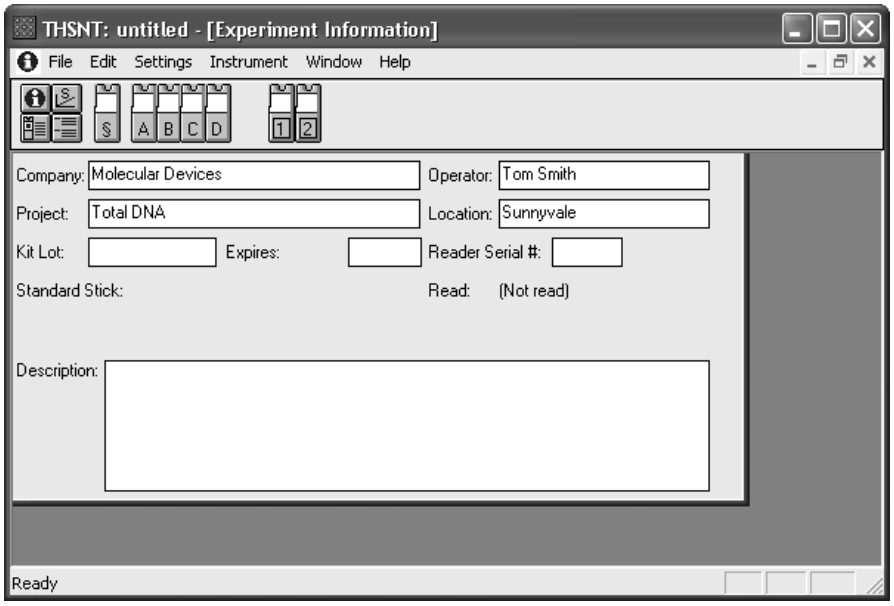


Figure 4-3: The Opening Screen

Creating a New Experiment

To create a new experiment, the user can simply stay on the Opening Screen that always defaults to a new experiment. The user can also select **File**, **New** (ALT, F, N) to bring up a New Experiment screen, with the current preferences settings.

Experiment Information Window

The user must enter or change the appropriate information for each field in the Experiment Information window.

Table 4-4: Experiment Information Window

Field	Change or Enter Information
Company:	Company name
Operator:	Your name
Project:	Project name
Location:	Instrument location
Kit Lot:	Reagent kit lot number from Certificate of Lot Analysis
Expires:	Reagent kit lot expiration date from Certificate of Lot Analysis
Reader Serial #:	Reader serial number – serial number (S/N) is located on the front of the Threshold Reader
Description:	Any detailed experiment information.

The **Standard Stick** and **Read** fields are not user-definable. These fields are automatically entered by the Threshold Software program from the Standard Stick.

Analysis Settings

Note: *Threshold Enterprise users must have the appropriate permission set in Enterprise Administrator to change the Analysis Settings.*

To open the Analysis settings:

1) Select **Settings**, **Analysis**... (ALT, S, A).
2) The Analysis Settings dialog opens (Figure 4-4).

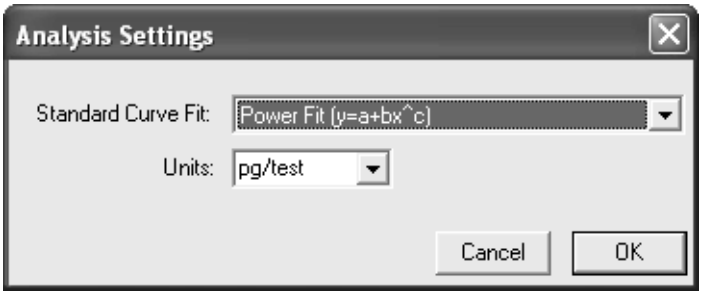


Figure 4-4: The Analysis Settings Dialog

3) Select the desired Standard Curve Fit and Units for the assay.

Standard Curve Fit: There are five available equations used to describe the standard curve:

Linear Fit [y=a+bx]
Power Fit [$y=a+bx^c$]
Extended Power Fit [$y=a+bx^c$] (Threshold Enterprise only)
Quadratic Fit [$y=a+bx+cx^2$]
4-Parameter Fit [$y=[[a-d]/[1+[x/c]^b]]+d$]
Piecewise Linear Fit

4) Select the desired Units for the assay.

Units: The concentration or value units of the sample. This field is displayed and printed on Stick Reports and Group Reports in the "Value (*Units*)" field. There are ten different units available in the menu:

fg/ml
pg/ml
ng/ml
µg/ml
fg/test
pg/test
ng/test
µg/test
units
molecules

Note: *The units set here are only text and are not used in rate analysis or quantitation. For example, changing the units from ng/ml to pg/ml will not affect any numerical calculation.*

5) If the unit required is not available, enter the appropriate unit measurement in the units box.

Note: *Text entered in the Units box does not take effect if the drop-down menu is open.*

6) Click **OK** (ENTER).

Stick Count Settings

Note: *Threshold Enterprise users must have the appropriate permission set in Enterprise Administrator to change the Stick Count settings.*

To open the Stick Count settings:

1) Select **Settings**, **Stick Count**... to open the Stick Count dialog, (ALT, S, S).
2) The Stick Count dialog opens (Figure 4-5). The Stick Count dialog consists of Number of Data Sticks and Number of Blank Sticks fields.

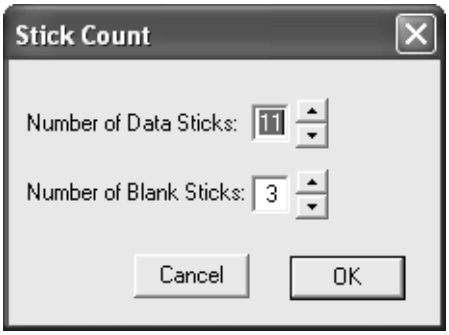


Figure 4-5: The Stick Count Dialog

The **Number of Data Sticks** can be set at any value between 1 and 24.

The **Number of Blank Sticks** can be set at any value between 1 and 9.

3) Click **OK** (ENTER).
4) The number of individual sticks ,selected in **Stick Count** from the **Settings** menu, is displayed in the Toolbar (Figure 4-2).

Preferences

Preferences are default settings defined by the user. Defaults set in Preferences are applied when the software is started and every time a new experiment is created, and can also be applied to the current file. These settings can be altered for the current file through the Analysis and Stick count dialogs, and by overwriting the default information in the information screen and Stick displays.

Note: *Threshold Enterprise users must have the appropriate permission set in Enterprise Administrator to change Preferences.*

To set preferences:

1) Select **Settings**, **Preferences**, (ALT, S, P).
2) The Preferences dialog opens (Figure 4-6).
3) Fill in the fields with the information to be used as defaults for new experiments.
4) The Preferences dialog consists of:
   a. Default curve fit
   b. Default units
   c. Decimal Places – An option for setting the number of decimal places to be used for the values fields. This should generally be left at one decimal place, except in certain ILA assays where the range of standard curve dilutions makes it convenient to express the values to more than 1 decimal place. For example, if the standards run from 10 ng/ml down to 50 pg/mL of analyte, this could be expressed as 10,000 to 50 pg/mL, or 10 to 0.05 ng/mL.
   d. Default user information:

      Company

      Operator

      Project

      Location

   e. Print Graphics – an option for setting the size of graphics printouts (Kinetic plots and Standard curves) to full, half, and quarter page
   f. Default Calibrators – default calibrator values for the standard curve and Zero- and Mid-Calibrators (* & #) for each stick
   g. Default Standards
   h. Default number of sticks
   i. Beep After Reading Stick – an option for setting the computer to beep at the end of stick reads
   j. Instrument COM Port – the COM port to be used to communicate with the instrument. This must be set to the COM port (serial port) that the instrument is plugged into.
5) To apply the settings to the current experiment, check the Apply preferences to the current experiment checkbox. Use the SPACE BAR to uncheck or check the checkbox.
6) Click **OK** (ENTER).

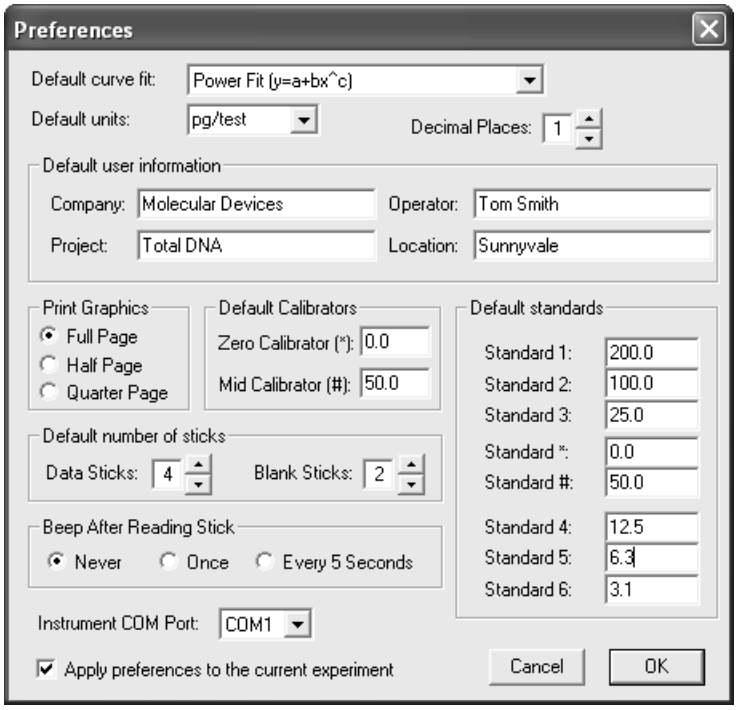


Figure 4-6: The Preferences Dialog

Opening An Existing .THS, THW, or .TH3 File

To open an existing file:

1) Select **File**, **Open**, (ALT, F, O).
2) The Open dialog opens (Figure 4-7).

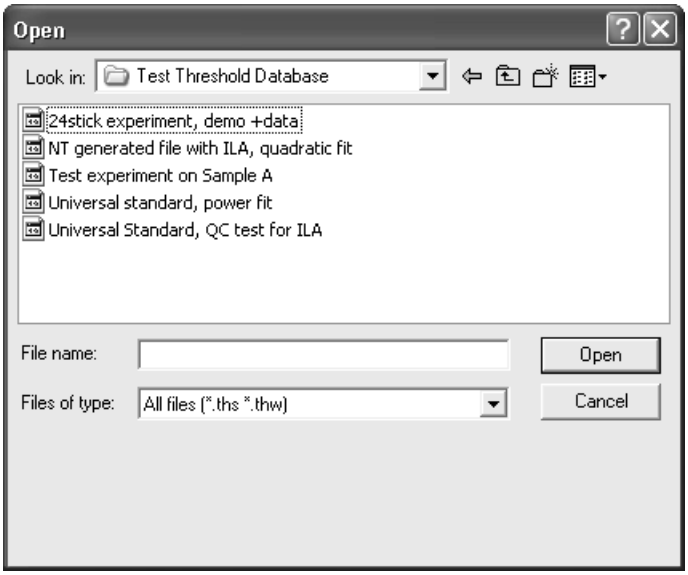


Figure 4-7: The Open Dialog

3) In the **File Name** box, enter a filename, or double-click the filename in the list above.
4) If the filename is not listed, select a new drive or directory in the **Look In** box, or select a different type of file from the **Files of Type** box.

   Note: *Threshold Software can open files created by DOS (.THS) and Windows (.THW) versions of the software. Threshold Enterprise can open all of these files, as well as files created by Threshold Enterprise (.TH3).*
5) Click **Open** (ENTER).

Step 3 - Instrument Self-Diagnostic Test

The Instrument Self-Diagnostic Test checks the status of the entire Threshold System. Each test is reported with results of Pass or Fail. If any test Fails, instructions for corrective action are displayed. For a description of the tests, see Appendix F, "Threshold Enterprise and Threshold Software Reference."

1) Select **Instrument**, **Test**, (ALT, I, T), (CTRL+T), or press F7.
2) The message "Performing instrument diagnostics..." is displayed with each test and results, "Pass" or "Fail" (Figure 4-8):

Figure 4-8: The Test Screen

3) If all the tests pass, the message "All tests completed at:," with a time/date stamp, is displayed in the lower left corner of the test window.
4) If a test fails, the Testing Finished dialog displays the name of the failed test, with an explanation and What to do instructions.
5) Click **Print** to print the test results.
6) Click **OK** (ENTER).

Step 4 - Stick Setup

The number of individual sticks selected in **Stick Count** from the **Settings** menu, are displayed as stick buttons in the Toolbar (Figure 4-9).

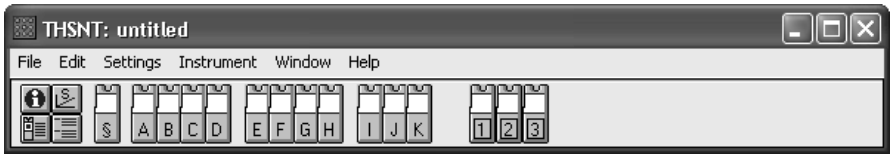


Figure 4-9: The Toolbar

To set up stick displays:

1) Click one of the stick buttons on the Toolbar to open the Stick Display window, (ALT, W, D).
2) View the Stick Display window (Figure 4-10) containing:
   - Description box
   - Identification text boxes (six samples, mid-level calibrator, zero calibrator)

- Value fields
- Signal and Stick Gain

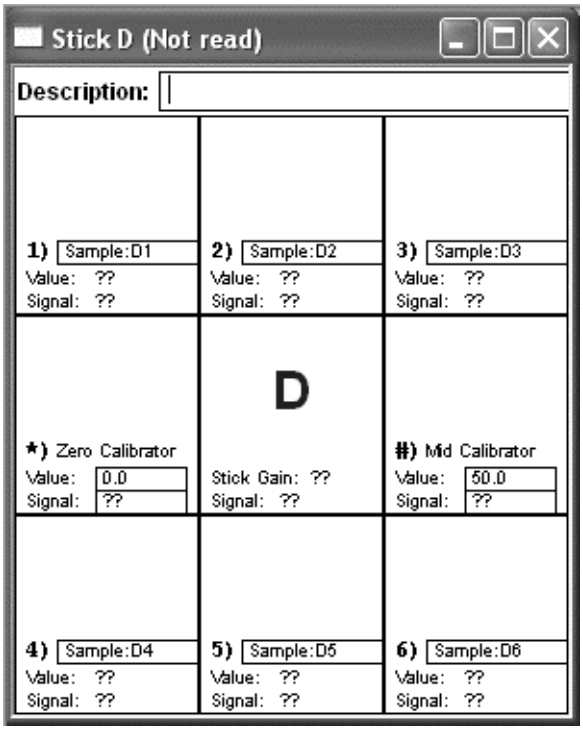


Figure 4-10: The Stick Display Window

3) Enter a general description of the stick contents in the **Description** field.
4) Enter specific identifications in the six (6) sample fields. The remaining fields are filled by the software, when a stick is read.

Table 4-5: Stick Setup Menu

Field	Change or Enter Information
Signal:	Raw data (slope of the kinetic plots in µV/sec) entered by Threshold Software, after the stick is read
Value:	Quantitation of the signal from the standard curve
Stick Gain:	The gain for each stick, determined by the rate difference between the * and # calibrator positions on the sample stick compared to the rate difference predicted for these two quantities based on the standard curve. (For a discussion of stick gain, see Chapter 6, "Calibration and Quantitation.")

Displaying Multiple Windows Using Cascade or Tile Options

By default, only one stick is displayed at a time in Threshold Software.

To display multiple sticks at the same time:

1) Hold down the CTRL key, SHIFT key, or ALT key, while either clicking the stick display button of each stick to be displayed, or selecting **Window**, **Display**, and selecting each stick in turn, (ALT, W, D).
2) Repeat until all desired sticks are on the desktop.
   - This method displays the Stick Display windows in cascade format.
3) To switch to a tiled display, select **Window**, **Tile**, (ALT, W, L).

Step 5 - Transfer and Filtration of Samples

When incubation is complete, and samples have been transferred into the filtration units, the **Instrument**, **Filter** command is used to control the Threshold Workstation vacuum. The **Filtration Rate** Menu (Figure 4-11) consists of Set vacuum to Low, High, Off, and Special options.

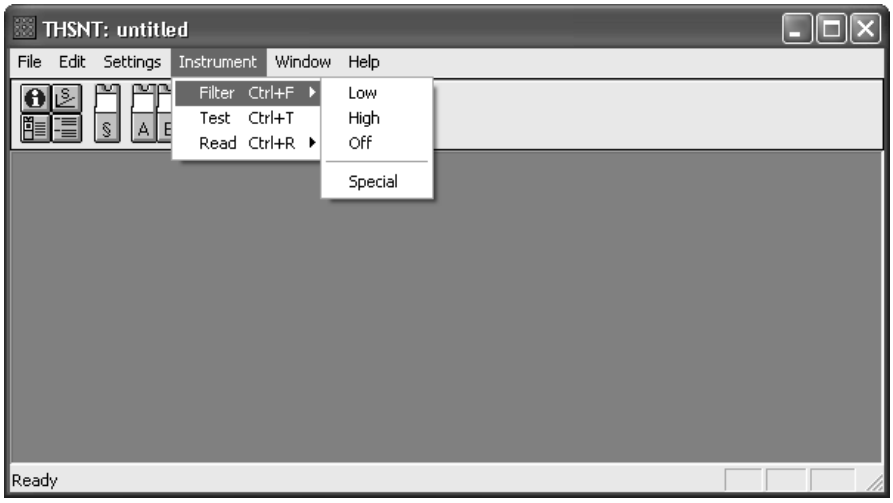


Figure 4-11: The Filtration Rate Menu

To set vacuum levels:

1) Select **Instrument**, **Filter**
2) Select the desired vacuum level (**Low**, **High**, **Off**, and **Special**), (ALT, I, F).

Table 4-6: Filter Vacuum Level Menu

Use	Speed Key	For
Low	ALT, I, F, L	Samples
High	ALT, I, F, H	Washes
Off	ALT, I, F, O	Turn filter off, drain on
Special	ALT, I, F, S	1/2 of Low filtration rate

3) The status selected is displayed on the left side of the Status Bar.
4) In addition, a timer is started and displayed in the Status Bar for each of the filtration levels.

Note: *Turning the vacuum off starts the drain.*

Step 6 - Reading Sticks

Sticks are read one at a time. As each stick is being read, the data points are plotted in each of the eight sample positions in the Stick Display window. After the stick is read, nine blue dots appear on the membrane portion of the stick button.

Note: *Threshold Enterprise users must have the appropriate permission set in Enterprise Administrator to read sticks.*

To read a stick:

1) Select **Instrument**, **Read**, and highlight the name of the stick to be read (ALT, I, R), (Figure 4-12).

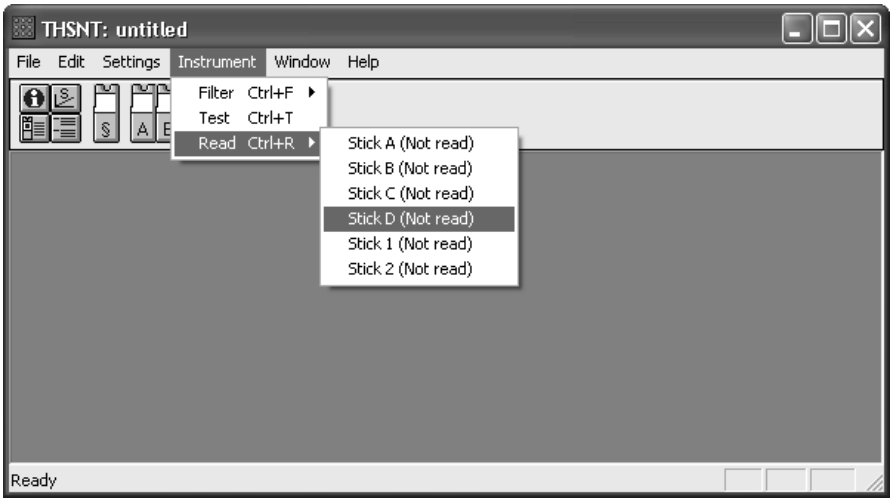


Figure 4-12: The Stick Read Menu

- Data sticks are labeled with alphabetic characters.
- Blank sticks are labeled with numeric characters.
- [Not read] changes to [Time] [Date], when sticks contain read data.

2) The Stick Display window for the selected stick appears on the desktop (Figure 4-10).
3) The Instrument Diagnostics dialog opens over the Stick Display window. The message "Performing instrument diagnostics..." is displayed while each test with results flashes briefly on the screen.
4) When the tests are complete, follow the detailed instructions that appear on the screen (Figure 4-13).
5) Put the stick in the Reader.
6) Make sure the stick clicks in place.

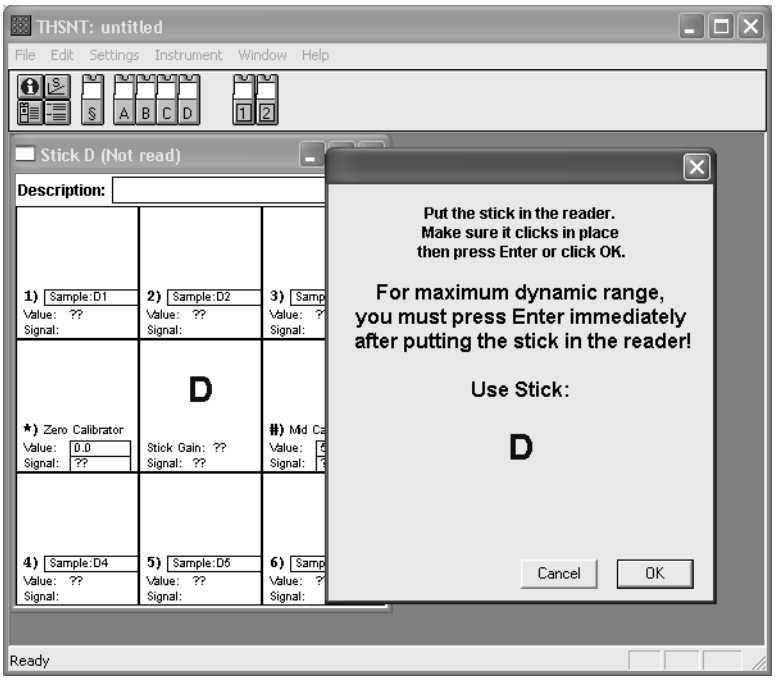


Figure 4-13: The Stick Display Window for the Stick to Be Read

7) Click **OK** (ENTER).

Note: *For maximum dynamic range, you must press* Enter *immediately after putting the stick in the Reader.*

8) After the stick is read, nine blue dots appear on the membrane portion of the stick button in the Toolbar (Figure 4-14).

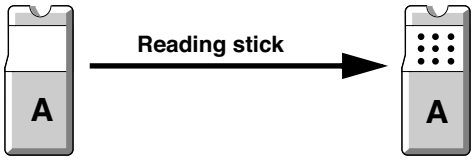


Figure 4-14: The Effect of Reading a Stick on the Tooxbar Stick Buttons

9) The time and date of read appears in the stick title bar.

10) Follow the instructions displayed in the Please remove the stick from the Reader now dialog.

11) Click **OK** (ENTER).

12) The stick is displayed with the kinetic plots and the signals for each sample and calibrator position (Figure 4-15). If a standard curve has been loaded, then the value and stick gain fields will be completed by the software.

13) Repeat Steps 1-12 for all remaining sticks.

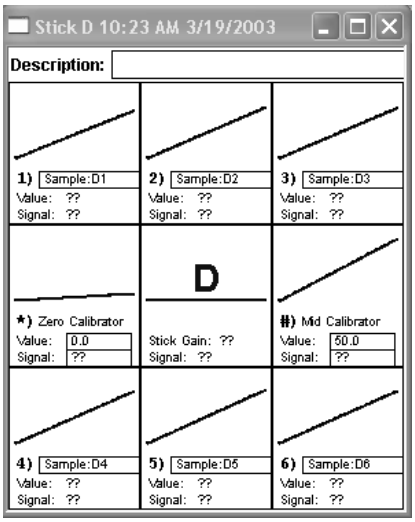


Figure 4-15: The Stick Display Window for a Stick That Has Been Read

Step 7 - Copying Stick to Standard

The standard curve calibrators are run on one of the data sticks (A-X). To generate a standard curve, the data from this stick has to be copied to the standard stick (Stick §).

Note: *Threshold Enterprise users must have the appropriate permission set in Enterprise Administrator to copy a stick to Standard*

To copy data from a stick to the standard stick:

1) Select **Edit**, **Copy Stick to Standard**, and highlight the stick to be copied to the standard stick (ALT, E, S).

2) The current stick data points, signals, sample identification, time and date of read are copied to the standard stick. Nine blue dots appear on the membrane portion of the standard stick button, similar to reading a stick (Figure 4-14).

3) The following warning message appears if the standard stick already has valid data: "The standard stick already has valid data. If you continue, the data will be overwritten."

4) Choose **Cancel** to retain the current standards and NOT accept the copied information.

5) Or, choose **OK** to overwrite the current standards with the copied information.

6) The standard stick information is then used to generate the standard curve and quantitate values on all other sticks.

Viewing the Standard Curve

To view the standard curve:

1) Click the Standard Curve button on the Toolbar, or select **Window, Standard Curve**, (ALT, W, S).
2) The Standard Curve window opens (Figure 4-16).

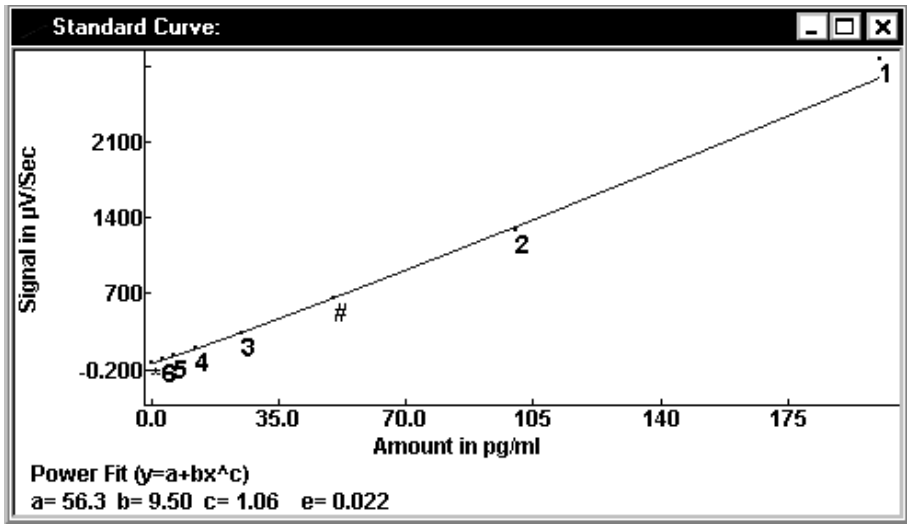


Figure 4-16: The Standard Curve Window

3) The Standard Curve window displays the standard stick signals and values plotted on a graph, with the calculated curve fit.
4) The fit type and the fit parameters are displayed at the bottom of the graph.

Importing a Standard Curve

A standard curve can also be imported from a saved file created in:

- Threshold Software for DOS (.THS)
- Threshold Software for Windows (.THW)
- Threshold Software for Windows 95/NT (.THW)
- Threshold Enterprise for Windows 2000/XP/7/10 (.TH3) (Threshold Enterprise

Note: *Threshold Enterprise users must have the appropriate permission set in Enterprise Administrator to import a standard curve.*

To import a standard curve:

1) Select **File, Import Standard Curve**, (ALT, F, I).
2) The Open dialog opens (Figure 4-17).

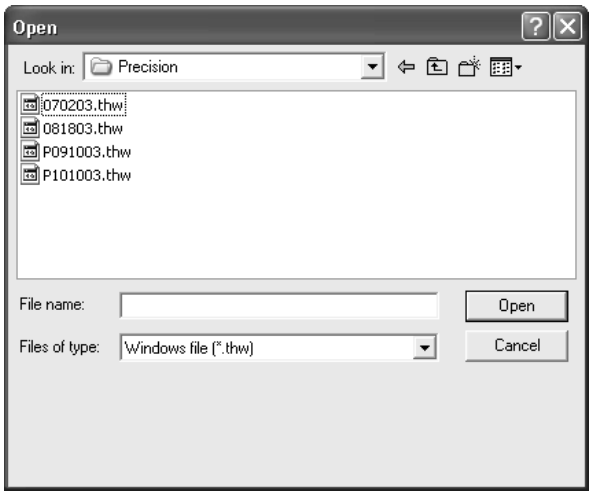


Figure 4-17: The Open Dialog for Importing Standard Curves

3) In the **File Name** box, enter a filename, or double-click the filename in the list above the **File Name** box. If the filename is not listed, select a new drive or directory in the **Look In** pull-down menu, or select a different type of file from the **Files of Type** pull-down menu.
   Threshold Software can import standard curves from files created by Windows 3.1 (.THW) and Windows 95/NT (.THW). Threshold Enterprise can also import standard curves from files created in Windows 200/XP/7/10 (.TH3).
4) Click **Open**. (ENTER)
5) The Stick § window (Standard Curve stick) opens with the time and date of the imported standard curve, and all of the associated standards data.

Additional Stick Options in the Edit Menu

There are a number of other options, available on the **Edit** menu, that allow all the information on a stick to be moved or erased. The following points should be noted:

- The number of sticks in the **Stick** menu correspond to the number of sticks selected in the Stick Count dialog.
- Data sticks are labeled with alphabetic characters and Blank sticks are labeled with numeric characters.
- [Not read] changes to [Time] [Date] when sticks contain read data.
- The word [modified] is displayed to the right of the date when any signal on the standard curve (Stick §) is changed, or when the signal for the * and # positions of any data stick (A-X) is changed.

Table 4-7: Edit Menu Stick Options

Edit Menu Item	What It Does	How to Use
Copy Stick	Copies stick information to the Clipboard.	1) Select **Edit**, **Copy Stick**, and highlight the stick to be copied, (ALT, E, O). 2) The stick information is now available to be pasted into another stick, or another Microsoft Windows program.
Paste Stick	Pastes stick information from the Clipboard to the selected stick, replacing the current stick information. ***Note:*** *Threshold users must have the appropriate permission set in Enterprise Administrator to copy sticks.*	1) Select **Edit**, **Paste to Stick**, and highlight the stick to be overwritten with the Clipboard contents, (ALT, E, A). 2) If the new stick already has data, then a warning dialog opens before the data is overwritten. 3) After the stick information is pasted into the new stick, nine blue dots appear on the membrane portion of the new stick button on the Toolbar (if the stick on the Clipboard has valid data). 4) The time and date of the actual read appears in the stick title bar. 5) If the data is modified after the read, [modified] is displayed to the right of the date.
Clear Stick Data	Clears the current stick data points, signals, time and date of read but retains the Sample identification. Use this function to repeat the same experiment with the same samples at a later date. ***Note:*** *Threshold users must have the appropriate permission set in Enterprise Administrator to copy sticks.*	1) Select **Edit**, **Clear Stick Data**, and highlight the stick to be cleared, (ALT, E, L). 2) If the stick already has data, then a warning dialog opens before the data is cleared. 3) All stick information is cleared, and the nine blue dots disappear on the membrane portion of the cleared stick's Toolbar button.

Table 4-7: Edit Menu Stick Options (Continued)

Edit Menu Item	What It Does	How to Use
Reset Stick	Resets the current stick data points, signals, time and date of read, and sample identification to the default settings. ***Note:*** *Threshold users must have the appropriate permission set in Enterprise Administrator to copy sticks.*	1) Select **Edit**, **Reset Stick**, and highlight the stick to be reset, (ALT, E, R). 2) A warning dialog opens before the stick is reset. All stick information is reset, and the nine blue dots disappear on the membrane portion of the reset stick's Toolbar button.

Step 8 - Save and Save As

After reading all the sticks, it is important to save the data files. Use the **Save** or **Save As** command to save a copy of the current file. Both commands access the Windows Save As dialog, so as to prevent accidentally overwriting a file.

Note: *Threshold Enterprise users must have the appropriate permission set in Enterprise Administrator to save the data files.*

To save a file:

1) Select **File**, **Save** or **Save As**, (ALT, F, S, or ALT, F, A).
2) The Save As dialog opens (Figure 4-18).

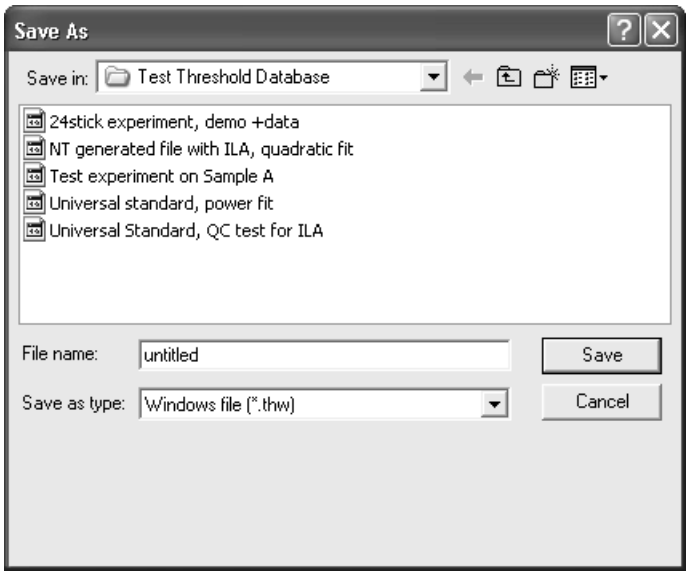


Figure 4-18: The Save As Dialog

3) Click the **Save in** box or drop-down arrow, to navigate to the desired drive and directory.
4) Enter the desired filename in the **File Name** box.

Threshold Software supports long filenames, but they cannot include punctuation, except for the period between the filename and extension. Threshold Software automatically adds the .THW default extension. Threshold Enterprise automatically adds the .TH3 default extension.

5) Click **Save**.

Step 9 - Printing Reports

There are two print options in Threshold Software. These are Print Window, which prints the current active window, Print Reports, which enables the printing of several report formats at once.

Printing a Report Using Print Window

1) Select the file you want to print, e.g., Stick Report, so that the report is visible in the main window.
2) Select **File**, **Print Window** (CTL+P).

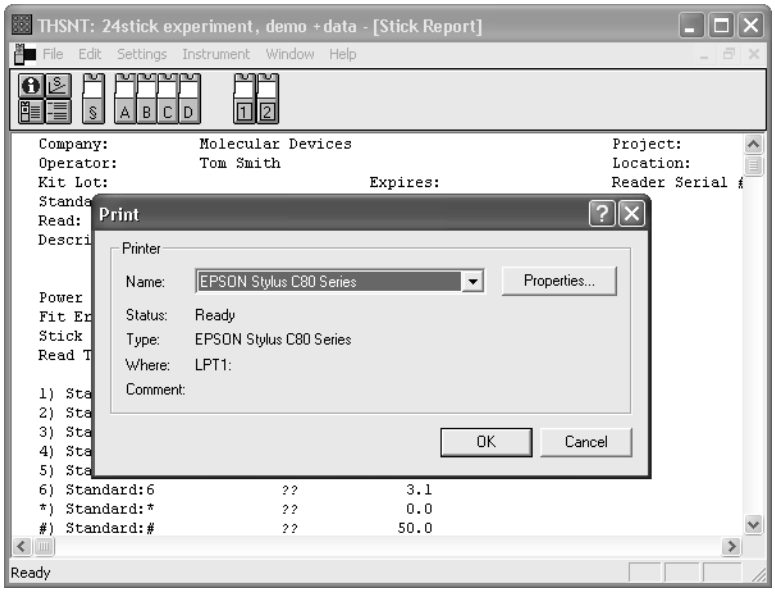


Figure 4-19: The Print Window Dialog

3) Select **Printer** and click OK.

Printing a Series of Reports Using Print Report

1) Select **File**, **Print Reports**, (ALT, F, R).
2) The Print Reports dialog opens (Figure 4-20).

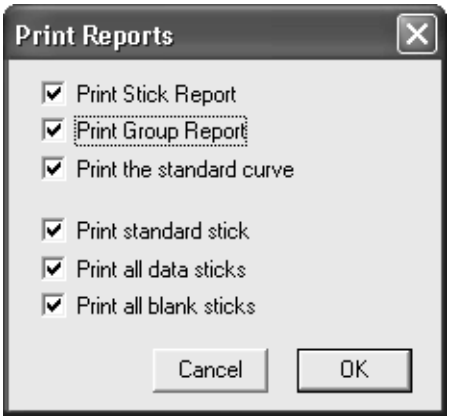


Figure 4-20: The Print Reports Dialog

3) Select the desired reports (TAB, SPACEBAR) (see Figure 4-20). Any or all of the reports can be selected (see Table 4-8)
4) Click **OK** (ENTER).
5) The Print dialog opens. Choose **OK** to print (ENTER) or choose the **Properties** button to set up a printer before printing.

Table 4-8: Print Report Menu

Type of Report	Report Contains
Print Stick Report	• All experiment information • Selected curve fit • Sample identification and position • Sample Signal (mV/sec) • Quantitated Value in selected units • Stick Gain for each stick
Print Group Report	• All experiment information selected curve fit • Sample identification and position • Raw and adjusted sample Signal (mV/sec) • Quantitated Value in selected units • Statistical evaluation for all groups of sample replicates
Print standard curve	• Graphical analysis of the standard curve • Graphical analysis of the selected curve fit
Print standard stick	• Standard Curve stick (Stick §) kinetic plot display

Table 4-8: Print Report Menu (Continued)

Type of Report	Report Contains
Print all data sticks	• All data sticks' kinetic plot displays, even if the sticks have not been read
Print all blank sticks	• All blank sticks, even if the sticks have not been read

Step 10 - Exporting Data

Exporting to Files

Threshold reports can easily be exported to files, with word processing or spreadsheet software, using the Export Window command.

To export a report to a designated file:

1) Select the Window to be exported, e.g., the Stick Report.
2) Select **File**, **Export Window**, (ALT, F, E).
3) The Save As dialog opens (Figure 4-21).

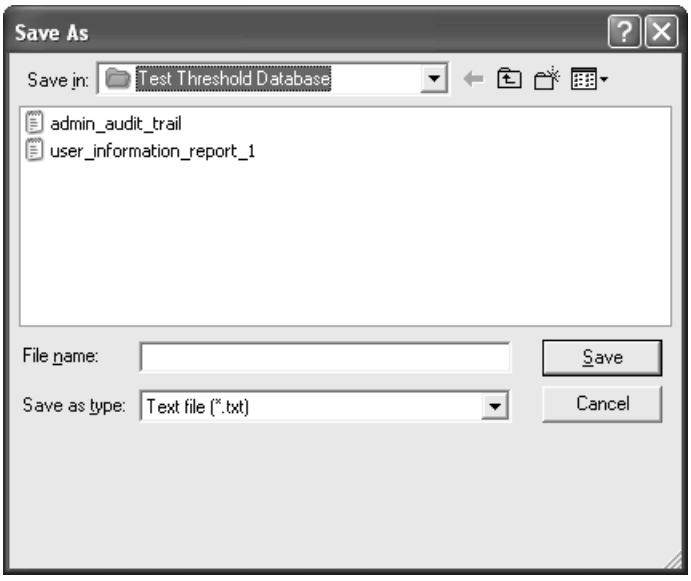


Figure 4-21: The Save As Dialog

4) Select a drive and directory. Enter in a filename.
5) The data will be saved as a .TXT file, which can be opened in word processing and spreadsheet softwares.

Note: *Exporting a Stick Display, in this way, exports the numerical data that was used to generate the kinetic plots. To transfer graphics, such as kinetic plots or standard curves, use the Windows Clipboard.*

Using the Clipboard to Transfer Data Between Applications

The Microsoft Windows Clipboard provides the temporary storage of information, when transferring data or graphics from Threshold Software to another application. The Clipboard can hold only one item at a time.

To transfer data between applications:

1) Open the appropriate window, e.g., the Stick Display.
   - Select **Edit**, **Copy** (ALT, E, C) for Stick and Group Reports.
   - Select **Edit**, **Copy Stick**, (ALT, E, O) for Stick Displays.
2) The data is copied to the Clipboard.
3) Paste the data into another open application, such as a word processing or spreadsheet application.

To transfer graphics, such as kinetic plots or standard curves:

1) Press ALT+PRINT SCREEN.
2) The graphic is copied from the current window to the Clipboard.
3) Paste the graphic into another open application

Step 11 - Exiting the Software

Threshold Software always has one file open, so it is not possible to exit the application until the open file has been saved (see "Step 8 - Save and Save As" on page 4-23).

To exit Threshold Software:

1) Save the current file.
2) Select **File**, **Exit**, (ALT, F, X).

   If the current file has been modified since the last time it was saved, and **File**, **Exit** is selected, a warning dialog opens. Select **Cancel** and save the current file before exiting again.

Chapter 5– Threshold Enterprise

5

Introduction

Threshold Enterprise 1.0 is a Microsoft® Windows™ application that controls, collects, and analyzes data from the workstation. Chapter 4, "Threshold Software" presents basic skills and concepts that are common to both Threshold Enterprise and Threshold Software.This chapter focuses on features that are unique to Threshold Enterprise.

Threshold Enterprise works together with Enterprise Administrator to provide 21 CFR Part 11 compliance features. A user has an account and a set of permissions that are administered by using Enterprise Administrator. Each user should coordinate with the administrator for Threshold Enterprise and Enterprise Administrator to initiate a new user account.

Further details of the menus and commands can be found in Appendix F, "Threshold Enterprise and Threshold Software Reference."

Compliance Features of Threshold Enterprise

Threshold Enterprise includes features to comply with FDA requirements for 21 CFR Part 11. These features include:

- Individual Passwords and User IDs
- Permission-Dependent Access to Major System Features
- Audit Trail of Logon and Log-Off Activities, and All User Actions that Modify the File
- Signed Electronic Statements

Security Menu (On Menu Bar)

The security features of Threshold Enterprise are accessed through the Security menu. A user group administrator needs to be identified by the company to set up and maintain user accounts for Threshold Enterprise. The user group administrator sets up and maintains user accounts, initiates passwords, provides permissions to individual users, and creates the link between Threshold Enterprise and Enterprise Administrator. A complete description of these actions and other features of Enterprise Administrator are provided in Chapter 8, "Enterprise Administrator."

Set Administration File...

This menu item prompts the Threshold Enterprise administrator to locate the Enterprise Administrator user group file (a file with a ".EDB" extension) that will link a user group to the running copy of Threshold Enterprise. Once the file is located and opened, the administrator will be prompted for the file's administrative user ID and password to establish the link.

Log On…

This menu item prompts the user to enter his/her assigned user ID and password. Once logged on, the user has access to all features within the limits of the permissions set by the user group administrator. A user must log on when beginning to work with Threshold Enterprise, and when resuming work with Threshold Enterprise after logging off, or after being automatically logged off Threshold Enterprise in response to an administrator-defined idle period.

Log Off

This menu item logs the user off Threshold Enterprise. Users should log off or exit the program when they are finished using Threshold Enterprise.

Change Password…

This menu item opens a dialog allowing the current user to change his / her password.

Show Audit Trail

This menu item opens a window displaying a list of all user activities performed on the current file. This information can be printed using **File**, **Print Window** or copied to the Clipboard using **Edit**, **Copy**.

Show Permission Information…

This menu item opens a dialog showing the user's name and user ID, the active administrative file, and the permissions for the user.

Sign Document…

This menu item opens a dialog prompting the user to enter his / her user ID and password. A statement can also be added. This statement initializes to "Accept." After signing a document, the signature, the time that the signature was added and the statement appear at the bottom of the Experiment Information window.

Add Confirmation Signature…

This menu item is only available after a document has been signed. This opens a dialog prompting the user to enter his / her user ID and password. A statement can also be added. This statement initializes to "Confirmed." After adding a confirmation signature, the signature, the time that the signature was added and the statement appear at the bottom of the Experiment Information window.

Remove Signatures

This menu item is only available after a document has been signed. A warning dialog opens asking whether the user wants to remove signature. Clicking **OK** will remove all signatures. The dialog can also be canceled.

The Spike Recovery Report

Threshold Enterprise includes a new report, the Spike Recovery Report. Spike Recovery can be used to assess quantitation accuracy by measuring the recovery of known quantities of analyte that are added to a sample prior to pretreatment or testing. Additional information on pretreatments and spike recovery is provided in Chapter 19, "Total DNA Sample Pretreatment."

It is important to realize that the Spike Recovery analysis needs to link the samples, controls and pretreatment controls with their associated spiked versions. This is done by requiring that the names of all spiked samples and controls be named exactly the same as the associated unspiked samples and controls, with the addition of a '+' character at the end of the name to indicate that it is spiked.

Note: *If this is not done, the samples and controls will not be included in the Spike Recovery report.*

Table 5-1: Naming Conventions for Spiked and Unspiked Samples

Unspiked	Spiked
ZC	ZC+
Sample 47	Sample 47+
My Sample	My Sample+
Pretreatment A	Pretreatment A+

Note: *There should not be a space between the end of the sample name and the '+' character.*

Enabling a Spike Recovery Report

To include a Spike Recovery Report in a Threshold data file, make sure the Enable Spike Report selection of the Settings menu is checked. If it is not, select Enable Spike Report.

Setting up a Spike Recovery Report

To inititalize a Spike Recovery Report:

1) Select **Setup Spike Recovery Report** from the **Settings** menu.
2) The Spike Recovery Report Setup dialog opens (Figure 5-1).

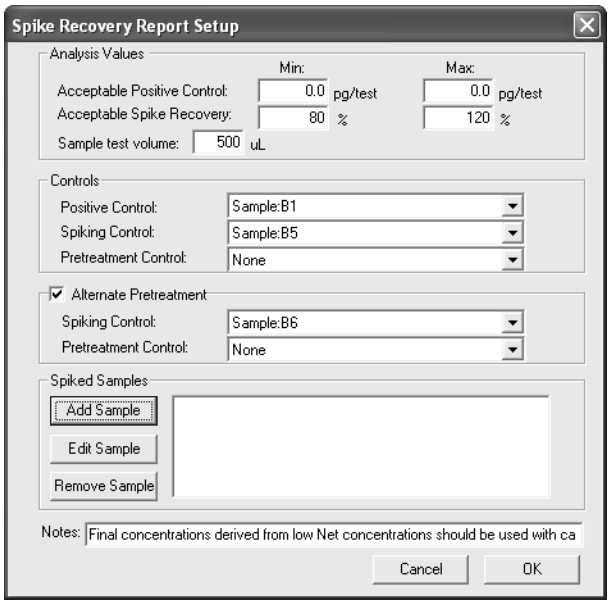


Figure 5-1: Spike Recovery Report Dialog

3) Within the Analysis Values box, enter:
   a. The minimum and maximum values for the acceptable range for positive controls. If you are using the Total DNA Assay Kit, refer to the Certificate of Analysis for the Positive Control range.

b. The minimum and maximum percentages for acceptable spike recovery and the sample test volume.

4) Within the Controls box, select the sample names for the Positive Control, Spiking Control and Pretreatment Control from the drop-down menus.
   - If any of these controls are not to be included, select **None** in the appropriate drop-down menu.
   - If an Alternative Pretreatment is included, click the Alternative Pretreatment checkbox.
5) Select the Spiking Control and Pretreatment Control from the drop-down menus.
   - If any of these controls are not to be included, select **None** in the appropriate drop-down menu.
6) In the Spiked Samples box:
   a. Click **Add Sample**.
   b. This opens the Add Spiked Sample dialog (Figure 5-2).

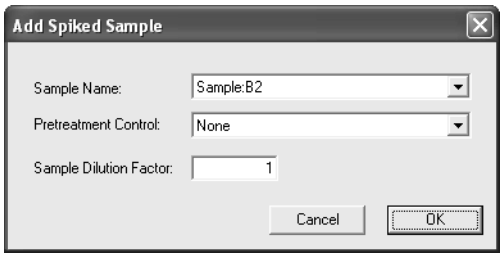


Figure 5-2: Add Spiked Sample Dialog

   c. In this dialog, select the first Sample name and Pretreatment control for that sample from the drop-down menus.
   If a Pretreatment control is not included for the sample being added, select **None** from this drop-down menu.
   d. Type in the sample dilution factor for this sample. Click **OK**.
7) Continue adding samples until all the Spiked samples are listed in the text box.
   - If an inappropriate sample has been added, it can be removed from the list by highlighting it in the text box and clicking Remove Sample.
   - If a sample was inappropriately defined when added, it can be corrected by highlighting the sample name in the text box and clicking **Edit Sample**. The **Edit Spiked Sample** dialog opens, allowing you to edit the information on this sample.

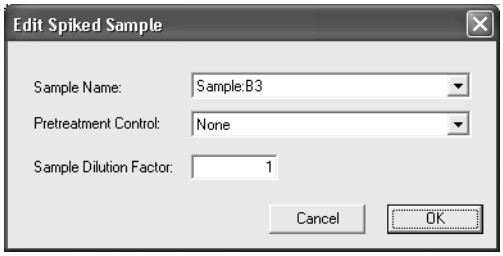


Figure 5-3: Edit Spiked Sample Dialog

8) A note can be added at the bottom of the **Spike Recovery Report Setup** dialog. This note will be included at the bottom of the Spike Recovery Report.
9) Click **OK** to complete the Spike Recovery Report setup.

Viewing the Spike Recovery Report

To view the Spike Recovery Report, select **Spike Recovery Report** from the **Window** menu.

Printing the Spike Recovery Report

The spike recovery report can be printed in two ways.

1) Only this report:
   a. Make the Spike Recovery Report window active
   b. Select **Print Window**… from the **File** menu.
2) This report with other reports:
   a. Select **Print Reports**… from the **File** menu

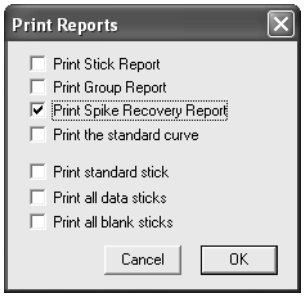


Figure 5-4: The Print Reports Dialog

   b. Select Spike Recovery Report.

Exporting the Spike Recovery Report

The Spike Recovery Report can be exported:

1) Make the Spike Recovery Report window active
2) Select **Export Window**… from the **File** menu.
3) A Save As… dialog is opened
4) Select the name and location for the exported report.

Extended Power Fit

In Threshold Enterprise, there are six functions that are available for fitting a standard curve to the data on a standard stick:

- Linear
- Power
- Quadratic
- Four-Parameter
- Piecewise Linear
- Extended Power

The Extended Power Fit was added to the previously-available fitting routines to extend the range of negative quantitation with a power fit. With the Extended Power Fit, all sample values are included for quantitation. The Power Fit continues to include samples, with signals down to 10% below the background signal.

To use the Extended Power Fit:

1) Choose **Analysis** from the **Settings** menu.
2) In the **Analysis Settings** dialog, choose **Extended Power Fit** from the **Standard Curve Fit** drop-down menu.
3) Click **OK**.

Chapter 6– Calibration and Quantitation

Introduction

In order to measure the amount of an analyte (or to quantitate the analyte) in unknown samples, the Threshold Assay must be calibrated. To calibrate an assay, a stick containing a range of known concentrations of analyte (standards) is run. The concentration of analyte for each standard is then recorded in Threshold Enterprise / Software.

The Threshold System measures the signal for each standard. Threshold Enterprise / Software then matches the signals with the known concentration of analyte. This process produces a standard curve. Each point in the standard curve represents a known concentration of analyte and the corresponding signal. Threshold Enterprise / Software automatically uses this standard curve to convert signals from test samples into quantities of analyte.

The standard curve should be within the dynamic range of the assay. The standards should be selected to span the entire dynamic range of the assay, or only part of the range. A sample outside the range of the standard curve should not be quantitated.

Two known concentrations of analyte, called the * (zero) calibrator and # (mid-level) calibrator, are on every stick containing unknown samples (on-board calibrators). These calibrators allow Threshold Enterprise / Software to adjust the signals for this stick, to compensate for changes in the assay conditions (for example, flow rate through the membrane, or temperature).

The following definitions will be used for the remainder of this chapter:

- ***Precision***. This is the reproducibility of measurements, often measured by the coefficient of variation (%CV), %CV = 100 x standard deviation/mean result.
- ***Accuracy***. This is the ability of the assay to report the correct amount of an analyte.
- ***Background***. Background is the signal due to nonspecific interactions of the enzyme reagent with assay components in the absence of analyte.
- ***Interference***. This causes decrease or increase in signal due to components in the sample (for example, salts, detergents, excess analyte).

Guidelines for Calibration

Following are the basic guidelines for Threshold Assay calibration and quantitation procedures:

- Each standard consists of the known concentration of standard and the corresponding signal (µV/sec) measured by the Threshold workstation. The known concentration for each standard is entered in Threshold Enterprise / Software.
- The correct curve fit should be selected to define the standard curve. Threshold Enterprise / Software also allows editing of the data (for example, removal of erratic standards), to give the best fit standard curve.
- Threshold requires a standard curve to quantitate unknown samples. Once the standard curve is specified, Threshold Enterprise / Software uses that curve to quantitate all subsequent sticks in that experiment, unless a new standard curve is specified.
- Threshold Enterprise / Software saves the calculated standard curve, used to quantitate a stick, in the same data file that contains the assay results for that stick. When a data file is recalled, the standard curve is included with the data, automatically replacing any previous standard curve.

- The on-board calibrators (* and #) ***must*** be included on all sticks containing unknown samples. These calibrators are made from the same dilutions as the standard curve. Threshold Enterprise / Software uses these calibrators to adjust the measurements of unknown samples for stick-to-stick variations so that the standard curve produces accurate results (see Stick Gain on page 6-9).

Setting Up a Standard Curve

For specific Threshold Assays see Chapter 9, "Immuno-Ligand Assay Introduction," and Chapter 16, "Total DNA Assay Introduction."

After running the Threshold Assay with one or more sticks, containing standards, there are two options:

- Use the results from one stick to create the standard curve.
- Print reports for standards on two or more replicate sticks and average the results. Also, average duplicate values from one concentration on one stick. Enter in these numbers for the standard curve.

Note: *If the user changes the signals for a standard curve, Threshold Enterprise / Software shows the time the Standard Stick was read. However, if the signal is changed, the stick is marked as "Modified."*

Procedure for One Standard Stick

After measuring the standard samples on a stick (A-X), use this data as the new standard curve. To do this:

1) Select **Edit**, **Copy Stick to Standard**.
2) Highlight the stick to be copied to the Standard Stick §.
3) To display the standard curve, either click on the standard curve button on the Toolbar or select **Window**, **Standard Curve**.
4) Confirm that the curve shows the correct concentrations of analyte. The descriptions, concentrations, or signals of the standards may be changed in the Standard Stick (Stick §) window.
5) Using the standard curve window, confirm the curve fits the standards and the resulting error term is acceptable.

Procedure for Averaging More Than One Standard Stick

After reading each Standard Stick, display the results:

1) Select **Window**, **Stick Display**.
2) Record the results for each standard (µV/sec for each concentration of analyte).

To print the results:

1) Select **File**, **Print Reports**.
2) The Print Reports dialog opens.
3) Choose **Print Stick Report** and **Print Group Report** by marking the appropriate boxes with an "X".

4) Click **OK** or press ENTER.
5) Calculate the average for replicate values, or use the mean of raw signals generated in the Group Report printout.
6) Click the Standard Stick § button to open the Standard Stick window.
7) Enter the average values for signal (µV/sec), and the analyte quantity, in the appropriate fields.
8) Examine the standard curve, its equation, and the error term, displayed in the standard curve window, to confirm they are suitable.

Signal Determinations

Data Acquisition

The Threshold System measures voltage at evenly-spaced time intervals for each sample on a stick. As a stick is read, Threshold Enterprise / Software displays a plot of voltage (µV) on the Y-axis and time in seconds (s) on the X-axis. The slope of this plot is the rate or signal (µV/s). The signal is proportional to the rate of change of pH which is dependent on the quantity of analyte.

Signal Calculations

An initial point, taken before the plunger presses the stick membrane against the sensor, establishes the initial voltage, $V_{i(init)}$. This corresponds to the unperturbed pH of the bulk Substrate Solution in the Reader. After the workstation presses the membrane against the sensor, it makes 60 measurements at each position. Of the 60 points, the assay uses only those falling within a specified voltage window from $V_{i(init)}$ to 59 mV (1.0 pH unit) above $V_{i(init)}$.

Note: *Threshold Enterprise / Software requires two or more data points to calculate a signal, in addition to $V_{i(init)}$, the initial voltage point.*

The kinetic plot should be linear for an accurate signal calculation. Non-linear plots can result from a high starting pH in the Reader. While reading a stick, the pH can increase to a level that is non-optimal for enzyme (urease) activity. If the data plot is non-linear, take these precautions:

- Check for correct pH in the Wash and Substrate Solutions.
- Be sure to start reading immediately after inserting the stick into the Reader. A delay of even 5 to 10 seconds can cause non-linearity.

Threshold Enterprise / Software determines the signals or rates, R_i, for each sample or calibrator position (i), by unweighted least-squares fit to this linear equation:

$$V_i = A_i + (R_i \cdot t)$$

In other words, this expression is minimized with respect to deviations from a line with intercept A_i and the slope R_i:

$$\sum_{j=1}^{60} d_{ij} \cdot ((A_i + R_i) \cdot (t_i - V_{ij}))^2$$

Here d_{ij} picks out the voltage point within the window: $d_{ij} = 1$ if $V_{low} \leq V_{ij} \leq V_{high}$ and otherwise $d_{ij} = 0$. V_{low} is defined as the starting potential and V_{high} is defined as the starting potential + 59 mV. It is not necessary to use d_{ij} in the actual calculation; it is here to simplify notation. The intercept A_i is unused.

Threshold Enterprise / Software stops and discards the calculation of R_i if fewer than two of the remaining data points are in the voltage window.

Types of Standard Curves

Threshold Software includes the following functions for fitting a standard curve to the data on the Standard Stick:

- Linear
- Power
- Extended Power (Threshold Enterprise Only)
- Quadratic
- Four-Parameter
- Piecewise Linear

Linear Fit

Select "Linear Fit" from the drop-down menu in the **Settings**, **Analysis** dialog. This is the equation Threshold Enterprise / Software uses to calculate the standard curve for a linear fit:

$$Y = a + (b \cdot X)$$

where X is the "value" or quantity of analyte (Q) and Y is the signal or rate (R), in µV/sec, thus the equation becomes:

$$R = a + (b \cdot Q)$$

Threshold Enterprise / Software uses the data from the Standard Stick § to find the coefficients (**a**, **b**) that give the best fit to this equation, using standard linear regression.

Power Fit

Select "Power Fit" from the drop-down menu in the **Settings**, **Analysis** dialog. This is the equation Threshold Enterprise / Software uses to calculate the standard curve for a power fit:

$$Y = a + (b \cdot X^c)$$

where X is the "value" or quantity of analyte (Q) and Y is the signal or rate (R) in µV/sec, thus the equation becomes:

$$R = a + (b \cdot Q^c)$$

Threshold Enterprise / Software uses the data from the Standard Stick § to find the coefficients (**a**, **b**, **c**) that give the best fit to this equation. The coefficients are determined in this way:

1) Subtract **a** from both sides of the equation:

$$R' = R - a = b \cdot Q^c$$

a is the y -intercept, and corresponds to the background signal in the absence of analyte. Threshold Enterprise / Software determines **a** from the * calibrator on the Standard Stick §.

For calculating the standard curve, Threshold Enterprise / Software uses only positions on the Standard Stick § that have signals greater than the signal of the * calibrator on the standard curve stick.

2) Take the logarithm of each side of the equation. This yields:

$$ln\ (R') = ln(b) + c\ ln\ (Q)$$

3) Determine **b** and **c** by calculating the least-squares fit to this equation, using the values of Q_i and R'_i from the sites on the Standard Stick §.

The least-squares fit determines values for **b** and **c**, which minimize this expression:

$$\sum_{i=1}^{8} [ln(b) + C \cdot ln(Q_i) - ln(R_i')]^2$$

4) To eliminate the slight positive bias in the mean quantitation of samples with zero analyte that arises by only taking positive values, the curve is extrapolated to enable quantitation of near-zero negative values. The curve is extrapolated by an X-Y reflection around the Y-intercept. Negative quantitation is only allowed for samples with signals down to 10% below the background signal (Y-intercept, or **a** term). For the extended power fit (Threshold Enterprise only) full negative quantitation is allowed.

Note: *The algorithm used for the power curve fit in Threshold Enterprise / Software for Windows 2000/XP/7/10 is identical to that used in Threshold Software for Windows 95/ NT. For positive quantitation, the algorithm is identical to that used in Threshold Software for Win-dows and Threshold Software for DOS (THS 1.0). The algorithm only differs in that it enables near-zero negative quantitation, which will eliminate the slight positive bias in the mean quantitation of samples with zero analyte. This change may result in minor differences in quantitation for data in Threshold Enterprise / Software for Windows 2000/ XP/7/10 and Threshold Software for Windows or Threshold Software for DOS. To achieve identical quan-titation to the older versions of Threshold Software when using the power fit, simply treat any negative values as "??", and recalculate the mean values accordingly.*

Extended Power Fit (Threshold Enterprise only)

Select Extended Power Fit from the drop-down menu in the **Settings, Analysis** dialog. This curve Fit uses the same equations as the Power Fit, but extends the range of negative quantitation to include all sample values. The Power Fit continues to include samples with signals down to 10% below the background signal.

Quadratic Fit

Select "Quadratic Fit" from the drop-down menu in the **Settings**, **Analysis** dialog. This is the equation Threshold Enterprise / Software uses to calculate the standard curve for a quadratic fit:

$$Y = a + (b \cdot X) + (C \cdot X^{\mathbf{2}})$$

where X is the "value" or quantity of analyte (Q) and Y is the signal or rate (R) in µV/sec, thus the equation becomes:

$$R = a + (b \cdot Q) + (c \cdot Q^{\mathbf{2}})$$

Threshold Enterprise / Software uses the data from the Standard Stick § to find the coefficients (**a**, **b**, **c**) that give the best fit to this equation, by solving the normal equations of the generalized least-squares technique. Please see "Four-Parameter Fit" on page 6-8 for a complete reference.

Four-Parameter Fit

Select "4-Parameter Fit" from the drop-down menu in the **Settings**, **Analysis** dialog. Use the 4-parameter fit for sigmoidal curves. This is the function Threshold Enterprise / Software uses to calculate the standard curve for a 4-parameter fit:

$$Y = \frac{a - d}{\mathbf{1} + (X/c)^{b}} + d$$

where X is the "value" or quantity of analyte (Q) and Y is the signal or rate (R), thus the equation becomes:

$$R = \frac{a - d}{\mathbf{1} + (Q/c)^{b}} + d$$

The four parameters (**a**, **b**, **c**, **d**) may be thought of in this way: the first parameter, **a**, is the asymptote for the standard curve at low values of the x axis (that is, at low quantities of analyte). The fourth parameter, **d**, is the asymptote for the standard curve at high values of the x axis (that is, at high quantities of analyte). The parameter **c** is the x-value corresponding to the midpoint between **a** and **d**. The parameter **b** is determined by how rapidly the curve makes its transition from the asymptotes to the center of the curve. A large (absolute) value of **b** describes a sharper transition.

The algorithm for the 4-parameter curve fit in Threshold Software for Windows and Threshold Enterprise / Software for Windows 2000/XP/7/10 differs from that used in Threshold Software for DOS (THS 1.0). The current curve fitting algorithm is based on the Levenberg-Marquardt Method. Discussion of this method can be found in *"Numerical Recipes in C: The Art of Scientific Computing"*, by William H. Press, Brian P. Flannery, Saul A. Teukolski and William T. Vetterling, published by Cambridge University Press, New York, 1988. This change in the algorithm may result in minor differences in quantitation for data in Threshold Software for DOS and Threshold Enterprise / Software for Windows 2000/XP/7/10.

Note: *A 4-parameter fit does not work for linear data. The data needs to contain an inflection point.*

Piecewise Linear Fit

Select "Piecewise Linear Fit" from the drop-down menu in the **Settings**, **Analysis** dialog. This fit has no equation. Threshold Enterprise / Software determines the "value" or quantity of an unknown sample by interpolating between the two nearest standards (after performing zero suppression and gain adjustment, as with other fits). If there is more than one possible interpolation for a signal (because the curve is non-monotonic), then Threshold Enterprise / Software returns the leftmost value (the lowest "value" or quantity). There is no error measurement, because the fit is exact.

Calculations For Quantitation

Stick-to-Stick Variations

There are two main reasons for stick-to-stick variations

Background Signals

Threshold Enterprise / Software corrects for variations in non-specific background signals, or rates, by using the * position on each stick to zero offset the signals of other positions, before interpolation. Zero offset or zero suppression sets both sides of the equation to the same baseline. This may be done either by subtracting the background from both sides thus setting the baseline to zero; or by adding the background to one side so that both sides have the same non-zero baseline.

Stick Gain

Threshold Enterprise / Software corrects the signals of sample positions by comparing the signal (rate) difference between the * and # calibrator positions on the sample stick, $[R_{\#} - R_{*}]$, to the signal (rate) difference predicted for these two quantities based on the standard curve, $[f_{\#} - f_{*}]$. In the recommended protocol, * and # are dedicated to zero and mid-level calibrators, respectively.

The Stick Gain is:

$$\frac{[R_{\#} - R_{*}]}{[f_{\#} - f_{*}]}$$

Adjusting the Signal

The following equation describes the adjusted signal (corrected rate) for an unknown sample run in position **i**, adjusted for background and Stick Gain:

$$R_i^{corr} = \left((R_i - R_*) \cdot \frac{[f_\# - f_*]}{[R_\# - R_*]} \right)$$

where R_i^{corr} is the value that Threshold Enterprise / Software reports as in the Group Report as adjusted signal.

For competitive assays, the signal is inversely related to the quantity of analyte. The standard curve decreases from a high signal for the * calibrator to a lower signal for the # calibrator. Although the value for $[f_\# - f_*]$ has a negative sign, Threshold Enterprise / Software uses the same method to correct for stick-to-stick variations.

Computing the Quantity of Analyte From the Rate

Threshold Enterprise / Software calculates the quantity of analyte for each sample position (**i**) on the sample stick, using the standard curve equation that has been selected (linear, power, quadratic, 4-parameter or piecewise linear), and the adjusted signal for that position.

Linear Fit

Threshold Enterprise / Software uses the following equation for the linear curve fit:

$$R = a + (b \cdot Q)$$

When this equation is corrected for zero suppression and Stick Gain, it becomes:

$$R_i^{corr} = (b \cdot Q_i)$$

where Q_i is the quantity of analyte to determine, and R_i^{corr} is the signal (rate) measured at the sample position **i**, corrected for background and Stick Gain. Thus, R_i^{corr} is the adjusted signal. Threshold Enterprise / Software determines values of the coefficient **b** from the standard curve.

To solve this equation for Q_i, the quantity of analyte, divide both sides of the equation by **b**:

$$\frac{R_i^{corr}}{b} = Q_i$$

Substituting in the adjusted signal (R_i^{corr}) for a sample and the **b** term from the standard curve gives the desired "value" or quantity, Q_i.

Power Fit and Extended Power Fit

Threshold Enterprise / Software uses the following equation for the power curve Fit.

$$R = a + (b \cdot Q^c)$$

When this equation is corrected for zero suppression and Stick Gain, it becomes:

$$R_i^{corr} = b \cdot Q_i^c$$

where Q_i is the quantity of analyte to determine, and R_i^{corr} is the signal (rate) measured at the sample position **i**, corrected for background and Stick Gain. Thus, R_i^{corr} is the adjusted signal. Threshold Enterprise / Software determines values of the coefficients **b** and **c** from the standard curve.

To solve this equation for Q_i, the quantity of analyte:

1) Divide both sides of the equation by **b**:

$$\frac{R_i^{corr}}{b} = Q_i^c$$

2) Raise both sides of the equation to the power 1/**c**:

$$\left[\frac{R_i^{corr}}{b}\right]^{1/c} = Q_i$$

Substituting in the adjusted signal (R_i^{corr}) for a sample and the **b** and **c** terms from the standard curve gives the desired "value" or quantity, Q_i.

Threshold Enterprise / Software solves the power fit equation for values of R_i^{corr} greater than or equal to zero and for near-zero negative values. Negative adjusted signals indicate that the signal of the sample is less than the signal at the * calibrator position for that stick. For near-zero negative adjusted signals (within 10% of the standard curve zero or **a** value), this is probably random noise around a true zero measurement. Ignoring these near-zero negative adjusted signals and only taking positive values will cause a slight positive bias in the mean quantitation of samples with zero analyte. To eliminate this bias, near-zero negative values are quantitated. Highly negative values imply analyte concentrations lower than the zero calibrator, or sample interference in the assay. Therefore, these are not quantitated, and are reported as "??" to indicate that the adjusted signal is anomalous. If Threshold Enterprise / Software displays no quantity for a position (only "??" appears on the screen or printed report), this indicates that the adjusted signal for the position is less than 90% of the standard curve zero signal, or **a** term, or that the kinetic plots did not contain at least two points.

Quadratic Fit

Threshold Enterprise / Software uses the following equation for the quadratic curve fit:

$$R = a + (b \cdot Q) + (c \cdot Q^2)$$

When this equation is corrected for zero suppression and Stick Gain, it becomes:

$$R_i^{corr} = (b \cdot Q_i) + (c \cdot Q_i^2)$$

where Q_i is the quantity of analyte to determine, and R_i^{corr} is the signal (rate) measured at the sample position **i**, corrected for background and Stick Gain. Thus, R_i^{corr} is the adjusted signal. Threshold Enterprise / Software determines values of the coefficients **b** and **c** from the standard curve.

To solve this equation for Q_i, the quantity of analyte, the quadratic formula is used:

$$Q_i = \frac{-b \pm \sqrt{b^2 + 4 \cdot R_i^{corr} \cdot c}}{2c}$$

Substituting in the adjusted signal (R_i^{corr}) for a sample and the **b** and **c** terms from the standard curve gives two solutions to this equation, for the desired "value" or quantity, Q_i. Threshold Enterprise / Software chooses the solution that lies closest to the average of the standard curve calibrators.

4-Parameter Fit

Threshold Enterprise / Software uses the following equation for the 4 parameter curve fit:

$$R = \frac{a - d}{1 + (Q/c)^b} + d$$

When the equation is adjusted for background and Stick Gain, this equation becomes:

$$R_i^{corr} + a = \frac{a - d}{1 + (Q_i/c)^b} + d$$

where Q_i is the quantity of analyte to be determined and R_i^{corr} is the rate measured at the sample position **i**, corrected for background and Stick Gain. Thus, R_i^{corr} is the adjusted signal. Threshold Enterprise / Software determines the values of the coefficients **a**, **b**, **c**, and **d** from the standard curve.

To solve the equation for Q_i:

$$Q_i = C \cdot \left(\frac{a - d}{(R_i^{corr} + a - d)} - 1 \right)^{1/b}$$

Substituting in the adjusted signal (R_i^{corr}) for a sample and the **a**, **b**, **c**, and **d** terms from the standard curve gives the desired "value" or quantity, Q_i.

Spike Recovery

The Threshold System can measure quantities of analytes, down to the picogram level. At these levels, quantitation accuracy may be affected by the presence of interfering substances in the sample or that are introduced during pretreatment of samples. Spike recovery can be used to assess quantitation accuracy, by measuring the recovery of known quantities of analyte that are added to a sample prior to pretreatment or testing.

It is important to realize that the Spike Recovery analysis needs to link the samples, controls and pretreatment controls with their associated spiked versions. This is done by requiring that the names of all spiked samples and controls be named exactly the same as the associated unspiked samples and controls, with the addition of a '+' character at the end of the name to indicate that it is spiked.

Note: *If this is not done, the samples and controls will not be included in the Spike Recovery report.*

Table 6-1: Naming Conventions for Spiked and Unspiked Samples

Unspiked	Spiked
ZC	ZC+
Sample 47	Sample 47+
My Sample	My Sample+
Pretreatment A	Pretreatment A+

Note: *There should not be a space between the end of the sample name and the '+' character.*

Two types of spike recovery control may be used:

- **Spike Controls.** Used to define the expected spike value for the samples. A buffer control or zero calibrator is tested with, and without, a known quantity of added analyte (spike). The background control (non-spiked) value is then subtracted from the spiked control value, to provide a net spike value. This value is then defined as providing 100% spike recovery.

$$Netspike = BufferSpiked - Buffer$$

- **Pretreatment Controls.** Used to determine the effect of sample pretreatment on assay accuracy. A buffer control or zero calibrator is set up with, and without, the same known quantity of added analyte as the spike controls. The pretreatment controls are then pretreated and tested. The background pretreatment control value (non-spiked) is then subtracted from the spiked pretreatment control value to provide a net pretreatment spike value. This net value is then divided by the value, for 100% spike recovery, to provide a percentage spike recovery for the pretreatment control, which is a measure of the effect of pretreatment on assay accuracy.

Net Pretreatment Spike:

$$Netspike\mathbf{Pr} = BufferSpiked\mathbf{Pr} - Buffer\mathbf{Pr}$$

Percentage Pretreatment Spike Recovery:

$$\%SR = \frac{Netspike\mathbf{Pr}}{Netspike} \times \mathbf{100}$$

Sample Spike Recovery

To measure spike recovery of a sample at a given dilution, the same known quantity of analyte is added to the sample. A background value measured from an unspiked sample, at the same dilution, is then subtracted from the measured value of the spiked sample, providing a net spike value. This net value is then divided by the value for 100% spike recovery that was determined with the Spike Controls, to provide a percentage spike recovery for the sample.

Net Sample Spike:

$$NetspikeSample = SampleSpiked - Sample$$

Percentage Sample Spike Recovery:

$$\%SR = \frac{NetspikeSample}{Netspike} \times \mathbf{100}$$

Final Concentration After Spike Recovery

If the percentage of spike recovery for the sample is found to be within a user-defined acceptable range, the final concentration of analyte in the sample can be determined. First, the non-spiked value, measured from the appropriate pretreatment control, is subtracted from the measured value of the non-spiked sample, thereby removing the known background signal. This net sample concentration is then multiplied by the dilution factor of the sample and divided by the sample test volume, to provide a final concentration of analyte per mL.

Net Sample Concentration:

$$NetConcSample = Sample - Buffer\mathbf{Pr}$$

Note: *Where pretreatment is set to "None" in the* ***Spike Recovery Report*** *setup, no background subtraction is performed, so NetConcSample = Sample.*

Final Sample Concentration:

$$FinalConc = \frac{NetConcSample \times DilutionFactor}{TestVolume}$$

Alternate Pretreatments

For more complex experiments, where samples pretreated in two different ways are tested, an alternate set of Spiking and Pretreatment Controls can be defined. The calculations performed are identical to those above, and the derived values are used only for the samples where the alternate pretreatment is assigned. Note that where pretreatment is set to "None" in the Spike Recovery Report setup, the primary spiking control is always used for determining the spike recovery and no background subtraction is performed.

Threshold Order of Calculations

Order of Calculations

Threshold Enterprise / Software uses a specific sequence of calculations to arrive at values reported in the Stick, Group, and Spike Recovery reports. Although a Standard curve can be specified after stick signals have been recorded, this change will affect each of the other calculations, which are then recomputed. The basic order of calculations is:

1) Creation of a Standard curve.
2) Signal calculation, using a linear fit to raw data from the initial voltage to 59 mV above the initial voltage for each sample on each stick.
3) Stick gain calculation for each stick.
4) Adjusted signal for each sample on each stick.
5) Quantitation of analyte for each sample on each stick.
6) Statistical analysis of raw signals, adjusted signals and quantitation values for each stick.
7) Spike recovery for selected samples.

Error Term, E

When Threshold Enterprise / Software fits a curve to standard points, it calculates and reports an error term, E, as the average relative error. E is an estimate of the accuracy of how well the standard curve fits the standard points.

Threshold Enterprise / Software first calculates the relative error for each point, taking the absolute value:

$$e_{point} = \frac{|measured\ \mu V/sec - calculated\ \mu V/sec|}{measured\ \mu V/sec}$$

The E term is an average of these values:

$$E = \frac{1}{number\ of\ points} \sum_{N=1}^{No.\ of\ Points} e_{point}$$

Interpreting E

This method of calculating E gives more importance to errors in standards with low signals. A low error term ***may*** indicate a good fit to the data at the low end of the curve, with a poor fit at the high end of the curve. Be sure to look at the curve (select **Window**, **Standard Curve** or click the standard curve button on the Toolbar) to check that the fit is satisfactory.

If the E term, for example, is 0.04 for an eight point standard curve, all eight points may deviate 4% from the calculated values. Alternatively, seven points could be the same as the calculated points, with one point deviating 32%.

Procedures For Standard Curve Fitting

Select a curve fitting method:

Table 6-2: Curve Methods

If the standard points follow:	Then, select:
A straight line	Linear Fit
An S-shape or have an inflection point	4-Parameter Fit
A curve, in only one direction	Quadratic or Power Fit

Interpreting Standard Curves

Examine the standard curve obtained with each fitting method:

- Inspect how well standard points fall on the calculated curve. Notice points that are far from the fitted curve. These are called “outliers.”
- Examine quantitation of the standard curve against itself.
- Check the E term.

Troubleshooting Standard Curves

Deviations of standard curves from standard points or high E values are due to the following factors:

- An inappropriate curve fit was selected.
- There are errors in the assay procedure, such as dilution errors.
- There are random errors in the standard points. View the standard curve and note standards that are far from the fitted curve. Display the Standard Stick § and delete these points, by deleting the value field. This procedure may improve the curve fit.

Conditions Leading to Quantitation Failure

The following conditions can lead to failure of quantitation:

Threshold Enterprise / Software cannot calculate an acceptable rate for the sample.

- One or more of the first two points on the kinetic plot is outside the pH window (1.0 pH units above initial pH). This affects only the sample position in question. These samples may contain a very high enzyme concentration.

Threshold Enterprise / Software cannot calculate an acceptable rate for one or more on-board calibrators (* or #).

- Quantitation is impossible for any sample on this stick. The * and # data can be changed to overcome errors. It is possible to generate estimates of quantitation in these cases by changing the * and # rates on this stick. For example, use average rates for * and # positions on other sticks in the same experiment. If using this correction, interpret the quantitation of unknowns with caution. The sticks will be marked as 'modified.'

Threshold Enterprise / Software cannot adjust errors in sample rates with the * or # calibrators.

- This can occur if $R_{\#} = R_{*}$ (signal values for * and # are equal), or if the * and # samples were placed in the wrong positions on the stick. Quantitation is impossible for any sample on this stick. If * and # calibrators were placed in the wrong positions on the stick, they may be edited and accurate quantitation is possible. The sticks will be marked as 'modified.'

Threshold Enterprise / Software cannot construct an acceptable standard curve.

- In the absence of a standard curve, quantitation is impossible for any sample on the stick. If Threshold Enterprise / Software is able to construct an acceptable standard curve, it displays both the curve and its equation, otherwise it displays the reason for the error.

Further Information

Further information on the error term, standard curves, Stick Gain, and quantitation (including step-by-step examples of the calculations) is available from Molecular Devices Technical Support.

Chapter 7– System Troubleshooting

Introduction

This chapter contains solutions to possible problems encountered during routine use of the Threshold System (workstation, Reader, diagnostics, filtering, general assay signals and results). The following tables list effects, with possible causes, along with solutions for each cause.

For problems with the computer, monitor or printer, please consult the appropriate user manual.

If you need further assistance, call Molecular Devices Technical Support. Please have this information available so that we can help you efficiently:

- Reagent Kit Lot number.
- Workstation serial number.
- Reader serial number.
- Exact nature of the problem.

Note: *Please obtain a Returned Goods Authorization Number from Molecular Devices before returning materials or parts.*

Effects, Possible Causes and Solutions Workstation

Table 7-1: Workstation Troubleshooting

Effect	Possible Cause	Solution	Reference
Threshold Enterprise / Software Cannot Communicate With the Workstation.	The Workstation is off.	Turn the Workstation ON and make sure the green "power" light on the Workstation is lit. To check communications in Threshold Enterprise / Software, select the **Instrument** Menu and choose **Test**.	Chapter 2, Chapter 5
	The fuse.	If the Workstation is turned ON, but the green "power" light is not on, replace the fuse as described in Chapter 3, "Operating Procedures and Maintenance."	Chapter 3
	The cable connecting the computer to the Workstation is unplugged.	Make sure the cable between the computer and the Workstation is plugged in securely on both ends. To check communications in Threshold, select the **Instrument** Menu and choose **Test**.	Chapter 2
	Incorrect communications port specified in Threshold Enterprise / Software.	In Threshold Enterprise / Software, select the **Settings** menu and choose **Preferences**. Check that the communication port that is chosen under **Preferences** is the same port that the Workstation is connected to, on the back of the computer.	Chapter 2
Liquid is Running Out the Bottom of the Workstation.	There is leakage from either the tubing or the reservoir inside the Workstation.	Turn the Workstation OFF and call Molecular Devices Technical Support. **Caution: If the liquid is a biohazard, contain the spill and disinfect, as needed.**	

Reader

Table 7-2: Reader Troubleshooting

Effect	Possible Cause	Solution	Reference
A "Reader not connected" Error Message Opens.	The Reader contains much less than 30 ml of fluid.	Fill the Reader to the fill mark, as described in Chapter 3, "Operating Procedures and Maintenance."	Chapter 3
	The Reader is not completely pushed into the Reader housing.	Push the Reader into the Reader housing until it clicks into place.	Chapter 3
	Gold contacts in the Workstation housing are bent or broken.	Call Molecular Devices Technical Support.	
	The Reader is malfunctioning.	Replace the Reader with a new, hydrated Reader.	
The Reader Leaks Substrate (other than from the 4 openings On Top of the Reader).	If Reader S/N >R000-6000, the gasket may have been incorrectly aligned.	Empty the Reader, rinse it with deionized water, open the Reader, and reassemble it. Make sure the gasket is properly aligned and the screws appropriately tightened.	Chapter 3
	If Reader S/N >R000-6000, the Reader may have been overfilled.	Empty the Reader and check that it was not overfilled (over 30 ml substrate). Overfilling may cause the Reader to leak.	
	If Reader S/N <R000-6000, the Reader is defective.	Replace the Reader.	

Self-Diagnostics

Table 7-3: Self-Diagnostics Troubleshooting

Effect	Possible Cause	Solution	Reference
Reader Leakage "Fail"	The Reader is wet.	Any moisture on the gold contacts of the Reader or Workstation will cause a "Reader Leakage" error. Dry the Reader and the Workstation housing thoroughly: 1. Remove the Reference Electrode from the Reader by holding its white cap. Do not pull the wire attached to the electrode. Be careful not to drip liquid on the Reader or into the Workstation housing. 2. Put the electrode in the storage tube on the Workstation or in a beaker of 1X Wash Solution. Keep the electrode immersed at all times. Make sure the ceramic tip of the electrode is fully hydrated. 3. Release the Reader by pressing the triangular button to the left. Remove the Reader from the Workstation. 4. Carefully wipe the Reader dry with a laboratory wipe, especially around the recessed, gold-plated electrical contacts on the front of the unit. 5. Very carefully wipe the inside of the Workstation housing dry with a laboratory wipe, **only wiping downward** so as not to damage the gold contacts. 6. Reinsert the Reader in the Workstation housing making sure the Reader clicks into place, taking care not to spill any substrate. 7. Reinsert the Reference Electrode. 8. In Threshold Enterprise / Software, select the **Instrument** menu and choose **Test** to run the diagnostic tests again. If the "Reader Leakage" message persists, either the Reader is still wet or defective, or the Reference Electrode is defective. Try a new Reader. If the new Reader gives the same error message, the Reader housing of the Workstation may still be damp. Try drying the Workstation housing with warm, but not hot, air (e.g., from a hair dryer). **Caution: Keep the Workstation and the outside of the Reader absolutely dry at all times. Liquids and salts can short out the electrical contacts. Always blot the wet end of a stick before putting it in the Reader. When taking a stick out of the Reader, remove it slowly, being careful not to drip Substrate Solution on the Reader or into the Workstation housing.**	Chapter 3
	The Reference Electrode is defective.	Try refilling the Reference Electrode, replacing the clear PVC tube on the Reference Electrode, or replacing the whole Reference Electrode. See Reference Circuitry "Fails"7 , below.	Chapter 3

Table 7-3: Self-Diagnostics Troubleshooting

Effect	Possible Cause	Solution	Reference
"Silicon Photoresponse Fails"	The Reader is not full.	Check that the Reader is full by drawing out the Substrate Solution. If it is less than 30 ml, add more Substrate Solution.	Chapter 3
	The Reader is incorrectly inserted.	Push the Reader down until it clicks into place.	
	One of the nine holes on the front of the Reader (between the contacts) has fluid or other foreign matter in it.	Remove the Reader, without spilling liquid, and check that all nine holes are dry and clear of foreign matter. Clear out any material by gently blowing with compressed air (canned air).	
	The Reference Electrode is defective or needs filling.	1. Remove the Reference Electrode from the Reader, check the liquid level and fill, if necessary, as described in Chapter 3, "Operating Procedures and Maintenance." 2. If the ceramic junction is clogged or discolored, replace the PVC tube with the spare one provided in the original electrode kit box and hydrate it fully before testing again.	Chapter 3
	The silicon sensor is contaminated with protein (see "Assay Signals and Results" on page 7-10).	Replace the Reader, or call Molecular Devices Technical Support.	
	The silicon sensor in the Reader has failed.	Use a new Reader, or call Molecular Devices Technical Support.	
Reference Circuitry "Fails"	The Reference Electrode is not fully inserted into the Reader and plugged into the Workstation.	Check that the Reference Electrode is fully inserted into the Reader and that the cable is plugged into the back of the Workstation. In Threshold Enterprise / Software, select the **Instrument** menu and choose **Test**. If the error message persists, see next possible cause.	Chapter 2 Chapter 3
	The Reader is not fully inserted into the Workstation.	Press down on the Reader until it clicks into place. In Threshold Enterprise / Software, select the **Instrument** menu and choose **Test**. If the error message persists, see next possible cause.	Chapter 2 Chapter 3
	Reference electrode liquid is old, or level is low.	Refill the electrode's PVC tube with KCl-AgCl solution as described in Chapter 3, "Operating Procedures and Maintenance." In Threshold Enterprise / Software, select the **Instrument** menu and choose **Test**. If the error message persists, see next possible cause.	Chapter 3

Table 7-3: Self-Diagnostics Troubleshooting

Effect	Possible Cause	Solution	Reference
	The Reader is not filled with Substrate Solution.	Make sure the Reader is filled with Substrate Solution. Withdraw Substrate Solution from the Reader using a 30-ml syringe; if there is less than 30 ml of fluid, add more substrate to the Reader. See Chapter 3, "Operating Procedures and Maintenance," for instructions on withdrawing and adding fluid to the Reader. In Threshold Enterprise / Software, select the **Instrument** menu and choose **Test**. If the error message persists, see next possible cause.	Chapter 3
	The PVC tube of the Reference Electrode is bad.	Replace the PVC tube as described in Chapter 3, "Operating Procedures and Maintenance." In Threshold Enterprise / Software, select the **Instrument** menu and choose **Test**. If the error message persists, see next possible cause.	Chapter 3
	The Reference Electrode is clogged.	Clean the Reference Electrode by soaking the electrode tip in warm deionized water to remove any material that may be clogging the ceramic junction. In Threshold Enterprise / Software, select the **Instrument** menu and choose **Test**. If the error message persists, see next possible cause.	
	The Reference Electrode is defective.	Replace the Reference Electrode. Remove the Reference Electrode from the Reader and unplug it from the back of the Workstation. Hydrate a new Reference Electrode and install it as described in Chapter 3, "Operating Procedures and Maintenance." In Threshold Enterprise / Software, select the **Instrument** menu and choose **Test**. If the error message persists, see next possible cause.	Chapter 3
	The Reader is defective.	Replace the Reader. Hydrate a new Reader for one hour with 30 ml of Substrate solution and insert it in the Workstation. In Threshold Enterprise / Software, select the **Instrument** menu and choose **Test**. If the error message does NOT appear, the first Reader may be defective.	Chapter 3

Filtering

Table 7-4: Filtering Troubleshooting

Effect	Possible Cause	Solution	Reference
One or More of the Samples Takes More Than 30 Minutes to Filer AND the * and #Samples Filer in Less Time.	A component of the sample (probably protein) is clogging the membrane.	If the other samples in the filter unit have filtered correctly, remove the slow or non-filtering samples and disregard any measurement at these positions. **Caution: When a stick contains one or more samples that do not filter within 30 minutes, non-specifically captured protein can be transferred to the silicon sensor when the stick is read. Read a blank stick immediately after reading any stick, with slow-filtering samples, to determine if the Reader has become contaminated. Any position producing greater than 10 µV/sec for a blank stick indicates possible contamination and the Reader should be cleaned, see "Assay Signals and Results" on page 7-10 "Rates on a blank stick are greater than 10µV/sec".**	
All Samples (including * and #) Take More Than 30 minutes to Filter AND the "Filtering" Indicator Light is Blinking.	There is no stick in the filter base and block.	Assemble the filter unit with a stick.	Chapter 3
	A filter base has been left on a vacuum port without a stick or a filter block.	Remove the base.	Chapter 3
	The stick membrane has a hole or tear.	Cover the opening in the top of the filter block, for the sample positions where there is a hole or leak, with 2 layers of Parafilm, or remove the stick.	
	The vacuum pump or sensor is malfunctioning.	Call Molecular Devices Technical Support.	
	The vacuum tubing connecting the Workstation and auxiliary manifold is leaking.	Inspect for holes and replace the tubing, as necessary.	

Table 7-4: Filtering Troubleshooting

Effect	Possible Cause	Solution	Reference
After Turning the Vacuum OFF, the Screen Reports "Vacuum/ Drain" for More Than 3 to 4 Minutes, or the Collected Filtrate Drains Very Slowly or Not at All.	The waste tubing from the Workstation is obstructed or crimped.	Check that the tubing is not twisted or crimped and that liquid is flowing freely through the tubing. Clear obstructed tubes.	
	The Auxiliary Manifold is not turned on.	Turn the Auxiliary Manifold on.	Chapter 2
	The level sensor or the drain pump is malfunctioning.	Call Molecular Devices Technical Support.	

Assay Signals and Results

Table 7-5: Assay Signals and Results Troubleshooting

Effect	Possible Cause	Solution	Reference
Rates On a Blank Stick are Less Than -10 µV/sec.	The blank stick is not fully hydrated.	1. Put the stick in a beaker of 1X Wash Solution at room temperature for a further 10-15 minutes. 2. Re-read the blank stick.	Chapter 3
	The Wash Solution or Substrate Solution is not at room temperature.	1. Put the blank stick in a beaker of 1X Wash Solution. 2. Read the blank stick again when the Wash and Substrate Solutions have equilibrated to room temperature.	
	There is a pH difference between the Wash Solution used to equilibrate the blank stick and the Substrate Solution in the Reader.	1. Remove the Substrate Solution from the Reader and check for a pH difference in comparison to the Wash Solution used to hydrate the blank stick. 2. Add 30 ml of new Substrate Solution to the Reader and equilibrate for 15 minutes. 3. Hydrate a blank stick in the same Wash Solution used to prepare the new Substrate Solution. 4. Read the blank stick.	
	The Reference Electrode is malfunctioning.	See *"Self-Diagnostics" on page 7-6*	Chapter 2 Chapter 3

Table 7-5: Assay Signals and Results Troubleshooting

Effect	Possible Cause	Solution	Reference
	Protein contamination of the Reader.	**If the Reader S/N is >R000-6000:** 1. Clean the Reader: remove the substrate from the Reader, open it and clean the silicon sensor and titanium plunger. 2. Close the Reader, refill with the substrate and read a fresh hydrated blank stick. If the rates do not increase above -10 µV/sec call Molecular Devices technical support.	Chapter 3
		If the Reader S/N is < R000-6000: 1. Rehydrate a new blank stick and read it. In many cases, the contaminating material transfers to the membrane of the blank stick which cleans the silicon surface. If the signal for the second blank stick is higher than the first blank reading, but less than -10 µV/sec, hydrate and read additional blank sticks until the rates are above -10 µV/sec. 2. If the rates do not increase above -10 µV/sec, the silicon sensor will need to be cleaned. NOTE: Readers below serial number R000-6000 cannot be opened for cleaning and should be cleaned using the enzymic cleaner, hemazyme. Call Molecular Devices Technical Support for more information.	Chapter 3
The Rates on a Blank Stick areGreater Than 10 µV/sec.	There is a pH difference between the Wash Solution used to equilibrate the blank stick and the Substrate Solution in the Reader.	1. Remove the Substrate Solution from the Reader and check for a pH difference in comparison to the Wash Solution used to hydrate the blank stick. 2. Add 30 ml of new Substrate Solution to the Reader and equilibrate for 15 minutes. 3. Hydrate a blank stick in the same Wash Solution used to prepare the new Substrate Solution. 4. Read the blank stick.	Chapter 3

Table 7-5: Assay Signals and Results Troubleshooting

Effect	Possible Cause	Solution	Reference
	The stick or the Reader is contaminated with urease.	**If the Reader S/N is >R000-6000:** 1. Clean the Reader: remove the substrate from the Reader, open it and clean the silicon sensor and titanium plunger. 2. Close the Reader, refill with the substrate and read a fresh hydrated blank stick. If the rates do not drop below 10 µV/sec call Molecular Devices technical support.	Chapter 3
		If the Reader S/N is < R000-6000: 1. Rehydrate a new blank stick and read it. In many cases, the contaminating material transfers to the membrane of the blank stick which cleans the silicon surface. If the signal for the second blank stick is lower than the first blank reading, but greater than 10 µV/sec, hydrate and read additional blank sticks until the rates are below 10 µV/sec. 2. If the rates do not drop below 10 µV/sec, the silicon sensor will need to be cleaned. NOTE: Readers below serial number R000-6000 cannot be opened for cleaning, and should be cleaned using the enzymic cleaner, hemazyme. Call Molecular Devices Technical Support for more information.	Chapter 3
TheSignal Values for One or Two Spots on aTest Stick are -300 µV/sec or Lower.	There are bubbles in the Substrate Solution in the Reader.	Reread the stick: 1. Remove the stick and place it in a beaker of 1X Wash Solution for 1 to 2 minutes. 2. Read the stick again. **Caution: Re-reading a stick may give inaccurate results. (This does not apply to blank sticks).**	
Standard Curve Slope is Negative, Erratic, and/or Not Quantitating Correctly.	A previous standard curve is still being used. The present standard curve has not been "loaded" into memory.	1. Check that the signals on Stick § agree with those on the data stick containing the standards. 2. If the signals are not the same, in Threshold Enterprise / Software, select **Copy Stick to Standard** from the **Edit** menu. This will copy the data on the data stick to Stick § and replace the previous standard curve.	Chapter 5
Standard Curve Slope is Negative, Erratic, and/or Not Quantitating Correctly (continued).	The wrong calibrator values are entered on stick §.	Enter the correct calibrator values in the "value" fields on stick §.	
Very Poor Assay Precision; Coefficient of Variation (CV) is Consistently Greater Than 20% for Raw Signals.	There may be a problem with the Reader, assay chemistry, pipetting devices, or technique.	Call Molecular Devices Technical Support.	

Chapter 8– Enterprise Administrator

Introduction

Enterprise Administrator is used in conjunction with Enterprise-versioned software from Molecular Devices that provides tools for compliance with the FDA 21 CFR Part 11 Final Rule. Enterprise Administrator allows an administrator to create an administered group file that contains user accounts. Each user account holds the user's name, a unique User ID and password combination, and permissions that may limit access to specific features of the Enterprise-versioned software. The Threshold Enterprise administrator links Threshold Enterprise to the administered group file using the "**Set Administration File...**" menu item in the **Security** menu. The linked group file is then used, when a user logs onto Threshold Enterprise, to validate the user's identity and to retrieve the associated permission information.

Users have only limited access to the features of Threshold Enterprise until they have logged onto Threshold Enterprise. User permissions for Threshold Enterprise are discussed in detail in "A Guide to User Permissions" on page 8-9.

This chapter focuses on the steps that an administrator should follow to set up and maintain user accounts for Threshold Enterprise. Note that Enterprise Administrator may have been modified after publication of this manual. The description that follows is included here for reference purposes only. A more comprehensive overview of Enterprise Administrator is provided in the *Enterprise Administrator User Manual* accompanying Enterprise Administrator.

Using Enterprise Administrator

Enterprise Administrator is a Microsoft Windows™ 2000/XP/7/10 application that an administrator uses to set up and maintain user accounts. This chapter presents the basic skills and concepts needed to use Enterprise Administrator with Threshold Enterprise.

Enterprise Administrator can be operated by using the mouse or keyboard. In this chapter, instructions are provided for using the mouse, with keyboard alternatives in parentheses.

Menus can be selected with a single click of the left mouse button, or with the keyboard shortcut keys. Shortcut keys are underlined letters in the menus. To use a shortcut key, hold down the ALT key to activate the Menu Bar, then press the underlined character. Text fields and drop-down menus may be selected with a single click of the left mouse button, or by using the keyboard. Press TAB to move forward and SHIFT+TAB to move backward between fields. Use the up and down arrow keys to scroll through lists.

Enterprise Administrator Logon

When Enterprise Administrator is launched, the **Administrator Logon** dialog opens (Figure 8-1).

Figure 8-1: The Administrator Logon Dialog

To log on, the administrator follows these steps:

1) Enter an Admin User ID and Admin Password.
2) Review the default database file showing in the **Database Name** text box.
3) To select a different database, click the **...** (Browse) button. An **Open** dialog opens.
4) Select the desired database file.
5) Click OK.

Note: *If an incorrect entry has been made, an* ***Open Database*** *warning dialog opens.*

Creating a New Database File

More than one database file can be used with Enterprise Administrator. For example, separate database files can be used for different Molecular Devices' software packages.

To create a new database, the administrator follows these steps:

1) Open Enterprise Administrator and log on.
2) Click the **Create Database** button in the **Administrator Logon** window. This opens the **Create Database** window. (Figure 8-2)

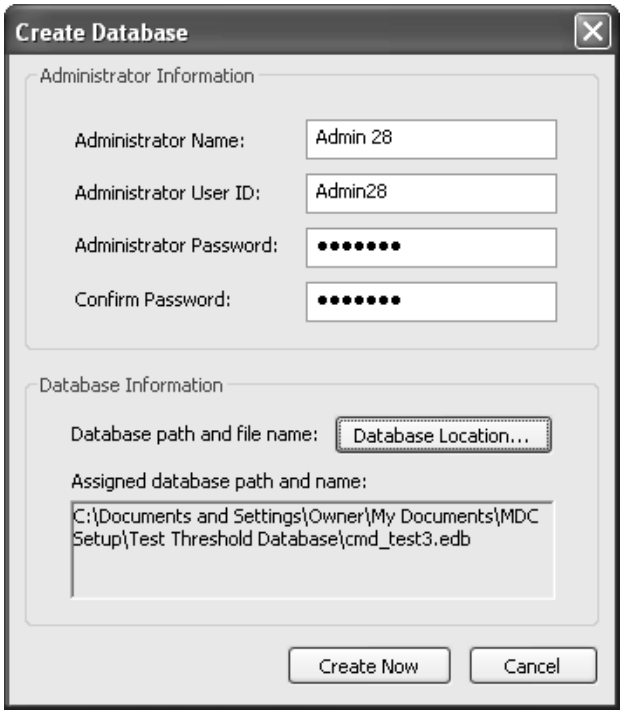


Figure 8-2: The Create Database Window

3) Enter the Administrator Name, User ID and Password. *(The password must be at least 6 characters long and include 1 number and 1 letter.)*
4) Click the **Database Location…** button to select the location for storing the database. This opens a **Save As** dialog.
5) Name the new database file and click **Save**.
6) Click **Create Now**. A **Database Creation** confirmation dialog opens when the database is successfully created.
7) Click **OK** to complete this process.
8) Click **Done** to exit Enterprise Administrator.

The Enterprise Administrator Main Screen

Enterprise Administrator's main screen provides access to four pages: Users, Templates, Report, and License. The administrator uses the four pages to make administrative changes to selected user database files. (Figure 8-3)

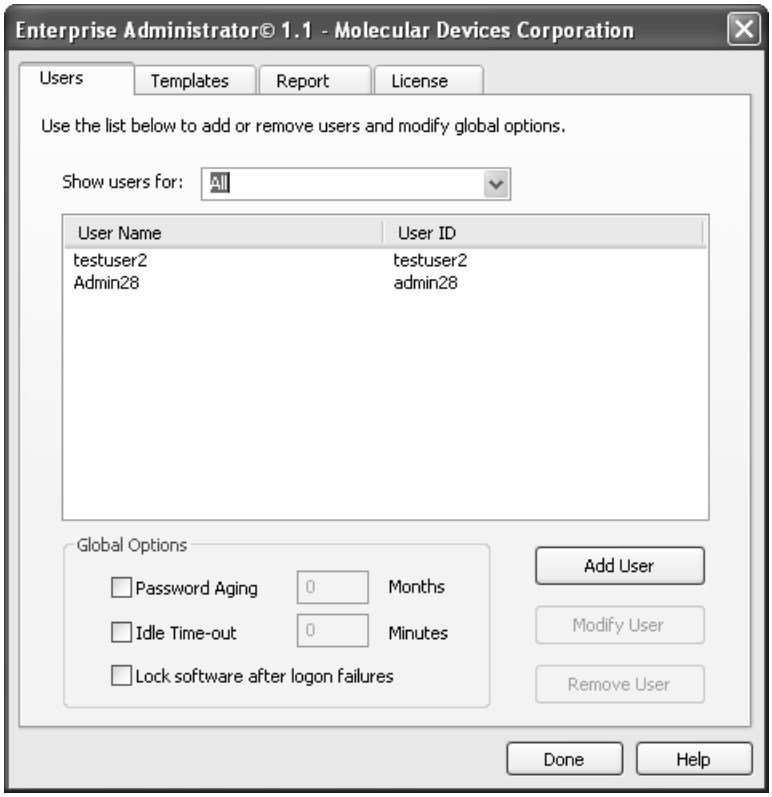


Figure 8-3: The Enterprise Administrator Main Screen

The Users Page

The function of the Users Page is to add new users, remove existing users and set global logon options (Figure 8-3). This page is also used to modify the permissions of existing users. The main window of the Users Page shows the user names and IDs for the accounts defined in a selected database. Initially, all user accounts are shown. If more than one Molecular Devices' software package is being administered with the selected database, the administrator can restrict the user list to show only the user accounts for one application. For example, to show users for Threshold Enterprise, the administrator selects **Threshold** in the **Show users for:**drop-down menu.

Setting Up a New User Account

To add a new user to the database:

1) Open Enterprise Administrator and log on.
2) On the default **Users Page**, click **Add User**. This opens the **User Information** dialog (Figure 8-4).

Figure 8-4: The User Information Dialog

3) Enter the User's name, a unique user ID and initial password. *This information must then be provided to the new user so that he / she can log on to Threshold Enterprise.*
4) Select the **Password Option** if you want the new user to be forced to change his/her initial password the first time he / she logs on.
5) Click **Next.** This opens the **Software Information** dialog (Figure 8-5).

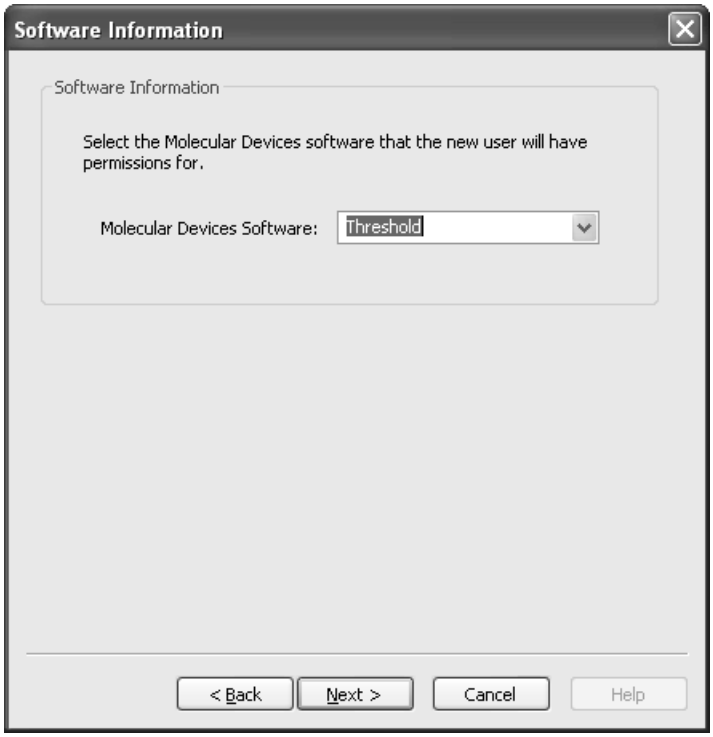


Figure 8-5: The Software Information Dialog

6) Select **Threshold** from the **Molecular Devices Software:** drop-down menu.
7) Click the **Next** button. This opens the **User Permissions** dialog (Figure 8-6).

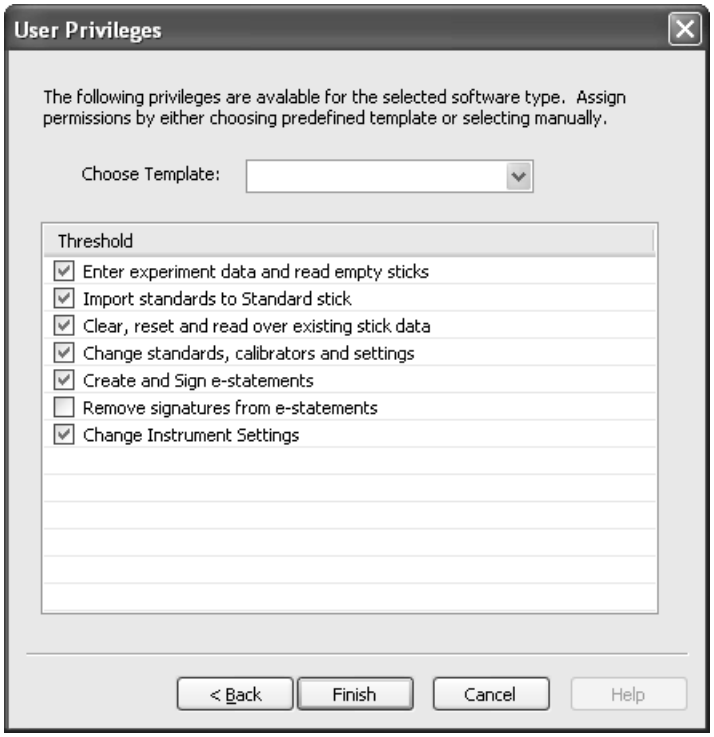


Figure 8-6: The User Permissions Screen

8) Define a new user's permissions by either:
   - Selecting one of the predefined templates in the **Choose Template** drop-down menu, or
   - Manually selecting each permission.
9) Click **Finish**, or step back through the previous windows to view and revise the settings.
10) Click **Done** to exit Enterprise Administrator.

A Guide to User Permissions

User Permissions define which Threshold Enterprise features are available to a user account. Seven permissions are available.

No Permissions

- Allows a user to open, view, print and save data files with new names or to new folders.
- Does not allow a user to modify a file.

Enter experiment data and read empty sticks

- Allows a user to perform all the basic functions that are necessary to run an assay.
- Allows a user to change text information in the **Experiment Information** window and **Stick Display** windows.
- Allows a user to read new sticks and copy stick data to the Standard Stick.

Note: *This permission is important for people running assays in the laboratory.*

Import standards to Standard stick

- Provides access to the **Import Standard Curve…** selection in the **File** menu.
- Allows a user to import a new standard curve.

Clear, reset and read over existing stick data

- Provides access to the **Paste to Stick, Clear Stick Data**, and **Reset Stick** selections in the **Edit** menu.
- Allows a user to change existing stick data without reading over the stick.

Change standards, calibrators and settings

Provides access to the **Analysis**, **Stick Count**, **Enable Spike Recovery Report** and **Preferences** selections in the **Settings** menu.

- Allows a user to change the **Standard Curve Fit** and **Analysis Units**.
- Allows a user to change the **Stick Count**.
- Allows a user to include a **Spike Recovery Report** in the file.
- Allows a user to change the preferences for new data files.

Note: *This permission is important for users that create new assay files.*

Create and Sign e-statements

- Provides access to the **Sign Document** and **Add Confirmation Signature** selections in the **Security** menu.
- Allows a user to add a signed e-statement to a file, or add a confirmation signature to a document.

Remove signatures from e-statements

- Provides access to the **Remove Signatures** selection in the **Security** menu.
- Allows a user to remove all signatures and e-statements from a file.

Change Instrument Settings

- Allows a user to change internal parameters in the instrument.

Note: *This permission should only be provided to expert users that need to run custom assays.*

Modifying An Existing User Account

Note: ***Modify User*** *is not supported by Windows 10. To make a user change,* ***Remove User*** *and then* ***Add User****. The changes save in the Audit Trail.*

To modify an existing user account:

1) Open Enterprise Administrator and log on.
2) Go to the **Users Page**.

3) Highlight the user's name.
4) Click the **Modify User** button. This opens the **Modify User** dialog (Figure 8-7).

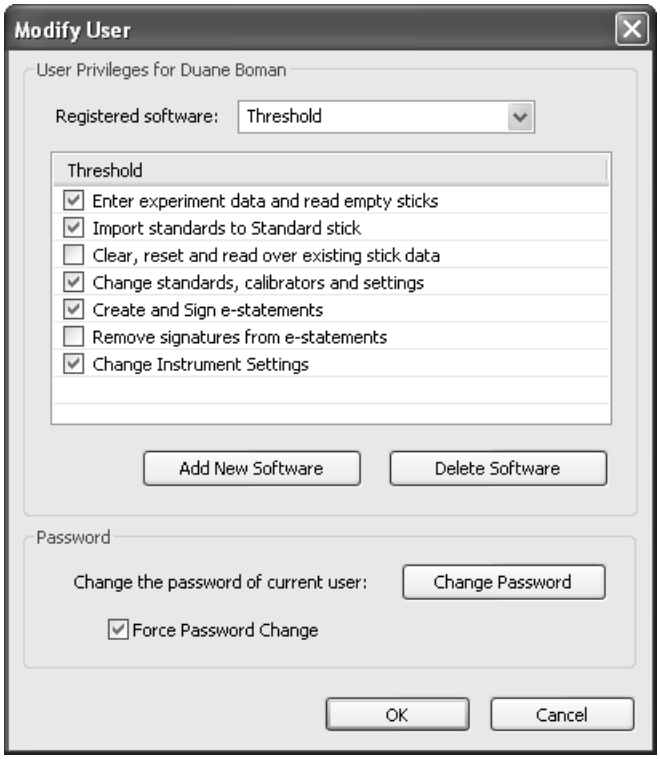


Figure 8-7: The Modify User Dialog

5) Check that **Threshold** is selected in the **Registered Software** drop-down menu.
6) Make any of the following changes to a user's account:
   - Add access to another Molecular Devices' software package.
   - Delete access to a Molecular Devices' software package.
   - Modify the list of permissions.
   - Change a user's password.
   - Force a user to enter a new password the next time he / she logs on to Threshold Enterprise.
7) Click **OK**.
8) Click **Done** to exit Enterprise Administrator.

Removing a User Account

To remove an existing user account:

1) Open Enterprise Administrator and log on.
2) Go to the **Users Page**.
3) Highlight the user's name.

4) Click the **Remove User** button.
5) The **Remove User** warning dialog opens (Figure 8-8).
6) Click **OK** to remove the user account from the database, or click **Cancel** to cancel this action.
7) Click **Done** to exit Enterprise Administrator.

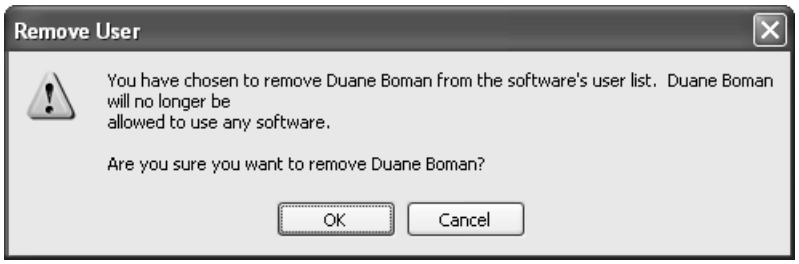


Figure 8-8: The Remove User Warning Dialog

Setting Global Options

The administrator can set three logon options that apply to ***all*** users in a database file: Password Aging, Idle Time-out and Lock software after logon failures. (Figure 8-9).

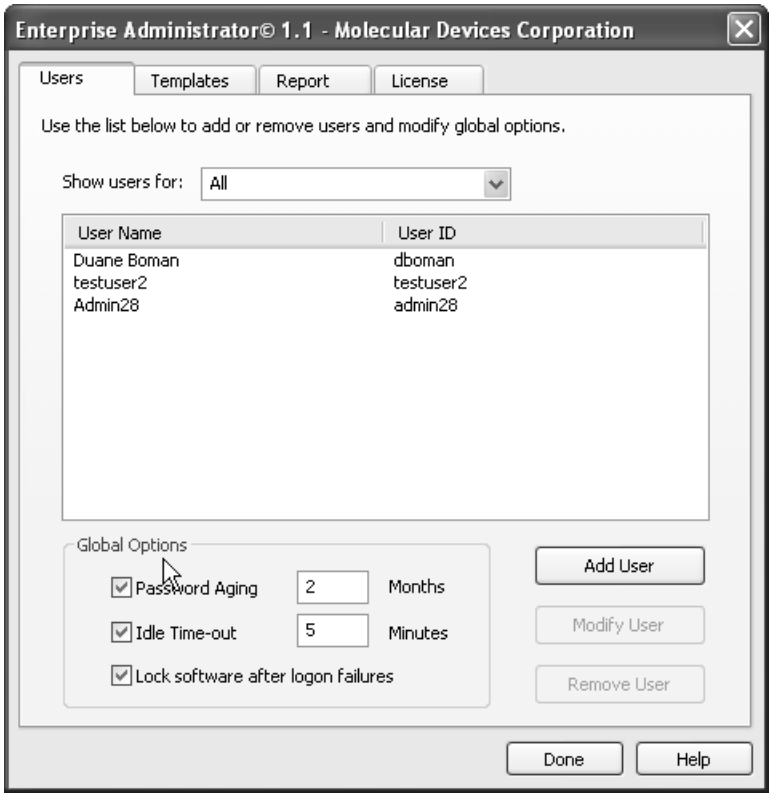


Figure 8-9: The Global Logon Options Screen

Password Aging

To set Password Aging for all users in a database file:

1) Open Enterprise Administrator and log on.
2) Go to the **Users Page**.
3) Click the **Password Aging** check box.
4) In the text box provided, enter the number of months after which a users' password will expire. *Users are warned about their password expiration 15 days prior to the expiration date.)*
5) Click **Done** to exit Enterprise Administrator.

Idle Time-out

To set Idle Time-out for all users in a database file:

1) Open Enterprise Administrator and log on.
2) Go to the **Users Page**.
3) In the **Global Options** area, click the **Idle time-out** check box.
4) In the text box provided, enter the number of minutes after which user sessions will expire.
5) Click **Done** to exit Enterprise Administrator.

Lock Software

By checking this option, users will be locked out of Threshold Software if they are unable to log on within three attempts. Once users are locked out, they will need to contact the administrator to have their accounts reactivated.

To set Lock Software for all users in a database file:

1) Open Enterprise Administrator and log on.
2) Go to the **Users Page**.
3) Click the check box named: **Lock software after logon failures**.
4) Click **Done** to exit Enterprise Administrator.

The Templates Page

Templates are sets of predefined permissions. Templates simplify the task of providing permissions for groups of users that should have similar accounts. The Templates page is used to create, modify and remove templates for Molecular Devices' software applications (Figure 8-10).

Figure 8-10: The Templates Page

Creating a New Template

To create a new template:

1) Open Enterprise Administrator and log on.
2) Go to the **Templates Page**.
3) Click **Add Template**. This opens the **Add Template** dialog (Figure 8-11).

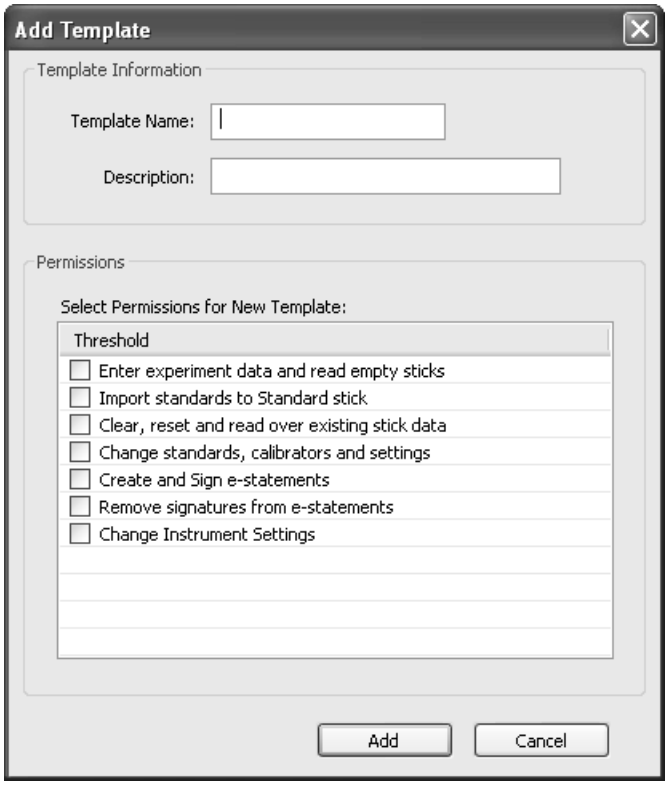


Figure 8-11: The Add Template Screen

4) Select **Threshold** from the **Show Templates for Software:** drop-down menu.
5) In the **Template Name** text box, enter a name for the new template.
6) In the **Description** text box, enter a short description of the new template.
7) Click the appropriate boxes to select the permissions desired.
8) Click **Add** or **Cancel**, to cancel this action
9) Click **Done** to exit Enterprise Administrator.

Editing an Existing Template

To edit a template:

1) Open Enterprise Administrator and log on.
2) Go to the **Templates Page**.
3) Highlight the template to be edited.
4) Click **Edit Template**. This opens the **Edit Template** dialog (Figure 8-12).

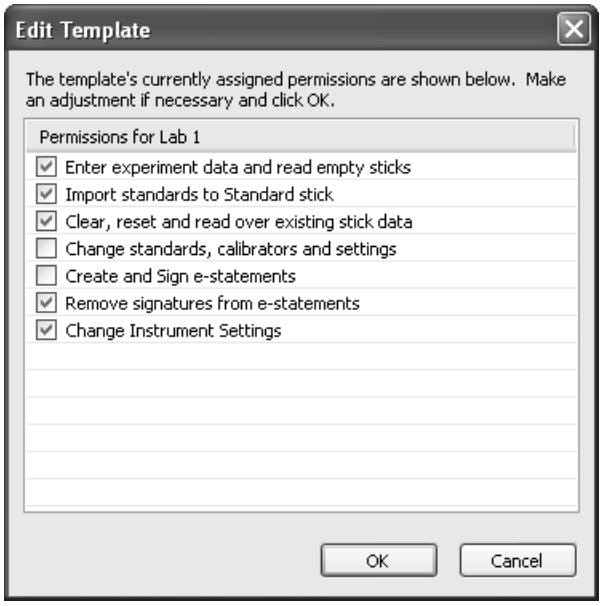


Figure 8-12: The Edit Template Screen

5) Click or unclick the appropriate boxes to select, or deselect, the permissions.
6) Click **OK**, or **Cancel**, to cancel this action.
7) Click **Done** to exit Enterprise Administrator.

Deleting a Template

To delete a template:

1) Open Enterprise Administrator and log on.
2) Go to the **Templates Page.**
3) Highlight the template to be deleted.
4) Click **Delete Template**. This opens the **Delete Template** dialog(Figure 8-13).

Figure 8-13: The Delete Template Dialog

5) Click **OK**, or **Cancel**, to cancel this action.
6) Click **Done** to exit Enterprise Administrator.

The Report Page

Two reports are available in Enterprise Administrator: User Information Report and Administrator Audit Trail. The User Report provides a list of users and their user names, and optionally their permission levels. The Audit Trail lists all operations that have been performed on the administrator database.

Generating a User Information Report

To generate a user information report on Threshold Enterprise users:

1) Open Enterprise Administrator and log on.
2) Go to the **Report Page**. (Figure 8-14)

Figure 8-14: The Report Page

3) In the **Export Options** area, select **Threshold** from the drop-down menu.
4) Select, or leave blank, **Include user name and user ID**.
5) Select, or leave blank, **Include user permission information**.
6) Click **Export**. This opens the **Save As** dialog. (Figure 8-15).

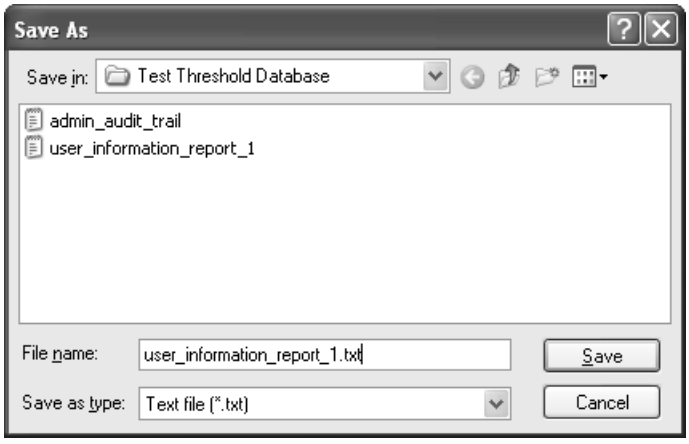


Figure 8-15: The User Information Save As Dialog

7) Name the file and select where the report is to be saved.
8) Click **Save**.
9) Click **Done** to exit Enterprise Administrator.

Generating an Administrator Audit Trail

To generate an audit trail report of all administrator actions:

1) Open Enterprise Administrator and log on.
2) Go to the **Report Page**. (Figure 8-15)
3) In the **Audit Trail** area, click **View Audit Trail**. This opens the **Audit Trail** window (Figure 8-16).

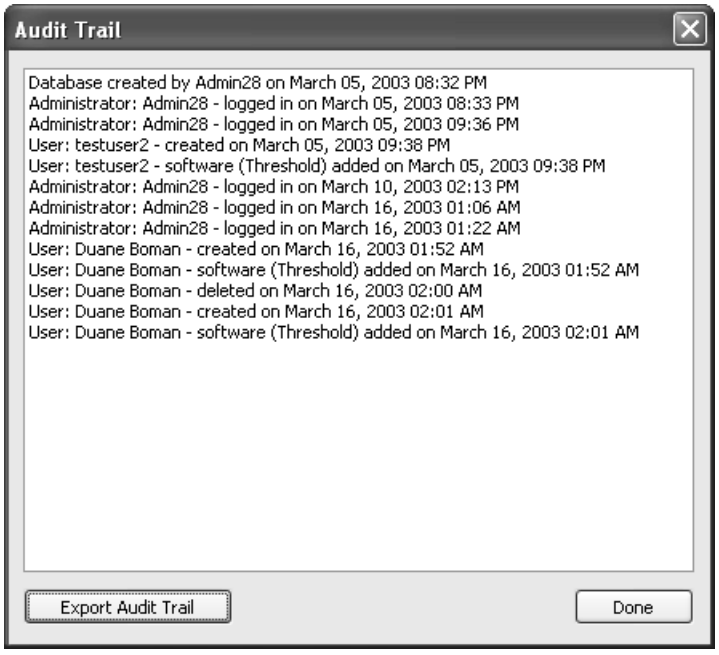


Figure 8-16: The Audit Trail Window

4) Click **Export Audit Trail**. This opens a **Save As** dialog. (Figure 8-17).

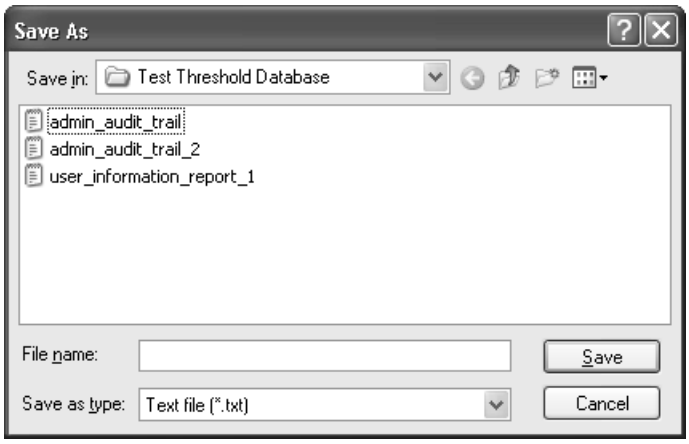


Figure 8-17: The Audit Trail Save As Dialog

5) Name the file and select where the report is to be saved.
6) Click **Save**.
7) Click **Done**.
8) Click **Done** to exit Enterprise Administrator.

The License Page

The License Page helps the administrator keep track of license information for Molecular Devices' software applications. This page shows information on licenses that have been entered into Enterprise Administrator. (Figure 8-18).

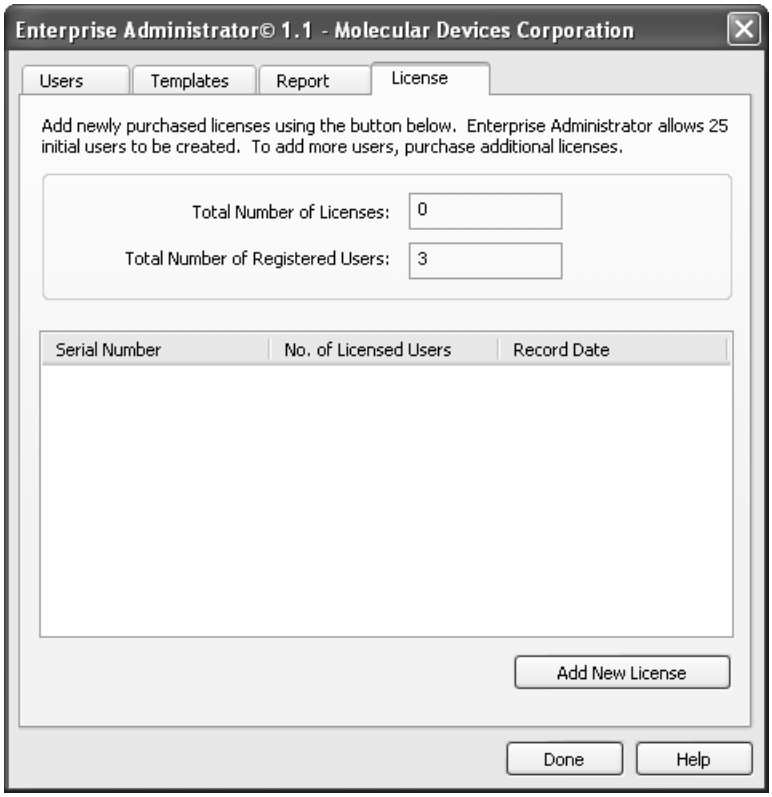


Figure 8-18: The License Page

The certificate number is provided by Molecular Devices with the purchase of Enterprise Administrator. This license can be used for up to 25 user accounts. For additional accounts, an additional license must be purchased. Further information on licensing options is available in the *Enterprise Administrator User Manual 1.1*.

Adding a New License

To add a new license:

1) Open Enterprise Administrator and log on.
2) Go to the **License Page**.
3) Click **Add New License**. This opens a New **License** dialog.
4) Enter the certificate number provided by Molecular Devices in the **Certification Number** box. (Figure 8-19).

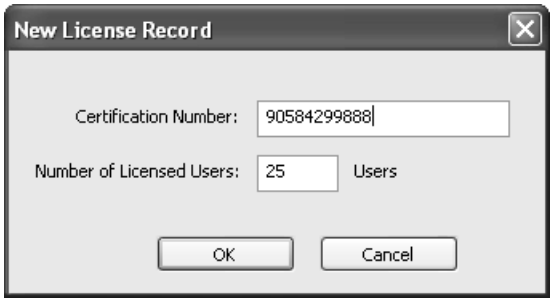


Figure 8-19: The New License Record Screen

5) Enter the number of licensed users (*up to 25)* in the **Number of License Users** box.
6) Click OK.
7) Click **Done** to exit Enterprise Administrator.

Linking Threshold Enterprise to a User Database

When Threshold Enterprise is first installed on a computer, the administrator must select the appropriate user account database from within Threshold Enterprise. Prior to this step, the user database must be created, using Enterprise Administrator.

User accounts in the database can be added, modified and removed after this initialization. Global options can also be modified after initialization.

To select a user database for Threshold Enterprise:

1) Open the **Threshold Enterprise** application.
2) Select the **Security** drop-down menu.
3) Click **Set Administration File…**
4) An Open dialog opens (Figure 8-20).

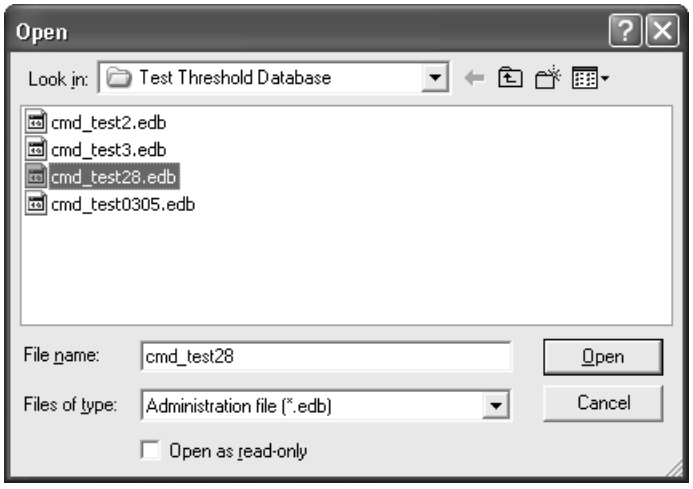


Figure 8-20: Open Dialog

5) Find the appropriate database file and click **Open**.

8

6) The **Log on as Permissions Administrator** dialog opens.

Figure 8-21: Log On as Permissions Dialog

7) Enter your Administrator ID and password.
8) Click **OK** to complete this action.

Chapter 9– Immuno-Ligand Assay Introduction

The Immuno-Ligand Assay

The Threshold Immuno-Ligand Assay (ILA) allows the user to perform a wide range of immunoassays, using the Threshold System. Immunoassays can measure a diverse range of analytes including drugs, proteins, micro-organisms for biopharmaceutical development and production, and for clinical diagnostics. Samples range from fermentation supernatants, samples from a purification process and other biochemical preparations, to serum or other bodily fluids. Traditional methods for performing immunoassays include EIA (enzyme immuno assay), ELISA (enzyme linked immunosorbant assay), and RIA (radioimmunoassay). The ILA is based on these traditional immunoassay techniques. ILA applications include interaction of antibodies with antigen, receptor with ligand, and lectin with carbohydrate. Theoretically, the ILA applies to any analyte-binding protein reaction meeting the following conditions:

- The reaction occurs with high affinity.
- Both analyte and binding proteins are soluble.
- Binding protein may be labeled with biotin or fluorescein for sandwich format assay, and the analyte may be labeled with biotin or fluorescein for the competitive format assay.

This chapter discusses stages of the ILA, format, protocols, assay configuration, and pretreatment of samples to be used in the ILA. The following table (Table 9-1 on page 3) contains the terminology used to describe the Immuno-Ligand Assay in this manual.

Table 9-1: ILA Terminology

Term	Meaning
Analyte	The analyte is the substance being measured in the ILA. The analyte may be an antigen, an antibody, a ligand, a receptor, or other proteins.
Asssay Format	Format of an ILA may be either sandwich or competitive.
Assay Protocol	ILA protocol may be either simultaneous incubation or sequential incubation.
Assay Configuration	Configuration of the ILA refers to which assay component is fluoresceinated and which assay component is biotinylated.
Binding Protein	The binding proteins bind specifically to the analyte. The binding proteins are most frequently antibodies, although they may also be receptors, an antigen, or a peptide.

Stages of Immuno-Ligand Assay

Immunoassays are either, sandwich assays where the analyte reacts with an excess of binding proteins or, competitive where labeled and unlabeled analyte compete for limited binding proteins. The Threshold ILA Assay may use either sandwich or competitive format. All immunoassays (both sandwich and competitive) consist of 4 stages:

- Reaction
- Separation
- Detection
- Analysis

These 4 stages of the Threshold ILA are illustrated in Figure 9-1. The analyte-binding protein complex forms in liquid phase during the reaction stage. The reaction complex is separated from free binding protein and captured on a nitrocellulose membrane by filtration. Detection takes place in the Threshold Reader, which measures the change in pH caused by the hydrolysis of urea by urease.

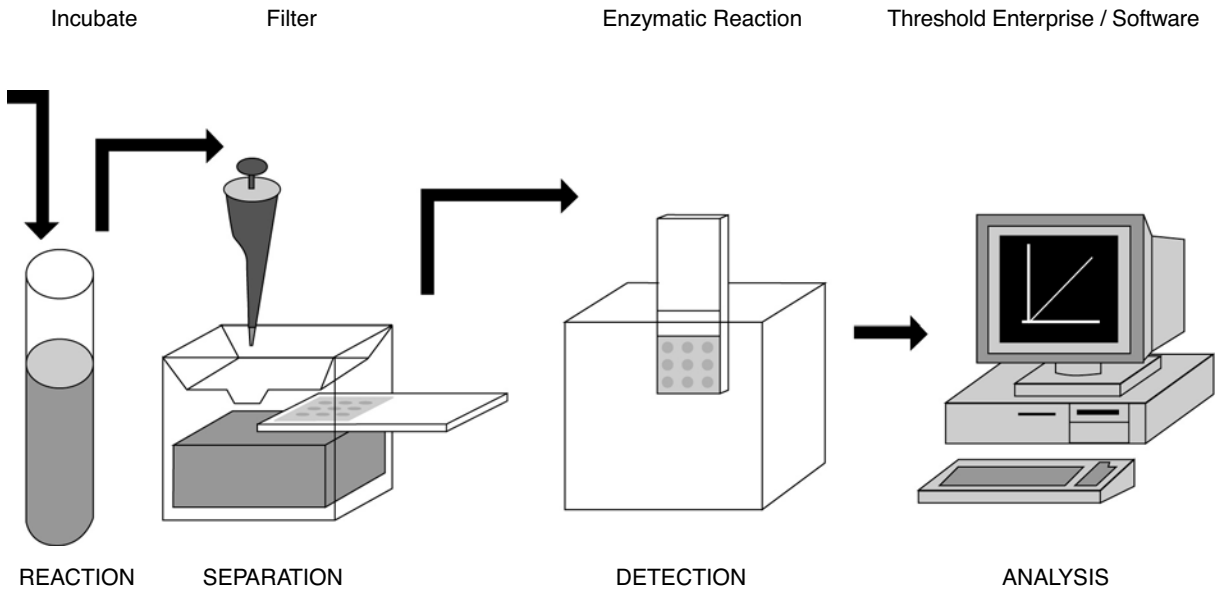


Figure 9-1: The Four Stages of the Threshold ILA

Reaction Stage

The ILA reaction stage involves formation of a complex between the analyte, binding proteins, streptavidin, and an anti-fluorescein:urease conjugate. The binding proteins and/or the analyte are labeled with biotin and fluorescein, thus providing the necessary links for complex formation with streptavidin and anti-fluorescein:urease. During the reaction stage, the components are in liquid phase, which allows the analyte and the binding proteins to interact in their native conformations.

Separation Stage

The ILA assay separates and concentrates the reaction complex onto a nitrocellulose membrane during the separation stage. The capture mechanism for ILA is the binding between biotin and streptavidin (which has multiple binding sites for biotin). Both the capture antibody and the nitrocellulose membrane are biotinylated. Streptavidin is added to the reaction mixture and reacts with the biotinylated antibody. When the reaction mixture is filtered on the Threshold Workstation, the reaction complex is actively captured on the membrane by the binding of biotin to streptavidin. This binding is very rapid and has a very high affinity (10^{-15}). Following filtration, the membrane is washed to remove nonspecifically-bound anti-fluorescein:urease.

Detection Stage

After the capture of the reaction complex on the nitrocellulose membrane, the sticks are removed from the filtration units and placed in the Threshold Reader. The ILA measures enzyme activity. The hydrolysis of urea by urease produces an increase in pH that is measured as a change in potential at the silicon sensor surface. The rate of change of surface potential is proportional to the amount of analyte present (Chapter 1, "The Threshold System.")

Analysis Stage

Threshold Enterprise / Software analyzes the detected signal and provides quantitation.

ILA Assay Format

An ILA may use either a sandwich or a competitive format and two protocols, simultaneous or sequential incubation, that can be used with both formats. The appropriate format and protocol of the assay depend on the analyte and binding proteins as well as on the assay requirements. This section discusses how to select the format and protocol for an assay. For discussion purposes, assays will be discussed using antibodies as the binding proteins and an antigen as the analyte.

Sandwich Assay Format

Traditional sandwich assays, such as an ELISA, involve immobilizing a "capture" antibody onto a solid support, such as a 96-well microplate. The antigen is added and binds to this capture antibody. A second antibody is added to bind the captured antigen. The second antibody can be conjugated to an enzyme for detection, or a third antibody-enzyme conjugate is added to bind to the second antibody.

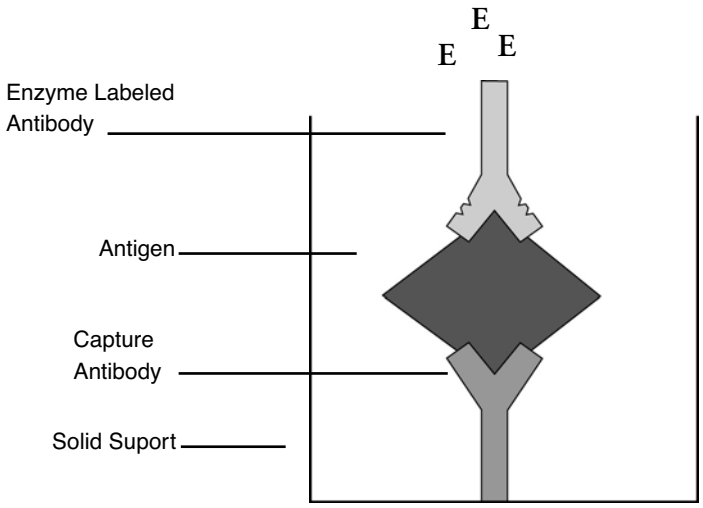


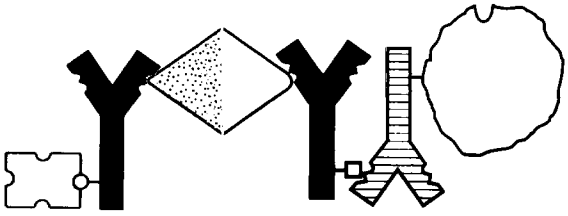
Figure 9-2: A Two-Site Sandwich ELISA

In the ILA, antigen and labeled antibodies (analyte and binding proteins) are combined in liquid phase. The antibodies are labeled with biotin (for capture) or fluorescein (for detection). The Enzyme Reagent (anti-fluorescein:urease) is also added to the reaction mixture at this time in a simultaneous incubation protocol.

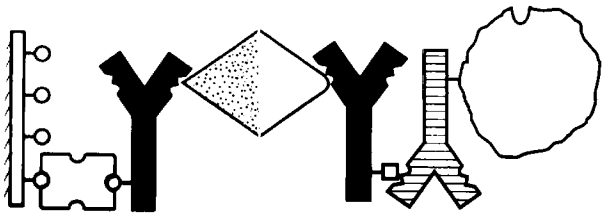
The ILA concentrates and separates the reaction complex onto a nitrocellulose membrane in the separation stage. The reaction complex is captured by the interaction between streptavidin and biotin. The membrane is washed to remove nonspecifically-bound complexes and free binding protein. In a sequential incubation protocol, the Enzyme Reagent is added after the complex is captured on the membrane.

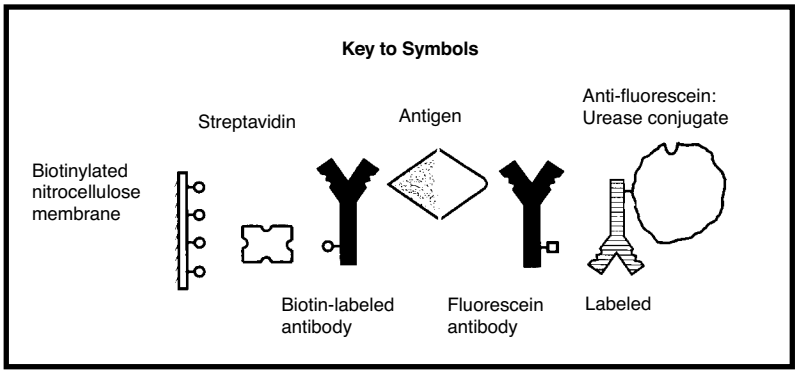
The ILA measures the reaction complexes enzymatically. The turnover of urea by urease causes an increase in pH that is measured as change in potential at the surface of a silicon sensor.

Reaction Stage



Separation Stage





Reaction Stage: streptavidin, b-Ab, Ag, f-Ab, and anti-fluorescein:urease bind to form this complex in solution.

Separation Stage: the complex is captured onto the biotinylated nitrocellulose membrane during filtration.

Figure 9-3: Threshold ILA Sandwich Format

Competitive Assay Format

Traditional competitive assays, such as RIAs, involve labeling an antigen with a radioactive isotope. A constant amount of radio-labeled antigen is combined with unlabeled antigen (sample or calibrator) and a limited amount of antibody in the reaction mixture. All components of the reaction mixture may be in liquid phase or one component may be immobilized on a solid support, such as a bead. Following an incubation period, the reaction complex is separated from unincorporated radiolabeled material. The reaction complex is detected by measuring the radioactivity. The signal generated will be inversely proportional to the amount of unlabeled analyte present. The radioactivity decreases with increasing concentrations of sample analyte.

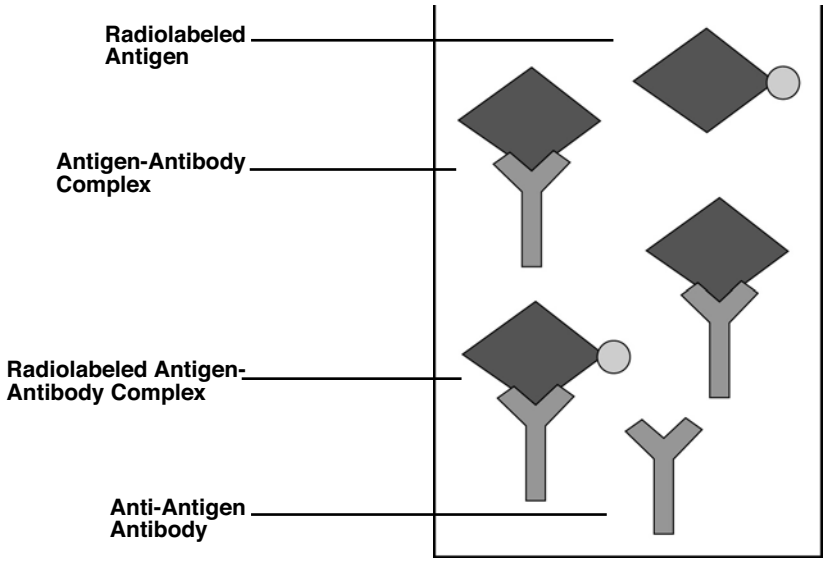
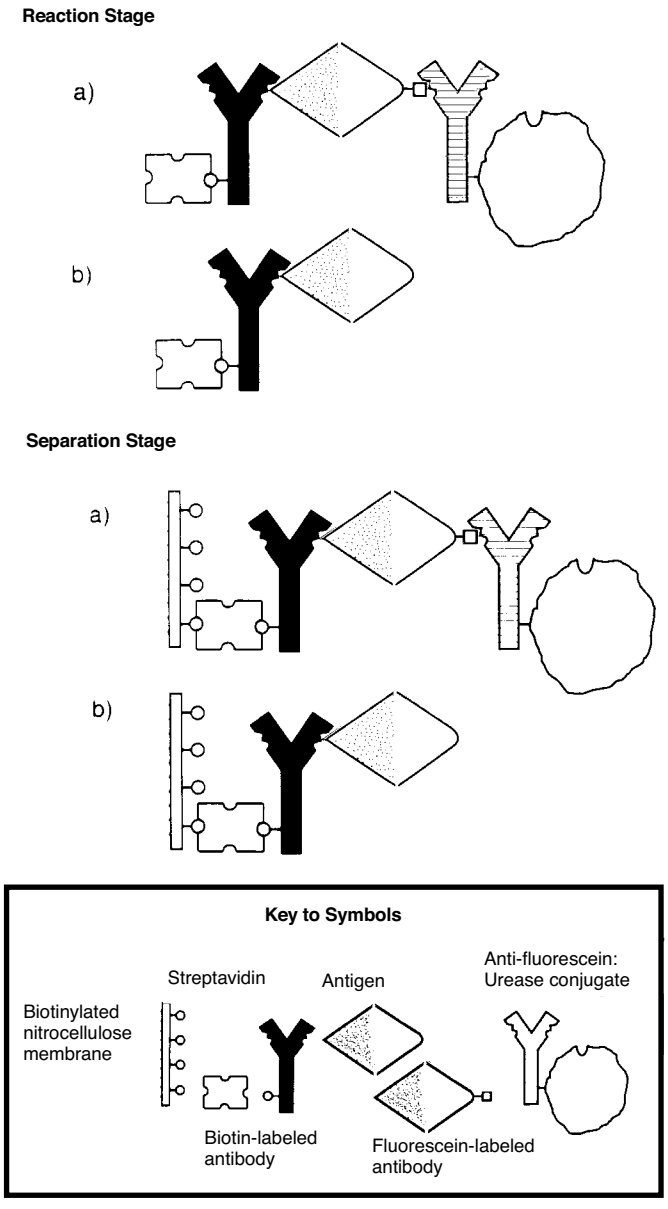


Figure 9-4: A Radioimmunoassay (RIA)

The competitive format of the ILA has two configurations. The antigen can be labeled with fluorescein for detection, and the antibody is labeled with biotin for capture; or the reverse, with the antigen labeled with biotin and the antibody is labeled with fluorescein. For both configurations, the enzymatic signal is inversely proportional to the amount of sample antigen: the assay rate decreases with increasing concentration of sample antigen.

Unlabeled Antigen Competing With Fluoresceinated-Antigen For Biotinylated-Antibody



Reaction Stage: streptavidin, b-Ab, f-Ag, Ag, and anti-fluorescein:urease interact in solution.
Following Incubation: two types of complexes are present.
Separation Stage: both complexes are captured onto the nitrocellulose membrane.

Figure 9-5: Fluoresceinated-Antigen/Biotinylated-Antibody Configuration

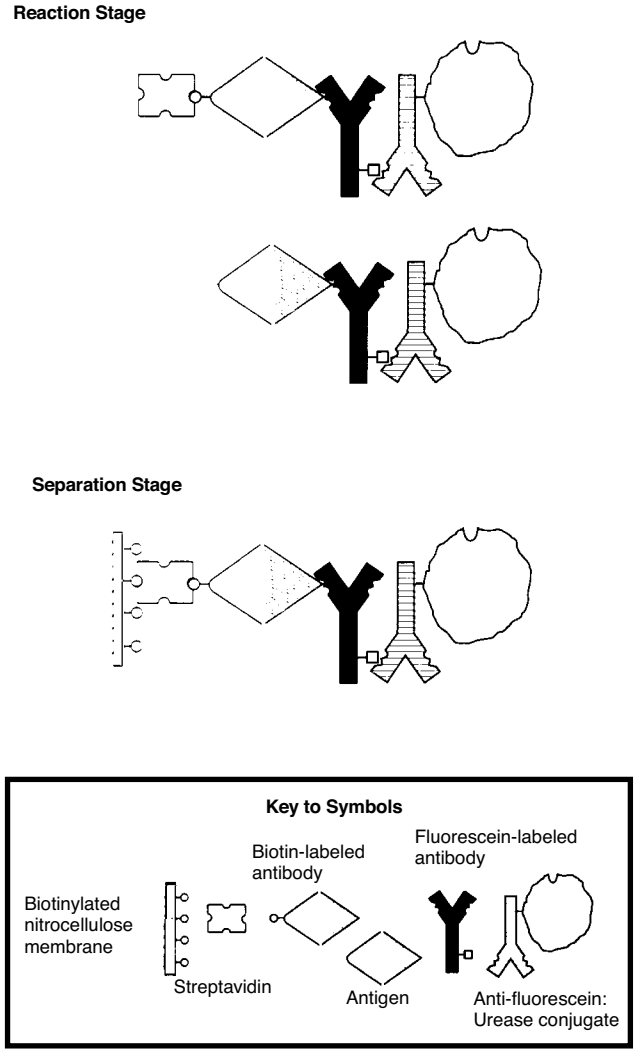
This configuration of the competitive format uses the antigen labeled with fluorescein (f-Ag) and the antibody labeled with biotin (b-Ab). Unlabeled antigen in the test sample competes with the constant amount of f-Ag to bind the limited amount of b-Ab.

Two types of complexes form in the reaction stage:

- One contains streptavidin, b-Ab, f-Ag, and anti-fluorescein:urease.
- The other complex contains only streptavidin, b-Ab, and unlabeled Ag.

The filtration step captures both complexes onto the biotinylated nitrocellulose membrane, but only the complex that contains the enzyme (anti-fluorescein:urease) is detected. The enzyme-containing complex predominates when the amount of unlabeled antigen (sample) is low. Conversely, when the amount of unlabeled antigen is high, little of the enzyme-containing complex forms.

Unlabeled Antigen Competing With Biotinylated-Antigen For Fluoresceinated-Antibody



Reaction Stage: streptavidin, Ag, b-Ag, f-Ab, and anti-fluorescein:urease interact in solution.
Following Incubation: two types of complexes are present.
Separation Stage: only the biotin-labeled complex is captured onto the nitrocellulose membrane.

Figure 9-6: Biotinylated-Antigen/Fluoresceinated-Antibody Configuration

This configuration of the competitive format uses the antigen labeled with biotin (b-Ag) and the antibody labeled with fluorescein (f-Ab). Unlabeled antigen in the test sample competes with a constant amount of b-Ag to bind with a limited amount of f-Ab.

Two types of complexes form in the reaction stage:

- One contains streptavidin, b-Ag, f-Ab, and anti-fluorescein:urease.
- The other complex contains Ag, f-Ab, and anti-fluorescein:urease. This second complex does not contain streptavidin because it does not contain b-Ag.

The filtration step captures only the complex containing streptavidin. The complex containing streptavidin predominates when the unlabeled antigen (sample) is low. Conversely, when the amount of unlabeled antigen is high, little of the streptavidin containing complex forms.

Choosing Sandwich or Competitive Format

The design of the ILA involves several considerations, the most important of which are: the number (and type) of antibodies available, the size of the antigen, and the number of independent epitopes on the antigen. Other considerations include purity of the reagents to be labeled, functional needs of the assay, and optimization of the assay.

Number (and Type) of Antibodies and Epitopes

The first consideration is the size of the antigen and the number of epitopes on the antigen. If the antigen is so small that only one antibody can bind, or there is only one available epitope (or determinant) for an antibody to bind, then the competitive configuration must be used. If there is a single repeating epitope or multiple epitopes to which antibodies may bind without substantial steric hindrance, then the sandwich configuration can be used.

The second consideration is the number and type of antibodies available. If there is only a single monoclonal antibody available, then the competitive configuration must be used. If there is (1) a single polyclonal, (2) one monoclonal and one polyclonal, (3) two monoclonals, or (4) two polyclonal antibodies available and the antigen is polyvalent, then the sandwich configuration is most frequently used.

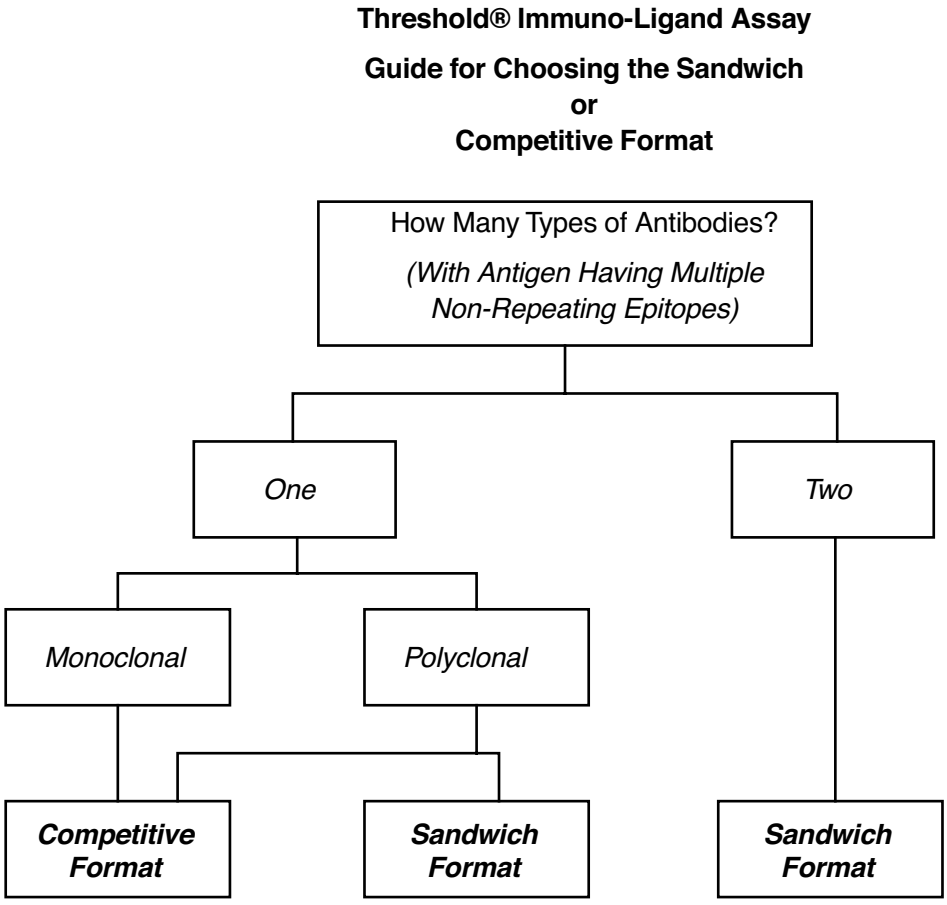


Figure 9-7: Guide for Choosing the Sandwich (Noncompetitive) or Competitive Format

Other Considerations

Other arrangements of assay components are possible. For example, the sandwich format can detect specific antibody as the analyte, using antigen and anti-immunoglobulin as the two binding proteins. If class-specific anti-immunoglobulin is used, this approach can detect the immunoglobulin class of a specific immune response.

Functional needs may sometimes dictate the assay design. For example, the competitive format can directly demonstrate antigenic identity between proteins, while the sandwich format may not. The competitive format may be preferred for analyzing the structure of a recombinant protein designed for use as a vaccine. The protein that mimics the antigenicity of the native protein should have maximum biological effectiveness.

Additional factors contributing to choice of the final assay design are presented in Chapter 10, “General ILA Procedure.” Chapter 12, “ILA Labeling Procedure,” discusses the purity of the reagents labeled with biotin or fluorescein and the effectiveness of labeling a

protein. Chapter 13, "ILA Sandwich Format Optimization," and Chapter 14, "ILA Competitive Format Optimization," discuss optimal use of the biotin-labeled and fluorescein-labeled proteins, incubation conditions, and dynamic range of the standard curve.

ILA Assay Protocols

There are two protocols for any format of the ILA:

- Simultaneous incubation of reactants.
- Sequential incubation of reactants.

Note: *The previous version of the manual referred to simultaneous incubation as Scheme A, and sequential incubation as Scheme B.*

Simultaneous Incubation Protocol

In the simultaneous incubation, the complex forms in solution, using the following protocol:

- Incubate the antigen and labeled antibodies together.
- Add Enzyme Reagent (anti-fluorescein:urease) to the mixture and incubate.
- Add Capture Reagent (streptavidin).
- Filter reaction complex onto nitrocellulose membrane on low vacuum.
- Wash on high vacuum.
- Read the stick.

The simultaneous incubation protocol can only be used when the fluoresceinated binding protein is greater than 90% specific to the analyte. If the binding protein is an antibody, then it must be either an immuno-affinity purified polyclonal or a monoclonal antibody. This protocol offers the advantage of incubating all the reactants together and forming a complete complex. To capture the complete complex, a single filtration of the reaction mixture is required, followed by a wash step.

The simultaneous protocol may be used only when the fluoresceinated binding protein does not exceed the anti-fluorescein:urease conjugate in each test. The concentration of the anti-fluorescein:urease is limited to minimize background rates. If the fluoresceinated binding protein exceeds the concentration of the anti-fluorescein:urease conjugate, the probability that each analyte/binding protein complex will contain a urease conjugate molecule is decreased and will decrease the observed signal. Limiting the fluoresceinated binding protein will also reduce the potential dynamic range of the assay. Sandwich assays require the analyte to be the only rate-limiting component, with the binding proteins in excess.

Note: *When using the simultaneous incubation and an antibody as the binding protein,* ***the fluoresceinated antibody should not exceed 25 ng per test*** *(approximately 167 x 10^{-12} moles /test). The biotinylated antibody should not exceed 8 µg per test (approximately 53 x 10^{-9} moles /test) to assure that the streptavidin remains in excess.*

Sequential Incubation Protocol

In the sequential incubation, a partial complex is formed in solution and captured on

the membrane. Complex formation is completed on the membrane:

1) Incubate the antigen and labeled antibodies together.
2) Add Capture Reagent (streptavidin) to the mixture.
3) Filter partial complex onto the nitrocellulose membrane on low vacuum.
4) Wash on high, and turn the vacuum OFF.
5) Add the Enzyme Reagent (anti-fluorescein:urease), and filter on low.
6) Wash on high vacuum.
7) Read the stick.

The sequential incubation protocol can be used when the fluoresceinated binding protein is less than 90% pure. This protocol allows use of higher concentrations of the fluoresceinated binding protein during the initial incubation with the analyte. The higher concentrations of binding protein can increase the potential dynamic range of the assay. Increased binding protein concentration can also enhance the binding kinetics to allow shorter incubation times with greater sensitivity.

The possibility of a sample component adversely affecting the enzyme activity is eliminated when using the sequential incubation protocol, because the anti-fluorescein:urease conjugate is not incubated with the sample. The sequential incubation protocol will also prevent any nonspecific signal that can occur if a sample component binds non-specifically to the enzyme conjugate. An example is Rheumatoid factor in human serum and plasma samples that non-specifically binds antibodies, and therefore can bind the anti-fluorescein:urease conjugate, causing a false positive signal.

The sequential incubation protocol requires two filtration steps. The first filtration captures the binding protein/analyte complex onto the nitrocellulose membrane, followed by a wash. The anti-fluorescein:urease conjugate is then filtered through the membrane to bind to the fluoresceinated binding protein contained in the captured complex, followed by a wash.

Note: *When using the sequential protocol and an antibody as the binding protein,* ***the fluoresceinated binding protein can exceed 25 ng per test*** *(approximately 167 x 10^{-12} moles /test). The biotinylated antibody should not exceed 8 µg per test (approximately 53 x 10^{-9} moles /test) to assure that streptavidin remains in excess.*

Comparison of Simultaneous and Sequential Incubation Protocols

Table 9-2: Simultaneous and Sequential Incubation Protocol Comparison

Steps	Simultaneous Incubation	Sequential Incubation
Mix reagents	Sample and labeled proteins	Sample and labeled proteins
Incubate	Time and temperature as optimized	Time and temperature as optimized
	Add 0.1 ml undiluted Enzyme Reagent	———
Incubate	1 hour at room temperature	———
Streptavidin	Add diluted Capture Reagent	Add diluted Capture Reagent
Filtering (capture)	Low vacuum	Low vacuum
Wash	Add 2 ml Wash Solution	Add 2 ml Wash Solution
	High vacuum	High vacuum
		Add 1 ml diluted Enzyme Reagent
		Low vacuum
		Add 2 ml Wash Solution
		High vacuum
Remove and read each stick		

Pretreatment

Samples may contain components that can interfere with the accurate measurement of the analyte of interest. Pretreatment of the sample may be required to remove the interference. A known amount of analyte should be added to a sample prior to pretreatment, then the sample is tested for recovery of the added analyte. This will evaluate the effectiveness of the pretreatment method and assure that the analyte is not reduced by the procedure. Substances in a biological sample that may interfere with the ILA include amino acids, carbohydrates, organic solvents, urine, protein denaturants, salts, and detergents.

Physical Interference

Particles and large aggregates in samples can slow the rate of flow, or clog the biotinylated nitrocellulose membrane (0.45 µm) on the stick. To prevent this, filter serum and other samples with a 0.22 µm "low protein-binding" polysulfone, PVDF, or cellulose acetate filter, before using them in the assay. Use disposable filter devices for microcentrifugation for higher volume recovery.

Chemical Interference

Specimens may need treatment to adjust the pH or ionic strength to optimal conditions (pH 7.0 in 150 mM NaCl for most analytes and binding proteins) or to remove interfering substances (such as chaotropic ions). Extremes in pH and high concentrations of salts can affect the binding reactions between the analyte and binding proteins, streptavidin and biotin, and the anti-fluorescein:urease and fluorescein. Samples may require pH adjustment, dilution or dialysis to allow optimum binding between analyte and binding protein. Monoclonal antibodies may have different pH optima, and the assay buffer may be modified to accommodate this requirement.

Note: *The pH range of the Assay Buffer is 6.8 to 7.2. If needed, adjust the sample solution to this pH range.*

Protein Effects

High concentrations of protein can interfere with ILA. The problem is a physical barrier to diffusion of immuno-reactants, which delays the formation of complexes. The results may bias quantitation. Possible remedies include:

- Diluting the sample.
- Increasing the concentration of labeled antibodies.
- Longer incubation for the reaction stage.
- Switching from simultaneous incubation protocol to sequential incubation protocol.

Biochemical and Serological Effects

Specimens containing serum may have constituents that bind the analyte, binding protein, streptavidin, or anti-fluorescein:urease conjugate.

Human serum may contain Rheumatoid factor or antibodies to immunoglobulins of other species (heterophile antibodies). For example, a high background might result from human anti-murine Ig antibodies, which could bind both biotin-labeled and fluorescein-labeled murine antibodies and form a bridge in a complete complex with streptavidin and anti-fluorescein:urease conjugate. The complex would form in the absence of analyte. This problem can be corrected by immunoabsorption of the human serum samples or by adding unlabeled protein, in this case murine immunoglobulin, to block the unwanted reaction.

Adding a carrier protein to the Assay Buffer may be useful to avoid nonspecific binding of the immunoreactants.

Tested Potential Sources of Interference

The following compounds have been tested for interference, using a murine IgG assay. The reported results are the maximum potential contaminant levels that allow greater than 80% recovery of added analyte. Samples of 100 µl were tested in both the simultaneous and sequential incubation protocols. These results are applicable only to the Threshold ILA.

Table 9-3: Immuno-Ligand Assay Interfering Substances

Compound	Simultaneous Incubation Sample Content >80% Spike Recovery	Sequential Incubation Sample Content >80% Spike Recovery
Salts		
Magnesium Chloride	20 mM	100 mM
Potassium Chloride	125 mM	300 mM
EDTA	50 mM*	100 mM
Sodium Chloride	300 mM	400 mM
Ammonium Sulfate	100 mM	150 mM
Protein Denaturants		
Guanidine-HCl	100 mM	200 mM
Potassium Thiocyanate	100 mM	100 mM
Urea	30 mM	1.0 M
Sodium Sulfite	75 mM	100 mM* (90% spike recovery)
Detergents		
CHAPS	0.75% (w/v)	1.0% (w/v)
N-Octyl Glucopyranoside	0.80% (w/v)	1.0% (w/v)

Table 9-3: Immuno-Ligand Assay Interfering Substances (Continued)

Compound	Simultaneous Incubation Sample Content >80% Spike Recovery	Sequential Incubation Sample Content >80% Spike Recovery
SDS	0.05% (w/v)	0.05% (w/v)
Triton X-100	1.5% (v/v)	2.0%* (v/v) (90% spike recovery)
Tween-20	1.0% (v/v)	1.0%* (v/v) (90% spike recovery)
Zwittergent 3-12	0.8% (w/v)	1.0% (w/v)
Amino Acids		
Arginine	100 mM	100 mM
Glutamine	250 mM* (90% spike recovery)	250 mM* (90% spike recovery)
Glycine	500 mM* (90% spike recovery)	500 mM* (90% spike recovery)
Histidine	60 mM	80 mM
Lysine	75 mM	100 mM (increase in background signal)
Carbohydrates		
Mannitol	5% (w/v)	5%* (w/v) (85% spike recovery)
Sorbitol	5%* (w/v) (85% spike recovery)	5%* (w/v) (90% spike recovery)
Sucrose	5%* (w/v) (85% spike recovery)	5%* (w/v) (85% spike recovery)
Trehalose	5%* (w/v) (85% spike recovery)	5%* (w/v) (85% spike recovery)
Organic Solvents		
Dimethylsulfoxide	3% (v/v)	4%* (v/v) (100% spike recovery)
Dimethylformamide	3% (v/v)	4%* (v/v) (100% spike recovery)
Other		
Biotin	10 ng	10 ng
Phenol Red	0.001% (w/v)	0.005%* (w/v) (100% spike recovery)

Table 9-3: Immuno-Ligand Assay Interfering Substances (Continued)

Compound	Simultaneous Incubation Sample Content >80% Spike Recovery	Sequential Incubation Sample Content >80% Spike Recovery
Urine (pooled)	5% (v/v)	50% (v/v)
Thimerosal	Not tested	0.005%* (w/v) (>90% spike recovery)
Gelatin	Not tested	0.05% (w/v) (>90% spike recovery)

* The maximum level tested

Chapter 10– General ILA Procedure

Introduction

This chapter describes materials provided in the Immuno-Ligand Assay (ILA) detection and labeling kits, materials provided by the customer, reagent preparation, sample preparation and assay performance. Starting the system, and preparing the Threshold Reader and Reference Electrode are discussed in Chapter 3, "Operating Procedures and Maintenance."

ILA Kit Components

There are two reagent kits required for the Immuno-Ligand Assay: the ILA Labeling Kit and the ILA Detection Kit.

ILA Labeling Kit

The ILA Labeling Kit contains the reagents stated below (Table 10-1).

Table 10-1: Threshold ILA Labeling Kit

Reagent	Quantity
N-hydroxysuccinimide ester of Biotin-DNP	2 vials, lyophilized
N-hydroxysuccinimide ester of Fluorescein	2 vials, lyophilized

ILA Detection Kit

The ILA Detection Kit contains reagents for 192 determinations (See Table 10-2).

Before using the kit:

1) Inspect the contents of the kit for shipping damage, discoloration, or cloudiness.
2) Do not use the kit, if it is defective or if it is expired.
3) Bring all the reagents to room temperature, before performing the assay.

Table 10-2: Threshold ILA Detection Kit

Reagent	Contents	Quantity
Assay Buffer Concentrate (10X)	PBS, BSA, and Triton X-100	1 bottle, solution
Wash Concentrate (10X)	PBS, Tween 20	1 bottle, solution
Capture Reagent	Streptavidin	1 bottle, lyophilized
Enzyme Reagent	Anti-Fluorescein:Urease	6 bottles, lyophilized
Substrate Concentrate	Urea	1 bottle, solution
Threshold Stick	Biotinylated nitrocellulose Nitrocellulose	24 assay sticks 6 blank sticks
Universal Standard Reagent	Fluorescein, biotin-labeled BSA	1 bottle, lyophilized
Filter Blocks and Bases*		8 blocks/bases per kit

*Sold separately from ILA Detection Kit

Materials Provided by the User

ILA Labeling

The following materials are required to perform an ILA assay.

- Protein to be labeled.
- Dimethylformamide (DMF, Part #20673, Pierce Chemical).
- Sephadex® G-25 columns (PD-10 columns, Pharmacia).
- Dialysis tubing (appropriate molecular weight cut-off, for antibodies the molecular weight cut-off is 14,000).
- Phosphate Buffered Saline (PBS: 10 mM Phosphate 150 mM NaCl, pH 7.0).
- Spectrophotometer.
- 4 syringes, 1 ml.
- 2 hypodermic needles (between 18-21 gauge).
- Precision pipets and disposable tips.
- Positive displacement pipettor for volumes less than 10 µl (recommended).
- Borosilicate glass test tubes (12 x 75 mm).
- Gloves (for handling DMF).

For a complete description of the labeling procedure please refer to Chapter 12, "ILA Labeling Procedure."

ILA Assay

The following materials are required to perform an ILA assay.

- Analyte to be measured.
- Binding proteins to the analyte (labeled with biotin and fluorescein).
- 100 ml beaker.
- Syringes, 30 ml, with Luer Lock fittings.
- Precision pipets and disposable tips.
- Eppendorf repeater pipettor (50 ml, 12.5 ml, 2.5 ml and 1.25 ml tips).
- Polypropylene tubes (12x75).
- 50 ml conical tubes (polystyrene).
- Test tube racks.
- Gloves (for biohazardous samples).

Preparing ILA Detection Kit Reagents

The Threshold ILA Detection Kit should always be stored at ***2-8°C***. Before using, bring all reagents to ***room temperature***.

Note: *The shelf life for the components listed (Table 10-3) is for reconstituted and diluted reagents. The expiration date of the ILA Detection Kit, prior to reconstitution or dilution of components, is given in the Certificate of Lot Analysis, provided with the kit.*

Table 10-3: Shelf Life of LA Detection Kit Components

Kit Component	Shelf Life
Assay Buffer	**3 months at 2-8°C**
Dilute 1 part Assay Buffer Concentrate with 9 parts deionized water. Check the pH is 7.0 ± 0.2. Filter through 0.2 µm or 0.45 µm membrane into a clean storage vessel.	
Wash Solution	**3 months at 2-8°C**
Dilute 1 part Wash Concentrate with 9 parts deionized water. Check that the pH is 6.5 ± 0.2. Filter through 0.2 µm or 0.45 µm membrane into a clean storage vessel.	
Capture Reagent	**3 months at 2-8°C**
Add 25 ml of 1X Assay Buffer to rehydrate the lyophilized reagent. Allow 10 minutes for equilibration. Mix by gentle swirling or inversion.	
Dilute this reagent further before use: for each stick being run, combine 1 ml of rehydrated Capture Reagent with 9 ml of 1X Assay Buffer. Use diluted Capture Reagent same day.	
An alternative dilution scheme is to combine 1 ml rehydrated Capture Reagent with 5 ml of 1X Assay Buffer and use 0.5 ml in the assay. Use diluted Capture Reagent same day.	
Enzyme Reagent	**1 week at 2-8°C**
Add 4 ml of 1X Assay Buffer per bottle to rehydrate the lyophilized reagent. Allow 10 minutes for equilibration. Mix by gentle swirling or inversion.	
If using sequential incubation (See Chapter 9, "Immuno-Ligand Assay Introduction.") a further dilution of Enzyme Reagent must be made: For each stick in the assay, dilute the reconstituted enzyme reagent 1 ml with 9 ml of 1X Assay Buffer.	
Substrate Solution	**Use Same Day**
Add 0.6 ml of Substrate Concentrate in 30 ml of 1X Wash Solution and mix.	
Threshold Stick	**Use When Opened**
The assay sticks are ready to use. Hydrate the blank stick before use.	

Table 10-3: Shelf Life of LA Detection Kit Components (Continued)

Kit Component	Shelf Life
Universal Standard Reagent	**3 months at 2-8°C**
Add 5 ml of 1X Assay Buffer per bottle to rehydrate the lyophilized reagent. Use this reagent and its dilutions, as recommended for the checkout procedure, or for troubleshooting.	

ILA Sample Preparation

Samples for ILA must meet the following general conditions for the assay:

- ***pH and ionic strength optimal for specific analyte and binding proteins.***
- ***Absence of interfering substances.***

Handling Recommendations

In addition to meeting the above criteria, there are four general recommendations for handling ILA samples:

Volume

This manual recommends a sample volume of 100 µl and a binding protein combination (biotinylated binding protein + fluoresceinated binding protein) volume of 100 µl. These volumes are recommended as a starting point, when developing an ILA, and have been used for convenience and continuity throughout the manual. However, final sample volume and binding protein combination volume should be chosen, as appropriate for each assay

Tubes

As a general recommendation, Molecular Devices advises using polypropylene tubes to minimize potential protein-tube interactions. If the analyte or binding protein can interact with polypropylene, appropriate alternative tubes should be used.

Pipetting Techniques

Because the reaction mixture volume is small (200 µl), special pipetting techniques are required. Do not dispense solutions to the side of the tube; dispense to the bottom of the tube and avoid splashing the solution.

Spike Recovery

Threshold ILA assays provide detection of analytes at extremely low concentrations. To ensure the accuracy of quantitation in the sample, Molecular Devices recommends the routine use of spike recovery. Samples and control buffer should be tested with, and without, a known quantity of added analyte. The amount of added analyte recovered in the sample can be compared to that recovered in the buffer to determine a percentage spike recovery in the sample. This is a measure of the accuracy of quantitation in the presence of sample.

Assay Performance

Setup

To set up each stick in the assay:

1) Set up a row of eight test tubes in a rack. For example, to run four sticks, set up four rows of eight tubes.
2) Label the tubes appropriately. Typically, Stick A (in Threshold Enterprise / Software) is used for the standard curve.
3) Set up the * position as a zero calibrator, the # position as a mid-level calibrator and the remaining six calibrators, as desired, for the standard curve.
4) On other sticks, assign unknowns to positions 1 through 6, * position as the zero calibrator, and # position as the midlevel calibrator.
5) Pipet 100 µl of the appropriate standard or sample solution into each sample tube.
6) Prepare a solution of labeled binding proteins in 1X Assay Buffer (see Chapter 11, "The ILA Checkout Assay.") Pipet 100 µl of the labeled binding protein solution into each sample tube.

Incubation

For Simultaneous Incubation: Add 100 µl of Enzyme Reagent to each sample tube.

For Sequential Incubation: the Enzyme Reagent is diluted 1 ml: 9 ml with 1X Assay Buffer and added after the filtration of sample and first wash.

1) Incubate the reaction mixture. The time and temperature of incubation will need to be optimized for each experimental protocol.
2) During the incubation, set up assay information in Threshold Enterprise / Software (Chapter 4, "Threshold Software," and Chapter 5, "Threshold Enterprise.").
3) Assemble the filtration units on the Threshold Workstation (Chapter 3, "Operating Procedures and Maintenance.")
4) During the incubation period, prepare a solution of streptavidin, by diluting 1 ml of rehydrated Capture Reagent with 9 ml of 1X Assay Buffer, for each stick.
5) At the end of the incubation, add 1 ml of diluted Capture Reagent to each sample tube.

Filtration

1) Immediately transfer the appropriate volume of the reaction mixture to the filtration units, on the Threshold Workstation.
2) Filter on low vacuum. Filter until all the wells are empty. Filtration rate averages 100 µl per minute through the membrane. Filtering may continue for up to 10 minutes after the wells are empty.
3) Examine for fluid remaining in the wells, noting that bubbles (foam) may remain.

Note: *If one or more wells, or a whole block, is filtering incorrectly, see Chapter 7, "System Troubleshooting." If filtering of unknown samples is excessively slow, or stopped, and the * and # samples are filtering faster, the samples may contain too much protein or particulates and may require prefiltering (using a 0.2 µm low protein binding filter).*

4) When the sample is completely filtered, add 2 ml of 1X Wash Solution to each well (an Eppendorf Repeating Pipettor with a 50 ml Combitip is recommended).
5) Change the vacuum to High.
6) The Wash Solution will filter through the membrane in approximately 4 minutes. Watch the sample wells from the top and the sides to determine when filtration is complete. Filtering may continue for up to 5 minutes after the wells are empty.
7) Turn the vacuum OFF.

Assay

Simultaneous Incubation Protocol

1) Remove the filter blocks, sticks and bases from the Threshold Workstation.
2) Place the sticks in a beaker of 1X Wash Solution.
3) Read the sticks in the Threshold Reader.

Sequential Incubation Protocol

1) Add 1 ml of diluted Enzyme Reagent (see "Preparing ILA Detection Kit Reagents" on page 10-5) to each well. (An Eppendorf Repeating Pipettor with a 12.5 ml Combitip is recommended.)
2) Turn the vacuum on low, and filter until the wells are empty. The filtration rate averages 100 µl per minute and may continue for up to 5 minutes after the sample wells are empty (bubbles or foam may remain).
3) Repeat the wash step, described above, and filter on high vacuum.
4) When all the wells are empty, turn the vacuum OFF and remove the filter blocks, sticks and bases from the Threshold Workstation.
5) Place the sticks in a beaker of Wash Solution.
6) Read the sticks in the Threshold Reader.

Completing the Assay

1) After the sticks have been read, print the report and save the data file (Chapter 4, "Threshold Software.").
2) Shut down the Threshold System (Chapter 3, "Operating Procedures and Maintenance.").

Chapter 11– The ILA Checkout Assay

Introduction

This chapter describes how to perform the checkout procedure to test the Threshold System, after installation, or as an initial training exercise for the Immuno-Ligand Assay (ILA). This procedure uses the Universal Standard Reagent (biotin- and fluorescein-labeled bovine serum albumin), which eliminates the need for specific labeled binding proteins. Successful completion of this assay confirms correct installation of the Threshold instrumentation and successful implementation of the Immuno-Ligand Assay.

After unpacking and installing the Threshold Instrument, review Chapter 10, "General ILA Procedure."

Materials

1) ILA Detection Kit.
2) 12x75 mm polypropylene test tubes (for example, Falcon #2002).
3) One 50-ml beaker.
4) Two 50-ml conical tubes (polystyrene).
5) Precision pipettors and tips.
6) Eppendorf repeating pipettor with:
   2.5 ml combitip
   12.5 ml combitip
   50 ml combitip
7) Multichannel Pipettor and Sample Organizer Rack from Molecular Devices (recommended).

Checkout Assay Protocol

Setup

1) Reconstitute reagents:
   a. Reconstitute Universal Standard Reagent with 5 ml 1X Assay Buffer.
   b. Reconstitute 1 vial of Enzyme Reagent with 4 ml 1X Assay Buffer.
   c. Reconstitute Capture Reagent with 25 ml 1X Assay Buffer.
2) Label tubes for preparing standard curve and precision solutions.
3) Label sample tubes.
4) Prepare standard curve.
5) Prepare precision samples.
6) Dispense 100 µl of standards and precision solutions into the appropriate sample tubes.
7) Add 100 µl of Enzyme Reagent to each sample tube (use the Eppendorf Repeating Pipettor with 2.5 ml Combitip).
8) Mix each tube GENTLY.

Incubation

1) Incubate for 30 minutes, at room temperature.
2) During the incubation:

a. Prepare Substrate Solution (0.6 ml of Substrate Concentrate + 30 ml 1X Wash Solution in 50 ml conical tube).
b. Remove old substrate from Reader and replace with fresh substrate.
c. Assemble filtration units with Threshold sticks on Threshold Workstation.
d. Place the blank stick in a beaker filled with 1X Wash Solution. The Wash Solution should completely cover the nitrocellulose membrane.
e. Dilute the reconstituted Capture Reagent in a 50 ml conical tube, 1 ml: 9 ml with 1X Assay Buffer for each stick. Therefore, for this assay mix 4 ml: 36 ml.
f. Input sample information for each stick (see Chapter 4, "Threshold Software,"and Chapter 6, "Calibration and Quantitation.").

3) Add 1 ml of the diluted Capture Reagent to each tube (use the Eppendorf Repeating Pipettor with 12.5 ml Combitip) and mix.
4) Transfer 1 ml from each sample tube to filter units (0.2 ml of solution will remain in tubes). Transfer, using the 8 Channel Pipettor from MDC, or a precision pipet with 1 tip.

Filtration

1) Filter on low vacuum, until the wells are empty.
2) Add 2 ml of 1X Wash Solution to each sample well (use the Eppendorf Repeater Pipet with 50 ml Combitip).
3) Filter on high vacuum, until wells are empty.
4) Turn the vacuum OFF.

Cleaning

1) Release the filter blocks and remove from the Threshold Workstation.
2) Remove the sticks and place in the beaker of Wash Solution with the Blank Stick.
3) Remove the filter bases from the Threshold Workstation. Put the filter blocks and bases aside, to be cleaned, as described in the ILA Product Insert.
4)
5) Read the standard curve and load it (See "Results and Data Analysis" on page 11-7).
6) Read sticks B, C, and D.
7) Save the data, print the report, and shut down the system.

Labeling Standard, Precision, and Reaction Mixture Tubes

This assay is a 4-stick assay, consisting of a standard curve, and precision samples (12 low concentration, precision samples and 6 high concentration, precision samples). The sticks will be labeled A,B,C,D. Positions on the filtration block, and stick, are identified as follows:

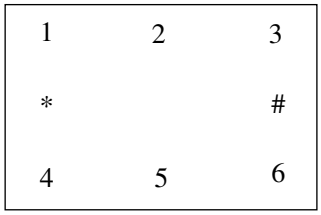


Figure 11-1: Positions on Filtration Block and Stick

Positions 1-6 contain samples, * = zero calibrator (100 µl of 1X Assay Buffer) and # = midlevel calibrator (for this assay the 4000 units/ml standard).

1) Label 6 tubes for the standard curve:

   4000 units/ml
   2000 units/ml
   1000 units/ml
   500 units/ml
   250 units/ml
   125 units/ml

2) Label 1 tube for preparation of the low concentration precision sample:

   3000 units/ml

3) There will be 32 tubes for reaction mixtures (8 tubes per stick). Label, and arrange these tubes as shown in the following diagram (Figure 11-2).

4) Using the arrangements shown in Figure 11-2, place the test tubes in a rack and dispense the sample mixtures into the filter blocks.

Filter Block Numbering Scheme

1	2	3
*		#
4	5	6

Stick A:

Standard Curve units/ml Universal Standard Reagent

8000	2000	1000
0		4000
500	250	125

Stick B:

Precision Test Samples, 3000 units/ml of Universal Standard Reagent

3000	3000	3000
0		4000
3000	3000	3000

Stick C:

Precision Test Samples, 3000 units/ml of Universal Standard Reagent

3000	3000	3000
0		4000
3000	3000	3000

Stick D:

Precision Test Samples, 8000 units/ml Universal Standard Reagent

8000	8000	8000
0		4000
8000	8000	8000

Figure 11-2: The ILA Test Tube and Filter Block Arrangements

Preparation of Standard Curve and Precision Solutions

Both the standard curve and precision solutions are prepared from the Universal Standard Reagent. The Universal Standard Reagent, when reconstituted with 5 ml of 1X Assay Buffer, is 8000 units/ml.

Standard Curve (Stick A)

8000 unit/ml standard may be pipetted directly from the Universal Standard Reagent vial into the labeled standard curve calibrator (Stick A) and 8000 unit/ml precision samples (Stick D) reaction mixture tubes.

1 ml (8000 units/ml) + 1 ml Assay Buffer	=	4000 units/ml
1 ml (4000 units/ml) + 1 ml Assay Buffer	=	2000 units/ml
1 ml (2000 units/ml) + 1 ml Assay Buffer	=	1000 units/ml
1 ml (1000 units/ml) + 1 ml Assay Buffer	=	500 units/ml
1 ml (500 units/ml) + 1 ml Assay Buffer	=	250 units/ml
1 ml (250 units/ml) + 1 ml Assay Buffer	=	125 units/ml

Precision Solutions (Sticks B, C, and D)

0.6 ml (8000 units/ml) + 1.0 ml Assay Buffer	=	3000 units/ml

For the precision test on **Stick D**, use the reconstituted Universal Standard reagent directly from the vial, 8000 units/ml.

Results and Data Analysis

Refer to Chapter 4, "Threshold Software," for instructions on entering sample information into sticks A, B, C, and D.

Reading the Blank Stick and Stick A

To load the standard curve:

1) Select **Copy Stick to Standard** from the **Edit** menu and highlight Stick A. This will place standard curve information onto Stick §.
2) To view the standard curve, click the **Standard Curve** button in the toolbar.
3) Read sticks B, C, and D.
4) All sticks (A, B, C, D) will be quantitated from the standard curve.
5) The quantitated results for sticks B, C, and D will be displayed, as they are read.

Printing and Saving Reports

To print reports of the data:

- Select **Print Reports** from the **File** menu.

To save the data:

1) Select **Save**, or **Save As**, from the **File** menu. For further information on using the Threshold Enterprise / Software refer to Chapter 4, "Threshold Software."
2) Review the Certificate of Lot Analysis, provided in the Threshold ILA Detection Kit.

3) Compare the Checkout Assay Standard Curve rates to those on the Certificate of Lot Analysis.

Note: *The coefficient of variation (% CV) for results from the precision test samples should be less than 10%. Threshold Enterprise / Software prints the CV (%) with the mean and standard deviations for all replicates. Replicates are samples entered in Threshold Enterprise / Software that have exactly the same names.*

12

Chapter 12– ILA Labeling Procedure

12

Labeling of Binding Proteins

This chapter describes how to covalently link (label) biotin and fluorescein to the binding proteins for the ILA. This is the first step in developing an ILA. The biotin allows capture of the reaction complex onto the nitrocellulose membrane and the fluorescein allows detection of the complex in the Threshold Reader. The biotin and fluorescein haptens are provided in the Threshold Immuno-Ligand Assay Labeling Kit as the N-hydroxysuccinimide (NHS) esters. The labeling reaction is a nucleophilic substitution in which the carbonyl carbon of the hapten reacts with an unprotonated primary amine (usually the epsilon amine of lysine) of the binding protein to form a stable amide bond. The succinimide group leaves and the hapten is covalently linked to the binding protein.

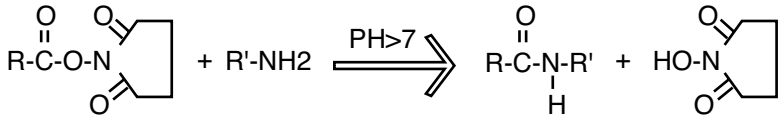


Figure 12-1: The NHS Ester Labeling Reaction

Materials for Labeling Binding Proteins

The following materials are required for labeling binding proteins:

- Threshold ILA Labeling Kit:
  - 2 vials of Biotin Label (DNP-biotin NHS).
  - 2 vials of Fluorescein Label (carboxyfluorescein NHS).
- Antibodies or antigen to label.
- Assay Buffer Concentrate (10x) provided in the Threshold ILA Detection Kit.
- PBS: 10 mM Phosphate, 150 mM NaCl, pH 7.0.[1]
- Anhydrous dimethylformamide (DMF, Sequanal grade), preferably stored with a molecular sieve (for example, Pierce Chemical Co., Cat. No. 20673).
- Pharmacia PD-10, Sephadex® G25 column or other appropriate gel filtration column.
- Dialysis tubing with a molecular weight cut-off of 14,000 for antibodies (for other proteins use appropriate tubing).
- Glass test tubes (12 x 75 mm).
- Pipetting aids; µl to ml volumes with ± 2% accuracy.
- 1 ml syringes and needles (or Hamilton type syringe).

---

1 To make 1 liter of 10X PBS:

4.608 gm	NaH_2PO_4:H_2O
9.454 gm	Na_2HPO_4
87.7 gm	NaCl

Dissolve in 1 liter of deionized water and adjust pH to 7.0.

- Syringe-type filters (low-protein-binding polysulfone, PVDF, or cellulose acetate.

  For filtration, do not use nitrocellulose, nylon, or other membranes that bind proteins. These filters may reduce yield of labeled protein.

- Quartz cuvettes.
- Spectrophotometer, capable of measuring at 280 nm, 362 nm, and 490 nm.
- Vortex mixer.

Abbreviated Labeling Procedure

This is a summary of the steps required to label antibodies.

1) Decide which hapten to place on the protein to be labeled.
2) Adjust the protein solution to appropriate conditions (PBS, pH 7.0-8.0, concentration 0.20 to 1.0 mg/ml).
3) Determine the Molar Coupling Ratio to be used.
4) Reconstitute the biotin and fluorescein haptens in DMF.
5) Incubate the binding protein and hapten together for 2 hours, at room temperature, in the dark.
6) Separate the unreacted hapten from the binding protein, using the PD-10 column or dialysis.
7) Determine the binding protein concentration, protein recovery and the Molar Incorporation Ratio by measuring the absorbance of the purified binding proteins using a spectrophotometer.

Types of Protein to be Labeled

Proteins to be labeled may be antibodies (monoclonal and polyclonal), other proteins, or the analyte for a competitive assay format. All antibodies should be purified from ascites or serum before labeling. This can be accomplished by using a Protein A or Protein G affinity column, or an antigen column. Selecting the proteins, to be labeled, will depend on the purity of the proteins and the assay format to be used (competitive or sandwich).

Amount of Protein to be Labeled

A minimum of 0.20 mg of protein should be used for a labeling to ensure accurate optical density measurements, after purification of labeled protein. For the initial labeling, 0.20 to 0.25 mg of protein provides sufficient material to determine the Molar Coupling Ratio and to adequately test the performance of the labeled proteins. After the initial evaluation, if required, larger amounts of protein can be labeled to provide assay reagents for many experiments.

Preparation of Proteins for Labeling

Efficient labeling of the protein with the haptens requires that the protein solution should be:

- Neutral pH (pH 7-8).
- Final concentration of 0.20-1.0 mg/ml.
- Free of interfering substances such as other nucleophiles.

The ideal buffer for the protein is phosphate-buffered saline (PBS, 10 mM sodium

phosphate, 150 mM NaCl, pH 7.0). If the pH is less than 7.0. the epsilon amine of a lysine will be protonated and no reaction will occur.

Nucleophiles such as sodium azide, Tris, and amino acids (for example, glycine) must be removed from the protein solution, prior to the labeling, because they will compete with the unprotonated primary amine of the protein and hydrolyze the haptens.

Remove any nucleophiles in the protein solution, by chromatography or dialysis, prior to labeling.

For concentrated proteins (0.7 to 1.0 mg/ml), a PD-10 column will allow a rapid method for removing interfering substances. This procedure, however, will result in an approximate 2-fold dilution of the protein. If the concentration of the protein is less than 0.7 mg/ml, dialysis against PBS, overnight at 4°C, is suggested for removing interfering substances, while minimizing dilution of the binding protein.

Choosing Hapten-Protein Configurations

The haptens used for the ILA are N-hydroxysuccinimide (NHS) esters of dinitrophenyl biotin (DNP-biotin) and carboxyfluorescein (fluorescein) (Figure 12-2). The DNP group is a chromophore that has been incorporated into the biotin-NHS ester, to allow determination of hapten incorporation spectrophotometrically. This section describes how to choose between labeling a protein with biotin or fluorescein, the amount of hapten to use in the labeling, and how to reconstitute the lyophilized esters.

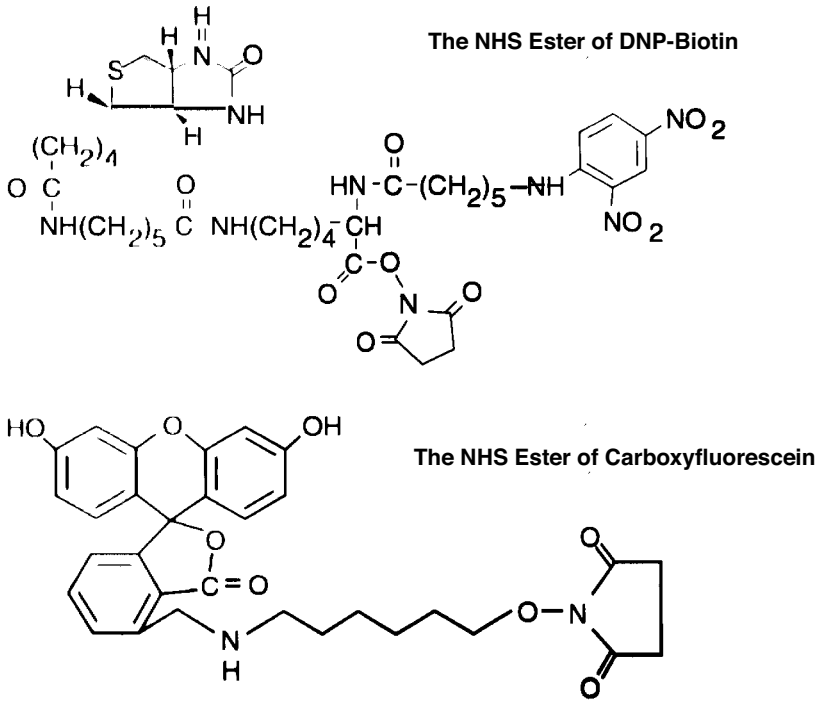


Figure 12-2: The Haptens

Sandwich Format

One Polyclonal Antibody: When using one polyclonal antibody binding to a polyvalent (multideterminant) analyte, label equal amounts of the antibody with biotin or fluorescein.

Two Different Polyclonal Antibodies: When using two different polyclonal antibodies that recognize different determinants, label the antibody with the greater purity and / or higher titer or affinity with fluorescein. This will allow lower concentrations of the fluoresceinated antibody to be used per test. Theoretically, because the signal generated corresponds to the fluoresceinated antibody, a lower concentration per test will result in lower background rates.

Purification of a polyclonal antibody, using a Protein A or Protein G column will result in an IgG solution that contains approximately 10%, or less, specific antibody. The remainder of the IgG antibodies will not be specific to the analyte. Purification of the antibody with an antigen specific column will result in an antibody solution that contains 90%, or greater, specific antibody. If both polyclonal antibodies are of equal purity and the titer or affinity is unknown, both configurations should be tested: aliquots of each antibody should be labeled with biotin and fluorescein so both configurations can be evaluated.

Two Different Monoclonal Antibodies: When using two different monoclonal antibodies that recognize different determinants, label the antibody with the highest affinity with fluorescein. If this configuration does not allow adequate assay performance, then test the reverse configuration.

Other Binding Proteins: When labeling proteins other than antibodies, label the purest and / or highest affinity binding protein with fluorescein, allowing lower concentrations of the signal generating binding protein per test to be used.

Increased amounts of the biotinylated component can also result in increased background rates. For optimum performance, the final configuration should be determined empirically.

Competitive Format

A competitive assay can be configured with the analyte labeled with fluorescein and the binding protein with biotin. Using this configuration, the fluoresceinated analyte competes with the unlabeled analyte to bind the biotinylated binding protein. The assay can alternatively be configured with the analyte labeled with biotin and the binding protein labeled with fluorescein. In this configuration, the biotinylated analyte competes with the unlabeled analyte to bind to the fluoresceinated binding protein. In both configurations, the labeled analyte and the labeled antibody are in limited quantities.

The most important factor to consider when choosing a configuration for a competitive, as well as a sandwich assay, is the purity of the analyte and binding protein. Fluoresceinate is the purest component.

The molecular weight and number of available lysines can also influence which configuration could be optimal. The fluorescein-NHS hapten tends to label slightly more efficiently than the DNP-biotin-NHS hapten. For small peptides or proteins with limited available lysines, labeling with fluorescein may result in better hapten incorporation.

However, the fluorescein labeled to a small peptide can sometimes affect the extinction coefficient of the peptide. This can result in erroneous calculations of concentration, often resulting in greater than 95% recovery of a fluoresceinated peptide after purification. If this should occur, test the reverse configuration.

Molar Coupling Ratio

The Molar Coupling Ratio (MCR) is defined as the number of moles of hapten per mole of protein used in the labeling reaction. The Molar Incorporation Ratio (MIR) is defined as the number of moles of hapten covalently bound to a mole of protein after the labeling reaction is complete.

The optimum MCR is dependent on many factors, including the molecular weight of the protein to be labeled, the number of available lysines on the protein, the concentration of the protein and the desired MIR. The first MCR to test when labeling a polyclonal antibody is 20:1 (20 moles of hapten per mole of antibody). This usually results in an MIR between 2 and 5.

The optimum MCR for monoclonal antibodies is not as consistent as the MCR for polyclonal antibodies. Monoclonal antibodies incorporate hapten with a wide range of efficiency. The recommended initial MCR to test is 10:1 (10 moles of hapten per mole of antibody). If the resulting MIR is below 2 then a higher MCR should be used. Conversely, if the MIR is above 5, then a lower MCR should be used. If adequate amounts of the monoclonal antibody are available, then multiple MCRs can be tested in one labeling experiment. For 0.2 to 0.25 mg aliquots of the monoclonal antibody, label with 5:1, 10:1 and 20:1 MCR. Determine the MIR for each labeling and select the antibody with the appropriate hapten number.

When labeling proteins other than antibodies, determine the optimal MCR and MIR empirically by testing different MCRs and evaluating the assay performance of the labeled protein. In general, proteins of molucular weight less than 160,000 (smaller than an antibody) may require lower MCRs, compared to polyclonal antibodies, although this depends on the number of available lysines and the concentration of the protein to be labeled. Increasing the pH of the protein solution to 8 can also increase the labeling efficiency. Proteins with a molecular weight greater than 160,000 may require MCR's greater than 20:1.

The volume of hapten, required for a specific MCR can be calculated, using the molarity of the hapten defined on the Certificate of Lot Analysis.

Equation for Molar Coupling Ratio:

$$h-NHS(\mu l) = \frac{\text{mM (protein)} \times MCR}{\text{mM (h-NHS)}} \times \frac{1 \times 10^6 \mu l}{\text{liter}}$$

h-NHS	=	hapten NHS ester
MCR	=	Molar Coupling Ratio
MW^h	=	molecular weight of hapten (µg/mmole)
MW^h, f-NHS	=	5.866 x10^5 µg/mmole

MW^h, b-NHS = 8.62×10^5 µg/mmole

Example: The volume of hapten required to label 1 mg of an antibody with a MCR of 20:1 would be calculated as follows:

$$\frac{(6.25 \times 10^{-6}\text{mM antibody})(20)(1 \times 10^{6}\mu\text{l/liter})}{5.8 \text{ mmole b-NHS/liter}} = 21.6\ \mu\text{l}$$

Table 12-1 lists the volumes of reconstituted hapten to use for different MCRs, when labeling 1 mg of antibody. This table assumes a molecular weight of 160,000 Daltons for a mole of antibody, and that the hapten-NHS has been reconstituted with 1 ml of DMF. If less than 1 mg of antibody is being labeled, use proportionally less hapten (for example, to label 0.5 mg of an antibody, use half the volume of hapten listed for the appropriate MCR).

Table 12-1: Coupling DNP-Biotin-NHS (b-NHS and Fluorescein-NHS (f-NHS) to 1 mg of Antibody

MCR	µl b-NHS	µl f-NHS
5:1	5.4	4.3
10:1	10.8	8.7
20:1	21.6	17.4
40:1	43.2	34.7

Reconstitution of Haptens

The haptens are reconstituted with anhydrous dimethylformamide (DMF) immediately prior to use. When labeling a protein other than an antibody, it is best to calculate the molar coupling ratio prior to reconstitution of the haptens. The ratio of the volume of reconstituted hapten / DMF to the volume of protein solution should be less than 5%, to prevent denaturation of the protein by the organic solvent (DMF). If a concentrated protein solution is to be labeled, or if high MCRs will be used, the hapten may be reconstituted in less than 1 ml of DMF, and the molarity of the hapten recalculated. This may be necessary so as to ensure that the volume of DMF is below 5% of the total solution volume. Conversely, if a very small amount of binding protein is being labeled, or very low MCRs will be used, additional dilution of the haptens may be required after reconstitution, so that the volume of hapten can be accurately pipetted.

To perform a reconstitution of haptens:

1) Check the color of the desiccant packet; it should be blue.

   Caution: *If the desiccant is pink, do not use the reagent. Call Molecular Devices Technical Support (800-635-5577).*

2) Remove the plastic cap from the vials, exposing the rubber septum.
3) Place a hypodermic needle into the rubber septum of each vial, to allow displaced air to escape when the DMF is injected.
4) Transfer 1 ml of DMF to each vial of hapten. ***(Wear gloves while handling DMF. It is a skin irritant)***.

5) Mix the hapten / DMF solution until the hapten is dissolved.

Note: *The final concentration of the hapten / DMF in the reaction mixture must be less than 5% (v/v).*

Haptens should be used within 24 hours of reconstitution, to assure consistent labeling efficiency. Reconstituted haptens are susceptible to hydrolysis by water.

The reconstituted haptens should be stored in the dark as they are light sensitive.

Labeling the Protein

To label the protein:

1) Place the appropriate amount of protein to be labeled in a glass test tube.
2) Use a 1-ml syringe to transfer an appropriate amount of reconstituted hapten from the vial into a separate test tube.
3) Using an appropriate pipet, dispense the hapten and add to the protein while vortexing gently. For volumes less than 10 µl, a positive displacement pipet is recommended.
4) Incubate the hapten / protein mixture for 2 hours, at room temperature, protected from light.

Purification and Quantification of Labeled Materials

The unreacted hapten can be separated from the protein by chromatography or by dialysis. Chromatography requires less time than dialysis and is the method of choice if the labeled protein is sufficiently concentrated. A Pharmacia PD-10 column is a convenient disposable column to use.

Purification of Material with PD-10 Column

Before use, you must equilibriate the PD-10 columns with PBS:

1) Remove the cap on the top end and pour the liquid into a waste container.
2) With scissors or a razor blade, snip off the pointed end of the column.
3) Wash and equilibrate the column with 25 ml of PBS.
4) Transfer the hapten / protein mixture to the column and allow it to flow through the fritted disk into the gel bed.
5) Add a volume of PBS sufficient to make the total volume equal to 2.5 ml:

   For example, 0.25 ml hapten / protein plus 2.25 ml of PBS will total 2.5 ml (void volume of the PD-10 column).

6) Allow the PBS to enter the column bed and the flow through to go into the waste container.
7) Transfer 0.5 ml aliquots of PBS to the column and collect the eluate (0.5 ml fractions). Collect five 0.5 ml fractions.

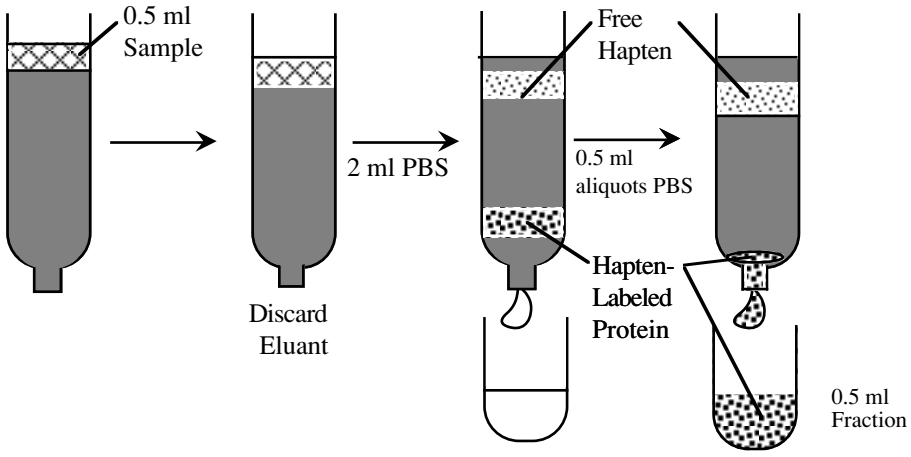


Figure 12-3: Elution of Hapten-Labeled Protein on a 10 ml Bed Volume Desalting Column

Purification of Material by Dialysis

Dialysis is an alternative method for separating the unreacted hapten from the protein, if the labeled protein concentration is very low, or if the size or other characteristics of the protein are not suitable for using the PD-10 column.

1) For very small proteins, use dialysis tubing of an appropriate molecular weight cutoff.
2) To stop the labeling reaction, add 0.1% sodium azide to the hapten / protein mixture. The sodium azide will hydrolyze the NHS ester.
3) Dialyze this mixture overnight at 4°C against PBS.
4) Measure the absorbance of the fractions collected from the column, or the protein solution after dialysis, in a spectrophotometer.
   - For biotinylated proteins, read the absorbance at 280 nm and 362 nm for protein and DNP-biotin, respectively.
   - For fluoresceinated proteins, read the absorbance at 280 nm and 490 nm for the protein and fluorescein, respectively.
5) Pool the fractions containing the greatest amount of labeled protein, and measure the pool at the appropriate wavelengths.

Calculations

Calculation of Molar Incorporation Ratio, Protein Recovery and Protein Concentration

1) When performing calculations, record the results on the Labeling Worksheet (Appendix C, "ILA Worksheets.")
2) If the extinction coefficient for the protein is not known, determine it by measuring the A_{280} of a solution of known concentration.
3) Calculate the extinction coefficient (ε) of the unlabeled protein at 1 mg/ml:

$$\varepsilon = \frac{A_{280}}{\text{mg/ml Protein}}$$

Biotin-Labeled Protein

1) Determine the concentration of biotin-labeled protein (b-P), using the following formula:

$$\text{mg/ml of b–P} = \frac{A_{280}^{\text{b-P}} - (0.32 \times A_{362}^{\text{b-P}})}{\varepsilon(1\ \text{mg/ml})}$$

For murine IgG, the extinction coefficient (ε) is 1.4. This number may differ slightly for IgG's from other species.

2) Determine the protein recovery:

$$\%\ \text{Protein Recovery} = \frac{\text{mg of Protein Recovered}}{\text{mg of Protein Labeled}} x 100$$

3) Determine the molar incorporation of biotin (b/p ratio) using these formulas:

$$\text{b/p} = \text{nmol b/nmol p} = \frac{(58 \times A_{362}^{\text{b-P}})\left(\varepsilon(1\ \text{mg/ml}) \times \frac{MW^P}{10^6}\right)}{A_{280}^{\text{b-P}} - (0.32 \times A_{362}^{\text{b-P}})}$$

4) For IgG, the molar incorporation equation simplifies to:

$$\text{b/IgG} = \frac{13 \cdot A_{362}^{\text{b-P}}}{A_{280}^{\text{b-P}} - (0.32 \times A_{362}^{\text{b-P}})}$$

For the derivation of the equation for molar incorporation of biotin, see Appendix A, "Incorporation of Biotin."

Fluorescein-Labeled Protein

1) Determine the concentration of fluorescein-labeled protein, using the following formula:

$$\text{mg/ml of f-P} = \frac{A_{280}^{\text{f-P}} - (0.18 \times A_{490}^{\text{f-P}})}{\varepsilon(1\ \text{mg/ml})}$$

2) Determine the Protein Recovery:

$$\%\ \text{Protein Recovery} = \frac{\text{mg Protein Recovered}}{\text{mg Protein Labeled}} \times 100$$

3) Determine the molar incorporation of fluorescein (f/p ratio), using these formulas:

$$\text{f/p} = \text{nmol f/nmol p} = \frac{(18 \times A_{490}^{\text{f-P}})\left(\varepsilon(1\ \text{mg/ml}) \times \frac{MW^{P}}{10^{6}}\right)}{A_{280}^{\text{f-P}} - (0.18 \times A_{490}^{\text{f-P}})}$$

4) For IgG, the molar incorporation equation simplifies to:

$$\text{f/IgG} = \frac{4 \times A_{490}^{\text{f-P}}}{A_{280}^{\text{f-P}} - (0.18 \times A_{490}^{\text{f-P}})}$$

For the derivation of the equations for molar incorporation of fluorescein, see Appendix B, "Incorporation of Fluorescein."

- If, during the labeling process, there is aggregation or some other effect on protein chemistry, that can cause a shift in the absorbance spectrum, incorrect values can result. In this case estimate that the recovery of the protein is 80%, and continue with the calculations.
- If the solution appears cloudy, filter through a low protein binding filter, such as PVDF or Durapore (both from Millipore), and measure the absorbance.

Storing Labeled Proteins

1) Labeled proteins should be diluted to 0.1 mg/ml or 0.01 mg/ml. Dilute with ILA Assay Buffer, which provides both a bulking protein (BSA at 1 mg/ml) and a preservative (0.1% sodium azide).
2) After testing for any effect from a freeze/thaw cycle, store the aliquots of the labeled proteins in a suitable manner for the individual protein (either 4°C or frozen at -20°).
3) Aliquot the protein in an amount sufficient to perform one assay.
4) To assure stability, monitor the performance of the labeled proteins in the ILA assay. Changes in the background, in the quantitation gain (net signal per unit of analyte measured), or noticeable aggregates in the solution of labeled protein, indicates loss of antibody activity.

13

Chapter 13– ILA Sandwich Format Optimization

ILA Sandwich Format Optimization

General Issues

There are several general considerations in the optimization of a sandwich format Immuno-Ligand Assay.

- In a sandwich assay, the binding proteins, streptavidin and anti-fluorescein:urease are in excess. The analyte is the rate-limiting component.
- The binding capacity of the streptavidin for the b-Ab is approximately 8 µg/test (approximately 53 x 10^{-9} moles/test) of b-Ab. Adding larger amounts of b-Ab may reduce the specific signal.
- The binding capacity of Enzyme Reagent (anti-fluorescein:urease) for f-Ab in a simultaneous incubation is approximately 25 ng/test (approximately 167 x 10^{-12} moles/test) of f-Ab. Using larger concentrations of f-Ab and a simultaneous incubation may reduce the specific signal. Using a sequential incubation allows use of higher concentrations of f-Ab.
- The extent of incorporation of biotin or fluorescein may affect assay performance. Generally, a molar incorporation of 2-4 moles of biotin or fluorescein per mole of binding protein is optimal (see Chapter 12, "ILA Labeling Procedure.")
- Following incubation, samples are filtered on the Threshold Workstation through a biotinylated nitrocellulose membrane. Large aggregates and particulate material in any of the added solutions may not pass through the membrane. This may clog the membrane or increase the background by binding specifically or non-specifically some of the reagents (see Chapter 7, "System Troubleshooting," and Chapter 15, "ILA Troubleshooting.".)
- A source of background is the nonspecific aggregation of b-Ab and f-Ab. This background may be time-dependent and may vary with the antibodies. Prepare the solution containing both these reagents shortly before use.
- If labeled reagents were prepared with more than one molar coupling ratio (MCR), first evaluate those that incorporated approximately 3 moles of hapten per mole of binding protein.

Note: *If using complex matrices, it is important to first optimize the assay conditions using purified proteins in Assay Buffer. This establishes that the labeled antibodies can detect the antigen under defined conditions. Then, determine whether ILA can detect accurately a known antigen introduced into the complex matrix.*

Optimization is the process of determining the assay conditions required to detect an analyte at a desired concentration.

This chapter outlines the experimental steps, necessary to establish the conditions for reagents and samples for the sandwich format ILA.

Prior to performing the experiments in this chapter:

- The binding proteins must be labeled (Chapter 12, "ILA Labeling Procedure,").
- The incubation protocol must be chosen (Chapter 9, "Immuno-Ligand Assay Introduction.")

For the purpose of clarity, the following experiments will describe the analyte as the anti-

gen, hapten-labeled proteins as antibodies, and use the sequential incubation protocol.

Note: *The amount of antigen is expressed as the concentration in units/ml. The amount of antibody combination is expressed in units/test. Typically 1 ml of antibody combination solution is needed for each stick run, and 100 µl of antigen solution is used per test in an assay.*

Experiment 1 Loading Study: Determination of the Amount of Labeled Binding Proteins Required

The objective of this experiment is to determine the optimal amount of biotinylated antibody and fluoresceinated antibody to use in the ILA. **This experiment should be repeated after each labeling.**

Binding Protein Concentrations

The amount of antibody per test depends on the type, purity of the antibodies, the affinity of the antibodies for the antigen, and the protocol to be used (simultaneous or sequential). The following tables may be used as a guide for selecting the concentrations of antibody to test in the loading study.

- In Table 13-2, Table 13-3, and Table 13-5, test each concentration of fluoresceinated antibody (f-Ab) listed with each concentration of biotinylated antibody (b-Ab) listed.
- In Table 13-1 and Table 13-4, test each row pair of b-Ab and f-Ab.

Test matched pairs:

Table 13-1: A Single Immunoaffinity Purified Polyclonal Antibody (> 90% specific antibody)

Sequential Incubation		Simultaneous Incubation	
Protocol		Protocol	
b-Ab	f-Ab	b-Ab	f-Ab
10 ng/test	10 ng/test	10 ng/test	10 ng/test
20 ng/test	20 ng/test	20 ng/test	20 ng/test
40 ng/test	40 ng/test	25 ng/test	25 ng/test
80 ng/test	80 ng/test		

Test all combinations:

Table 13-2: Two Immunoaffinity Purified Antibodies (> 90% specific antibody) That Recognize Different Determinants (i.e., 2 polyclonal antibodies, or 2 monoclonal antibodies, or 1 polyclonal antibody and 1 monoclonal antibody).

Sequential Incubation		Simultaneous Incubation	
Protocol		Protocol	
b-Ab	f-Ab	b-Ab	f-Ab
25 ng/test	10ng/test	25 ng/test	10 ng/test
50 ng/test	20 ng/test	50 ng/test	20 ng/test
100 ng/test	40 ng/test	100 ng/test	
	80 ng/test		

Test all combinations:

Table 13-3: One Antibody (f-Ab) > 90% Specific Antibody and One Antibody (b-Ab) < 90% Specific Antibody That Recognize Different Determinants (i.e. 2 polyclonal antibodies, or 1 monoclonal and 1 polyclonal antibody).

Sequential Incubation		Simultaneous Incubation	
Protocol		Protocol	
b-Ab	f-Ab	b-Ab	f-Ab
100 ng/test	10 ng/test	50 ng/test	10 ng/test
200 ng/test	20 ng/test	100 ng/test	20 ng/test
400 ng/test	40 ng/test	200 ng/test	
	80 ng/test	400 ng/test	

Test matched pairs:

Table 13-4: Single Protein A or G Purified Polyclonal Antibody (< 90% specific antibody).

Sequential Incubation		Simultaneous Incubation	
Protocol		Protocol	
b-Ab	f-Ab	b-Ab	f-Ab
100 ng/test	100 ng/test		
200 ng/test	200 ng/test	Not Applicable	
400 ng/test	400 ng/test		
800 ng/test	800 ng/test		

Test all combinations:

Table 13-5: Two Protein A or G Purified Polyclonal Antibodies (< 90% specific antibody) That Recognize Different Determinants.

Sequential Incubation		Simultaneous Incubation	
Protocol		Protocol	
b-Ab	f-Ab	b-Ab	f-Ab
100 ng/test	100 ng/test		
200 ng/test	200 ng/test	Not Applicable	
400 ng/test	400 ng/test		
800 ng/test	800 ng/test		

The antibodies are diluted in 1X Assay Buffer. The total volume of the b-Ab and f-Ab solution is 100 µl per test. For example, when preparing 1 ml of a combination of 100 ng/test of b-Ab and 40 ng/test f-Ab: if the b-Ab and f-Ab are both 0.01 mg/ml use 100 µl b-Ab + 40 µl f-Ab + 860 µl 1X Assay Buffer.

Antigen Concentrations

Test three concentrations of antigen: a negative control (0), a low concentration of antigen (+), and a high concentration of antigen (++). If an ELISA or other immunoassay is already in use, the higher concentration of antigen selected should be between the low and middle calibrators of the ELISA standard curve. The lower concentration of antigen should be 10-fold less than the higher concentration. If the antibodies have not been used in an assay, the lower concentration of antigen should equal the desired limit of detection and the higher concentration of antigen should be 10-fold higher.

Reagent Preparation

Each concentration of antigen should be tested in duplicate with each antibody combination. For example, when using a single polyclonal antibody as in
Table 13-4 on 13-5, there will be:

Four concentrations of antibody x 3 concentrations of antigen x 2 duplicates =
24 samples/8 tests per stick = 3 sticks.

This experiment will use only raw signals (µV/sec). Therefore the * and # positions may be used for samples rather than on-board calibrators. Samples may be filtered using two arrangements: (1) each stick contains a constant concentration of antigen with different antibody concentrations or, (2) a constant concentration of antibody with different antigen concentrations:

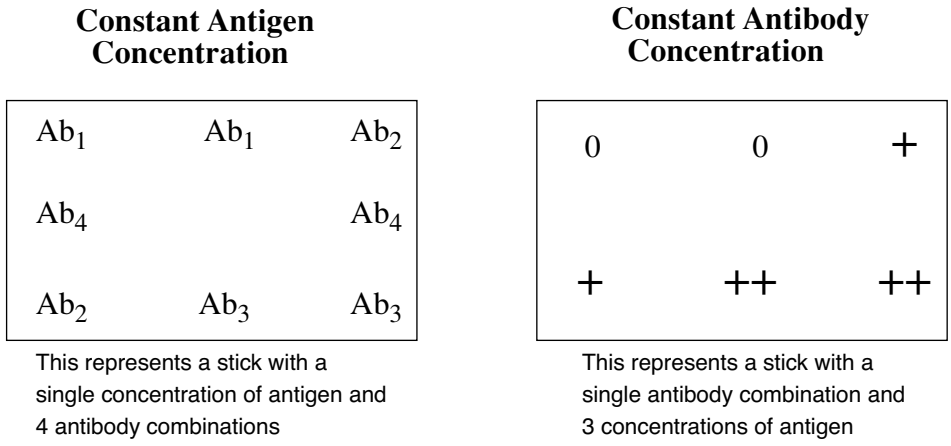


Figure 13-1: Stick Setup Alternatives for the Loading Study

1) Reconstitute and dilute the ILA Detection Kit reagents for the appropriate number of sticks.
2) Prepare the appropriate antigen concentration solutions.
3) Prepare the appropriate antibody combination solutions.
4) Label sample tubes.

Assay Procedure

1) Dispense 100 µl of antigen and 100 µl of antibody in the appropriate tubes. Vortex each tube briefly and gently.
2) Cover the tubes and incubate for 2 hours at room temperature. If the antibody and/or the antigen is not stable at room temperature, incubate at appropriate temperature. During the incubation, do the following:
   a. Make the Substrate Solution (600 µl of Substrate Concentrate + 30 ml of 1X Wash Solution).
   b. Remove the old Substrate Solution from the Reader.
   c. Place the new Substrate Solution in the Reader.
   d. Assemble the appropriate number of filtration units with sticks on the workstation.
   e. Hydrate a Blank Stick in a beaker of 1X Wash Solution.
   f. Dilute the reconstituted Capture Reagent 1:10 with 1X Assay Buffer.
   g. Dilute the reconstituted Enzyme Reagent 1:10 with 1X Assay Buffer.
   h. When the incubation is complete, add 1ml of diluted Capture Reagent to each tube.
3) Transfer the incubated mixtures to the filter blocks.
4) Filter on low vacuum until the wells are empty.
5) Add 2 ml of 1X Wash Solution to each well in the filter blocks, and filter on high vacuum until the wells are empty.
6) Turn off the vacuum.
7) Add 1 ml of diluted Enzyme Reagent to each well in the filter blocks. Filter on low vac-

uum until the wells are empty.

8) Add 2 ml of 1X Wash Solution to each well in the filter block and filter on high vacuum until the wells are empty.
9) Turn off the vacuum.
10) Unlock the filtration units and place the sticks in the beaker of 1X Wash Solution with the Blank Stick.
11) Read the sticks.

Data Analysis

Use the ILA Loading Study Data Summary Worksheet, in Appendix C, for summarizing the data for this experiment. The loading study analysis is based on the raw (µV/sec) signal. Figure 13-2 reproduces a portion of the worksheet.

To complete the worksheet:

1) Enter the concentrations of b-Ab, f-Ab, and antigen used.
2) In the appropriate column, record the mean signal for the negative control and each concentration of antigen.
3) Calculate the net signal for each concentration of antigen. The net signal is the mean rate (µV/sec) for each concentration of antigen (positive signal) minus the mean rate (µV/sec) of the negative control (background signal).
4) Calculate the quantitation gain (µV/sec/concentration of antigen/ml), by dividing the net signal by the antigen concentration.

				Mean µV/s positive sample			µV/s Net Signal			Quantitation Gain µV/s/unit		
Combination Number	f-P ng/test	b-P ng/test	Mean µV/s neg. sample	1	2	3	1	2	3	1	2	3
1												
2												
3												

Figure 13-2: Portion of the Loading Study Worksheet

5) Select the concentrations of labeled antibodies that exhibit an acceptable background signal and maximum separation between the zero and low concentration, as well as between the low and high concentrations of antigen (greatest slope). In most cases, the background signal should be below 150 µV/sec. Higher background rates may be acceptable, if the assay has sufficient dynamic range and sensitivity.

Experiment 2 Standard Curve and Positive Calibrator (#)

The objective of this experiment is to define the upper and lower limits of the standard curve for the assay (Figure 13-3). From Experiment 1, the concentration of antibodies to use was determined and an estimation of the lowest standard of the standard curve can be made.

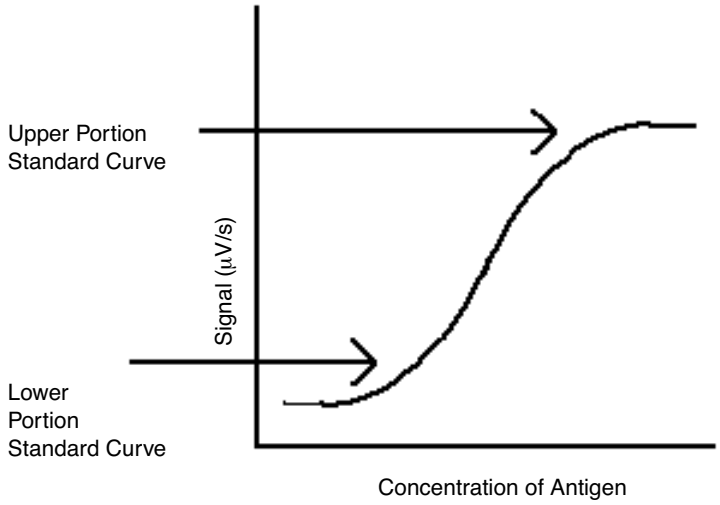


Figure 13-3: The Standard Curve

Determination of Standard Concentrations

If the background signal is 150 µV/sec or lower, the lowest standard should be the amount of antigen that produces a signal 30 µV/sec above background. If the background signal is greater than 150 µV/sec, the lowest standard should be the amount of antigen that produces a signal of 50 µV/sec above background. Use the quantitation gain (calculated in Experiment 1) to calculate the concentration of antigen for the lowest standard.

> For example: if the quantitation gain from Experiment 1 is 10 µV/sec/unit/ml, and the background signal is lower than 150 µV/sec, then the lowest standard should be 30 µV/sec above the background signal:

$$\text{Lowest Standard (S)} = \frac{30\ \mu\text{V/sec}}{10\ \mu\text{V/sec/unit/ml}}$$

$$= 3\ \text{units/ml}$$

To define the lower portion of the standard curve, use S, 2S, and 4S as standards. Typically, an ILA standard curve will have a 2 to 2.5 log (100 to 500 fold) dynamic range. Use 100S, 200S, and 500S as standards to define the upper portion of the standard curve. Choose an intermediate point (about 10S) for the mid-level calibrator (#). Use 1X Assay Buffer as the zero calibrator (*).

Note: *Maximum signal should not exceed 8,000 µV/sec for the highest calibrator.*

Reagent Preparation

This is a 1-stick experiment:

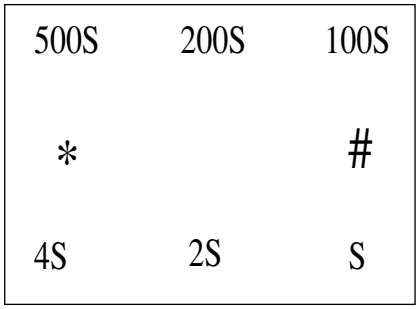


Figure 13-4: Stick Layout for Standard Curve

1) Reconstitute and dilute reagents from the ILA Detection Kit for 1 stick.
2) Prepare appropriate dilutions of the antigen for the standard curve at S, 2S, 4S, 10S, 100S, 200S, and 500S concentrations.
3) Prepare the antibody combination for 1 stick (1 ml), according to the loading study.

Assay Procedure

1) Dispense 100 µl of antigen and 100 µl of the antibody combination in the appropriate tubes. Vortex each tube briefly and gently.
2) Cover the tubes and incubate for 2 hours, at room temperature. If the antibody and/or the antigen is not stable at room temperature, incubate at appropriate temperature. During the incubation do the following:
   a. Make the Substrate Solution (600 µl of Substrate Concentrate + 30 ml of 1X Wash Solution).
   b. Remove the old Substrate Solution from the Reader.
   c. Place the new Substrate Solution in the Reader.
   d. Assemble the appropriate number of filtration units with sticks on the workstation.
   e. Hydrate a Blank Stick in a beaker of 1X Wash Solution.
   f. Dilute the reconstituted Capture Reagent 1:10 with 1X Assay Buffer.
   g. Dilute the reconstituted Enzyme Reagent 1:10 with 1X Assay Buffer.
   h. When the incubation is complete, add 1 ml of diluted Capture Reagent to each tube.
3) Transfer the incubated mixtures to the filter blocks.
4) Filter on low vacuum until the wells are empty.
5) Add 2 ml of 1X Wash Solution to each well in the filter blocks, and filter on high vacuum until the wells are empty.
6) Turn off the vacuum.
7) Add 1 ml of diluted Enzyme Reagent to each well in the filter blocks. Filter on low vacuum until the wells are empty.
8) Add 2 ml of 1X Wash Solution to each well in the filter block and filter on high vacuum until the wells are empty.

9) Turn off the vacuum.
10) Unlock the filtration units and place the sticks in the beaker of 1X Wash Solution with the Blank Stick.
11) Read the sticks.

Data Analysis

Threshold Enterprise / Software allows several curve fit options for the standard curve: power, quadratic, linear, 4-parameter and piecewise linear. The curve fits are described in Chapter 6, "Calibration and Quantitation." The appropriate standard curve fit must be determined, based on the curve shape, the error term (e), and quantitation of the standard curve from itself.

- If the upper and lower portions of the curve are well defined, and if the standards quantitate accurately from the curve within the linear portion, then proceed to Experiment 3.
- If the upper portion of the curve does not exhibit a plateau, (Figure 13-3), then repeat this experiment with a 3 log (1000-fold) dynamic range.

Experiment 3 Standard Curve 2

The objective of this experiment is to determine the optimal range of the standard curve.

Choose seven concentrations of antigen between the lower and upper standard limits selected in Experiment 2. If sensitivity is most important for this assay, include more standards in the lower portion of the curve. Otherwise, distribute the standards evenly along the curve.

Reagent Preparation

1) Prepare duplicate sticks of the standard curve.
2) Reconstitute and dilute reagents from the ILA Detection Kit for 2 sticks.
3) Prepare dilutions of the antigen for 2 standard curves.
4) Prepare antibody combination for 2 sticks (2 ml).

Assay Procedure

The protocol is the same as that used in Experiment 2.

Data Analysis

Choose the best fit for the standard curve based on the curve shape, the error term, and the quantification of the standard curve from itself. The quadratic and 4-parameter are the most common curve fits selected.

Experiment 4 Optimization of Incubation Time and Temperature

The objective of this experiment is to define the optimum incubation time and temperature using the standard curve from Experiment 3. Incubation time and temperature chosen will depend on desired sensitivity and total assay time.

Incubation Time

Choose five incubation times, for example, 30 min, 1 hr, 2 hr, 4 hr and overnight. The samples for the longest incubation period will be prepared first, so that all samples complete the incubation period at the same time.

Assay Preparation

This is a 5-stick assay.

1) Reconstitute and dilute reagents from the ILA Detection Kit for 5 sticks.
2) Prepare dilutions of the antigen for 5 standard curves.
3) Prepare antibody combination for 5 sticks.

Assay Procedure

1) Dispense 100 µl of antigen and 100 µl of the antibody combination in the appropriate tubes. Vortex each tube briefly and gently.
2) Cover the tubes and incubate at room temperature, according to the incubation times tested. Start the longest incubation first, so that all incubation times tested are complete at the same time.
   a. Make the Substrate Solution (600 µl of Substrate Concentrate + 30 ml of 1X Wash Solution).
   b. Remove the old Substrate Solution from the Reader.
   c. Place the new Substrate Solution in the Reader.
   d. Assemble the appropriate number of filtration units with sticks on the workstation.
   e. Hydrate a Blank Stick in a beaker of 1X Wash Solution.
   f. Dilute the reconstituted Capture Reagent 1:10 with 1X Assay Buffer.
   g. Dilute the reconstituted Enzyme Reagent 1:10 with 1X Assay Buffer.
   h. When the incubation is complete, add 1 ml of diluted Capture Reagent to each tube.
3) Transfer the incubated mixtures to the filter blocks.
4) Filter on low vacuum, until the wells are empty.
5) Add 2 ml of 1X Wash Solution to each well in the filter blocks, and filter on high vacuum until the wells are empty.
6) Turn off the vacuum.
7) Add 1 ml of diluted Enzyme Reagent to each well in the filter blocks. Filter on low vacuum until the wells are empty.
8) Add 2 ml of 1X Wash Solution to each well in the filter block and filter on high vacuum until the wells are empty.
9) Turn off the vacuum.
10) Unlock the filtration units and place the sticks in the beaker of 1X Wash Solution with the Blank Stick.
11) Read the sticks.

Data Analysis

The optimum incubation time depends on whether sensitivity or total assay time is most important for the assay. If total assay time is most important, choose the shortest incubation time that provides adequate sensitivity. If sensitivity is most important, choose the shortest time that provides the best sensitivity.

Incubation Temperature

The objective of this experiment is to determine if incubating at elevated temperature improves assay performance. From the incubation time experiment above, choose two incubation times. Incubate standard curves at room temperature and at 37°C.

Reagent Preparation

This is a 4-stick assay:

1) Reconstitute and dilute reagents from the ILA Detection Kit for 4 sticks.
2) Prepare dilutions of the antigen for 4 standard curves.
3) Prepare antibody combination for 4 sticks.

Assay Procedure

1) Dispense 100 µl of antigen and 100 µl of the antibody combination in the appropriate tubes. Vortex each tube briefly and gently.
2) Cover the tubes and incubate at the appropriate temperature. Start the longest incubation first, so that all incubation times tested are complete at the same time.
   a. Make the Substrate Solution (600 µl of Substrate Concentrate + 30 ml of 1X Wash Solution).
   b. Remove the old Substrate Solution from the Reader.
   c. Place the new Substrate Solution in the Reader.
   d. Assemble the appropriate number of filtration units with sticks on the workstation.
   e. Hydrate a Blank Stick in a beaker of 1X Wash Solution.
   f. Dilute the reconstituted Capture Reagent 1:10 with 1X Assay Buffer.
   g. Dilute the reconstituted Enzyme Reagent 1:10 with 1X Assay Buffer.
   h. When the incubation is complete, add 1 ml of diluted Capture Reagent to each tube.
3) Transfer the incubated mixtures to the filter blocks.
4) Filter on low vacuum until the wells are empty.
5) Add 2 ml of 1X Wash Solution to each well in the filter blocks, and filter on high vacuum until the wells are empty.
6) Turn off the vacuum.
7) Add 1 ml of diluted Enzyme Reagent to each well in the filter blocks. Filter on low vacuum until the wells are empty.
8) Add 2 ml of 1X Wash Solution to each well in the filter block and filter on high vacuum until the wells are empty.
9) Turn off the vacuum.

10) Unlock the filtration units and place the sticks in the beaker of 1X Wash Solution with the Blank Stick.

11) Read the sticks.

Data Analysis

Choose the incubation time and temperature which provides the adequate total assay time and sensitivity. Use this incubation time and temperature for future experiments.

Experiment 5 Sensitivity: Determination of the Limit of Detection

The objective of this experiment is to provide an initial estimation of the limit of detection of the assay. Limit of detection can be defined in many different ways and is often outlined by company policy. This is one method to evaluate potential limit of detection using adjusted signal. Test three concentrations of antigen and a negative control.

Concentrations of Antigen to Test

Using the data from Experiment 3:

1) Choose a concentration of antigen that generates a net signal of approximately 30 µV/sec above background, if the background rate is lower than 150 µV/sec.
2) Choose a concentration of antigen that generates 50 µV/s of net signal above background if the background rate is greater than 150 µV/sec. This experiment uses this concentration as the initial concentration (C).
3) In addition, test 1/2 of this concentration of antigen (0.5C) and twice this concentration of antigen (2C). Test three replicates for each concentration as well as a negative control (1X Assay Buffer). A larger number of replicates may be used.

Reagent Preparation

This is a 3-stick assay, consisting of a standard curve stick and two data sticks:

Stick A Standard Curve			Stick B			Stick C		
1	2	3	0.5C	C	2C	2C	0	0.5C
*		#	*		#	*		#
4	5	6	0	0.5C	C	C	2C	0

Figure 13-5: Stick Layout for Determination of Limit of Detection

1) Reconstitute and dilute ILA Detection Kit reagents for 3 sticks.
2) Prepare a standard curve (Experiment 3 above) and use the mid-level calibrator in the # position for all 3 sticks.
3) Prepare 500 µl of 0.5C, C, and 2C in 1X Assay Buffer.

4) Prepare antibody combination sufficient for 3 sticks (3 ml).

Assay Procedure

1) Dispense 100 µl of antigen and 100 µl of the antibody combination in the appropriate tubes. Vortex each tube briefly and gently.
2) Incubate at the temperature, and for the time, optimized in Experiment 3.
   a. Make the Substrate Solution (600 µl of Substrate Concentrate + 30 ml of 1X Wash Solution).
   b. Remove the old Substrate Solution from the Reader.
   c. Place the new Substrate Solution in the Reader.
   d. Assemble the appropriate number of filtration units with sticks on the workstation.
   e. Hydrate a Blank Stick in a beaker of 1X Wash Solution.
   f. Dilute the reconstituted Capture Reagent 1:10 with 1X Assay Buffer.
   g. Dilute the reconstituted Enzyme Reagent 1:10 with 1X Assay Buffer.
   h. When the incubation is complete, add 1 ml of diluted Capture Reagent to each tube.
3) Transfer the incubated mixtures to the filter blocks.
4) Filter on low vacuum until the wells are empty.
5) Add 2 ml of 1X Wash Solution to each well in the filter blocks, and filter on high vacuum until the wells are empty.
6) Turn off the vacuum.
7) Add 1 ml of diluted Enzyme Reagent to each well in the filter blocks. Filter on low vacuum until the wells are empty.
8) Add 2 ml of 1X Wash Solution to each well in the filter block and filter on high vacuum until the wells are empty.
9) Turn off the vacuum.
10) Unlock the filtration units and place the sticks in the beaker of 1X Wash Solution with the Blank Stick.
11) Read the sticks.

Data Analysis

Use adjusted signals to analyze data. Calculate the mean and standard deviation of the three antigen concentrations and the negative control. Using the equation below, determine the lowest antigen concentration that does not produce an overlap with the negative control. This antigen concentration is the estimated limit of detection in Assay Buffer.

$$X_{antigen} - 2\ \mathrm{SD}_{antigen} > X_0 + 2\ \mathrm{SD}_{X_0}$$

$X_{antigen}$ = mean antigen signal

X_o = mean background signal

SD = standard deviation

The following illustrates the concept of sensitivity:

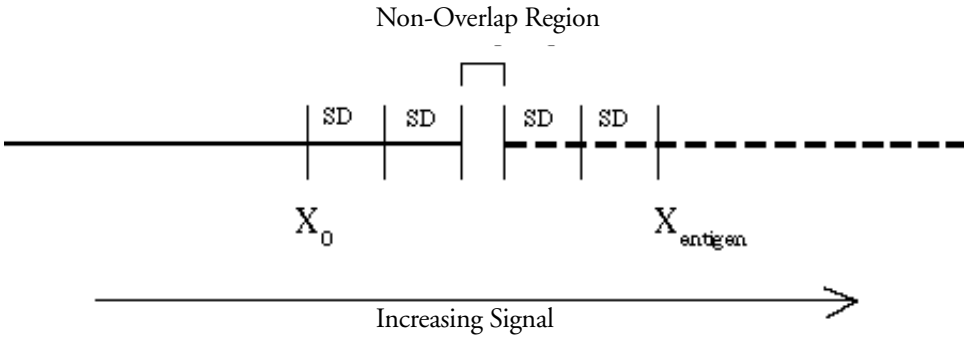


Figure 13-6: Four SD Separation Between Positive Signal and Background

Non-Overlap of Signal and Background

If all the antigen concentrations used produce signals that fail the limit of detection test and overlap with background signals, choose higher concentrations of antigen and repeat the experiment. If ($X_{antigen}$ - 2SD) is substantially greater than (X_o + 2SD), the limit of detection could be a lower concentration of antigen than those tested.

Chapter 14– ILA Competitive Format Optimization

14

ILA Competitive Format Optimization

General Issues

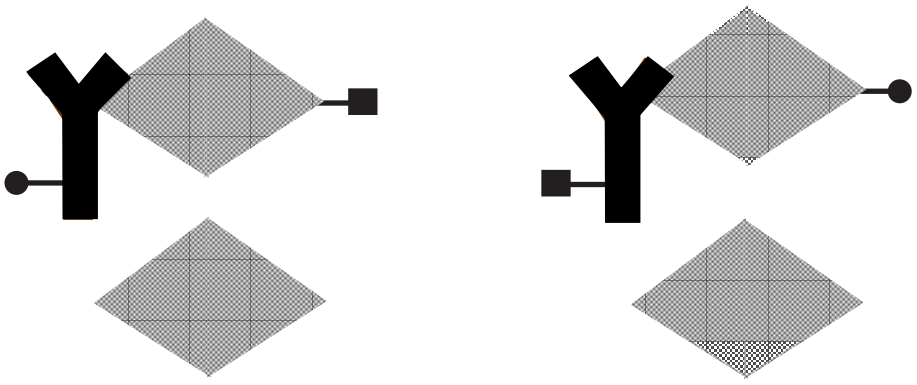
There are several general issues to be considered when optimizing a competitive ILA:

- In a competitive assay, the labeled binding protein and the labeled analyte are in limiting amount, while the streptavidin and anti-fluorescein:urease conjugate are in excess.
- The binding capacity of the b-Ab for the streptavidin is approximately 8 µg/test (approximately $53x10^{-9}$ moles/test). Adding larger concentrations of b-Ab may reduce the specific signal.
- The binding capacity of f-Ab for Enzyme Reagent (anti-fluorescein:urease) in a simultaneous incubation is approximately 25 ng/test (approximately $167x10^{-12}$ moles/test). Using larger concentrations of f-Ab, and a simultaneous incubation, may reduce the specific signal. Using a sequential incubation allows use of higher concentrations of f-Ab.
- The extent of incorporation of biotin or fluorescein may affect assay performance. Generally, a molar incorporation of 2-4 moles of biotin or fluorescein per mole of binding protein is optimal (See Chapter 12, “ILA Labeling Procedure,”, Appendix A, “Incorporation of Biotin,”and Appendix B, “Incorporation of Fluorescein.”).
- Following incubation, samples are filtered on the Threshold Workstation through a biotinylated nitrocellulose membrane. Large aggregates and particulate material in any of the added solutions may not pass through the membrane and can clog the membrane or increase the background by binding specifically or non-specifically some of the reagents (See Chapter 7, “System Troubleshooting,” and Chapter 15, “ILA Troubleshooting.”) A potential source of signal variation is the nonspecific aggregation of b-Ab and f-Ab. This signal variation may be time-dependent and may vary with the antibodies. Prepare the solution containing both these reagents shortly before use.
- If labeled reagents were prepared with more than one molar coupling ratio (MCR), first evaluate those that incorporated approximately 3 moles of hapten per mole of binding protein.

Note: *If using complex matrices, it is important to first optimize the assay conditions using purified proteins in Assay Buffer. This establishes that the labeled antibodies can detect the antigen under defined conditions. Then determine whether ILA can detect accurately a known antigen in a complex matrix.*

A competitive assay can be optimized by using two different configurations of labeled analyte and labeled binding protein (See Chapter 12, “ILA Labeling Procedure.”) For purposes of clarity, the analyte will be identified as an antigen, and the binding protein as an antibody; in this case, the compound to be quantified is the unlabeled antigen. The explanations can be reversed when the antibody is to be quantified.

- When measuring an antigen by a competitive assay, the two configurations are shown as follows (Figure 14-1):



a.

The Fluoresceinated antigen competes with the unlabeled antigen to bind the biotinylated antibody.

b.

The biotinylated antigen competes with the unlabeled antigen to bind the fluoresceinated antibody.

Figure 14-1: Measuring an Antigen

In both configurations, the labeled antigen and the labeled antibody are in limiting concentrations.

Purity of the antigen and antibody need to be considered when choosing between the two configurations. The purest component is usually fluoresceinated (See Chapter 12, "ILA Labeling Procedure.") If purity and / or possible interference does not allow determination of the configuration, label the antigen with fluorescein.

- When the configuration is chosen, two different protocols can be used: simultaneous incubation or sequential incubation. Potential of non-specific binding and interference is the most important criterion for choosing between the two protocols. (see Chapter 9, "Immuno-Ligand Assay Introduction.") Use the sequential protocol, unless previous experience with the antibody and antigen shows that the simultaneous protocol is the best. The overall performance of the assay is generally better with a sequential incubation protocol.

For better clarity, the following experiments will describe an assay with fluoresceinated antigen, and will use the sequential incubation protocol.

Experiment 1

Loading Study: Determination of the Amount of Labeled Binding Proteins Required

The loading study should be performed after each labeling experiment, in order to ensure that the labeled proteins generate comparable signals for each labeling.

The objective of the loading study is to react a fixed concentration of labeled antigen with varied concentrations of biotinylated antibody. There is no competition between labeled and unlabeled antigen in the loading study. The resulting plot will show an increase of signal with increasing concentrations of biotinylated antibody, until a plateau is reached.

- The fixed concentration of labeled antigen is conventionally four times the desired limit of detection. This concentration of labeled antigen will be used in the loading study as well, as in the final assay. The concentration of antigen, corresponding to the desired limit of detection, will be used in the standard curve as one of the lowest concentrations of unlabeled antigen.

  Example: The desired limit of detection is 0.5 ng of antigen. The amount of labeled antigen to use in this loading study is 2 ng. In the final assay, if 2 ng of labeled antigen competes with 0.5 ng of unlabeled antigen (desired limit of detection), the unlabeled antigen represents 20% of the total antigen present
  (2 ng + 0.5 ng = 2.5 ng). The unlabeled antigen should decrease the total signal by 20% when competing with the labeled antigen to form a complex. At this point of the assay optimization, it is assumed that the labeled and unlabeled antigen have comparable affinities for the antibody. A 20% decrease of the signal is a reasonable definition of the limit of detection: if the standard deviation of the zero antigen corresponds to 5%, four times this standard deviation will still be equal to the limit of detection (see Experiment 5).

  If the availability of the antigen and / or the antibody is limited, perform the loading study with only one concentration of labeled antigen (four times the desired limit of detection). If antibody and antigen availability is not a problem, test two different amounts of labeled antigen. In the previous example, it would be recommended to test 2 ng and 10 ng of the labeled antigen.

- The varied concentrations of biotinylated antibody should represent a wide range of concentrations, to produce a plateau in signal. In case of extremely high affinity between the antigen and antibody, one mole of antibody would theoretically bind one mole of antigen. Therefore, the two lowest concentrations of biotinylated antibody to use will be the equimolar amount of labeled antigen and half of this concentration.

  Be sure to test six higher concentrations, taking into account the purity of the antibody: if the antibody is affinity purified against the antigen, a 2 log (100-fold) range is probably enough to produce the plateau; if the antibody is not affinity purified, 2.5 log to 3 log (500 to 1000-fold) range may be necessary, because the percentage of specific antibody reacting with the antigen can be less than 10%.

  The eight antibody concentrations can be tested on 1 stick.

- In the present example, it is assumed that the antibody is affinity purified, and that the molecular weights of the antigen and the antibody are comparable. Then the two lowest

concentrations of biotinylated antibody would be 2 ng and 1 ng for 2 ng of labeled antigen. The higher concentrations of biotinylated antibody would be: 4 ng, 10 ng, 25 ng, 50 ng, 100 ng, 200 ng for a 2 log (100-fold) range (See Table 14-1).

- If more than one concentration of labeled antigen is being tested, the antibody concentrations should be adjusted to the amount of labeled antigen used.

Each concentration of f-Ag has to be tested with each concentration of b-Ab.

Table 14-1: Example of Loading Study (Antibody is > 90% Specific and Molecular Weights of Antigen and Antibody are Comparable)

f-antigen	b-antibody
2 ng/test	200 ng/test
10 ng/test	100 ng/test
	50 ng/test
	25 ng/test
	10 ng/test
	4 ng/test
	2 ng/test
	1 ng/test

Reagent Preparation

The number of sticks depends on the design of the loading study. Duplicate sticks are used per concentration of labeled antigen tested. If two concentrations of labeled antigen are tested, 4 sticks will be used.

1) Reconstitute and dilute reagents in the ILA Detection Kit for the number of sticks required.
2) Arrange and label the polypropylene tubes according to the design of the loading study as shown below. The Threshold Organizer Rack facilitates the transfer of the samples to the filtration units.

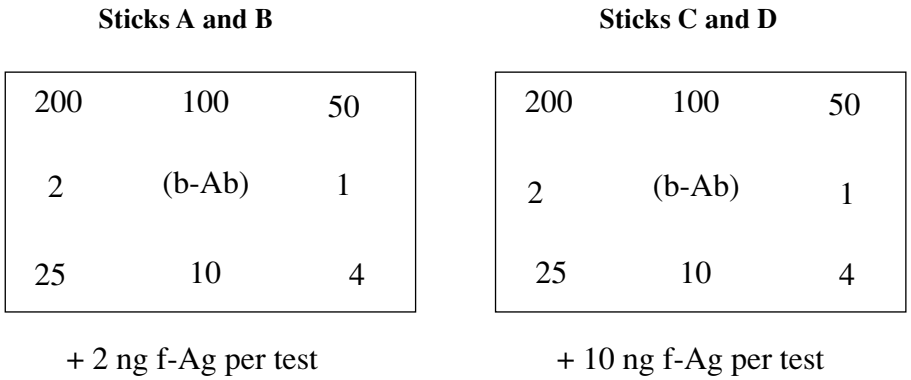


Figure 14-2: Stick Layout for the Loading Study

3) Dispense 100 µl of 1X Assay Buffer in each tube. This allows the volume of the reaction mixture to be the same as future experiments, in which the volume of unlabeled antigen will be 100 µl.
4) Prepare 2 ml of labeled antigen dilution in 1X Assay Buffer (1 ml per stick).

   Note: *The concentration unit used may be either per ml or per test. The concentration per ml is 10 times the concentration per test.*
5) Prepare 1 ml of each concentration of labeled antibody diluted in 1X Assay Buffer.

Assay Procedure

1) Dispense 100 µl of labeled antigen and 100 µl of labeled antibody to the appropriate tubes. Mix briefly and gently.
2) Cover the tubes, and incubate overnight at room temperature. If the antibody and / or the antigen is not stable at room temperature, incubate at appropriate temperature. Shortly before the end of the incubation, do the following:
   a. Make the Substrate Solution (600 µl of Substrate Concentrate + 30 ml of 1X Wash Solution).
   b. Remove the old Substrate Solution from the Reader.
   c. Place the new Substrate Solution in the Reader.
   d. Assemble the appropriate number of filtration units with sticks on the workstation.
   e. Hydrate a Blank Stick in a beaker of 1X Wash Solution.
   f. Dilute the reconstituted Capture Reagent 1:10 with 1X Assay Buffer.
   g. Dilute the reconstituted Enzyme Reagent 1:10 with 1X Assay Buffer.
   h. When the incubation is complete, add 1 ml of diluted Capture Reagent to each tube.
3) Transfer the incubated mixtures to the filter blocks.
4) Filter on low vacuum, until the wells are empty.
5) Add 2 ml of 1X Wash Solution to each well in the filter blocks, and filter on high vacuum, until the wells are empty.
6) Turn off the vacuum.

7) Add 1 ml of diluted Enzyme Reagent to each well in the filter blocks. Filter on low vacuum, until the wells are empty.
8) Add 2 ml of 1X Wash Solution to each well in the filter block and filter on high vacuum, until the wells are empty.
9) Turn off the vacuum.
10) Unlock the filtration units and place the sticks in the beaker of 1X Wash Solution with the Blank Stick.
11) Read the sticks.

Data Analysis

1) Plot the signal obtained in µV/sec on the y axis, and the concentration of antibody tested on the x axis. Use the mean signal for replicates.

   For each concentration of fluoresceinated antigen, the signal increases with increasing concentrations of biotinylated antibody until it reaches a plateau at the maximum signal.

2) Select the concentration of antibody corresponding to approximately 50 to 70% of the maximal signal plateau as the concentration of labeled antibody to use in subsequent assays.
3) In the standard curve experiment, test the different concentrations of labeled antigen used in the loading study associated with the corresponding concentration of biotinylated antibody.

- If the signal corresponding to 50-70% of the maximal signal plateau is below 500 µV/sec, the detection limit could be more difficult to achieve with the concentrations of antigen and antibody chosen. The loading study should be repeated with higher concentrations of labeled antigen.
- If the signal is very high (8000 µV/sec) the sensitivity may be improved by lowering the concentration of labeled antigen. The potential sensitivity of the assay depends on the affinity of the antibody for the antigen.

Experiment 2 Standard Curve 1

The objective of Standard Curve 1 experiment is to define the upper and lower portions of the standard curve. The concentrations of fluoresceinated antigen and biotinylated antibody chosen from the loading study will be used with a broad concentration range of unlabeled antigen.

The concentration of unlabeled antigen corresponding to the desired limit of detection will be called "S". Use 0.5S, 1S and 2S as the lower concentrations of unlabeled antigen. Typically, the standard curve will have a 1.5 to 2 log (50 to 100-fold) range. Use 50S, 100S and 200S for the higher concentrations. Choose an intermediate point (about 10S) as a midlevel calibrator (#). Use the Assay Buffer as the zero calibrator (*).

Reagent Preparation

1) Prepare 2 replicate sticks for this first standard curve. If two labeled antigen concentrations were tested in the loading study, test the second combination of antigen and anti-

body on 2 replicate sticks.

2) Reconstitute and dilute reagents in the ILA Detection Kit for 2 sticks.
3) Prepare 2 ml of fluoresceinated antigen in 1X Assay Buffer at the concentration chosen from the loading study.
4) Prepare 2 ml of biotinylated antibody in 1X Assay Buffer at the concentration chosen from the loading study.
5) From a known stock, prepare dilutions of the unlabeled antigen in 1X Assay Buffer, to obtain the concentrations: 200S, 100S, 50S 10S, 2S, 1S and 0.5S., knowing that S is the desired limit of detection. Prepare 500 µl of each dilution.
6) Arrange the tubes in a rack, as shown in Figure 14-3.

Sticks A and B

200S	100S	50S
0 (*)	(Ag)	(#) 10S
25	10	4

+ f-Ag (4 times desired limit of detection)

+ b-Ab (50-70% maximal signal)

Figure 14-3: Stick Layout for the Standard Curve

Assay Procedure

1) Dispense 100 µl of the different concentrations of unlabeled antigen in the tubes. Add 100 µl per tube of fluoresceinated antigen, then 100 µl of biotinylated antibody. Mix briefly and gently.

   It may be beneficial to preincubate the unlabeled antigen with the biotinylated antibody for 1 hour, prior to addition of the labeled antigen. This can sometimes improve the sensitivity of the assay. It will be tested in “Experiment 4” on page 14-11. Cover the tubes, and incubate overnight at room temperature. If the antibody and / or the antigen is not stable at room temperature, incubate at the appropriate temperature. Shortly before the end of the incubation, do the following:

   a. Make the Substrate Solution (600 µl of Substrate Concentrate + 30 ml of 1X Wash Solution).
   b. Remove the old Substrate Solution from the Reader.
   c. Place the new Substrate Solution in the Reader.
   d. Assemble the appropriate number of filtration units with sticks on the workstation.
   e. Hydrate a Blank Stick in a beaker of 1X Wash Solution.
   f. Dilute the reconstituted Capture Reagent 1:10 with 1X Assay Buffer.

g. Dilute the reconstituted Enzyme Reagent 1:10 with 1X Assay Buffer.

h. When the incubation is complete, add 1 ml of diluted Capture Reagent to each tube.

2) Transfer the incubated mixtures to the filter blocks.

3) Filter on low vacuum, until the wells are empty.

4) Add 2 ml of 1X Wash Solution to each well in the filter blocks, and filter on high vacuum until the wells are empty.

5) Turn off the vacuum.

6) Add 1 ml of diluted Enzyme Reagent to each well in the filter blocks. Filter on low vacuum, until the wells are empty.

7) Add 2 ml of 1X Wash Solution to each well in the filter block and filter on high vacuum until the wells are empty.

8) Turn off the vacuum.

9) Unlock the filtration units and place the sticks in the beaker of 1X Wash Solution with the Blank Stick.

10) Read the sticks.

Data Analysis

Typically, the standard curve for a competitive assay is sigmoidal (See Figure 14-4).

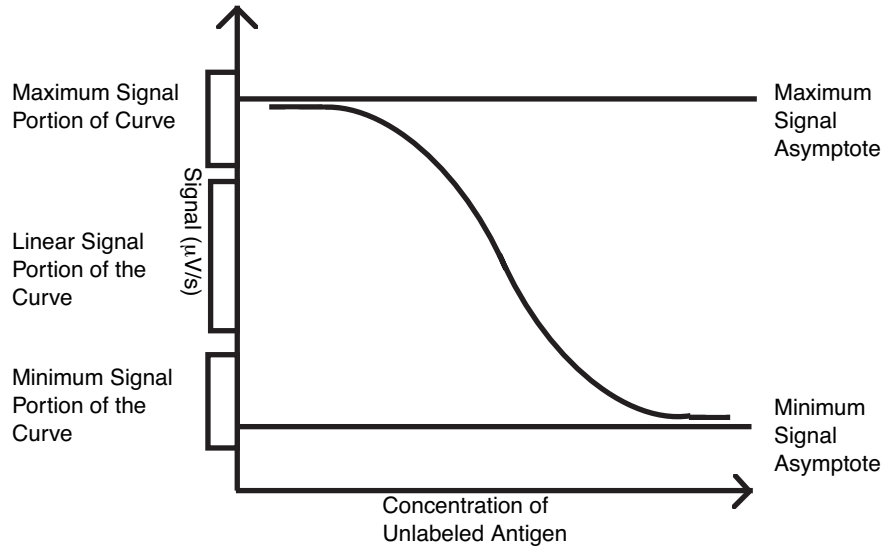


Figure 14-4: Sigmoidal Standard Curve

The zero concentration unlabeled antigen (Assay Buffer) generates the maximal signal, and a very high concentration of unlabeled antigen generates the minimal signal. The maximal and minimal signals may be difficult to determine from this experiment, if the concentrations used in the standard curve are not high or low enough to show the two horizontal asymptotes. Maximal and minimal signals may not be necessary to optimize

the assay. Nevertheless, determining maximal and minimal signals may be helpful to study the precision along the standard curve.

Precision of the data interpolated from the standard curve is best in the linear portion. Defining limits for this linear portion is recommended if limit of detection and dynamic range have to be studied. In the "low concentration" portion of the standard curve, precision of quantified data is frequently considered acceptable when the signal is 30 µV/sec lower than the maximal signal. In the "high concentration" portion of the standard curve, the limit for acceptable precision can vary. Determining the minimal signal may need large amounts of unlabeled antigen to define the dynamic range. In some cases, the linear portion of the standard curve may be defined as 30 µV/sec above the minimal signal and 30 µV/sec below the maximal signal.

The concentrations of unlabeled antigen corresponding to the estimated linearity limits will be used as the lowest and highest concentrations of the Standard Curve 2.

Experiment 3 Standard Curve 2

The objective of the Standard Curve 2 experiment is to determine the optimal range of the unlabeled antigen concentration, as standards for the standard curve.

1) Choose seven concentrations of unlabeled antigen, between the limits decided from the first standard curve experiment. If sensitivity is most important for this assay, include more standards in the lower concentrations. Otherwise, distribute the concentrations evenly between the limits required for acceptable precision.
2) Follow the same assay procedure, as in Experiment 2, using 2 replicate sticks.

   Detailed descriptions of the curve fits are given in Chapter 6, “Calibration and Quantitation.” The appropriate standard curve must be determined,based on the curve shape, the error term (e), and the quantification of the Standard Stick from itself. The best curve fit may be 4-parameter or quadratic.

Experiment 4 Optimization of Incubation Time and Temperature

The objective of the Optimization of Incubation Time and Temperature experiment is to test different incubation times and temperatures, using the standard curve from Experiment 3.

Choose three incubation times, including an overnight incubation. For example, 2 hours, 4 hours and overnight. Start the longest incubation first, so that all samples complete incubation at the same time.

Reagents Preparation

1) Reconstitute and dilute reagents in the ILA Detection Kit for 3 sticks.
2) Prepare 3 ml of fluoresceinated antigen at the same concentration, as in “Experiment 3” on page 14-11.
3) Prepare 3 ml of biotinylated antibody at the same concentration, as in “Experiment 3” on page 14-11.
4) Prepare 1 ml serial dilution of the unlabeled antigen from the known stock. Use the

same concentrations, as in "Experiment 3" on page 14-11.

5) Arrange the tubes in a rack, as in "Experiment 3" on page 14-11.

Assay Procedure

1) Dispense 100 µl of the different concentrations of unlabeled antigen in the tubes. Add 100 µl per tube of fluoresceinated antigen, then 100 µl of biotinylated antibody. Mix briefly and gently.
2) Cover the tubes, and incubate at room temperature for the times tested. Shortly before the end of the incubation, do the following:
   a. Make the Substrate Solution (600 µl of Substrate Concentrate + 30 ml of 1X Wash Solution).
   b. Remove the old Substrate Solution from the Reader.
   c. Add the new Substrate Solution to the Reader.
   d. Assemble the appropriate number of filtration units with sticks on the workstation.
   e. Hydrate a Blank Stick in a beaker of 1X Wash Solution.
   f. Dilute the reconstituted Capture Reagent 1:10 with 1X Assay Buffer.
   g. Dilute the reconstituted Enzyme Reagent 1:10 with 1X Assay Buffer.
   h. When the incubation is complete, add 1 ml of diluted Capture Reagent to each tube.
3) Transfer the incubated mixtures to the filter blocks.
4) Filter on low vacuum, until the wells are empty.
5) Add 2 ml of 1X Wash Solution to each well in the filter blocks, and filter on high vacuum, until the wells are empty.
6) Turn off the vacuum.
7) Add 1 ml of diluted Enzyme Reagent to each well in the filter blocks. Filter on low vacuum, until the wells are empty.
8) Add 2 ml of 1X Wash Solution to each well in the filter block and filter on high vacuum, until the wells are empty.
9) Turn off the vacuum.
10) Unlock the filtration units and place the sticks in the beaker of 1X Wash Solution with the Blank Stick.
11) Read the sticks.

Data Analysis

The optimum incubation time depends on whether sensitivity or total assay time is most important.

- If total assay time is most important, choose the shortest incubation time that provides adequate sensitivity.
- If sensitivity is most important, choose the shortest time that provides the best sensitivity.

1) Evaluate the loss of sensitivity between the overnight incubation and the 4-hour incuba-

tion. If the difference is within a factor of 2 or less, compare the 2-hour and 4-hour incubations at room temperature, in parallel with incubations at 37°C in a water bath.

2) Use the same dilutions for all four sticks, and keep the solutions at 4°C during the 2-hour delay. Elevated temperature incubation may improve sensitivity and allow shorter incubation time.
3) Test a preincubation protocol:
   a. Incubate the unlabeled antigen and the antibody for 1 hour.
   b. Add the labeled antigen and incubate for an additional 3 hours.
   c. Perform this in parallel with a 4-hour incubation without preincubation. The preincubation protocol can improve sensitivity by giving the unlabeled antigen the advantage of binding first and longer to the antibody.

Experiment 5 Sensitivity: Determination of the Limit of Detection

The limit of detection can be defined in many different ways and is often outlined by company policy. Here is one method to evaluate possible limit of detection.

The objective of the Senstivity: Determination of the Limite of Detection experiment is to test several low concentrations of unlabeled antigen, to evaluate the limit of detection.

Reagent Preparation

1) Reconstitute and dilute reagents in the ILA Detection Kit for 3 sticks.
2) Prepare 3 ml of fluoresceinated antigen at the same concentration, as in “Experiment 3” on page 14-11.
3) Prepare 3 ml of biotinylated antibody at the same concentration, as in “Experiment 3” on page 14-11.
4) Prepare 500 µl of each dilution of the unlabeled antigen to make the same standard curve, as in “Experiment 3” on page 14-11. The * and # solutions of the standard curve will be used for the 2 other sticks. From the same stock, make 500 µl of each dilution to reach a concentration "C" of unlabeled antigen corresponding to a separation of 30 µV/sec from the estimated maximum signal. Also, test 0.5C and 2C.
5) Arrange the tubes in a rack, as shown below in Figure 14-5.

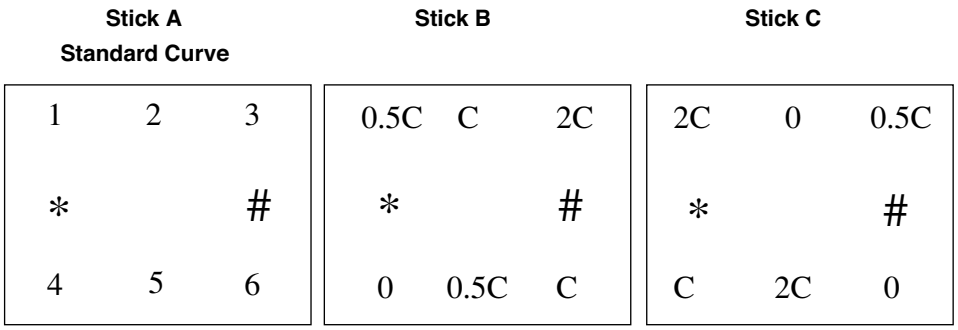


Figure 14-5: Stick Layout for Limit of Detection Determination

Assay Procedure

1) Dispense 100 µl of the different concentrations (0.5C, 1C and 2C) of unlabeled antigen in the tubes. Add 100 µl per tube of fluoresceinated antigen, then 100 µl of biotinylated antibody. Mix briefly and gently.
2) Cover the tubes, and incubate according to optimized time and temperature in Experiment 4. Shortly before the end of the incubation, do the following:
   a. Make the Substrate Solution (600 µl of Substrate Concentrate + 30 ml of 1X Wash Solution).
   b. Remove the old Substrate Solution from the Reader.
   c. Place the new Substrate Solution in the Reader.
   d. Assemble the appropriate number of filtration units with sticks on the workstation.
   e. Hydrate a Blank Stick in a beaker of 1X Wash Solution.
   f. Dilute the reconstituted Capture Reagent 1:10 with 1X Assay Buffer.
   g. Dilute the reconstituted Enzyme Reagent 1:10 with 1X Assay Buffer.
   h. When the incubation is complete, add 1 ml of diluted Capture Reagent to each tube.
3) Transfer the incubated mixtures to the filter blocks.
4) Filter on low vacuum, until the wells are empty.
5) Add 2 ml of 1X Wash Solution to each well in the filter blocks, and filter on high vacuum, until the wells are empty.
6) Turn off the vacuum.
7) Add 1 ml of diluted Enzyme Reagent to each well in the filter blocks. Filter on low vacuum, until the wells are empty.
8) Add 2 ml of 1X Wash Solution to each well in the filter block and filter on high vacuum, until the wells are empty.
9) Turn off the vacuum.
10) Unlock the filtration units and place the sticks in the beaker of 1X Wash Solution with the Blank Stick.

11) Read the sticks.

Data Analysis

Use the adjusted mean signals. Evaluate possible limit of detection as follows:

$$X_{antigen} - 2\ \mathrm{SD}_{antigen} > X_0 + 2\ \mathrm{SD}_{X_0}$$

$X_{antigen}$ = mean signal for each concentration of unlabeled antigen

X_0 = mean background signal (zero concentration of unlabeled antigen)

SD = standard deviation

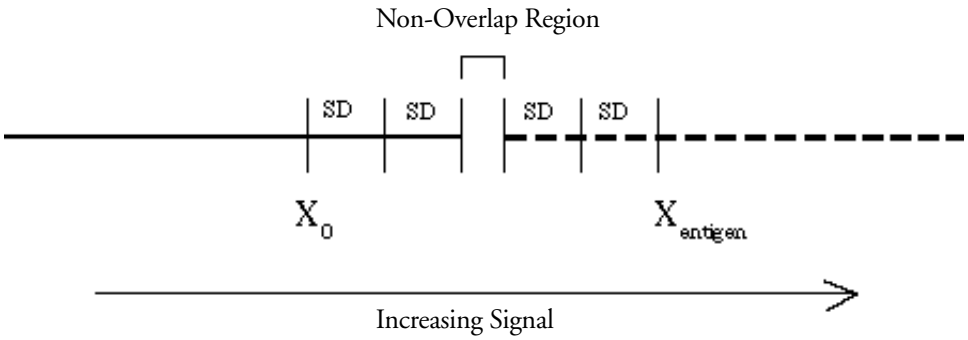


Figure 14-6: Four SD Separation Between Positive Signal and Background

Non-Overlap of Signal and Background

If all the concentrations tested fail the limit of detection, defined in the equation above, test higher concentrations of antigen. If (X - 2SD) is substantially greater than (X_0 + 2SD), the limit of detection could be a lower concentration of antigen. Repeat Experiment 5, using lower concentrations of unlabeled antigen.

Chapter 15– ILA Troubleshooting

15

Introduction

This chapter contains solutions to possible problems encountered during the biotin and fluorescein labeling procedures, and the optimization of the assay. The following tables list effects, with possible causes, along with solutions for each cause.

If you need further assistance, call Molecular Devices Technical Support.

Please have this information available so that we can help you efficiently:

- Reagent Kit Lot number.
- Workstation serial number.
- Reader serial number.
- Exact nature of the problem.

Note: *Please obtain a Return Goods Authorization Number from Molecular Devices before returning materials or parts.*

Effects, Possible Causes and Solutions

Labeling Proteins with Biotin and Fluorescein

Table 15-1: Labeling Proteins with Biotin and Fluorescein

Effect	Possible Cause	Solution	Reference
Protein Precipitates after labeling	The molar incorporation ratio is too high	Lower the molar coupling ratio.	Chapter 12
	Protein is denatured by DMF	Dissolve h-NHS in a smaller volume of DMF and use less volume during the labeling reaction.	
Molar Incorporation Ratio Too Low	Presence of a nucleophile or other interfering substance	Dialyze the protein to be labeled against PBS before labeling.	Chapter 12
	Few or inaccessible lysines	Increase the molar coupling ratio and/or increase the incubation time.	
Molar Incorporation Ratio Too High	Many lysines	Reduce the molar coupling ratio, and/or decrease the incubation time.	Chapter 12
Recovery of Labeled Protein < 50%	Aggregation or precipitation of labeled protein	1. Separate the unreacted hapten from the protein by dialysis, after stopping the labeling reaction with sodium azide. 2. If the molar incorporation ratio is high, reduce the molar coupling ratio.	Chapter 12
	Spectrophotometer not working properly	Reread the samples on another spectrophotometer.	
	Defective PD-10 column	Inspect the column and contact the vendor.	
	Protein interacts with the PD-10 column	Separate the unreacted hapten from the protein, using dialysis or other suitable chromatography.	
Recovery of Labeled Protein >100%	Aggregation of protein during the labeling reaction	Assume a protein recovery of 80% to calculate the protein concentration and test the performance of the labeled protein in a Labeled Binding Protein (loading) Study.	Chapter 12
	The spectrophometer is not working properly	Reread the samples on another spectrophotometer.	
	High molar incorporation ratio has caused aggregation	Relabel the protein, using a lower molar coupling ratio.	
	Incorporation of hapten (especially fluorescein), can shift the absorbance of the protein, particularly if a small peptide is labeled.	1. Assume a protein recovery of 80% to calculate protein concentration and test performance. 2. Label the protein with biotin. Determine the molar incorporation ratio and test performance of the labeled protein in a loading study.	

ILA Optimization Assay

Table 15-2: ILA Optimization Assay

Effect	Possible Cause	Solution	Reference
Background µV/sec Rates > 200 µV/sec	Labeled protein concentration per test is too high	Reduce the concentration of the labeled protein per test.	Chapter 13 Chapter 14
	Binding protein lacks specificity	1. Antibodies: purify by affinity chromatography with antigen coupled beads. 2. Other Proteins: purify by an appropriate method.	
	Aggregation of biotinylated protein fluoresceinated protein	Prepare the binding protein solutions immediately prior to use, and filter through a 0.22 µm low protein binding filter such as PVDF, Polysulfone or cellulose acetate. Reread the absorbance to correct for any change in concentration.	
Low Positive Signal (µV/sec/concentration of analyte)	Molar incorporation ratio too low or too high	Perform a new labeling.	Chapter 13 Chapter 14
	The binding protein does not have a high affinity for analyte	1. Increase the incubation time of the binding proteins with the analyte. 2. Increase the concentration of the binding proteins. 3. Find a binding protein with a high affinity for the analyte.	
	Analyte not stable	Use freshly prepared analyte. Store the analyte under appropriate conditions.	
	Analyte or binding proteins stick to storage container or test tubes	Use appropriate test tubes that do not bind the analyte or binding proteins.	
Poor Sensitivity	All of "Low Positive Signal" causes listed above	All the Low Positive Signal solutions.	Chapter 13 Chapter 14
	High background rates, which increase the standard deviation of the buffer control, and low concentrations of analyte	Use binding proteins with better specificity.	
	Using simultaneous incubation protocol, when the binding protein exceeds the concentration of anti-fluorescein-urease (> 25 ng/test)	Use the sequential incubation protocol and increase the concentration of binding proteins.	

Table 15-2: ILA Optimization Assay (Continued)

Effect	Possible Cause	Solution	Reference
Poor Precision	Pipetting technique	Carefully dispense the sample and binding protein to the bottom of the tube, without splashing. Do not dispense on the side of the tube.	Chapter 13 Chapter 14
	Silicon Sensor in Reader "fouled" with protein	Test the Reader for precision; if necessary purchase a new Reader.	
Poor Assay Reproducibility	Labeled proteins are not stable	Compare the stored labeled proteins with freshly labeled or freshly diluted proteins; make sure the labeled proteins are diluted in an appropriate buffer.	Chapter 13 Chapter 14
	Analyte is not stable	Compare stored analyte with a fresh aliquot of analyte.	
	Labeled proteins or analyte are sticking to storage or test containers	Use appropriate containers for storage and testing.	
	Labeled proteins or analyte are not being diluted the same for each experiment	Use careful and consistent technique to dilute assay reagents.	
Samples Filter Slowly or at Different Rates	Sample components are aggregated	Prefilter samples before dispensing, through a low protein binding 0.22 µm filter (PVDF, polysulfone or cellulose acetate).	

Chapter 16– Total DNA Assay Introduction

The Challenge of DNA Measurement

The use of recombinant DNA methods to produce therapeutic proteins has made it necessary to measure picogram quantities of DNA contamination.

DNA measurements are technically challenging because of these factors:

- ***Limited Sensitivity.*** Spectrophotometric analysis, using absorbance at 260 nm, or fluorometric analysis with ethidium bromide, cannot detect less than 10 ng/ml of DNA.
- ***Background***. There is usually a large excess of protein relative to DNA in samples.
- ***Time***. Nucleic acid hybridization assays can detect 10 pg of DNA, but they are labor intensive and time consuming. They also lack precision and reproducibility.

The Threshold Total DNA Assay quantitatively measures picograms of single-stranded DNA (ssDNA). Precision and reproducibility are high. Unlike hybridization techniques, which are limited to detecting DNA by its specific nucleic acid sequence, the Total DNA Assay measures DNA with broad sequence specificity. The assay does not detect RNA.

Threshold Total DNA Assay

The Total DNA Assay is specific for single-stranded DNA. The assay has three stages (See Figure 16-1).

Stage 1: Reaction Stage

In this stage, single-stranded DNA reacts with two binding proteins in the Labeling Reagent. One binding protein is a high affinity, single-stranded DNA binding protein (SSB), from *E. coli*, conjugated to biotin. Streptavidin, also present, binds tightly to the biotin on the SSB conjugate. The other binding protein is a monoclonal anti-DNA antibody against single-stranded DNA, conjugated to the enzyme urease. These binding proteins form a complex with DNA, in solution, at 37° C.

Stage 2: Separation Stage

This stage occurs on the Threshold Workstation. The DNA complex is filtered through a biotin coated nitrocellulose membrane. The biotin on the membrane reacts with the streptavidin in the DNA complex, capturing the complex. A rapid wash step removes nonspecific enzyme from the membrane.

Stage 3: Detection Stage

For this stage, the stick is placed in the Threshold Reader, which contains the substrate, urea. The enzymatic reaction changes the local pH of the substrate solution. A silicon sensor records a change in surface potential which is proportional to the pH change. The rate of change in surface potential is proportional to the amount of DNA.

The Threshold Workstation, the computer, and the Threshold Enterprise / Software monitor surface potential changes at each measurement site. The computer analyzes these kinetic measurements and quantitates the results, using a previously generated standard curve. Threshold Enterprise / Software computes the results for each sample in picograms of DNA.

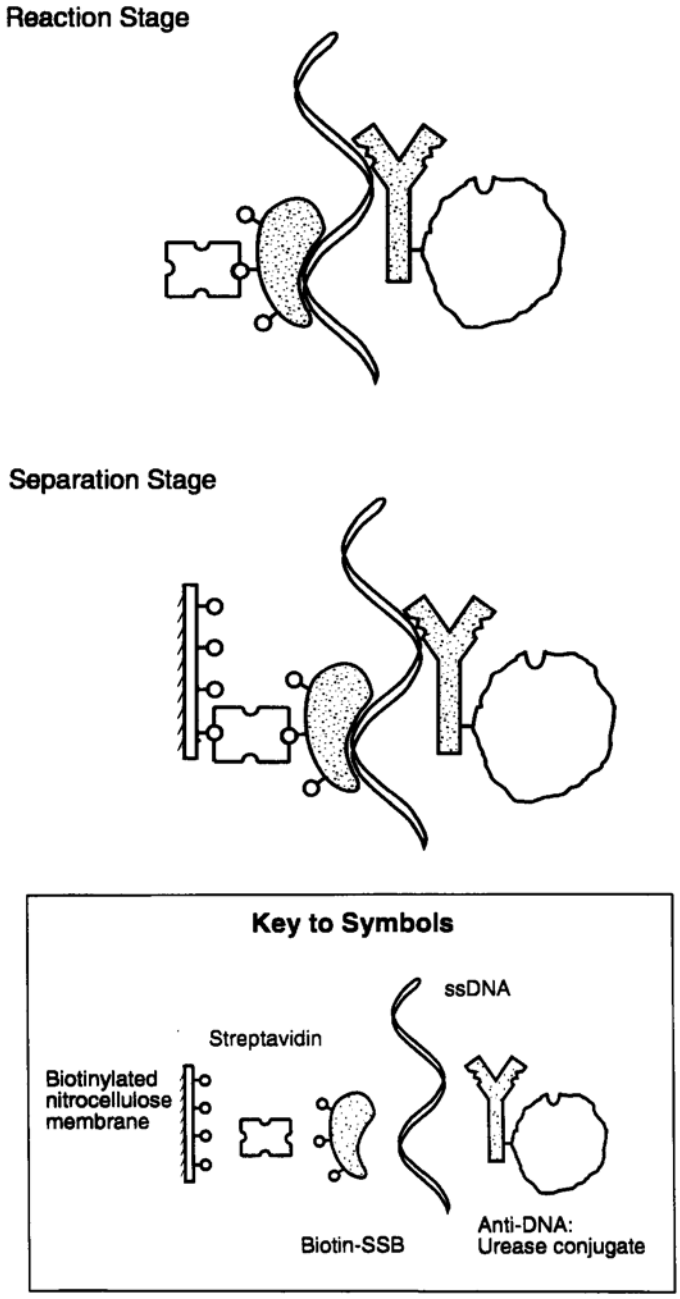


Figure 16-1: Total DNA Assay Reaction

Prerequisites For the DNA Assay

Compatible samples may be directly measured in the Total DNA Assay. Some samples may have to be pretreated to remove substances that can interfere with the assay.

Incompatibility of samples, composed largely of proteins, is often due to protein precipitation and aggregation. This occurs, before the Total DNA Assay, when samples are heated to denature DNA. Samples may also contain other interfering substances.

DNA can also inhibit the assay, either when present in large quantities (>1 nanogram per ml), or when there is a high concentration of short fragments (20 to 80 bases). Large quantities of DNA can inhibit the assay, either by generating signals too high for Threshold Enterprise / Software to accurately quantitate, or by consuming all of the binding conjugates. Very short fragments of DNA competitively inhibit the assay, by preventing the formation of complete reaction complexes.

Chapter 12, "Total DNA Sample Pretreatment," lists the optimal properties of samples, describes how to evaluate samples, and recommends pretreatment procedures.

References

Briggs, J., Kung, V.T., Pontis, G., Nagainis, P., Panfili, R.P., Chow, C., Smith, T., Sheldon, E., Ross, D., Smethers, R., Gomez, B., Lacy, M., Drbal, V., King, R. and Zuk, R., A total DNA assay system, *American Biotechnology Laboratory*, 7, 34-38 (1989).

Chase, J.W., Single-stranded DNA binding proteins required for DNA replication, *Ann. Rev. Biochem.*, **55**, 103-136 (1986).

Hafeman, D.G., Parce, J.W. and McConnell, H.M., Light addressable potentiometric sensor for biochemical systems, *Science*, **240**, 1182-1185 (1988).

Kung, V.T., Panfili, P.R., Sheldon, E.L., King, R.S., Nagainis, P.A., Gomez, Jr, B., Rose, D.A., Briggs, J., and Zuk, R.F., Picogram Quantitation of total DNA using DNA binding proteins in a silicon sensor-based system, *Anal. Biochem.*, **187**, 220-227 (1990).

Sheldon, E.L., Nagainis, P.A. and Kung, V.T., Detection of total DNA with single-stranded DNA binding protein conjugates, *Biochem. Biophys. Res. Comm.*, **156(1)**, 474-480 (1989).

Stollar, D.B., Antibodies to DNA, *CRC Critical Reviews in Biochem.*, **20**, 1-35 (1986).

Chapter 17– Total DNA Assay Procedure

Total DNA Assay Procedure

Introduction

This chapter describes the Total DNA Assay procedure. The Total DNA Assay Kit has supplies for 8 DNA sticks. Each stick allows for eight tests—six unknowns and two calibrators. Typically, one stick contains samples for the standard curve. The Total DNA Assay Kits are also available in a bulk kit, providing 100 sticks.

These are the steps in the assay:

- Pretreat your sample (if necessary).
- Set up the assay.
- Prepare for filtration.
- Filter the incubated assay mixtures.
- Read the sticks.
- Save the results and print the report.
- Shut down the system.

Required Reagents and Equipment

Threshold Reagents and Disposables

One Total DNA Assay Kit contains these reagents, sufficient for 8 sticks.

Note: *Inspect the contents of the kit for shipping damage, discoloration, or cloudiness. Do not use the kit, if it is defective, or if it is beyond its expiration date.*

- DNA Labeling Reagent (L), eight vials.
- Reconstitution Buffer (R), one vial.
- Substrate Concentrate (S), one vial.
- Wash Solution (W), two bottles.
- Zero Calibrator/Control (*), three vials.
- Positive Control (C), one vial (See the Certificate of Analysis in the Reagent Kit for DNA concentration).
- High Calibrator (H), one vial (Contains calf thymus DNA, 5 ng/ml).
- Ten Threshold sticks.
- Eight disposable, single-use, Threshold filter units, packaged as separate filter blocks and filter bases.

Caution: *Store the Assay Kit in the refrigerator. Equilibrate the Wash Solution to room temperature, before reading a stick.*

Standard Laboratory Equipment Supplied by User

- One 50 ml beaker.
- Syringes, 30 ml, with Luer Lock fittings.
- 10 ml sterile, disposable pipets with bulb.

17

- Sterile, disposable centrifuge tubes, 15 ml and 50 ml.
- Precision pipets with sterile, individually wrapped, disposable tips.
- Test tubes and caps:

  Sterile, screw-cap tubes (2.0 ml), for example, SARSTEDT ™ tubes.

  13x100 mm or 12x75 mm disposable borosilicate glass tubes.
- Standard water bath incubator at 37°C ± 1°C.
- Heat block at 105°C.
- Test tube racks.
- Gloves.
- Eight-channel pipettor.
- Sample organizer rack.
- SARSTEDT tube rack.

Checking the Threshold System

1) To start up the system.
2) Run diagnostics.
3) Read a blank stick (See Chapter 3, "Operating Procedures and Maintenance.")

Preparing Samples

Samples must meet these conditions prior to adding the labeling reagent:

- pH of 6.8 - 8.0.
- Ionic strength of 100 - 300 mM.
- Sample volume of 500 µl.
- DNA concentration ≤ 400 pg/ml.
- Absence of interfering substances.

Preparing Reagents

Most of the reagents provided in the Total DNA Assay Kit are ready to use.

1) Bring all reagents to ***room temperature***.
2) Just before use, reconstitute the DNA Labeling Reagent with 10 ml of Reconstitution Buffer for each vial and pool, as needed, in a sterile, disposable container.

   After reconstitution, the DNA Labeling Reagent should be used the same day. Do not use reusable laboratory glassware.

Setting Up the Assay

The Total DNA Assay quantitates the amount of DNA in samples by comparing results to a standard curve. Prepare the standard curve by making dilutions of heat-denatured High Calibrator (5 ng/ml calf thymus DNA).

1) Use Stick A for the standard curve, and subsequent sticks for unknown samples.
2) Run calibrators on each stick: the zero calibrator at the * position and the 50 pg calibrator at the # position.

Denature the Pretreated Unknowns, Positive Controls, and High Calibrator

Caution: *It is crucial to avoid environmental contamination of tubes and tips before the denaturation step. Use sterile, individually-wrapped pipet tips, gloves, 2.0 ml sterile screw-cap tubes and technique, appropriate to avoid contamination.*

To prepare the standards:

1) Pipet about 300 µl of High Calibrator into a 2.0 ml sterile screw cap tube (for example, SARSTEDT) and close the cap.
2) Transfer 500 µl of each sample or Positive Control to 2.0 ml, sterile screw-cap tubes and close the cap.
3) Heat the tubes containing samples or High Calibrator in the heat block at 105°C for ≥15 minutes.

   Caution: *Be sure to use the recommended heating block and heating block inserts. The heat block inserts must fit tightly around the 2.0 ml tube to provide adequate heat transfer at 105°C to denature the DNA completely.*

4) Immediately cool the tubes on ice for 5 minutes, until they reach room temperature. Rapid cooling prevents the DNA from renaturing. Denatured samples of linear DNA can be kept on ice for up to 6 hours before use in the DNA assay.

Prepare Standard Curve Solutions

The DNA concentration of each point in the standard curve is selected so that the concentrations are at regular intervals within the linear range of the Threshold Total DNA assay (2 to 200 pg). For testing very low DNA final product samples, the range of the standard curve can be reduced to focus on the lower portion of the curve. For discussion purposes, the 2 to 200 pg standard curve range will be used. Another source of DNA (instead of High Calibrator) may be used for the standard curve.

Caution: *Dilute denatured DNA in Zero Calibrator, that has not been boiled ,to minimize the risk of measuring contaminating DNA.*

The dilution scheme below provides standards (in pg DNA per 500 µl) at 0, 3.1, 6.3, 12.5, 25, 50, 100, and 200, by serial, 2-fold dilution. This scheme provides sufficient volumes for one standard stick, plus the calibrators (* and #) for three additional sticks. If more than 4 sticks are used in one experiment, adjust the serial dilution so there will be sufficient volume of the # Calibrator (usually 50 pg) for each stick that will contain samples to be quantitated from the standard curve.

1) Label eight glass test tubes (13 x 100 mm or 12 x 75 mm) as 200, 100, 50, 25, 12.5, 6.3, and 3.1.
2) With a precision pipet, add 1800, 1200, 1800, 600, 600, 600, and 600 µl of Zero Calibrator into the labeled test tubes (Table 17-1 on page 6). Add the solution in increments of 600 µl.

Table 17-1: Preparation of DNA Standards*

Add Zero Calibrator			
Tube (pg/0.5ml)	**No. of 600 µl aliquots**	**= Total µl**	**Serial Dilution of Denatured DNA (µl)**
200	3	1800	157 of High Calibrator
100	2	1200	1200 of 200 pg
50	3	1800	1800 of 100 pg
25	1	600	600 of 50 pg
12.5	1	600	600 of 25 pg
6.3	1	600	600 of 12.5 pg
3.1	1	600	600 of 6.3 pg

* This recipe makes enough mid-calibrator (50pg/0.5mL standard) to run on 5 sticks. For larger experiments, the volumes for the 200, 100, and 50 standards can be doubled, adding 314µl of denatured High Calibrator. This will generate enough mid-calibrator for 12 sticks.

3) Add the Zero Calibrator to the tubes.
4) Then add denatured High Calibrator DNA to the 200 pg tube, mix, and dilute serially with the indicated volumes. Transfer volumes in increments of 600 µl.
5) Add 157 µl of denatured High Calibrator to the 200 pg tube. Mix well.
6) Perform serial, 2-fold dilutions of denatured calibrator DNA (Table 17-1, "Preparation of DNA Standards*," on page 17-6). Pipet 1200 µl (in two 600 µl aliquots) from the 200-pg tube and add it to the 100-pg tube. The 100-pg tube should contain 1200 µl of Zero Calibrator.
7) Continue the serial dilutions, noting that, for each subsequent tube, the volume of Zero Calibrator in the tube equals the amount the diluted DNA added. Mix each dilution well, before transferring the aliquot to the next tube. The Zero Calibrator for the * position can be transferred directly from the container to the 2.0 ml tube.

Reconstitute the Labeling Reagent and Add to Samples

1) Use a precision pipet to dispense 500 µl of each sample (calibrators for the standard curve and unknowns) into labeled 2 ml screw cap (Sarstedt) tubes. Samples, in 500 µl aliquots, may be left in the Sarstedt boiling tubes.
2) Arrange the tubes according to the positions in the filter block (see Figure 17-1 and Figure 17-2). For the 37°C incubation, the samples can be arranged in a Sarstedt rack which allows easy removal of the screw cap prior to filtration.

3) Reconstitute the DNA Labeling Reagent in each vial with 10 ml of Reconstitution Buffer. Mix gently. Allow one vial per stick. If more than one vial is used, pool all the reconstituted DNA Labeling Reagent into a disposable beaker before dispensing. Reusable laboratory glassware should be avoided for pooling the reagent as residual detergent can cause elevated background rates.
4) Dispense 1000 µl of reconstituted DNA Labeling Reagent into each labeled tube. A repeater pipet, such as an Eppendorf with a Combitip™, simplifies this addition.
5) Cap the tubes and mix by inverting three to five times. An entire rack of tubes can be covered and inverted to save time over inverting individual tubes.

Incubate Samples

Incubate the assay mixtures at 37°C ± 1°C for 60 minutes, the water bath level should be above the meniscus of the sample mixtures.

Preparing for Filtration

Perform the steps in this section, while the samples are incubating.

Enter Assay Information in Threshold Enterprise / Sofware

Enter the assay information in the Experiment Information window. For additional details on the Experiment Information window, see Chapter 4, "Threshold Software."

Enter Descriptions of Sticks and Samples

1) Bring up the display window for each stick in the assay. Do this by selecting **Window, Stick Display** or by clicking the appropriate stick button on the toolbar.
2) Enter descriptions of the sticks in the fields labeled "Description," in each display window for the corresponding stick.
3) Enter descriptions of the samples in the fields labeled "1" to "6." corresponding to the arrangement of samples 1 to 6 (Figure 17-1) for each stick. Use this arrangement later when transfering the sample mixtures in the filter blocks.

Figure 17-1: Arrangement of Samples, Zero Calibrator/Control (*), and 50 pg Calibrator (#)

Threshold Enterprise / Software automatically fills in the calibration fields, "*" and "#" with values entered during the previous assay. If the concentrations are different from those displayed, edit the numbers by dragging to highlight the fields, deleting, and typing in a new value.

4) If a stick is used for a standard curve, enter descriptions of the standards in the fields labeled "1" to "6" in the display window for the standard stick, corresponding to the arrangement of standard quantities of DNA (Figure 17-2). Use this arrangement later when transfering the standard mixtures in the filter blocks.
5) Edit the values of the standards to correspond with the concentrations of standards you have prepared and the positions. To edit a value:
   a. Drag to select the value.
   b. Type in the new value.
6) Close the Standard Stick window by double-clicking in the upper-left corner of the window.

200	100	25
0		50
12.5	6.3	3.1

Figure 17-2: Arrangement of Standard Curve Samples in the Filter Block (pg DNA/0.5mL)

7) Confirm the analysis settings (Standard Curve Fit):
   a. Select **Analysis** from the **Settings** menu.
   b. In the **Analysis Settings** dialog (Figure 4-4 on page 4-9), select from the drop-down menus:

      For the Standard Curve Fit, choose pg/test.

      For the desired Units, choose Power.
   c. Click OK to confirm the selection.

Assemble Filter Units on the Workstation

Assemble filter units with sample sticks and label the sticks.

Filtering the Incubated Assay Mixtures

Filtering the assay mixture allows the DNA-enzyme complexes to bind specifically to sites on the membrane.

Transfer the Incubated Assay Mixtures

1) After the 60-minute incubation, remove the assay mixtures from the 37°C water bath.
2) In the Sarstedt rack, remove all tube lids. If an eight-channel pipettor will be used to transfer the samples to the filtration unit, remove the tube caps and transfer the Sarstedt tubes to the special "Sample Organizer Rack."
3) Begin to transfer the contents of the assay mixtures to each sample well in the filter blocks, within 10 minutes of removal from the bath.

Transfer all of the assay mixtures to the filter block by either of these methods:

- Using a 1 ml precision pipet set at about 800 µl, transfer (in two steps) all the assay mixture for each sample to one well.
- Using an eight-channel pipettor set at about 800 µl, transfer (in two steps) all the assay mixtures for one stick.

Filter the Assay Mixtures

1) Select **Instrument**, **Filter**, and select Low from the menu.
2) The status bar will show the vacuum status as Low, and start a count-up timer.
3) Filter until all the wells are empty. Examine for fluid remaining in the well, noting that bubbles of foam may remain.
   - The filter pump takes an average of 15 minutes to draw the assay mixture through the membrane.
   - Filtering for up to 10 minutes after the wells are empty will not affect assay results.

   Caution: *If one or more wells or a whole block is not filtering correctly, see Chapter 7, "System Troubleshooting." If filtering of unknown samples is excessively slow (>30 min) or stopped, the samples may contain too much protein or particulates.*

Wash the Filters

1) When every sample well is empty, dispense 2 ml of Wash Solution into each well.
2) Select **Instrument**, **Filter**, and select High from the menu.
3) The filtering rate will increase. The status bar will show the vacuum status as High and restart the countup timer.
   - The filter pump takes about four minutes to draw the Wash Solution through the filter membranes. Look at the sample wells repeatedly, from both the top and the sides, to keep track of how much Wash Solution remains in them.
   - Filtering for up to 5 minutes after the sample wells are empty will not cause any damage to the membrane.

Remove the Sticks from the Filter Units

1) When every well is completely empty, select **Instrument, Filter**, and select Off from the menu.
2) The vacuum will shut off and the drain pump comes on briefly (typically 1 minute). The status bar will show the vacuum status as Draining.
3) Unlock the filter units, remove the sticks, and place them in a beaker of Wash Solution, as explained in Chapter 3, "Operating Procedures and Maintenance."

Reading the Samples

Set Up Threshold Enterprise / Sofware for Reading a Stick

1) Select **Instrument**, **Read**, and highlight the name of the stick to be read.(Figure 17-3). Read the standard stick before reading the sticks containing unknowns. After a stick is read and data is stored under that heading, the date and time it was read appear next to the stick in the menu (instead of "not read"). Also, the Stick button on the toolbar shows a nine-dot pattern on its label.

Caution: *Reading a new stick replaces the data for that stick.*

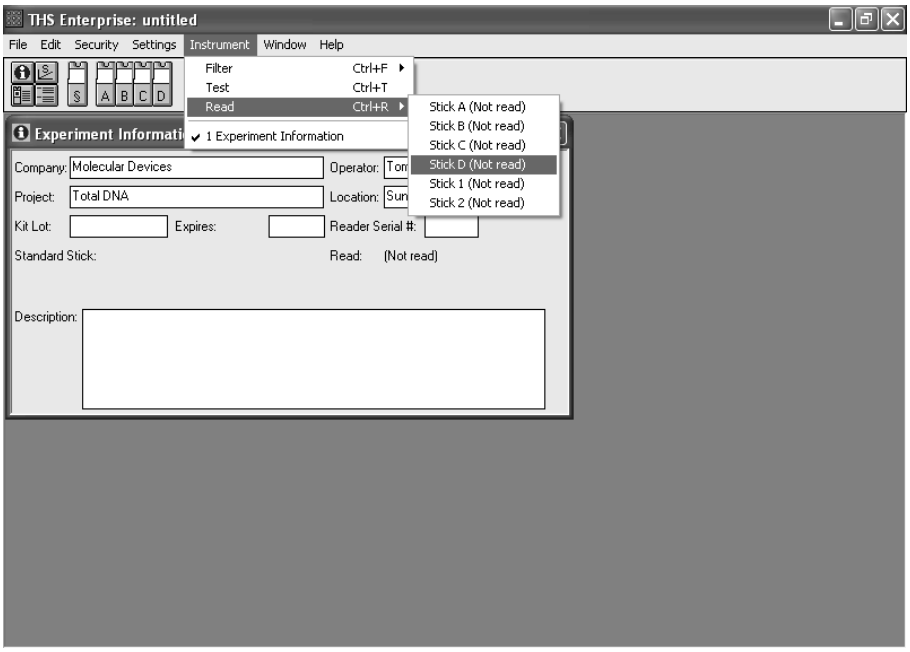


Figure 17-3: The Stick Read Menu

2) The message "Performing Instrument Diagnostics" appears.

Read a Stick

1) Wait for the message dialog "Put a Stick in the Reader..."
2) Holding the stick vertically, gently blot the notched end of the stick to remove drops of excess Wash Solution.
3) Insert the stick in the Reader with the notched end down, and the stick label and filter membrane facing you.
4) Press down lightly on the stick until it snaps softly into place.
5) Press Enter or click OK immediately.

   Caution: *Press* Enter *or click* OK *immediately after inserting the stick in the Reader to obtain the maximum dynamic range.*

6) After pressing Enter or clicking OK Threshold Enterprise / Sofware displays the Stick Display window, showing plots of the signal measurements for the stick. When it fin-

ishes reading the stick, Threshold Enterprise / Sofware displays the completed plots, signal values, and DNA quantities. Do not remove a stick from the Reader while it is being read.

7) Wait for the red indicator light "Reader Locked" to go off. Remove the stick from the Reader, with care to prevent liquid from dripping into the Reader housing.
8) Return the stick to the beaker of Wash Solution or discard it.

Load a Standard Curve

To use data from a stick as the standard curve, copy its data to the standard stick §:

1) Select **Copy Stick to Standard** from the **Edit** menu.
2) Choose the stick to be used as the standard curve from the drop-down menu.
3) The data from the selected stick will be copied to the standard stick §.
4) To view the standard curve, select **Standard Curve** from the **Window** menu.

The equation for the Power Fit under the standard curve describes the curve that is fit to the data points. The number to the right of the equation (for example, 0.034) is the error term (e). This describes the average relative error for fitting the data points to the curve.

Correct the Standard Curve (Optional)

If necessary, make changes in calibrator concentration or position:

1) Bring up the Stick Display window for the standard stick § by clicking on the **Standard Stick** button (§) on the toolbar or by selecting **Stick Display** from the **Window** menu, and then choosing the **Standard Stick §** from the menu.
2) Delete all incorrect values by highlighting the number and then pressing BACKSPACE. Enter the correct values in the appropriate fields.

Read the Remaining Sticks

Read the other sticks in the same way and return them to the beaker of Wash Solution, or discard them.

Recording the Results

Save the results and print the desired report according to the software instructions in Chapter 4, "Threshold Software."

Shutting Down

For complete shutdown procedures, see Chapter 3, "Operating Procedures and Maintenance."

Chapter 18– Total DNA Checkout Assay

The Total DNA Checkout Assay

Introduction

This chapter describes how to perform the Total DNA Checkout Assay to test the Threshold System, after installation, or as an initial training exercise for the Total DNA Assay.

The Checkout Procedure

Starting the Threshold System

1) Assemble the Total DNA Assay Kit, other required materials, and start the Threshold System.
2) Set a heating block to 105°C and a water bath to 37°C.
3) When the heating block and water bath reach these levels, proceed with the checkout procedure.

Setting Up the Checkout Assay

The 4 sticks in the checkout assay contain the samples shown in Table 18-1.

Table 18-1: Stick Samples

Stick	Samples
Stick A	Eight calibrators for the standard curve.
Stick B	Three aliquots of the Positive Control (labeled PC). Refer to the Certificate of Analysis, in the Total DNA Assay Kit, for the quantity of DNA in the Positive Control. Three aliquots of denatured Zero Calibrator, the Negative Control (labeled ZC).
Stick C	Six aliquots of a 40 pg DNA/0.5 ml buffer sample diluted from the High Calibrator.
Stick D	Same as Stick C.

1) Label and arrange the test tubes to receive the samples.
2) Using the arrangements shown in Figure 18-1, place the test tubes in a rack and dispense the sample mixtures into the filter blocks.

Filter Block Numbering Scheme

1	2	3
*		#
4	5	6

Stick A:

Standard Curve (pg DNA/ 0.5 mL)

200	100	25
*0		#50
12.5	6.3	3.1

Stick B:

Positive Controls and Zero Calibrator

PC	PC	PC
*0		#50
ZC	ZC	ZC

Stick C:

Precision Test Samples, 40 pg/0.5 mL

40	40	40
*0		#50
40	40	40

Stick D: Precision Test Samples, 40 pg/0.5 mL

40	40	40
*0		#50
40	40	40

Figure 18-1: Test Tube and Filter Block Arrangements

Prepare the Single-Stranded DNA Solutions

Caution: *Use sterile, individually wrapped pipet tips, sterile tubes, and aseptic technique to avoid contamination by other sources.*

a. Denature double-stranded DNA in High Calibrator and samples:

- Pipet 500 µl of High Calibrator (5 ng/ml of calf thymus DNA) into a 2.0 ml sterile screw-cap tube.
- Transfer three 500 µl aliquots of Positive Control into three 2.0-ml sterile screw cap tubes labeled PC.
- Transfer three 500 µl aliquots of Zero Calibrator into three 2.0 ml sterile screw cap tubes labeled ZC.
- Place all seven tubes in the heating block at 105°C for 15 minutes.
- Transfer the tubes to an ice bath and chill for at least 5 minutes before using these DNA solutions. The solutions may be used for up to 6 hours if kept on ice.

b. Standard curve: Prepare the standard solutions of ssDNA: 0, 3.1, 6.3, 12.5, 25, 50, 100, and 200 pg/0.5 ml, (Chapter 10, "Total DNA Assay Procedure.").

c. Precision test: To prepare 40 pg/ 0.5 ml, pipet 114 µl of denatured High Calibrator into 7.0 ml of non-denatured Zero Calibrator/ Control and mix well.

Mix Reagents and Incubate

- Reconstitute four vials of DNA Labeling Reagent and pool the contents.
- Pipet 500 µl of each calibrator into the appropriate tubes, including four aliquots of the 50 pg Calibrator for the # sample and four aliquots of the non-denatured Zero Calibrator for the * sample.
- Pipet 500 µl aliquots of the 40 pg calibrator into twelve tubes labeled "40."
- Add 1000 µl of reconstituted DNA Labeling Reagent to each tube including the 3 Positive Controls and the 3 Zero Calibrators. Cap the tubes. Invert three to five times to mix and avoid foaming.
- Incubate the assay mixtures at 37°C for 60 minutes in a water bath. The water level must be above the meniscus of the sample mixtures.

Transfer Solutions to Threshold Workstation

Transfer the contents of each assay mixture to a filter block.

Table 18-2: Stick Assignments

Stick	Samples
Stick A	Standard curve
Stick B	Positive Controls and Zero Calibrators
Sticks C and D	Precision test samples using 40 pg DNA/0.5 ml

Filter, wash, and read the sticks.

Results and Data Analysis

1) Read Stick A.
2) In Threshold Software, select **Edit**, **Copy Stick to Standard** and select Stick A. This will copy the data in Stick A to Stick §.
3) Select **Analysis** from the **Settings** menu.
4) In the **Analysis Settings** dialog (Figure 4-4 on page 4-9), select from the drop-down menus:

   For the Standard Curve Fit, choose pg/test.

   For the desired Units, choose Power.
5) Read Sticks B, C, and D.
6) Choose the **File** menu and select **Save** or **Save As** to save the results.
7) Choose the **File** menu and select **Print Reports** to print the results.

   For more information about Threshold Software see Chapter 5, "Threshold Software."

   The quantities of DNA measured for the PCs on Stick B should be within the range indicated on the Certificate of Lot Analysis provided in the Total DNA Assay Kit. If these values are substantially different from each other, the heating block may not be heating uniformly. If the values differ from those in the Certificate of Lot Analysis, review the accuracy of the standard curve.

   The quantity of DNA measured for the denatured ZCs on Stick B should be less than 2 pg/0.5 ml. If these values are higher than this, it is likely that the ZC was contaminated with DNA prior to the denaturation step. Careful attention should be paid to aseptic handling and to the use of appropriate disposables (sterile individually wrapped pipet tips, Sarstedt tubes, etc.) that come into contact with the samples prior to the denaturation step.

   For Sticks C and D, the coefficient of variation (% CV) for results from precision test samples should be less than 15%. The CV (%) is printed with the mean and standard deviations for all replicates on the group report. Replicates are those samples that have the exact same name, in the sample identification fields.

Chapter 19– Total DNA Sample Pretreatment

Introduction

The Threshold Total DNA Assay measures picogram amounts of DNA in samples from a wide variety of sources. Some samples may be assayed without pretreatment, but most samples require some pretreatment to remove interfering substances.

This chapter describes a general pretreatment scheme. These procedures provide an efficient and reliable path to validate pretreatment for a previously untested protein. This chapter describes sample requirements, limitations, specific pretreatment methods, and evaluation tests to qualify these methods in detail.

Optimal Sample Properties and Potential Interferences

Before adding DNA Labeling Reagent and performing the Total DNA Assay, samples must meet these requirements:

- pH between 6.8 - 8.0
- Total salt concentration between 100 - 300 mm
- Total sample volume of 500 µl
- DNA concentration ≤ 400 pg/ml
- Small DNA fragments (< 100 bases long) must be below inhibitory concentrations

pH

The optimal pH of the Total DNA assay mixture (sample plus Labeling Reagent) is 6.8 - 8.0 (Figure 19-1). The contribution of the sample to final pH (0.5 ml sample plus 1.0 ml of Labeling Reagent) depends on the buffering capacity of the sample protein and buffer. The pH of the Labeling Reagent is 7.4.

1) Test the final pH by mixing 1 part of sample with 2 parts of Labeling Reagent.
2) If the pH is out of range, dilute the sample in Zero Calibrator, adjust the pH of the sample, or exchange the buffer using ultrafiltration.

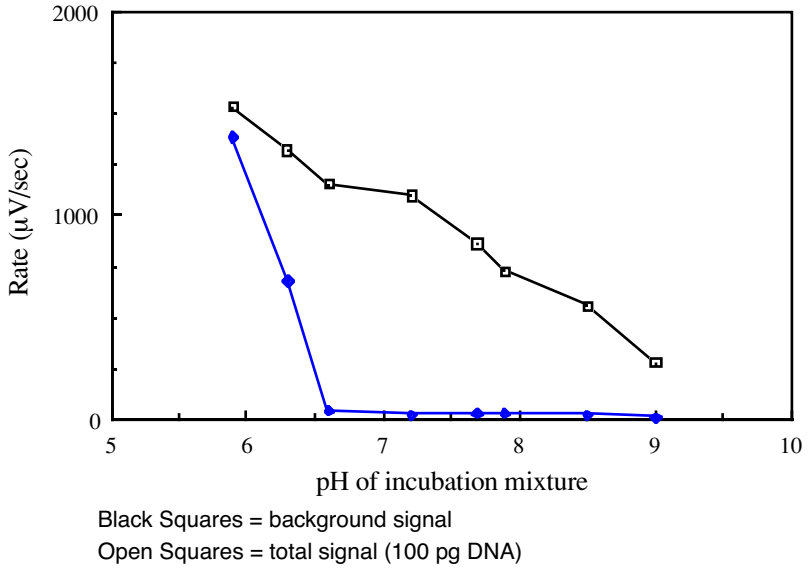


Figure 19-1: pH Dependence of Background and Total Signal

19

Total Salt Concentration

The salt concentration can affect the Total DNA Assay (Figure 19-2).

Dilute or use buffer exchange techniques (for example, Centricon-30), to bring total salt concentration within the plateau region—between 100-300 mM NaCl.

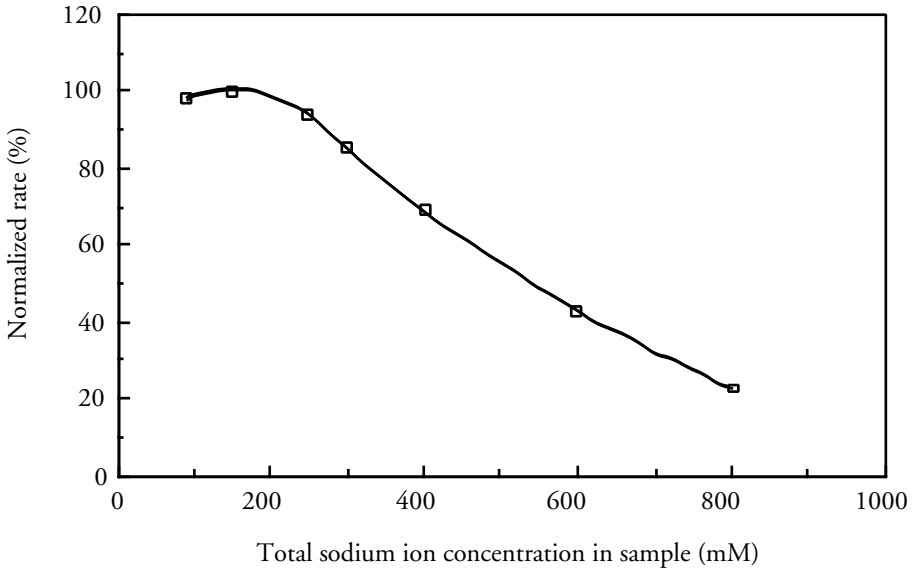


Figure 19-2: Salt Dependence (NaCl) of Relative Total DNA Assay Signal (50 pg Calf Thymus DNA in Assay Buffer)

Sample Volume

For samples less than 500 µl, add Zero Calibrator to adjust total volume to 500 µl. Reduce larger volumes by using a concentration method.

Total DNA Concentration

High DNA concentrations inhibit the Total DNA Assay (Figure 19-3). Excess DNA (1 ng/ml) inhibits the assay by decreasing the probability that any DNA fragment can bind to both DNA-binding protein conjugates, necessary for detection in the assay.

The acceptable range for the Total DNA Assay is 2 to 200 pg per sample (0.5 ml). Dilute the sample with Zero Calibrator to bring DNA within this range. When very high levels of DNA are present, several serial log (10-fold) dilutions may be necessary.

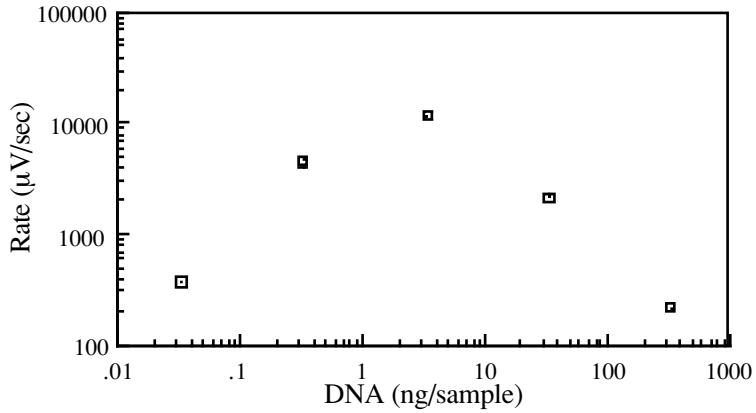


Figure 19-3: Assay Signal as a Function of Total DNA Concentration

Small DNA Fragments

High concentrations of DNA fragments less than 100 bases can cause significant inhibition of the signal and interfere with the Total DNA Assay. This inhibition is due to the reduced binding capacity of both labeling proteins to the same DNA fragment (Figure 19-4).

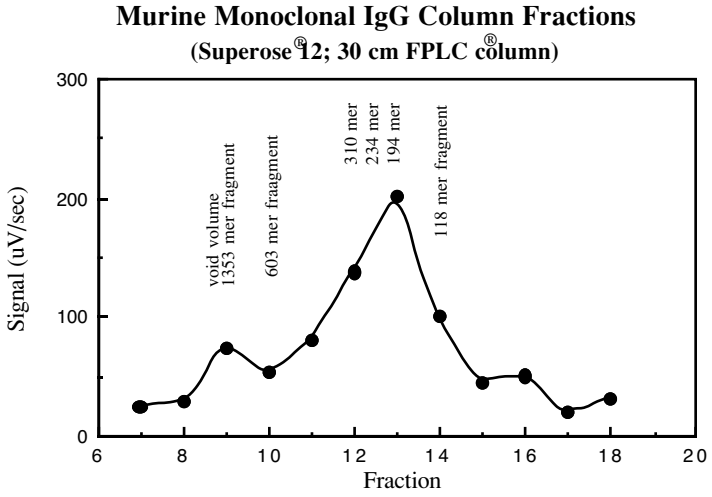


Figure 19-4: DNA Size Distribution in a Preparation of Murine Monoclonal Antibody

Summary

The Total DNA Assay is suboptimal, if the sample does not meet the conditions described in the previous section. Dilution into Zero Calibrator may correct for pH, salt

concentration, and concentration of nucleic acids. In some cases, it may be necessary to desalt and exchange buffer with an ultrafiltration device.

Table 19-1, "Total DNA Assay Interfering Substances," on page 19-15 at the end of this chapter, lists some common components of biological therapeutic samples and their effect, or potential interference, in the Threshold Total DNA Assay.

Guidelines For Pretreatment

Samples must be evaluated prior to testing to establish suitable sample conditions and, if required, an effective pretreatment method. During sample evaluation and development of a pretreatment protocol, it is important to follow these general guidelines:

- Use sterile (DNA-free) materials and techniques for all steps prior to the DNA denaturation step.
- Make all stock solutions, such as Proteinase K and SDS, in Zero Calibrator to avoid introduction of DNA. Dispense each stock solution into small aliquots, suitable for a single assay. To make stock solutions, weigh out the appropriate materials, and dilute in a sterile container. **DO NOT** use the original containers for dissolving these reagents, since the containers or stoppers may be contaminated with DNA.
- Use the same DNA source for standard curves and Spike Recovery studies. The High Calibrator provided in the kit, or a specific cell line DNA may be used. If a specific cell line DNA is used, characterize and qualify the DNA preparation (as outlined in DNA Series Application Note: Preparation and Qualification of DNA Standards for Use in the Threshold Total DNA Assay).
- A sample volume of 0.5 ml will be used, unless otherwise noted.

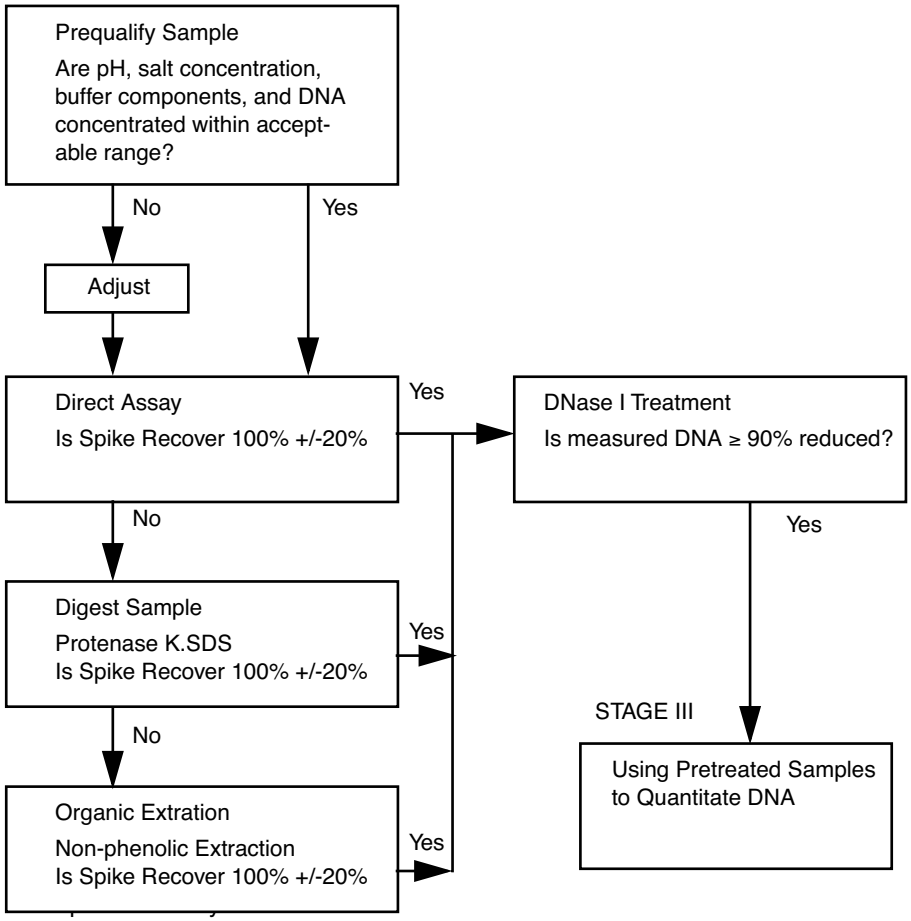


Figure 19-5: Scheme of Pretreatment Development

Spike Recovery

In general, the effectiveness of methods outlined in this section, is evaluated by "spike recovery", or the quantitation of a known amount of DNA, that has been added to a sample prior to pretreatment or testing. Monitoring recovery of added DNA demonstrates the accuracy of the sample DNA measurement and the presence of any inhibitory substances. A suggested experimental design for determining spike recovery is outlined below:

1) Test Zero Calibrator, in duplicate, as an experimental control. Label two sterile tubes "ZC", and two other tubes "ZC+" ("+" denotes added DNA). Sterile, screw-cap 2 ml Sarstedt tubes are recommended.
2) Test samples in duplicate. Label two sterile tubes "S", and two other tubes "S+".
3) Dispense 500 µl of Zero Calibrator (ZC) into their appropriate tubes.
4) Dispense 500 µl of the samples into their appropriate tubes.
5) Prepare a DNA solution of a 1:5 dilution of High Calibrator (HC) in a sterile tube, adding 200 µl HC to 800 µl ZC.
6) Add 50 µl of the diluted HC solution to each of the samples and Zero Calibrator controls marked with a "+". The DNA solution may be dispensed with an Eppendorf Repeater Pipettor and a sterile, 1.25 ml Combitip fitted with a sterile, individually wrapped pipet tip.
7) Heat all the samples and controls for 15 minutes at 105°C, and cool on ice for 5 minutes.
8) Assay the samples using the Total DNA Assay as outlined in Chapter 10, "General ILA Procedure," filtering the spiked and unspiked pairs on the same stick.
9) If using Threshold Enterprise / Software, the spike recovery can be automatically calculated by using the Spike Recovery Report (See "The Spike Recovery Report" on page 5-4 in Chapter 5, "Threshold Enterprise."). If you do not have Threshold Enterprise / Software, calculate the spike recovery by using the mean DNA concentrations for the duplicates, as follows:

$$\%\ \text{Recovery} = 100 \cdot [(S^* - S)/(ZC^* - ZC)]$$

Caution: *Raw rates (µV/sec) should not be used to calculate spike recovery, use only quantitated values.*

During the initial evaluation experiments, a previously-generated standard curve may be used for quantitation. Using a previously-generated standard curve will allow accurate evaluation of the percentage of spike recovery, and eliminate the need for one stick. However, the picogram quantitation of DNA may not be accurate, because the "*" and "#" samples are not made from the same dilution series as the standard curve on file. For maximum accuracy of quantitation, a new standard curve should be prepared for each experiment.

A 50 pg DNA standard can be prepared for the # position of each stick by diluting the denatured High Calibrator DNA to 100 pg/ml with Zero Calibrator. For an experiment using four sticks, 50 µl High Calibrator should be diluted with 2450 µl Zero Calibrator. Zero Calibrator can be used in the * position of each stick.

Sample Evaluation

It is important to evaluate the sample matrix before testing a sample. Compare the sample buffer constituents to Table 19-1, "Total DNA Assay Interfering Substances," on page 19-15 to determine if a sample constituent, other than protein or DNA, could cause low spike recovery. Denaturation and dilution tests for sample evaluation should be completed prior to sample testing.

Denaturation Test

The heating step to denature DNA can also denature and precipitate protein in the sample. The precipitated protein can interfere with the Total DNA Assay in several ways.

- The precipitated protein can entrap sample DNA, so it cannot be bound by the single-strand binding protein and anti-DNA antibody.
- Precipitated protein can prevent proper filtration of the sample and capture of the complex.
- Precipitated protein, trapped on the membrane, can be transferred to the silicon sensor surface during the "Read" step, resulting in inaccurate quantitation and damage to the sensor surface.

The maximum concentration of sample protein that can be heated, without producing a visible precipitate, must be determined.

1) Heat 500 µl volumes containing 200, 100, 50, and 10 µg of sample protein.
2) After cooling the samples, examine them for precipitation. Precipitation can easily be observed as a pellet after centrifuging the tubes. The highest concentration of sample protein that does not form a precipitate after heating is the maximum concentration that can be tested without pretreatment.

Dilution Test

Samples may have high DNA concentrations that exceed the dynamic range of the Total DNA Assay. Process samples may require dilution over several orders of magnitude (10^3-10^8) to reduce the DNA concentration to less than 200 pg in a 0.5 ml sample. Dilution of the sample can also reduce sample buffer interference.

- Several dilutions of the sample should be run in duplicate, with and without a DNA spike.
- Dilutions should be made with a DNA-free solution of proper ionic strength and pH, such as Zero Calibrator.
- If dilution does not improve spike recovery, or if the concentration of protein that allows acceptable spike recovery is less than desired, see the following section, "Pretreatment."

Pretreatment

Spike recovery of 100% ± 20% indicates that a pretreatment method is successful. Recovery of less than 80% of the spike in the sample indicates one, or more, of the following conditions:

- Sample protein interference.
- Sample buffer component interference.
- Excessive amounts of DNA (over 1 ng/ml).
- Presence of small fragments of DNA at inhibitory concentrations.

A staged approach is the most economical and efficient means of determining an appropriate pretreatment procedure. The pretreatment methods described below should be approached sequentially.

Proteinase K/SDS Digestion

Preparation of Stock Solutions

Before use with a sample, stock solutions of Proteinase K and SDS must be prepared and tested:

1) Dissolve Proteinase K from Boehringer Mannheim in Zero Calibrator in a sterile, clean tube to make a stock solution with a final concentration of 2 mg/ml.
2) Dispense small aliquots sufficient for one experiment into sterile tubes (2.0 ml Sarstedt tubes). Store at -20°C.
3) Dissolve SDS (Electrophoresis Grade Purity) in Zero Calibrator in a clean, sterile tube to make a stock solution of 2% (w/v). Aliquot and store at room temperature.
4) Test the Proteinase K and SDS stocks for DNA concentration, by adding 25 µl of each to 500 µl samples of Zero Calibrator. The final concentration of each will be 100 µg/ml and 0.1%, respectively.
5) The samples should be assayed with and without a DNA spike, to determine the level of contaminant DNA in each of the components, and the effect on spike recovery.

Pretreatment of Samples with Proteinase K/SDS

Digestion of a sample protein with Proteinase K and SDS may be required, to test higher concentrations of protein. The following experiment will demonstrate the maximum concentration of digested sample protein that will allow accurate DNA quantitation.

1) Choose four concentrations of the sample protein using, as the lowest concentration, the maximal amount of intact protein that can be heated without producing a visible precipitate (See “Denaturation Test” on page 19-9).
2) If required, dilute samples before the protease digestion. In the first experiments, do not exceed 2 mg/ml of sample protein. Spike 50 pg of DNA into aliquots of each protein concentration before digestion. It is important to include controls of Zero Calibrator or sample buffer that have been treated in the same manner as the sample. A sample buffer may be used in place of Zero Calibrator only if it has been previously evaluated for DNA concentration and spike recovery. Spike recovery, relative to an initial buffer control (B), can be calculated by using the Threshold Enterprise / Software Spike Recovery

Report (See "The Spike Recovery Report" on page 5-4) or, as follows:

$$\% \text{ Recovery } = 100 \cdot [(S^* - S)/(B^* - B)]$$

3) Digest 500 µl of sample by adding 25 µl of a 2 mg/ml Proteinase K stock and 25 µl of 2% SDS stock. Incubate overnight at 55°C.

4) Test six samples (Zero Calibrator or sample buffer with and without Proteinase K and SDS, and four concentrations of the protein sample), spiked and unspiked, in duplicate (24 samples in total).

5) After performing the assay, organize the samples and filter onto the sticks, using the following arrangement:

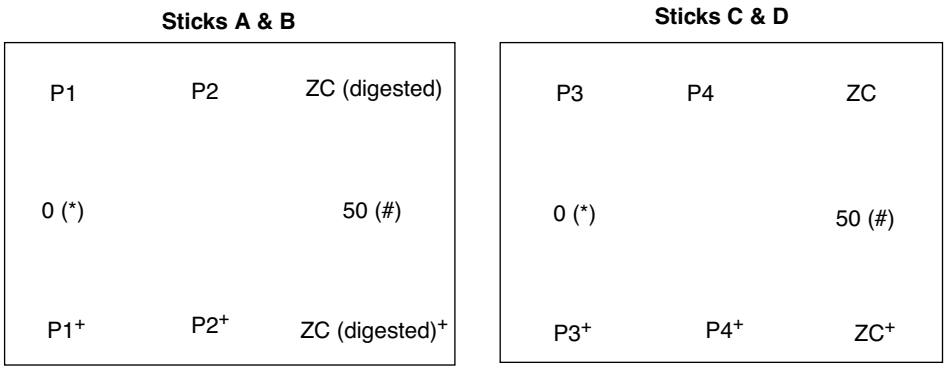


Figure 19-6: Stick Assignment for Digested Samples

P1-P4	=	Various concentrations of sample protein to be digested.
$P1^+$-$P4^+$	=	Same concentrations of samples, with a DNA spike.
ZC (digested)	=	Proteinase K and SDS in Zero Calibrator, or sample buffer, without a DNA spike.
ZC $(digested)^+$	=	Proteinase K and SDS in Zero Calibrator, or sample buffer, with a DNA spike.
ZC, ZC^+	=	Zero Calibrator or sample buffer with and without a DNA spike.

6) The maximal amount of digested protein that can be accurately tested in the Total DNA Assay is the highest concentration that filters within 30 minutes, and allows a spike recovery of 100 ± 20%.

Efficiency of the digest should be evaluated, using independent analytical methods to characterize the residual protein and peptides after a digestion. For example, a successful digestion method leaves no intact protein and only residual fragments that are very small (less than 3000 daltons). An appropriate analysis system may include PAGE, HPLC, or other chromatographic techniques. Always prepare an extra aliquot of a sample to evaluate by other analytical methods.

Digestion may be optimized by altering the sample pH, time and temperature of the incubation, and concentration of Proteinase K and SDS, as well as addition of other materials, such as DTT (dithiothreitol). If Proteinase K/SDS digestion does not allow adequate spike recovery at the desired concentration of sample, protein or peptide removal by an extraction method may be required. Two extraction procedures are described below.

Phenol-Free DNA Extraction

This DNA extraction method was evaluated, using a commercially-available extraction kit (see References) for pretreatment of biopharmaceutical samples to be tested in the Threshold Total DNA Assay. The procedure incorporates a chaotrope (NaI), a detergent, and isopropanol to co-precipitate nucleic acids with a polysaccharide carrier, glycogen. This precipitation allows the separation of DNA from sample components (proteins, lipids, and buffers) that may interfere with the Total DNA Assay. For some proteins, this extraction technique can replace a conventional overnight digest with Proteinase K and SDS, and/or a phenol/chloroform extraction. The following extraction protocol is more thoroughly detailed in the Threshold DNA Application Note: *A Phenol-Free DNA Extraction.*

The extraction procedure is performed, prior to heat denaturation of the DNA; therefore, it is important to use aseptic technique to minimize DNA contamination. Sterile, wrapped pipet tips, sterile tubes, and gloves should be used throughout this procedure.

To perform the phenol-free extraction:

1) Add 64 µl of Glycogen Solution to Sodium Iodide Solution.

   Caution: *Crystals that may appear in the Sodium Iodide Solution, during storage at 4°C, can be redissolved by warming the solution to 50°C for a short period of time.*

2) Add 2 µl of Glycogen Solution to Washing Solution B, and swirl the bottle to mix thoroughly.

   Caution: *The Washing Solution B is stable for only one week, at 4°C, after addition of the Glycogen Solution. If the entire kit is not used within a week, the desired volume of Washing Solution B should be transferred to a sterile tube, followed by the addition of 2 µl of Glycogen Solution.*

3) Dispense 500 µl of sample into a sterile 2-ml Sarstedt microfuge tube with cap.
4) Add 20 µl of Sodium N-Lauroyl Sarcosinate Solution to the tube and mix for 5-15 seconds with a vortex mixer. An Eppendorf Repeater Pipettor with a sterile, 0.5 ml Combitip may be used.
5) Add 500 µl of Sodium Iodide Solution (containing Glycogen Solution) to the mixture, vortex for 5-15 seconds, and incubate at 40°C for 15 minutes. A sterile, 12.5 ml Combitip and an Eppendorf Repeater Pipettor may be used.
6) Add 900 µl isopropanol (2-propanol) to the mixture with a 1 ml Pipetman with sterile tip. Little or no color change should occur. Vortex for 10-15 seconds, and let stand at room temperature for 15 minutes.
7) Centrifuge (10,000 x g) for 15 minutes in a microfuge to pellet the DNA. In some cases, a white pellet may be evident. Gently pour off the resulting supernatant, and blot

tubes on absorbent paper to remove as much residual solution from the tube walls as possible.

8) Add 800 µl of Washing Solution A to the tube, and mix for 5-15 seconds on a vortex mixer to resuspend the pellet in the tube.
9) Centrifuge for 5 minutes in a microfuge (10,000xg), and gently pour the resulting supernatant from the tube.
10) Add 1500 µl of Washing Solution B containing Glycogen Solution to the tube with a sterile, 12.5 ml Combitip and an Eppendorf Repeater Pipettor. Mix vigorously on the vortex mixer for 5-15 seconds.
11) Centrifuge for 5 minutes in a microfuge (10,000xg) and gently pour off the supernatant, and drain the tube well by blotting on a clean paper towel.
12) Resuspend the remaining pellet (containing glycogen and DNA) in 0.5 ml Zero Calibrator.
13) The sample is then heated at 105°C for 15 minutes to denature the DNA, and cooled on ice for 5 minutes before being tested in the Threshold Total DNA Assay.

Phenol/Chloroform DNA Extraction

An alternative method for removing residual proteins is the classic phenol/chloroform extraction, modified to be compatible with the Total DNA Assay. Since the extraction procedure is performed before heat denaturation, it is important to use aseptic technique and sterile materials. Sterile, individually-wrapped Eppendorf Combitips are convenient for dispensing the phenol and chloroform solutions.

To perform the phenol/chloroform extraction:

1) Digest samples with Proteinase K and SDS as previously described, except dispense 600 µl of each sample and control, rather than 500 µl. Add 30 µl of Proteinase K and 30µl of a 20% SDS stock, and 60 µl of the DNA spiking solution (1:4 dilution of High Calibrator in Zero Calibrator) to the appropriate tubes. A 10-fold increase of SDS can improve the digest but can only be used when the sample will be phenol/chloroform extracted after the digestion. To those tubes that will not be spiked, it is important to add 60 µl of Zero Calibrator to maintain the same volume. This extraction procedure requires an additional control. An extra Zero Calibrator or buffer control must be prepared, but not digested or extracted. Effects on spike recovery or background due to the extraction may be detected by comparing this control to one that is extracted.
2) Digest the samples overnight at 55°C.
3) Add approximately 2 volumes (1250 µl with a 12.5 ml Combitip) of "70% phenol/water/chloroform" mixture (Applied Biosystems, part #400765) that has been equilibrated to room temperature, and mixed thoroughly, to your digested sample in a 2 ml sterile, screw-cap tube from Sarstedt. Mix the tubes vigorously for 5 minutes to obtain a fine emulsion, which aids extraction efficiency. Centrifuge for 5 minutes at 2000 x g to separate the phases.
4) Using a precision pipettor and sterile, individually-wrapped pipet tip, transfer 650 µl of the top, aqueous phase containing the DNA, to another labeled, sterile tube. Holding the tube at a 45° angle can facilitate removing the top, aqueous phase without disturb-

ing the bottom, organic phase. The top, aqueous phase contains the extracted DNA. Be careful not to remove any of the interface or organic phase.

5) Add approximately 2 volumes (1250 µl with a 12.5 ml Combitip) of chloroform (Applied Biosystems or Fisher) to the aqueous sample. Shake vigorously for 5 minutes to obtain a fine emulsion, and centrifuge for 5 minutes at 2000 x g to separate the phases.
6) Using a precision pipettor and sterile, individually-wrapped tips, transfer 600 µl of the top, aqueous phase into a labeled, sterile tube.
7) Denature the samples at 105°C for 15 minutes, and cool on ice for 5 minutes. Assay the denatured samples in the normal manner.

   Caution: *The sample may turn slightly cloudy when the Labeling Reagent is added; this usually does not interfere with sample quantitation, unless the sample is very cloudy.*
8) Dispense the samples onto the sticks in the following arrangement (See Figure 19-7).

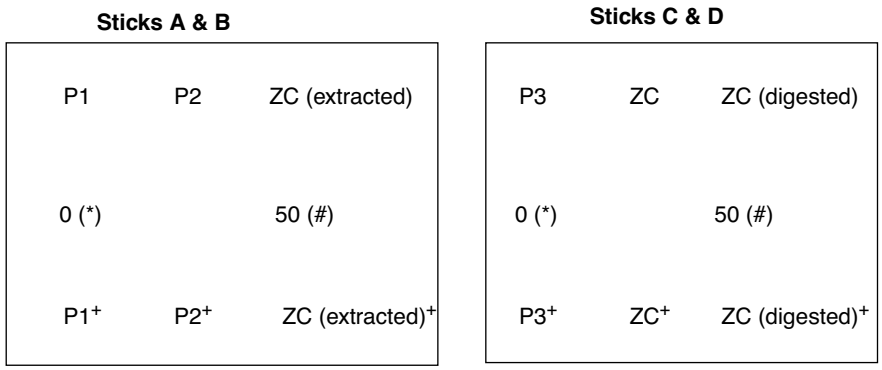


Figure 19-7: Stick Assignment for Digested and Extracted Samples

P1-P3	=	Samples at several protein concentrations, extracted.
$P1^+$-$P3^+$	=	Samples at several protein concentrations, spiked and extracted.
ZC, ZC^+	=	Non-extracted Zero Calibrator, or qualified sample buffer.
ZC (digested)	=	Zero Calibrator, or qualified sample buffer, with Proteinase K and SDS, unspiked.
ZC (digested)$^+$	=	Zero Calibrator, or qualified sample buffer, with Proteinase K and SDS, spiked.
ZC (extracted)	=	Zero Calibrator, or qualified sample buffer, with Proteinase K and SDS, extracted, unspiked.
ZC (extracted)$^+$	=	Extracted Zero Calibrator control, or qualified sample buffer, with Proteinase K and SDS, spiked.

DNase I Test

The DNase I test can confirm that the positive signal, observed in the Total DNA Assay, is due to DNA. A DNase I treatment should eliminate over 90% of the DNA that the Total DNA Assay measures above background. The DNase I test can also be used as a tool to determine if inhibition of spike recovery is due to the presence of small DNA fragments. After DNase I treatment, the samples should yield full spike recovery when tested in the Total DNA Assay. For more details on performing this procedure consult the DNA Application Note: *DNase I Treatment.*

Interfering Substances

Many substances are compatible with the Total DNA Assay up, to the levels specified in the following table (Table 19-1). To test these substances, they were added directly to the labeling reaction, without any pretreatment. Validate any of the substances that may interfere using your pretreatment method.

Use this list (Table 19-1) only as a guide to determine the compatibility of potential interfering substances. Even when a substance is listed, evaluate the compatibility of all potential interfering substances for your particular assay.

Table 19-1: Total DNA Assay Interfering Substances

Potential Contaminant	Concentration	Spike Recovery/Comments
Salts		
Magnesium chloride	10 mM** 20 mM*	82 % 60 %
EDTA	10 mM*	100 %
$MgCl_2$/EDTA	20 mM/40 mM*	100 %
Ammonium acetate (diluted from 2M stock)	50 mM** 100 mM	90 % 60 % (Ionic strength too high)
Ammonium sulfate (diluted from 2M stock)	50 mM** 100 mM	126 % 52 % (Ionic strength too high)
Protein Denaturants		
Proteinase K	200 µg/ml*	88 %
Dithiothreitol	50 mM*	100 %
Guanidine HCl	0.1 M 0.5 M	102 % 43 % (Ionic strength too high)
Urea	10 mM** 100 mM	80 % 45 %
Polymers and Surfactants		
Dextran	5 %*	100 %
Polyethylene glycol	5 %*	112 %

Table 19-1: Total DNA Assay Interfering Substances (Continued)

Potential Contaminant	Concentration	Spike Recovery/Comments
Dextran sulfate	1 %**	62 %; causes high background
Triton X-100	2 %	100 %
SDS	0.1 %** 0.2 %	80 - 90 % 50 - 80 %
Spermine	50 µg/ml	80 %
Acids		
Trifluoroacetic acid	1 %	High background; pH too low
Acetic acid	0.1 %	High background; pH too low
Organics		
Methanol	20 %*	94 %
Ethanol	20 %*	90 %
Phenol	1 %** 5 %*	92 % Cloudy, does not filter
Chloroform	Saturated*	94 %
Dimethylsulfoxide	5 %** 10 %*	90 % 60 %
Acetonitrile	20 %** 50 %*	100 % 0 %; Filtration rate increased
Mineral oil	Saturated*	90 %
Glutaraldehyde	0.0025 %	90 %
Amino Acids		
Arginine	10 mg/ml*	92 %
Glutamine	10 mg/ml*	97 %
Glycine	10 mg/ml*	81 %
Histidine	5 mg/ml	90 - 100 %
Lysine	5 mg/ml**	90 - 100 %
Carbohydrates		
Glucose	10 mg/ml*	93 %
Mannitol	10 mg/ml*	86 %
Sucrose	10 mg/ml*	89 %
Glycogen	400 µg/ml*	100 %; (Glycogen not boiled)

Table 19-1: Total DNA Assay Interfering Substances (Continued)

Potential Contaminant	Concentration	Spike Recovery/Comments
Other		
Acetonitrile/TFA	20 %/0.1 %**	95 %
DNase I	25 µg/ml	100 %
$MgCl_2$/DNase I	5 mM/12.5 µg**	0 % (desired); higher levels clog the filter
tRNA	≤ 200 ng/ml** 2 mg/ml*	≥ 80 % 54 %; possible DNA contamination
HSA or BSA	1000 µg/ml**	100 %; direct assay, no pretreatment
Phenol Red	1000 µg/ml*	100 %

* The maximum level tested
** The maximum acceptable level

References

Wako DNA Extractor Kit (part #295-50201)-50 tests/kit.

Wako Chemical USA, Inc., 1600 Bellwood Rd., Richmond, VA 23237, USA; Tel (800)992-9256; Telex 293208 Wako UR (RCA); Fax (804)271-7791

Wako Pure Chemical Industries, Ltd.: 1-2, Doshomachi 3-Chome, Chuio-Ku, Osaka 541, Japan; Tel (06)203-3741; Telex 65188 wakoos j; Fax (06)203-3741.

Wako Chemicals GmbH: Nissanstr. 2, W-4040 Neuss 1, Germany; Tel (02131)311-0; Telex 8517001 wako d; Fax (02131) 31100.

Chapter 20– Total DNA Assay Troubleshooting

Introduction

This chapter contains solutions to potential problems encountered during the Total DNA Assay. The following table lists effects with possible causes, along with solutions for each cause.

If you need further assistance, call Molecular Devices Technical Support.

Please have this information available so that we can help you efficiently:

- Reagent Kit Lot number.
- Workstation serial number.
- Reader serial number.
- Exact nature of the problem.

Note: *Please obtain a Returned Goods Authorization Number from Molecular Devices, before returning materials or parts.*

Effects, Causes and Solutions

Table 20-1: Sample Pretreatment for the Total DNA Assay Troubleshooting

Effect	Possible Cause	Solution	Reference
Spike Recovery <80% or >120%	Buffer interference (test buffer without sample protein)	1. If the pH is out of range, adjust the sample buffer pH after heat denaturation. 2. If the salt concentration is < 100 mM, add salt after heat denaturation. 3. If the salt concentration is > 300 mM, dilute the sample buffer with DNA free water (water for injection). 4. If there is an interfering buffer component (Table 18-1), extract the DNA using the phenol-free extraction or exchange the buffer by ultrafiltration.	Chapter 19
	Buffer interference in presence of sample	1. If the pH is out of range, adjust the sample pH after heat denaturation. 2. If the salt concentration is < 100 mM, add salt after heat denaturation. 3. If the salt concentration is > 300 mM, dilute the sample with DNA free water (water for injection). 4. If the salt concentration is > 300 mM and the DNA concentration in the sample is < 100 pg/0.5 ml, extract the DNA from the sample using the phenol-free extraction. 5. If there is an interfering buffer component (Table 18-1), extract the DNA using the phenol-free extraction or exchange the buffer by ultrafiltration.	
	Sample protein interference	Reduce and / or remove protein by: 1. Digestion with Proteinase K/**0.1**% SDS. 2. Extract the DNA with the phenol-free extraction method. 3. Digestion with Proteinase K/**1.0**% SDS and extract with the phenol-free extraction method. 4. Digestion with Proteinase K/**1.0**% SDS and extract using the phenol/chloroform method.	Chapter 19
	pI of sample protein >7.0	1. Adjust the pH of the sample above the pI, then digest with Proteinase K/**0.1**% SDS; readjust the pH to 7.0 after heat denaturation. 2. Adjust the pH of the sample above the pI, then extract the DNA using the phenol-free extraction. 3. Adjust the pH of the sample above the pI, then digest with Proteinase K/**1.0**% SDS; followed by extraction of the DNA, using the phenol-free extraction.	Chapter 19
	Sample protein glycosylated	Treat the sample with endoglycosidases to remove the sugar moiety from the protein.	
	High concentration of DNA	Dilute the sample to reduce the DNA to < 200 pg/0.5 ml.	Chapter 19

Table 20-1: Sample Pretreatment for the Total DNA Assay Troubleshooting (Continued)

Effect	Possible Cause	Solution	Reference
Spike Recovery <80% or >120%	DNAinterference at high concentration of small fragments of DNA	1. Confirm by digesting the sample DNA with DNase I, then spike the sample with DNA. 2. Modify the purification process to remove small fragment DNA. **NOTE: To differentiate between protein interference and DNA interference, perform the Spike Recovery Test in samples previously treated with DNase I and denatured at 105°C for 15 minutes. Acceptable recovery indicates previous interference was DNA-related.**	Chapter 19
Spike Recovery < 80% in Zero Calibrator Controls	SDS concentration too high	Remake the SDS stock solution and / or reduce the SDS to 0.08%.	
Positive Control Quantitates AboveSpecified Range	Absolute concentration of DNA, in standard curve calibrators, is lower than specified.	1. Check the accuracy of pipets and dilutions. 2. Compare the raw rates (µV/sec) of the standards and Positive Control to the Certificate of Lot Analysis.	
	Denaturation of high calibrator DNA is incomplete	1. Check that the temperature of the heating block is 105°C using mineral oil. 2. Check that the heating block inserts fit tightly around tubes.	
	Positive Control solution or tube contaminated with DNA	Use aseptic technique for sample handling prior to denaturation.	
Positive Control Quantitates Below Specified Range	Absolute concentration of DNA, in standard curve calibrators, is higher than specified.	1. Check the accuracy of pipets and dilutions. 2. Compare the raw rates (µV/sec) of the standards and Positive Control to the Certificate of Lot Analysis.	
High Background DNA in Controls	Contamination of ZC or buffer, Proteinase K/SDS stock, or extraction reagents	Qualify the buffer, SDS & Proteinase K stocks or extraction reagents.	Chapter 19

Table 20-1: Sample Pretreatment for the Total DNA Assay Troubleshooting (Continued)

Effect	Possible Cause	Solution	Reference
Unexplained High DNA in One or Two Samples	Contamination with non-sample DNA	Possible sources of contamination: 1. Tubes used for diluting the samples. 2. Pipet tips used for transferring the sample or adding pretreatment reagents. 3. Heat denaturation tubes. **Caution: Sterile supplies are not necessarily DNA-free.** 4. Pipetting technique that causes liquid to touch the pipettor tip. 5. Pipetting technique that causes the transfer of a small amount of solution from one tube to another. 6. Leaving tubes containing sample (before the heat denaturation step) open and / or tube caps facing up. **Caution: Use good aseptic lab techniques for any sample handling before denaturing the sample.**	
High µV/secRates in the * Position	Residual powder from gloves	Remove gloves and wash hands before preparing the Labeling Reagent.	
Filtration Time Greater Than 30 Minutes	Protein clogging the filter (Compare the filtration rate to *, #, ZC controls)	Reduce or remove protein (See "Sample Protein Interference." above.	Chapter 19
	If the sample is DNase I treated: aggregation of Mg^{++} with the binding proteins and / or DNase I	Add the correct amount of EDTA to chelate free Mg^{++} in the sample after heat denaturation.	
Cloudy Sample Mixture	If the sample is phenol extracted, cloudiness may be due to residual phenol **NOTE: A cloudy sample mixture does not *usually* cause problems, unless an excessive amount of phenol is present.**	Do not remove the entire aqueous phase to avoid transfer of phenol.	
	If the sample is not extracted: precipitated protein may cause cloudiness	Reduce or remove protein (See "Sample Protein Interference" above).	

Appendix A- Incorporation of Biotin

Incorporation of Biotin

The equations for the incorporation of biotin are derived from the Beer-Lambert Law:

$$\frac{\text{Absorbance}}{\text{Concentration}} = \text{Constant}$$

Biotin cannot be measured spectrophotometrically. Therefore a chromophore, dinitrophenol (DNP), has been conjugated to biotin-NHS. DNP absorbs at both 280 nm and 362 nm. Proteins absorb at 280 nm (for these equations to be valid it is necessary to confirm that the protein does not absorb significantly at 362 nm). The biotin- labeled proteins (b-P) are measured spectrophotometrically at 280 nm and 362 nm. The concentration of biotinylated protein is determined by correcting the $A_{280}^{b\text{-}P}$ for the absorbance of DNP at 280 nm. The biotin concentration is determined directly from the measured A_{362}. For all equations and formulas below, the following definitions apply:

MIR	=	Molar Incorporation Ratio (moles biotin per mole protein: b/P)
ε	=	absorbance of 1 mg/ml of protein at 280 nm (extinction coefficient)
ε'	=	absorbance of 1 nM protein at 280 nm (nanomolar extinction coefficient)
MW^P	=	molecular weight of protein (mg/mmol)
A_{280}^{P}	=	absorbance due to the protein at 280 nm
A_{280}^{b}	=	absorbance due to the biotin at 280 nm
$A_{280}^{b\text{-}P}$	=	absorbance of the biotinylated protein at 280 nm
$A_{362}^{b\text{-}P}$	=	absorbance of the biotinylated protein at 362 nm

Calculating the Molar Incorporation Ratio of Biotin to Protein

The following formulas are used to derive the molar incorporation ratio of biotin (MIR, moles of biotin incorporated per mole of protein). Formulas **d** and **e** were derived experimentally, using the optical density of DNP-biotin (b) at 280 nm and 362 nm.

a.) $\varepsilon' = \varepsilon \cdot (MW^P / 10^9)$

b.) $\text{nM P} = A_{280}^{P} / \varepsilon'$

c.) $A_{280}^{P} = A_{280}^{b\text{-}P} - A_{280}^{b}$

d.) $\text{nM b} = A_{362}^{b\text{-}P} / (17 \cdot 10^{-6})$

e.) $A_{280}^{b} = 0.32 \cdot A_{362}^{b\text{-}P}$

Substituting for A_{280}^{b} from formula **e** into formula **c** gives formula **f**.

$$\text{f.)}\quad A_{280}^{P} = A_{280}^{b\text{-}P} - (0.32 \cdot A_{362}^{b\text{-}P})$$

Molar incorporation ratio (MIR) is defined as:

$$1.\quad \text{MIR} = \frac{\text{nM b}}{\text{nM P}}$$

Substituting for nM b and nM P from equations **b** and **d**:

$$2.\quad \text{MIR} = \frac{A_{362}^{b\text{-}P}}{(17 \cdot 10^{-6})} \cdot \frac{\varepsilon'}{A_{280}^{P}}$$

Substituting for ε' and A_{280}^{P} from equations **a** and **f**:

$$3.\quad \text{MIR} = \frac{A_{362}^{b\text{-}P}}{(17 \cdot 10^{-6})} \cdot \frac{\varepsilon \cdot (MW^{P}/10^{9})}{(A_{280}^{b\text{-}P} - (0.32 \cdot A_{362}^{b\text{-}P}))}$$

Rearranging:

$$4.\quad \text{MIR} = \frac{(58 \cdot 10^{3}) \cdot A_{362}^{b\text{-}P} \cdot \varepsilon \cdot (MW^{P}/10^{9})}{A_{280}^{b\text{-}P} - (0.32 \cdot A_{362}^{b\text{-}P})}$$

Canceling out the 10^{3}:

$$5.\quad \text{MIR} = \frac{58 \cdot A_{362}^{b\text{-}P} \cdot \varepsilon \cdot (MW^{P}/10^{6})}{A_{280}^{b\text{-}P} - (0.32 \cdot A_{362}^{b\text{-}P})}$$

For IgG's, (ε = 1.4 and MW = 160,000) this equation is further simplified:

$$\text{MIR} = \frac{13 \cdot A_{362}^{b\text{-}P}}{A_{280}^{b\text{-}P} - (0.32 \cdot A_{362}^{b\text{-}P})}$$

Calculating the Biotin-Labeled Protein Concentration (mg/ml)

From the Beer-Lambert Law:

$$\text{mg/ml} = \frac{A_{280}^{P}}{\varepsilon}$$

Substituting for A_{280}^{P} from equation **f**:

$$\text{mg/ml} = \frac{A_{280}^{b\text{-}P} - (0.32 \cdot A_{362}^{b\text{-}P})}{\varepsilon}$$

For IgG's, (ε = 1.4) this equation becomes:

$$\text{mg/ml} = \frac{A_{280}^{b\text{-}P} - (0.32 \cdot A_{362}^{b\text{-}P})}{1.4}$$

B

Appendix B- Incorporation of Fluorescein

Incorporation of Fluorescein

The equations for the incorporation of fluorescein are derived from the Beer-Lambert Law:

$$\frac{\text{Absorbance}}{\text{Concentration}} = \text{Constant}$$

Fluorescein absorbs at both 280 nm and 490 nm. Proteins absorb at 280 nm (for these equations to be valid it is necessary to confirm that the protein does not absorb significantly at 490 nm). The fluorescein labeled proteins (f-P) are measured spectrophotometrically at 280 nm and 490 nm. The concentration of the fluoresceinated protein is determined by correcting the $A_{280}^{f\text{-}P}$ for the absorbance of fluorescein at 280 nm. The fluorescein concentration is determined directly from the measured A_{490}. For all equations and formulas below, the following definitions apply:

MIR	=	Molar Incorporation Ratio (moles fluorescein per mole protein: f/P)
ε	=	absorbance of 1 mg/ml of protein at 280 nm (extinction coefficient)
ε'	=	absorbance of 1 nM protein at 280 nm (nanomolar extinction coefficient)
MW^P	=	molecular weight of protein (mg/mmol)
A_{280}^P	=	absorbance due to the protein at 280 nm
A_{280}^f	=	absorbance due to the fluorescein at 280 nm
$A_{280}^{f\text{-}P}$	=	absorbance of the fluoresceinated protein at 280 nm
$A_{490}^{f\text{-}P}$	=	absorbance of the fluoresceinated protein at 490 nm

Calculating the Molar Incorporation Ratio of Fluorescein to Protein

The following formulas are used to derive the molar incorporation ratio of fluorescein (MIR, moles of fluorescein incorporated per mole of protein). Formulas **d** and **e** were derived experimentally, using the optical density of fluorescein (f) at 280 nm and 490 nm.

a.) $\varepsilon' = \varepsilon \cdot (MW^P / 10^9)$

b.) $\text{nM P} = A_{280}^P / \varepsilon'$

c.) $A_{280}^P = A_{280}^{f\text{-}P} - A_{280}^f$

d.) $\text{nM f} = A_{490}^{f\text{-}P} / (56 \cdot 10^{-6})$

$$e.)\ A_{280}^{f} = 0.18 \cdot A_{490}^{f\text{-}P}$$

Substituting for A_{280}^{f} from formula **e** into formula **c** gives formula **f**.

$$f.)\ A_{280}^{P} = A_{280}^{f\text{-}P} - (0.18 \cdot A_{490}^{f\text{-}P})$$

Molar incorporation ratio (MIR) is defined as:

$$1.\quad MIR = \frac{nM\ f}{nM\ P}$$

Substituting for nM f and nM P from equations **b** and **d**:

$$2.\quad MIR = \frac{A_{490}^{f\text{-}P}}{(56 \cdot 10^{-6})} \cdot \frac{\varepsilon'}{A_{280}^{P}}$$

Substituting for ε' and A_{280}^{P} from equations **a** and **f**:

$$3.\quad MIR = \frac{A_{490}^{f\text{-}P}}{(56 \cdot 10^{-6})} \cdot \frac{\varepsilon \cdot (MW^{P}/10^{9})}{(A_{280}^{f\text{-}P} - (0.18 \cdot A_{490}^{f\text{-}P}))}$$

Rearranging:

$$4.\quad MIR = \frac{(18 \cdot 10^{3}) \cdot A_{490}^{f\text{-}P} \cdot \varepsilon \cdot (MW^{P}/10^{9})}{A_{280}^{f\text{-}P} - (0.18 \cdot A_{490}^{f\text{-}P})}$$

Canceling out the 10^{3}:

$$5.\quad MIR = \frac{18 \cdot A_{490}^{f\text{-}P} \cdot \varepsilon \cdot (MW^{P}/10^{6})}{A_{280}^{f\text{-}P} - (0.18 \cdot A_{490}^{f\text{-}P})}$$

For IgG's, (ε = 1.4 and MW = 160,000) this equation is further simplified:

$$MIR = \frac{4 \cdot A_{490}^{f\text{-}P}}{A_{280}^{f\text{-}P} - (0.18 \cdot A_{490}^{f\text{-}P})}$$

Calculating the Fluorescein-Labeled Protein Concentration (mg/ml)

From the Beer-Lambert Law:

$$\text{mg/ml} = \frac{A_{280}^{P}}{\varepsilon}$$

Substituting for A_{280}^{P} from equation **f**:

$$\text{mg/ml} = \frac{A_{280}^{f\text{-}P} - (0.18 \cdot A_{490}^{f\text{-}P})}{\varepsilon}$$

For IgG's, (ε = 1.4) this equation becomes:

$$\text{mg/ml} = \frac{A_{280}^{f\text{-}P} - (0.18 \cdot A_{490}^{f\text{-}P})}{1.4}$$

C

Appendix C- ILA Worksheets

Threshold Immuno-Ligand Assay Labeling Worksheet

BIOTIN LABELING
Date: _____

Protein:

Protein for labeling: _____

Protein purification: _____

Buffer exchange: _____

Volume:	_____	_____	_____	_____
mg/mL:	_____	_____	_____	_____

Biotin Label

MCR:	_____	_____	_____	_____
µL for reaction:	_____	_____	_____	_____
Incubation time:	_____	_____	_____	_____

Purified Biotin-labeled Protein:

A_{280}:	_____	_____	_____	_____
A_{260}:	_____	_____	_____	_____
MIR:	_____	_____	_____	_____
Protein concentration:	_____	_____	_____	_____
Percent recovery:	_____	_____	_____	_____

FLUORESCEIN LABELING
Date: _____

Protein:

Protein for labeling: _____

Protein purification: _____

Buffer exchange: _____

Volume:	_____	_____	_____	_____
mg/mL:	_____	_____	_____	_____

Fluorescein Label

MCR:	_____	_____	_____	_____
µL for reaction:	_____	_____	_____	_____
Incubation time:	_____	_____	_____	_____

Purified Fluorescein-labeled Protein:

A_{280}:	_____	_____	_____	_____
A_{260}:	_____	_____	_____	_____
MIR:	_____	_____	_____	_____
Protein concentration:	_____	_____	_____	_____
Percent recovery:	_____	_____	_____	_____

ILA Loading Study Data Summary Worksheet

Combination Number	f-P ng/test	b-P ng/test	Mean µV/s neg. sample	Mean µV/s positive sample 1	2	3	µV/s Net Signal 1	2	3	Quantitation Gain µV/s/unit 1	2	3
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												
13												
14												
15												
16												

This worksheet is for Chapter 13, "ILA Sandwich Format Optimization," Experiment 1. Quantitation gain (µV/s/unit is the specific signal (µV/s Net Signal), divided by the amount of analyte (positive sample) tested.

Appendix D- Total DNA Recommended Supplies

D

Recommended Supplies

Molecular Devices recommends the following supplies for the Total DNA Assay. Other suppliers' products may be suitable.

Table D-1: General Supplies

Item	Details / Catalog No.	Supplier	Phone[a]
Sterile syringes with Luer Lock fittings	30 ml for substrate addition and removal from Reader, and 1 ml with 26 gauge needle for filling the Reference Electrode		
5 and10 ml pipets	Sterile, individually wrapped		
Water bath incubator	For 37°C incubation		
Precision pipets with sterile disposable tips	Use sterile individually wrapped tips for all protein pretreatment and sample handling steps before denaturing DNA.	Brinkman Instruments, Inc.	(800)645-3050
	Eppendorf Biopur Tips 1000µl tips – Cat. # 22-49-115-6 200µl tips – Cat. # 22-49-114-8	Fisher Scientific	(800) 766-7000
Eppendorf repeating pipetr	Repeater pipetr – Cat. # 22-26-000-6 or Repeater Plus – Cat # 22-26-020-1	Brinkman Instruments, Inc.	(800)645-3050
50 ml adaptor	50 ml adaptor – Cat. #22-26-670-5	Fisher Scientific	(800) 766-7000
Sterile individually-wrapped tips for pipetr –	Combitips, 12.5 ml – Cat. # 22-49-520-8, --for Plus: 10mL – Cat. # 22-49-612-3 Combitips 1.25 ml – Cat. # 22-49-510-1 --for Plus: 2.5mL – Cat. # 22-49-608-5 Combitips, 0.5mL – Cat. # 22-49-604-2 Combitips 50 ml, non-sterile – Cat. #22-26-660-8		
Screw-cap tubes	2.0 ml sterile – Cat. #72.693.605	Sarstedt Inc.	(800) 257-5101
Rack for 48 screw-cap tubes	Cat. #93.1428		
Sterile polypropylene screw-cap centrifuge tubes, 15 ml and 50 ml. For example Falcon, or Corning:	15 ml – Cat. # 05-538-51A 50 ml – Cat. # 05-538-55A	Fisher Scientific	(800) 766-7000
Disposable borosilicate glass test tubes--16 x 125mm or 13 x 100mm (for standard curve dilutions)	16 x 125 mm – Cat. # 14-961-30 13 x 100 mm – Cat. # 14-961-27	Fisher Scientific	(800) 766-7000

D

Table D-1: General Supplies (Continued)

Item	Details / Catalog No.	Supplier	Phone[a]
Heat block (for DNA denaturation at 105°C)	Reacti-Therm III heating module – Product # 18835 Three insert racks 'VI' holding microfuge tubes – Product # 18819	Pierce Chemical Company	(815) 968-0747

a. Most of these phone numbers are for United States suppliers. To obtain supplier information in other countries, contact your nearest Molecular Devices office or consult our Web site, www.moleculardevices.com.

Table D-2: Pretreatment Supplies

Item	Details / Catalog No.	Supplier	Phone[a]
DNase I	Cat. # 776 785	Roche Applied Science	(800) 262-1640
Proteinase K	PCR Grade Cat. # 1964 372	Roche Applied Science	(800) 262-1640
Sodium Dodecyl Sulfate (SDS)	Electrophoresis Purity grade	VWR/Sigma	
Wako DNA Extractor Kit	Cat. #295-50201 (50 tests/kit)	Wako Chemical USA, Inc	(800) 992-9256
Centrifuge with rotor for 2 ml microtubes	Capable of spinning at 2,000g for phenol/ chloroform extraction and 10,000g for Wako extraction		
70% Phenol H_2O Chloroform	Cat. # 400765	Applied Biosystems, Inc	(800) 545-7547
Chloroform	Nucleic Acid Purification Grade Cat. # 400459	Applied Biosystems Inc. Fisher Scientific	(800) 545-7547 (800) 766-7000

a. Most of these phone numbers are for United States suppliers. To obtain supplier information in other countries, contact your nearest Molecular Devices office or consult our Web site, www.moleculardevices.com.

Appendix E- Software Installation

Introduction

This chapter describes the system requirements and installation instructions for Threshold Software for Windows, Threshold Enterprise and Enterprise Administrator.

Threshold Software and Threshold Enterprise

System Requirements

Threshold Software

Recommended

·IBM-compatible computer, with a Pentium IV-compatible microprocessor running at 800 MHz, or greater.

·Windows XP, 7, and 10 operating systems.

·2 MB free disk space.

·CD-ROM drive.

·1 serial port.

Minimum

·IBM-compatible computer, with a Pentium-compatible microprocessor running 100 MHz, or greater.

·Windows 98, NT, 2000.

·2 MB free disk space.

·CD-ROM drive.

·1 serial port.

Threshold Enterprise

Recommended

- IBM-compatible computer, with a Pentium IV (or later)-compatible microprocessor running at 800 MHz, or greater.
- Windows XP, 7, and 10 operating systems.
- 2 MB free disk space.
- CD-ROM drive.
- 1 serial port.

Minimum

- IBM-compatible computer, with a Pentium III-compatible microprocessor running at 300 MHz, or greater.
- Windows 2000 operating system.
- 2 MB free disk space.
- CD-ROM drive.
- 1 serial port.

E

Unpacking Instructions

When unpacking a new Threshold System, please check the packing list. If parts are missing, contact Molecular Devices. Contact information is given in the front of this manual

Installing Threshold Enterprise and / or Software

Installation

The Threshold Software Setup program, included on the Threshold Software Setup CD, is used to install both Threshold Software and Threshold Enterprise.

To install the software:

1) Close any programs that are running.
2) Insert the **Threshold Software Setup** CD into your CD-ROM drive.
3) Click OK.
4) The **Threshold Software Setup** wizard opens (Figure E-1)

Figure E-1: Threshold Software Setup Welcome Screen

5) Click Next.
6) Please read the License Agreement completely (Figure E-2).

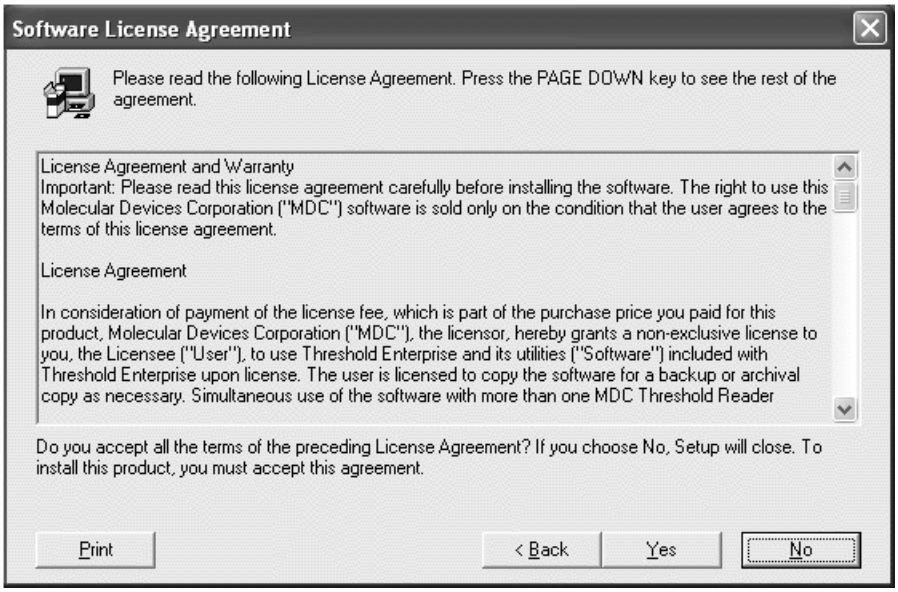


Figure E-2: Software License Agreement Screen

7) If you agree to the terms, choose **Yes**.
8) Click **Yes** to continue, or **No** to cancel.
9) If you choose Yes, the **Choose Destination Location** screen opens (Figure E-3).

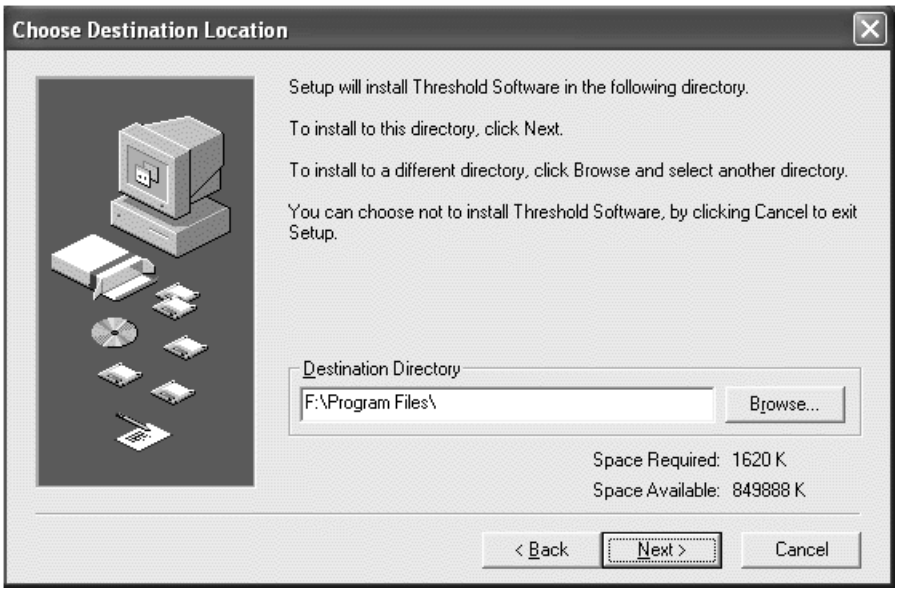


Figure E-3: Software Destination Screen

Threshold Software will be installed, by default, in the standard Windows program directory, **Program Files**. If you wish to install the program in a different directory, choose one of these options:

- Click **Browse** and select another directory.
- Enter the directory path directly into the **Destination Directory** text box.

10) When satisfied with the directory location, click **Next**.

11) In the **Setup Type** screen, choose the software program you wish to install (Figure E-4)

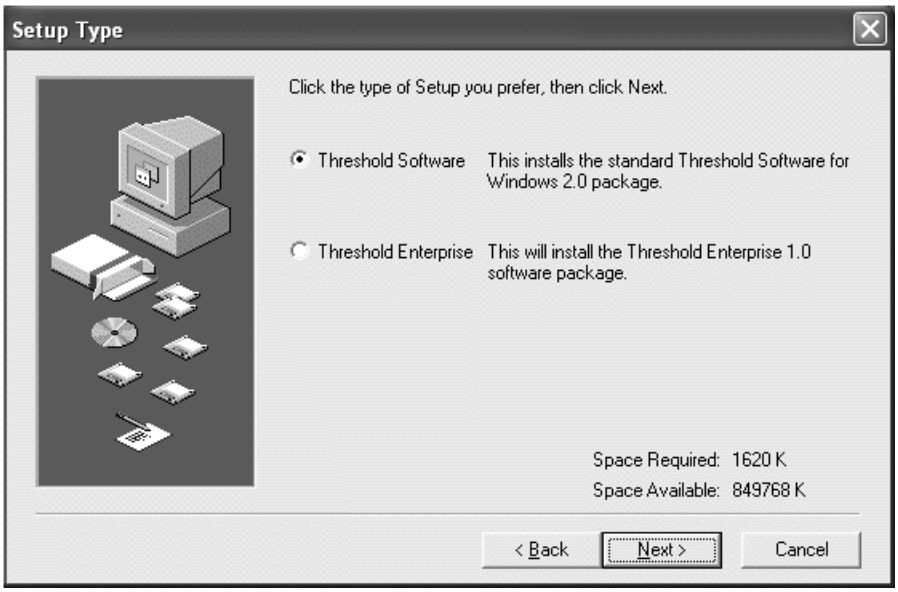


Figure E-4: Setup Type Screen

12) Click the button next to the software name (Threshold Software or Threshold Enterprise) and click **Next.**

13) The **Select Program Folder** screen opens (Figure E-5)

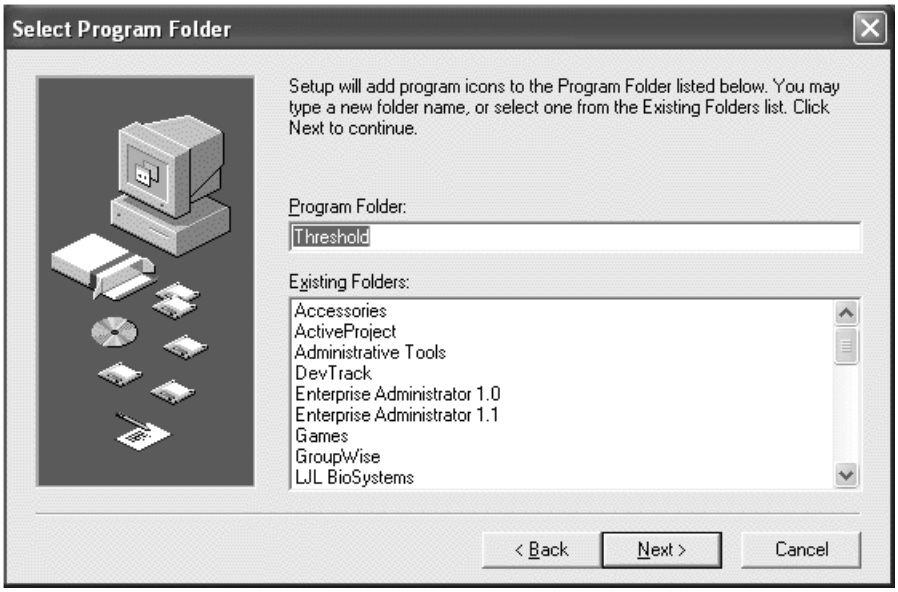


Figure E-5: Select Program Folder Screen

You may use this dialog to specify where to install the Program Folder in the **Start** menu. By default, the installer will create a folder named **Threshold**. This will allow you to launch either Threshold Software or Threshold Enterprise by clicking the **Start** menu, selecting the Threshold hierarchical menu item, and selecting the Threshold Software or Enterprise package icon.

You can do one of the following:

- Accept the default name.
- Create a new name.

14) Accept the Program Folder name or enter a new one.

15) When you have made your choice of folder, click **Next**.

16) The **Ready to Install** information window opens (Figure E-6).

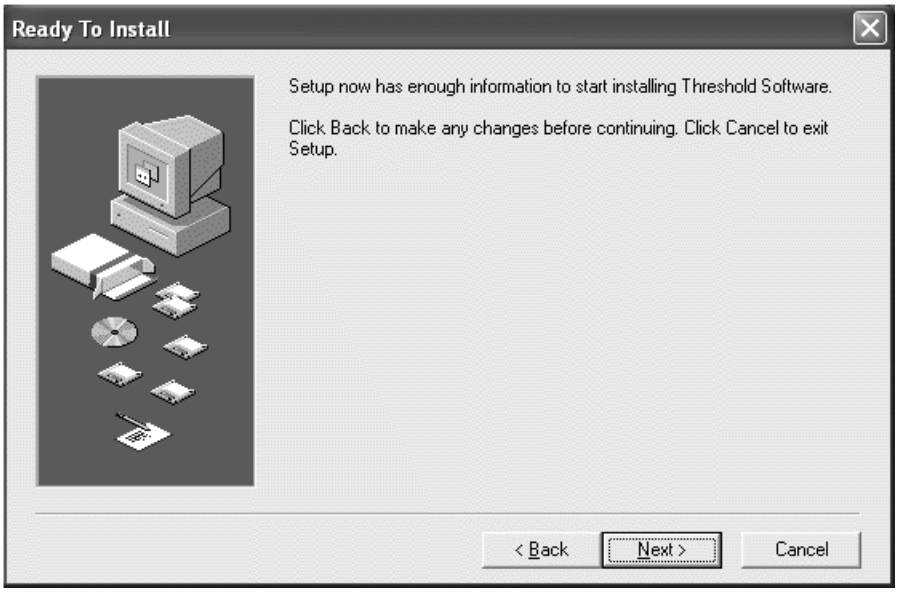


Figure E-6: Ready to Install Screen

- Click **Back** to make any changes before continuing.
- Click **Cancel** to exit Setup.
- Click **Next** to start the Threshold Software installation.

17) When installation is complete, two shortcut icons appear in the **Threshold** folder:

For Threshold Software (Figure E-7):

- Threshold Software
- Remove Threshold Software

For Threshold Enterprise (Figure E-8):

- Remove Threshold Software
- Threshold Enterprise

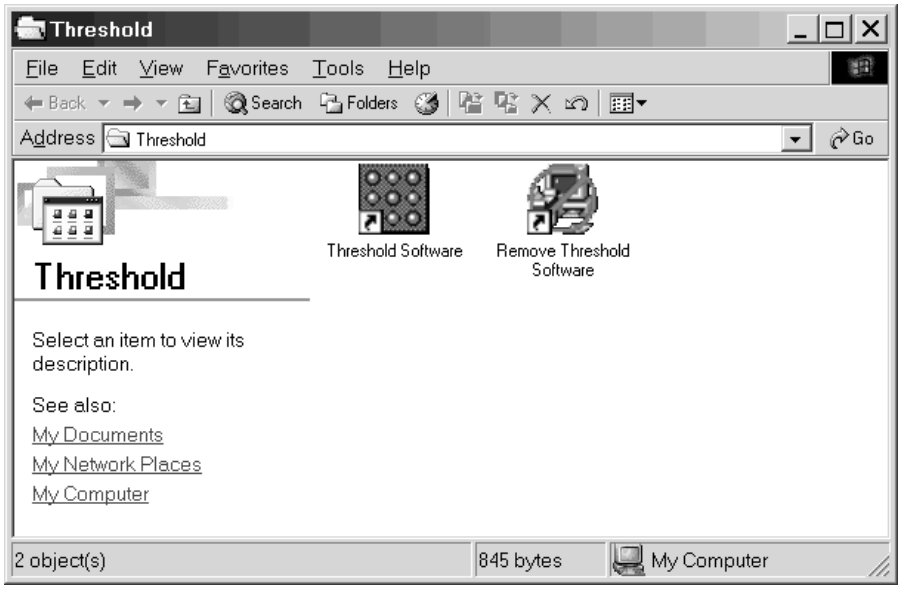


Figure E-7: Threshold Software Installed Files

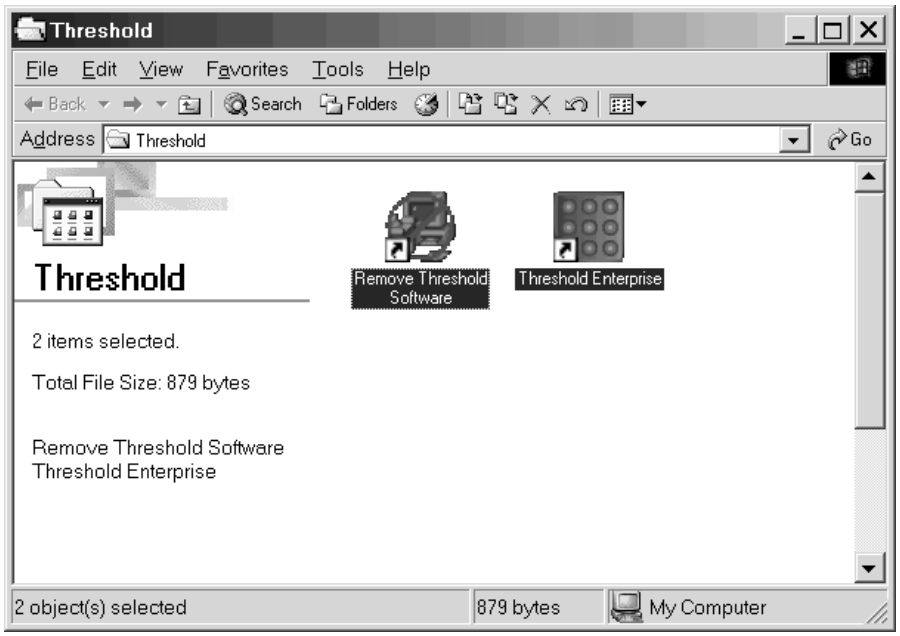


Figure E-8: Threshold Enterprise Installed Files

Threshold Enterprise Registration

If you installed Threshold Enterprise, the application will automatically launch.

At this point, you need to register the software in the **Software Registration** window (Figure E-9).

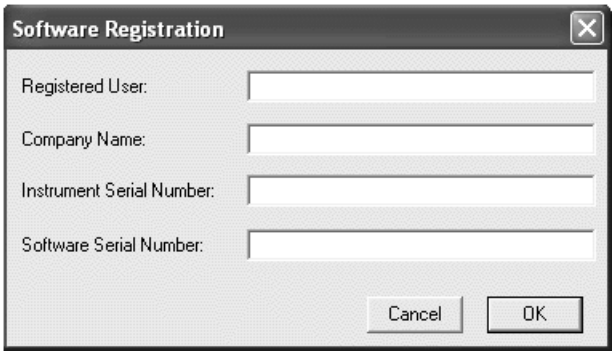


Figure E-9: Threshold Enterprise Registration Form

For more information, refer to Chapter 4, “Threshold Software.”

Installing Enterprise Administrator

This section describes how to install Enterprise Administrator.

System Requirements

- Pentium 4 or equivalent CPU.
- Windows XP, 7, and 10 operating systems.
- 128 MB RAM or better.
- 10 MB free disk space.

Installation

To install the software:

1) Insert the Enterprise Administrator CD into the CD-ROM drive.
2) Open **My Computer** and then double-click the CD-ROM drive icon, to display its contents.
3) Double-click the **Setup** icon and follow the screen prompts.
4) The **Welcome** screen suggests that you:
   - Close all other programs before beginning installation.
   - Disable any virus protection software you may have running.
5) The **Choose Destination Location** screen opens (Figure E-10).

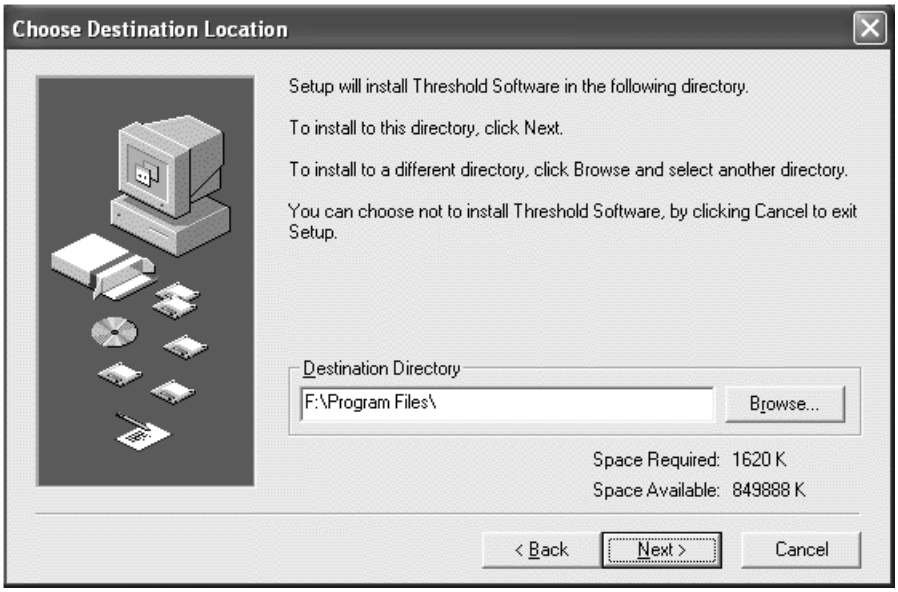


Figure E-10: Choose Destination Location Screen

E

6) Select the drive where you want the software to be installed. A default location will be shown on the screen.
7) Choose one of the following:
   - Change the destination to another disk.
   - Accept the default location.
8) The installation package will install the following files (See Table E-1)

Table E-1: Enterprise Administrator Files

File	Description
EnterpriseAdmin 1.1.exe	The application file
EnterpriseAdmin 1.1 Manual.pdf	This manual in portable document format (.pdf), which is readable using Adobe Acrobat Reader.
Enterprise Admin.chm	The Help file used by the Enterprise Administrator application.

9) When Installation is complete, these three shortcut cons appear in the Enterprise Administrator folder:
   - Enterprise Administrator
   - Help File
   - Manual

Figure E-11: Enterprise Administrator Installed Files

Appendix F- Threshold Enterprise and Threshold Software Reference

F

Introduction

This appendix summarizes the menus and options for both Threshold Enterprise and Threshold Software (Threshold Enterprise / Software). All the features of Threshold Software are included in Threshold Enterprise. Features that are unique to Threshold Enterprise will be specified as Threshold Enterprise-only.

Instructions for installing either of these applications are to be found in Appendix E, "Software Installation.".

Controlling System Functions

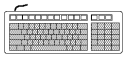
Both Threshold Software and Threshold Enterprise use the standard Microsoft Windows 2000 / XP / 7 / 10 user interface which utilizes windows, menus, dialogs, and the mouse. The mouse and keyboard are used to respond to the window displayed on the screen.

Using the Mouse

The mouse controls the pointer on the screen. To move the pointer, slide the mouse over a flat surface in one direction. The pointer moves in the same direction as the mouse.

The mouse has two buttons. The left button is used for all tasks in Threshold Enterprise / Software.

Using the Keyboard



To provide keyboard information, the Threshold Enterprise / Software / Enterprise doc-umentation uses Windows conventions.

- This manual abbreviates the names of the keys on the keyboard. For example, the CONTROL key is shown as CTRL and the ESCAPE key is shown as ESC.
- Keys are often used in combinations or sequences. For example, SHIFT+TAB means to hold down the SHIFT key while pressing the TAB key and CTRL+R means to hold down the CONTROL, key while pressing the letter R key.
- Shortcut keys are underlined letters in the menus. To use a shortcut key, press the ALT key, then press the underlined character. For example, (ALT, F, O).
- Arrow keys is the collective name for the UP ARROW, DOWN ARROW, LEFT ARROW, and RIGHT ARROW keys.

Menu Items / Commands

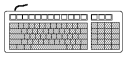
The menu commands are activated by either pointing and clicking the mouse or pressing a key combination on the keyboard. In Threshold Enterprise / Software / Enterprise, key combinations are provided after the menu and command names. For example, if a procedure step states "Select **File**, **Open** (ALT, F, O)" use the mouse and / or keyboard, as described in the following procedure:

To Choose a Command

1) Point to the **File** menu, and then click the left mouse button.
2) Point to the **Open** command, and then click the left mouse button.

OR

F



1) Press the ALT key to activate the menu bar and then press F to open the **File** menu.
2) Press O to choose the **Open** command.

Special Menu Symbols

The following symbols may be seen next to certain items in the menus:

Table F-1: Special Menu Symbols

Symbol	Meaning
Ellipses (•••)	Indicates that a dialog, requesting more information, will appear upon execution of the menu item.
Right-pointing triangle (⏵)	Indicates that a cascade menu (submenu) will appear when the cascade menu name is highlighted.
Checkmark (✓)	Indicates the active window in the list of open windows in the Window menu.
Grayed-out menu items	Indicates the menu option is unavailable.

Function Keys and Shortcuts

The twelve function keys, F1-F12, all have assigned shortcut functions in Threshold Software and Threshold Enterprise (Table F-2). In addition, there are a number of key-stroke combinations that give direct access to software functions without having to open the corresponding menus (Table F-3).

Table F-2: Function Key Shortcuts

Function Key	Function
F1	Starts Threshold Software Help
F2	Opens the Save As dialog
F3	Opens Vacuum Setting dialog
F4	Opens Read Stick dialog
F5	Displays Standard Curve graph
F6	Opens Stick Display dialog
F7	Starts instrument diagnostic test
F8	Opens Print Reports dialog
F9	Opens Clear Stick Data dialog
F10	Quits software, warning is given if data not already saved
F11	Opens software registration screen

Table F-2: Function Key Shortcuts

Function Key	Function
F12	Displays Threshold Software Title screen

Table F-3: Direct Keystroke Shortcuts

Keystroke	Function
CTRL+N	Creates a new experiment
CTRL+O	Opens an existing experiment
CTRL+S	Saves the current experiment
CTRL+P	Prints the current window
CTRL+Z	Undoes the most recent text edit
CTRL+X	Cuts the highlighted text, saves to Clipboard
CTRL+C	Copies the highlighted text to the Clipboard
CTRL+V	Pastes the Clipboard contents to the current field
CTRL+SHIFT+S	Opens the Copy Stick to Standard dialog
CTRL+SHIFT+C	Opens the Copy Stick dialog
CTRL+SHIFT+V	Opens the Paste Stick dialog
CTRL+SHIFT+X	Opens the Clear Stick Data dialog
CTRL+SHIFT+R	Opens the Reset Stick dialog
CTRL+F	Opens the Vacuum Setting dialog
CTRL+T	Starts instrument diagnostic test
CTRL+R	Opens Read Stick dialog

Threshold Enterprise / Software Screen

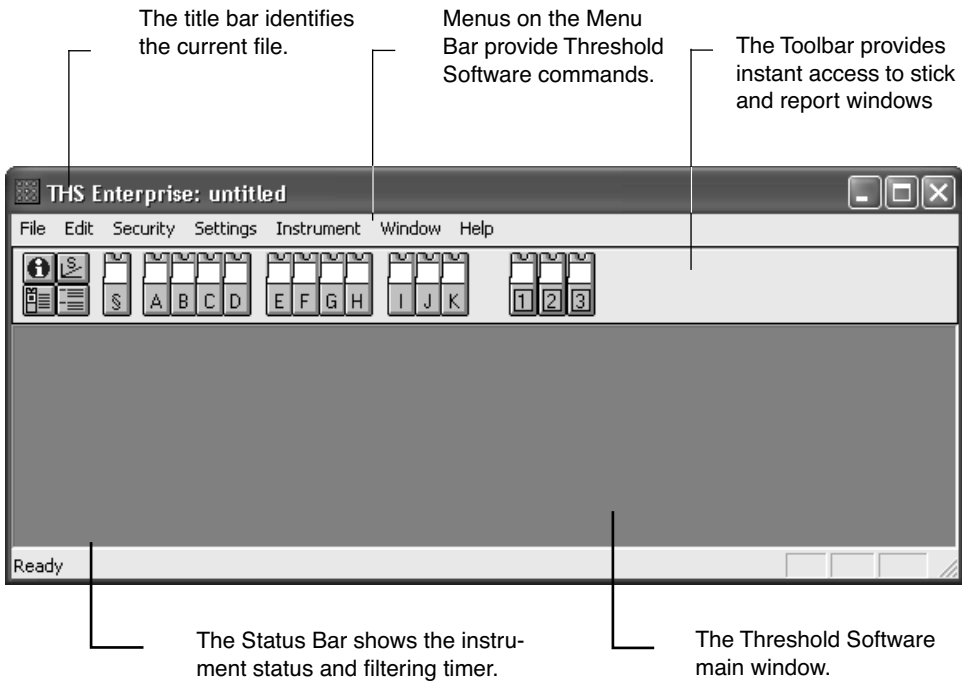


Figure F-1: The Threshold Enterprise / Software Screen

The Toolbar Buttons

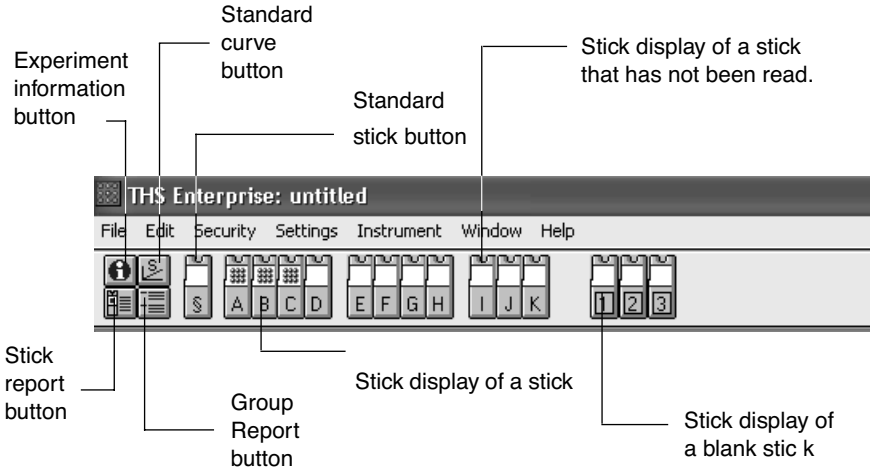
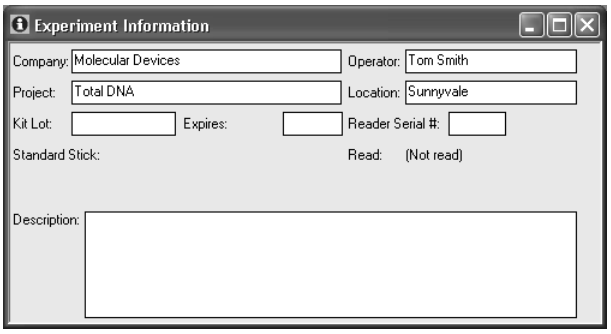


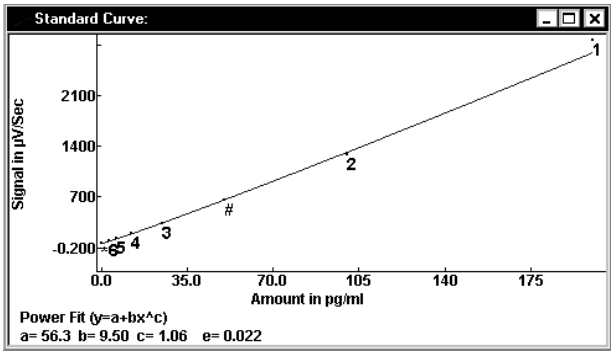
Figure F-2: The Toolbar

Click the Toolbar buttons to display the following windows:

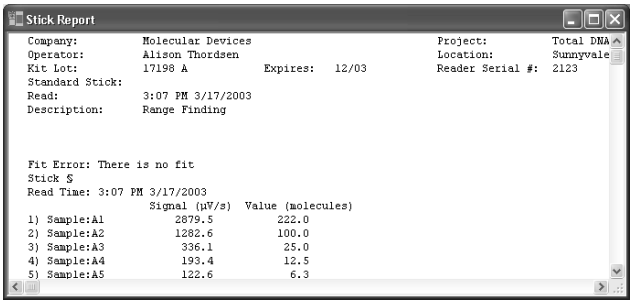
Click	To Display Experiment Information



Click	To Display the Standard Curve

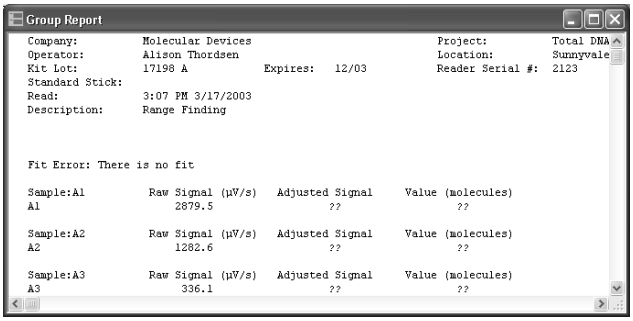


Click	To Display the Stick Report

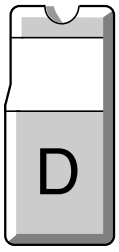


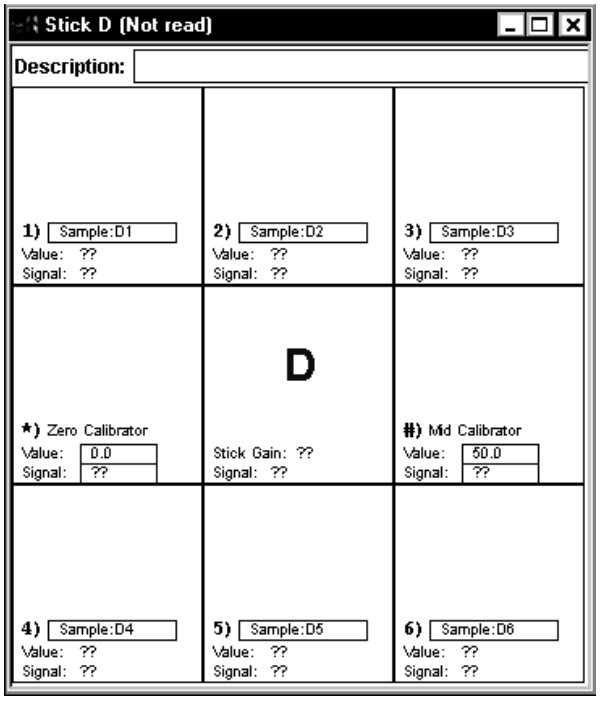
F

Click	To Display the Group Report



Click	To Display a Stick





Messages From Threshold Enterprise / Software

Threshold Enterprise / Software displays messages in dialogs which open in the middle of the screen. The types of messages found in Threshold Enterprise / Software are:

Information Messages

Information messages provide instructions, important information, and tips about working with the Threshold System and software. See Figure F-3 below for an example.

Figure F-3: An Example Information Message

Confirmation Messages

Confirmation messages ask for confirmation that the current action is the desired action. See Figure F-4 for an example.

Figure F-4: An Example Confirmation Message

Error Messages

Error messages are displayed to alert the user of a problem. See Figure F-5 for an example.

Figure F-5: An Example Error Message

Most error messages suggest a way to fix a problem. For additional information on a particular message, see Chapter 7, "System Troubleshooting."

Quick Reference To Menu Items

This section contains quick reference information for the menu items in Threshold Software (Figure F-6) and Threshold Enterprise (Figure F-7).

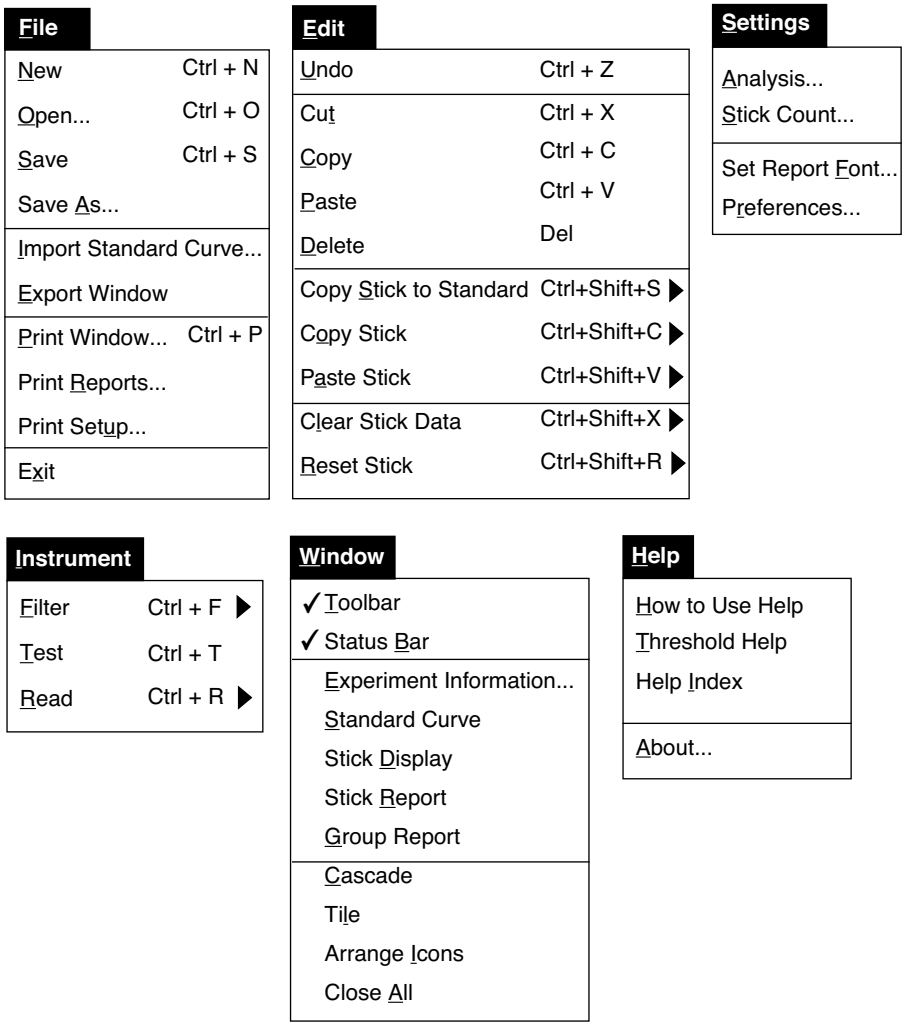


Figure F-6: Threshold Software Menus

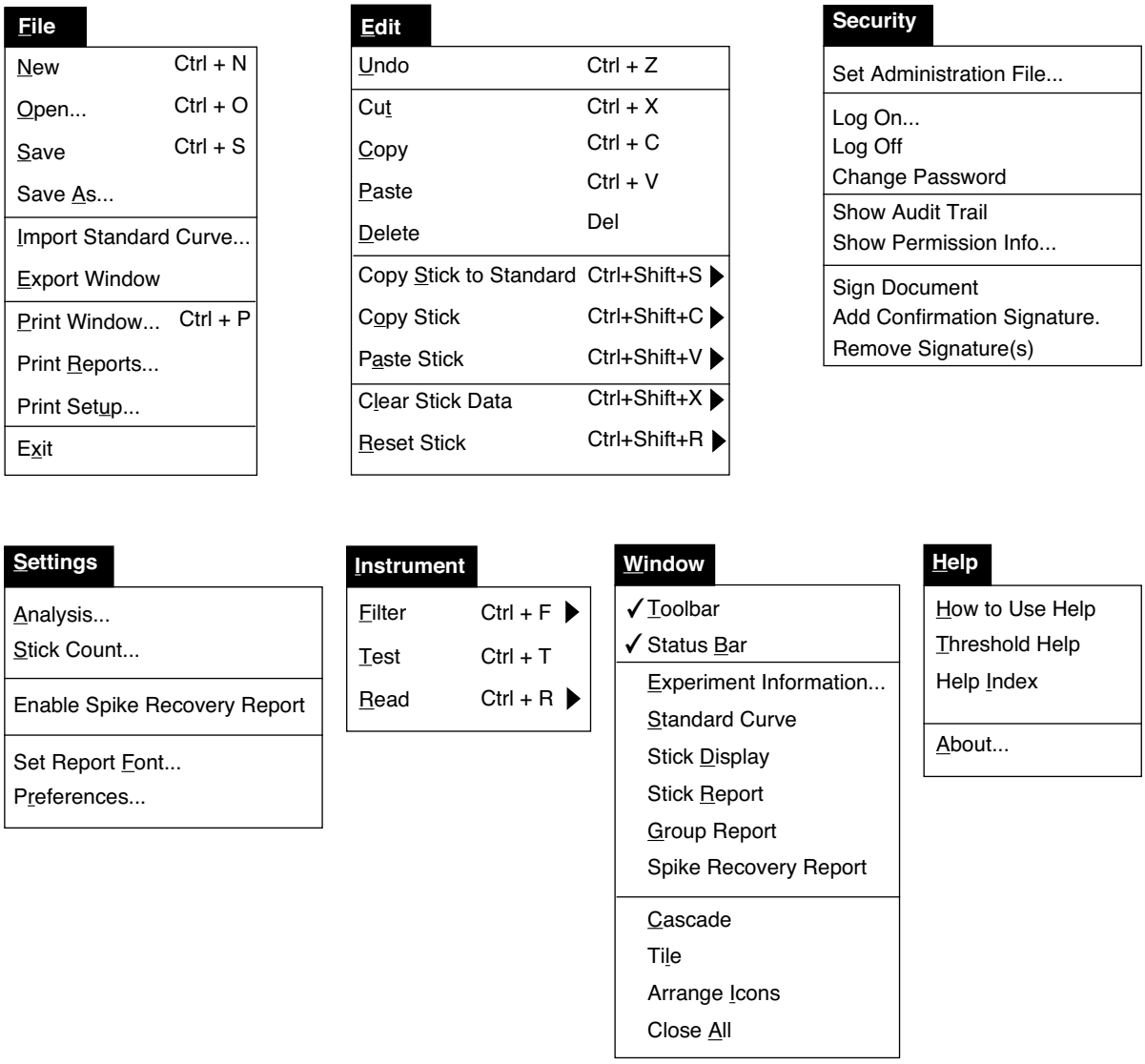


Figure F-7: Threshold Enterprise Menus

F

File Menu

The **File** menu contains selections used to create / open files, save files, import standard curves, export data, and print windows and reports (Table F-4).

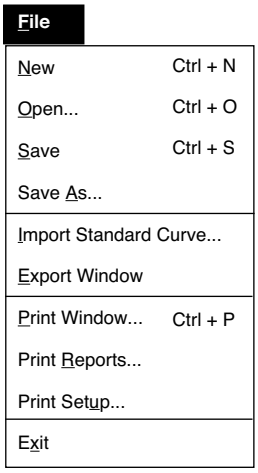


Table F-4: File Menu

File Menu Item	Action
New	Creates a new file to receive experiment information and data.
Open...	Brings up the Open dialog which lists existing data files to open; either Windows files (`.thw` and `.th3`) or DOS files (`.ths`).
Save	Saves the current file, and allows a new name or location to be given.
Save As...	Saves the current file, and allows a new name or location to be given.
Import Standard Curve...	Retrieves standard curve data from another Threshold experiment (`.thw` and `.th3` files only) for use in the current experiment.
Export Window	Saves the current window to a file, and allows a new name or location to be given
Print Window	Prints the currently-active window (front-most window).
Print Reports...	Prints any of six report choices selected from the Print Reports dialog.
Print Setup...	Allows choice of printer and printer settings to be used with Threshold Enterprise / Software.
Exit	Quits Threshold Enterprise / Software.

Edit Menu

The **Edit** menu contains Windows commands (**Undo**, **Cut**, **Copy**, **Paste**, **Delete**) with Clipboard access, as well as several commands specific to Threshold Enterprise / Software, such as **Copy Stick** and **Paste Stick** (Table F-5).

The Clipboard is a Windows application that functions as a holding place for what was last cut or copied. Information on the Clipboard can be pasted onto other sticks within Threshold Enterprise / Software, or into other documents. After selecting any of the commands for copying, pasting, clearing, or resetting a stick, a menu will open, showing a list of the sticks to choose from. Warning messages are always given if a stick editing operation will result in the loss of data.

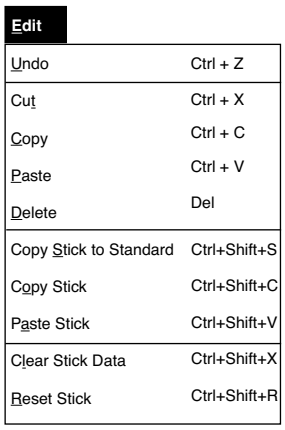


Table F-5: Edit Menu

Edit Menu Item	Action
Undo	Undoes the most recent text edit.
Cut	Cuts selected item and places it on the Clipboard.
Copy	Copies selected item to the Clipboard.
Paste	Pastes item from the Clipboard into the current text field.
Delete	Cuts selected item without placing it on the Clipboard.
Copy Stick to Standard	Copies the selected stick information directly to the standard stick.
Copy Stick	Copies the selected stick information to the Clipboard.
Paste Stick	Pastes the stick information on the Clipboard to the selected stick, replacing the current stick information.
Clear Stick Data	Clears the selected stick data.
Reset Stick	Resets all the stick information on the selected stick to the default settings.

F

Security Menu (Threshold Enterprise only)

The **Security** menu provides commands for security features, logging on and off the software, changing user passwords, showing audit trails and user permissions, and adding and deleting signatures from documents.

(Threshold Enterprise only)

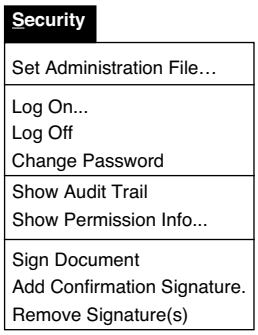


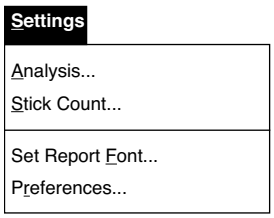
Table F-6: Security Menu

Security Menu Item	Content
Set Administration File…	Used by the administrator to select the Enterprise Administrator database file for Threshold Enterprise.
Log On	Allows a user to log on to the software.
Log Off	Logs a user off the software.
Change Password	Allows a user to change his / her system password..
Show Audit Trail	Brings up the Audit Trail window.
Show Permission info…	Brings up a window showing the permissions for the present software user.
Sign Document…	Allows a user to add an e-signature to a document.
Add Confirmation Signature…	Allows a user to add a confirming e-signature to a document
Remove Signature(s)	Removes all e-signatures from a document.

Settings Menu

The **Settings** menu allows you to define the basic settings that will apply for the current experiment such as the standard curve fit, the units, the number of sticks and blanks, and the fonts to be used on reports (See Table F-7). In addition, the **Preferences** command allows the default values that Threshold Enterprise / Software uses upon startup to be changed.

(Threshold Software only)



(Threshold Enterprise only)

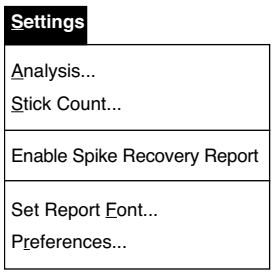


Table F-7: Settings Menu

Settings Menu Item	Action
Analysis...	Defines the standard curve fit and units.
Stick Count...	Defines the number of data and blank sticks in the experiment.
Enable Spike Recovery Report	(Threshold Enterprise only) Includes a Spike Recovery Report in the file.
Set Report Font...	Defines fonts for screen and printed reports.
Preferences...	The default values Threshold Enterprise / Software uses upon startup. Includes curve fit and units, user information, number of sticks, calibrator and standard values, and COM port used for the instrument.

Instrument Menu

The **Instrument** menu contains commands that directly control the instrument, such as setting the filtration rate, running diagnostics, and reading Threshold sticks (See Table F-8).

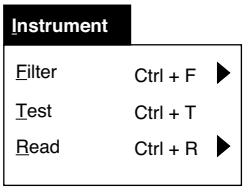


Table F-8: Instrument Menu

Instrument Menu Item	Action
Filter	Brings up a menu with vacuum pump control choices **High**, **Low**, **Special**, and **Off** used for filtering and washing samples.
Test	Performs diagnostic check on the entire Threshold System.
Read	Begins the process for analyzing sticks.

Testing Screen

The Testing screen is shown in Figure F-8.

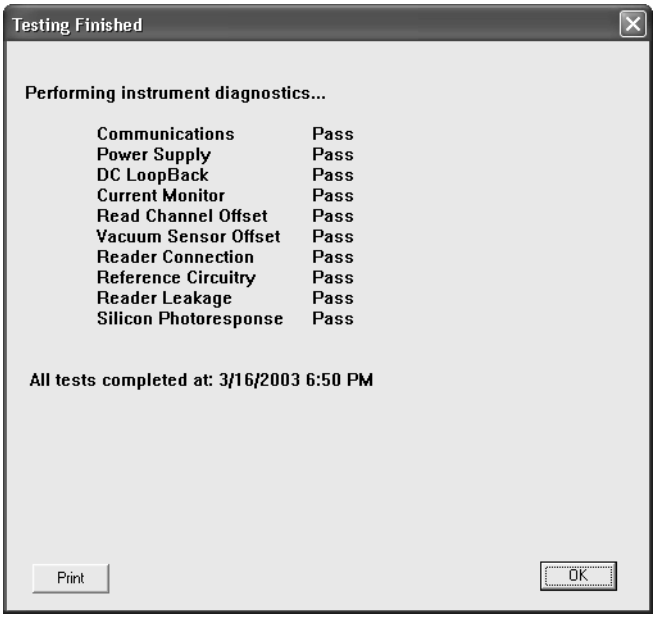


Figure F-8: Testing Finished Screen

The functions of each self-diagnostic test are:

Table F-9: Self-Diagnostic Test Functions

Test	Meaning
Communications	Checks that cables are connected and the workstation is on, and that information moves between the computer and workstation, appropriately.

F

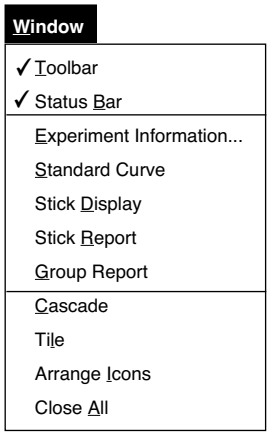
Table F-9: Self-Diagnostic Test Functions (Continued)

Test	Meaning
Power Supply	Checks that the power supply is working within specifications.
DC Loopback Current Monitor Read Channel Offset	These three tests check the workstation's analog circuits.
Vacuum Sensor Offset	Checks that the vacuum sensor is working properly.
Reader Connection	Checks that the Reader is properly inserted.
Reference Circuitry	Checks that the Reference Electrode is inserted in the Reader, connected to the workstation, and is working properly.
Reader Leakage	Checks for current leakage across the gold contacts in the Reader.
Silicon Photoresponse	Checks that the silicon sensor in the Reader is working correctly.

Window Menu

The **Window** menu allows you to view the data, report, or information windows in Threshold Enterprise / Software, as well as change their appearance or arrangement on the screen (See Table F-7). The five primary report and display windows can also be opened by clicking on the corresponding button on the toolbar.

(Threshold Software Only)



(Threshold Enterprise Only)

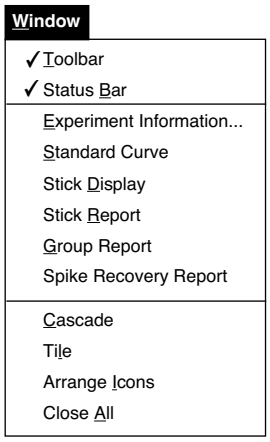


Table F-10: Window Menu

Window Menu Item	Action
Toolbar...	Switches between displaying and hiding the Toolbar.
Status Bar	Switches between displaying and hiding the Status Bar.
Experiment Information...	Brings up the Experiment Description window, which contains information such as kit lot, Reader number, and operator.
Standard Curve	Brings up the standard curve window.
Stick Display	Brings up the Stick Display window for a selected stick or sticks.
Stick Report	Brings up the Stick Report window showing stick data in tabular form.
Group Report	Brings up the Group Report window showing sample replicate data in tabular form.
Spike Recovery Report	(Threshold Enterprise only) Brings up the Spike Recovery Report window showing spike recovery data in tabular form.
Cascade	Arranges the current open windows in a cascade pattern, that allows viewing of the window title bars and the front-most window.
Tile	Arranges the current open windows in a tile pattern, that allows viewing of all the windows simultaneously.
Arrange Icons	Arranges all minimized window icons in rows at the bottom of the screen.
Close All	Closes all open windows.

Help Menu

The **Help** menu has commands used to access reference information for operating the Threshold System and software (See Table F-8).

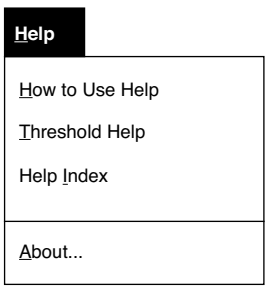


Table F-11: Help Menu

Help Menu Item	Content
How to Use Help	Help information for using the Windows help system.
Threshold Help	Detailed help information for using Threshold Enterprise / Software.
Help Index	An index of the Threshold Enterprise / Software Help information.
About	Brings up the Threshold Enterprise / Software Title screen.

F

E

F

G

H

I

L

M

N

O

P

Q

R

S

T

U

V

W

MOLECULAR DEVICES

SALES OFFICES

United States
Molecular Devices LLC
Tel. 800-635-5577

www.moleculardevices.com

ISO 9001
ANSI-RAB QMS ACCREDITED
Registered Company
TUV Rheinland of North America, Inc.