ProteinChip® System Users Guide

CIPHERGEN

Ciphergen Biosystems, Inc.
6611 Dumbarton Circle
Fremont, CA 94555, USA

Main Office: 510-505-2100
Toll Free: 888-864-3770
Fax: 510-505-2101
Email: support@ciphergen.com
www.ciphergen.com

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Chapter 1

Introducing the ProteinChip® System

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The ProteinChip System

Ciphergen's ProteinChip System is comprised of a ProteinChip Reader (series PBS II) integrated with ProteinChip Software and a personal computer to analyze proteins captured on ProteinChip Arrays. The ProteinChip System detects and accurately calculates the mass of compounds ranging from small molecules and peptides of less than 1000 Da up to proteins of 500 kDa or more based on measured time-of-flight. The System is compact enough to fit into almost any lab space, allowing researchers direct access to precision mass analysis of important peptides and proteins from complex biological samples.

ProteinChip Arrays

Ciphergen's ProteinChip Arrays distinguish this technology from other mass spectrometry-based analytical systems. ProteinChip Arrays provide a variety of surface chemistries that allow researchers to optimize protein capture and analysis. The surface chemistries of the arrays include a series of classic chromatographic chemistries and specialized affinity capture surfaces. Classic chromatographic surfaces include normal phase for generic protein binding; hydrophobic surfaces for reversed-phase capture; cation- and anion-exchange surfaces; and immobilized metal affinity capture (IMAC) for metal-binding proteins. Specific proteins of interest can be covalently immobilized on pre-activated surface arrays, enabling customized experiments to investigate antibody-antigen, DNA-protein, receptor-ligand, and other molecular interactions.

The ProteinChip Reader (Series PBS II)

The ProteinChip Reader is a laser desorption/ionization time-of-flight mass spectrometer that uses state-of-the-art ion optic and laser optic technology. The laser optics maximize ion extraction efficiency over the greatest possible sample area, and thus increase analytical sensitivity and reproducibility. The Reader's ion optics incorporate a four-stage, time-lag-focusing ion lens assembly that provides precise, accurate molecular weight determination with excellent mass sensitivity.

The ProteinChip Software

Ciphergen's ProteinChip Software controls all aspects of the ProteinChip Reader and facilitates data collection and analysis. The software uses a Microsoft Windows NT interface, and contains numerous features, including automatic reading of ProteinChip Arrays; multiple spectrum comparison for differential protein display and biomarker discovery; several alternative viewing formats for data; and a user-friendly interface.

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How the ProteinChip System Works

This section contains a general overview of how the ProteinChip System works. In this overview, the terms “analyte” and “protein” are used somewhat interchangeably. Most ProteinChip System applications involve the study of proteins; however, the System is also capable of analyzing other analytes, including peptides and small molecules.

Most protocols involve a short series of binding and washing steps on the chip. After preparation of the researcher’s biological sample, a few microliters are placed on the active surface of the ProteinChip Array. Proteins or other analytes are captured on the surface of the ProteinChip Array by means of specific surface chemistries or by biomolecules covalently bound to the array (Figure 1-1).

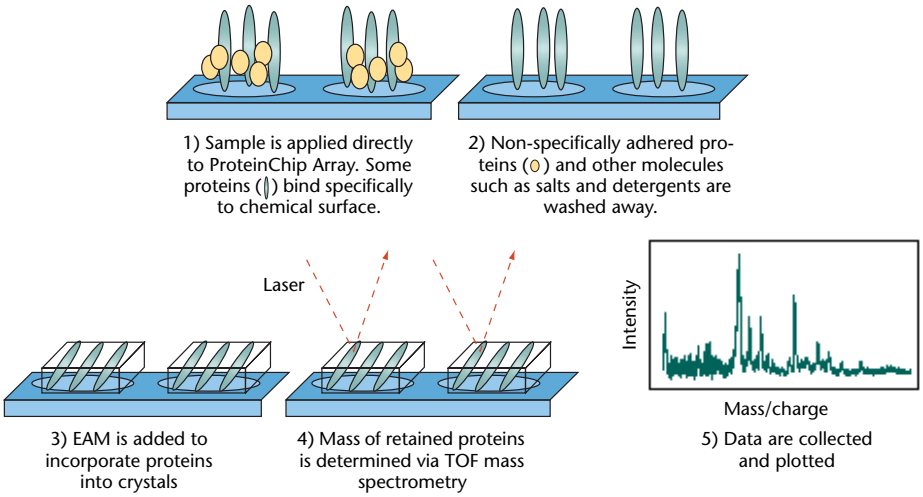

Figure 1-1: A schematic diagram of ProteinChip Array experiments, based on Surface-Enhanced Laser Desorption/Ionization (SELDI).

Specific capture of a subset of proteins in the sample occurs through simple chemical interactions or protein-protein interactions. Following binding and washing, an *Energy Absorbing Molecule* (EAM) solution is applied to the sample retained on the chip surface. Application of EAM in organic solvent causes the protein to dissolve into a solution with the EAM. When this solution dries on the chip surface, a very crude *crystal* forms that includes both the protein (or other analyte of interest) and a large molar excess of EAM molecules. The EAM molecules are essential to mediate ionization of the sample (see Figure 1-1). After the crystals of EAM and analyte have formed on the ProteinChip Array, it is placed into the ProteinChip Reader for mass spectrometric analysis.

The lid of the Reader is opened and the array is placed in the *sample handler*. The sample handler moves the array up or down to allow reading of a particular sample spot by query with a fixed laser beam. As shown in Figure 1-2, the energy from a single shot of the laser beam is tightly focused on a small area of the spot (“focused laser beam”). Thus each spot contains multiple, addressable positions (a few example

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positions are numbered 20, 50 and 80 in the figure). The ProteinChip Software is used to precisely control the laser query process.

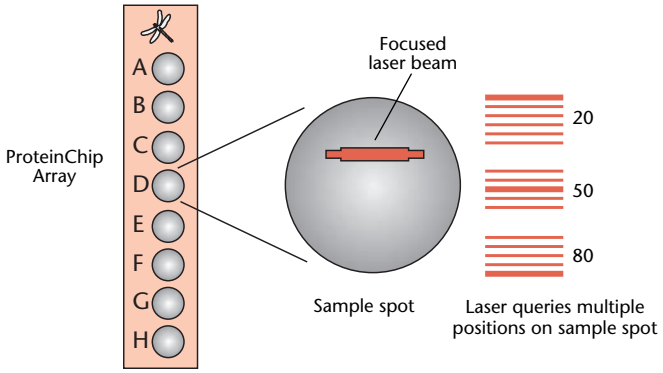

Figure 1-2: Multiple precisely-defined positions on the spot are queried by the laser.

When the laser is turned on or "fired," it illuminates the sample, beginning the process of *ionization* and *desorption*. Ionization of the analyte results from a poorly-understood interplay between the laser energy, the EAM and the analyte. In short, the laser energy induces both protein ionization and a change of state from the solid, crystalline phase into the gas phase.

After the laser beam is fired, two things happen. First, the analyte becomes charged. Second, the analyte is transformed into the gaseous phase; it can therefore be caused to move very rapidly, or "fly," upon application of a voltage differential. As shown in Figure 1-3, proteins with a positive charge are induced to fly away from a metal chip that also has a positive charge. The voltage differential applies the same energy to all of the analytes in the sample, thus resulting in flight times that depend upon the mass (remember: $e = mv^2$ where e = energy, m = mass, v = velocity). The ProteinChip Reader records the time-of-flight (TOF) of the analyte and from this measurement, a highly accurate and precise mass is derived.

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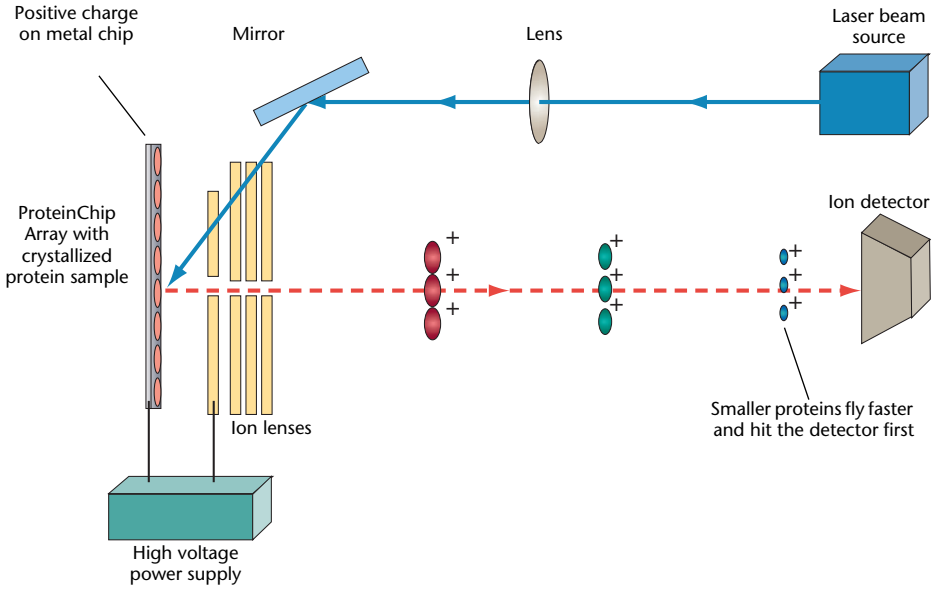

Figure 1-3: A schematic of the ProteinChip Reader.

The ProteinChip Software controls all aspects of data collection from the samples on the ProteinChip Arrays. The user specifies a few basic parameters before beginning data collection, then the run begins with a click of the mouse. The data can be compiled and presented in a number of formats to facilitate data analysis and interpretation and communicating results to others. Rapid comparison between data sets is also readily achieved — a feature particularly useful for protein differential display. Finally, the original data remains part of the data file no matter what analytical and visual changes have been made, allowing the original data to be retrieved at any time.

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Chapter 2

PBS II Operational Differences and Software Overview

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The new PBS II has a different detector, an ion deflector and more flexibility in setting the digitizer rate.

The detector operates between 2800 to 3100 volts (a higher voltage than the previous model). A typical starting detector voltage is 2900. At this detector voltage setting, a laser intensity setting of ~240 begins to saturate the detector with ions.

Because of the high detector response, you may encounter ion overload. When this happens, the baseline becomes artificially high, a "wave" may appear in the data, and signal strength, as measured by peak height, appears to drop as laser energy increases, due to the increased baseline. Refer to Figures 2-1 through 2-5 for sample data illustrating the effect of ion overload.

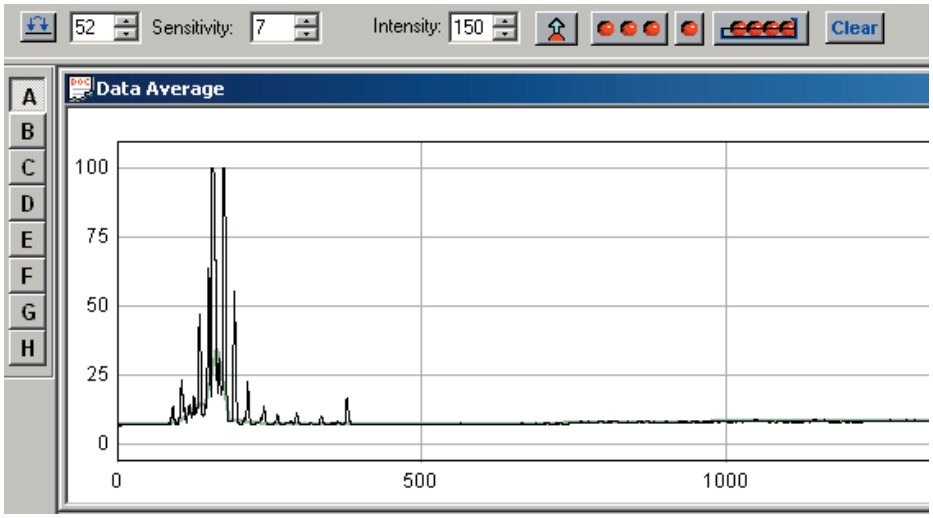

Figure 2-1: Effect of ion saturation on low MW samples. **Intensity** set to 150.

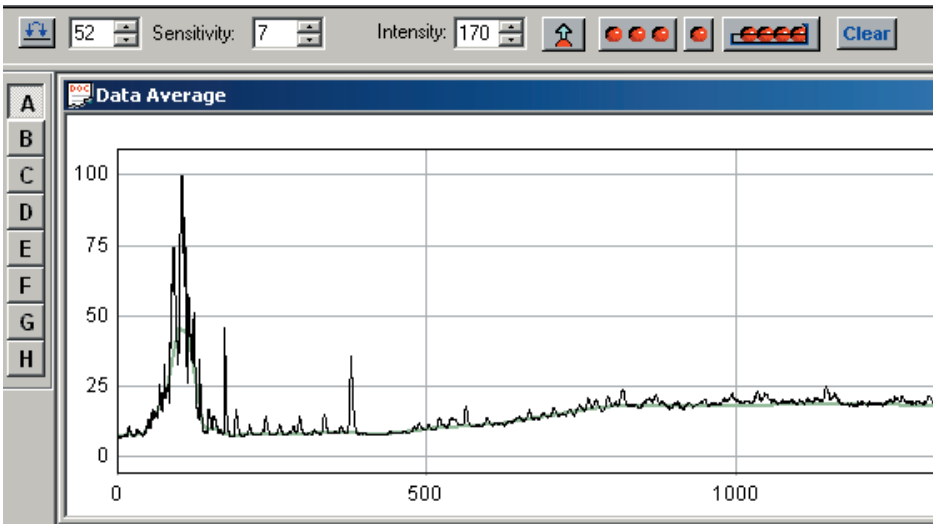

Figure 2-2: Effect of ion saturation on low MW samples. **Intensity** set to 170 — note the change in the shape of the matrix peaks, and the increasing baseline.

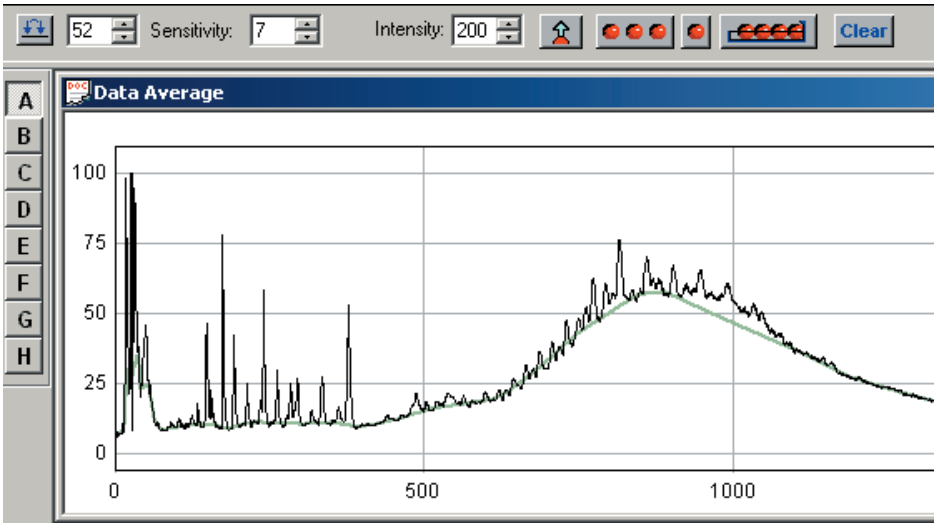

Figure 2-3: Intensity set to 200, **Mass Deflector** setting 2 (see below) – note the baseline "hump" from ions saturating the detector.

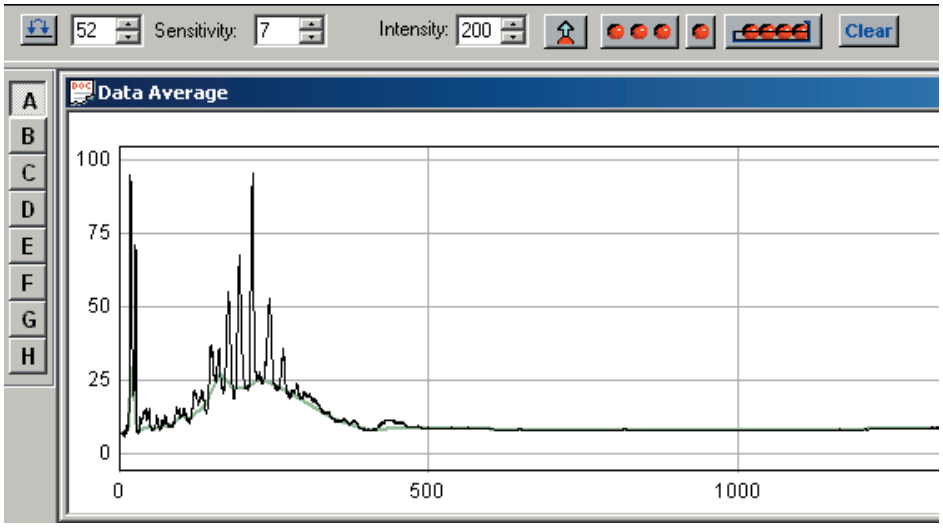

Figure 2-4: Intensity set to 200, **Mass Deflector** setting 10 (see below) — most of the lower MW ions are deflected away from the detector, preventing overload and resulting in a flattened baseline.

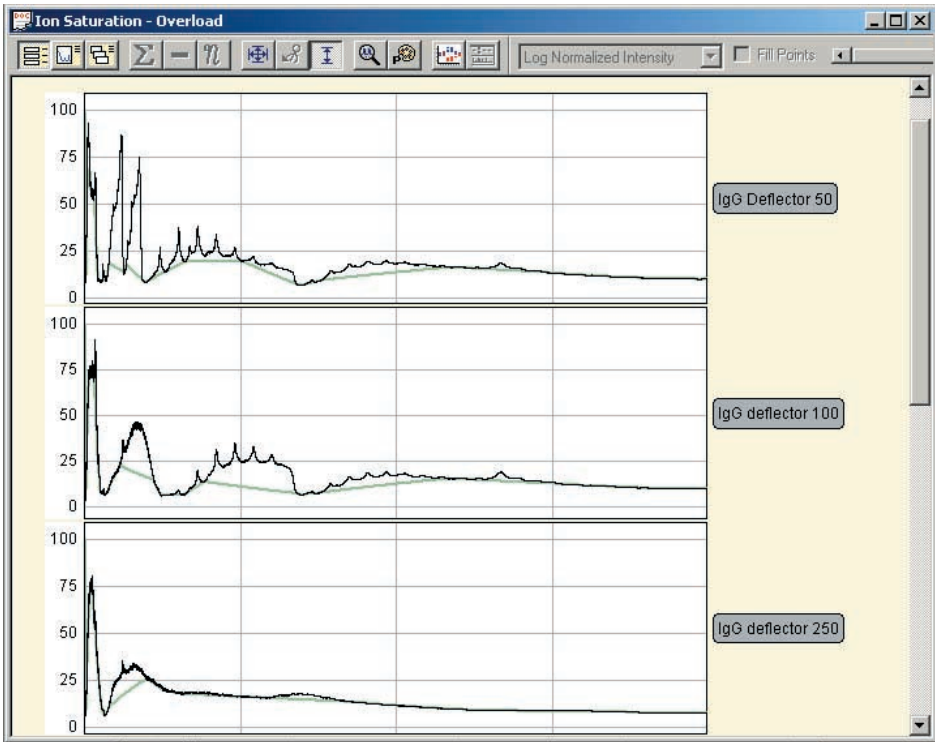

Figure 2-5: Detector overload (ion saturation). Increasing the **Mass Deflector** setting deflects more ions from the detector. Note the IgG peak disappears when the mass deflector is set to 250.

A mass deflector was added to the PBS II to compensate for ion overload, and to prevent overly abundant smaller MW ions (e.g. matrix ions) from obscuring the signal from more interesting analytes. The deflector prevents most of the ions below a certain MW from striking the detector.

The proper mass deflector setting depends on the MW region of interest. The lowest setting is 2; setting 12 corresponds to Insulin (5.8 kDa), and large MW ions are more easily detected with a setting of 40 to 80. You should experiment with the **Mass Deflector** setting in order to achieve the best results for your samples.

NOTE: *The deflector setting influences the time of flight (similar to the TLF setting). This means you will have to recalibrate the instrument when you change the* ***Mass Deflector*** *setting.*

Changing the Mass Deflector Setting

1. Select **Instrument | Configuration** to open the **Instrument Configuration** dialog, which contains the **Detector** and **Mass Deflector** settings (Figure 2-6).

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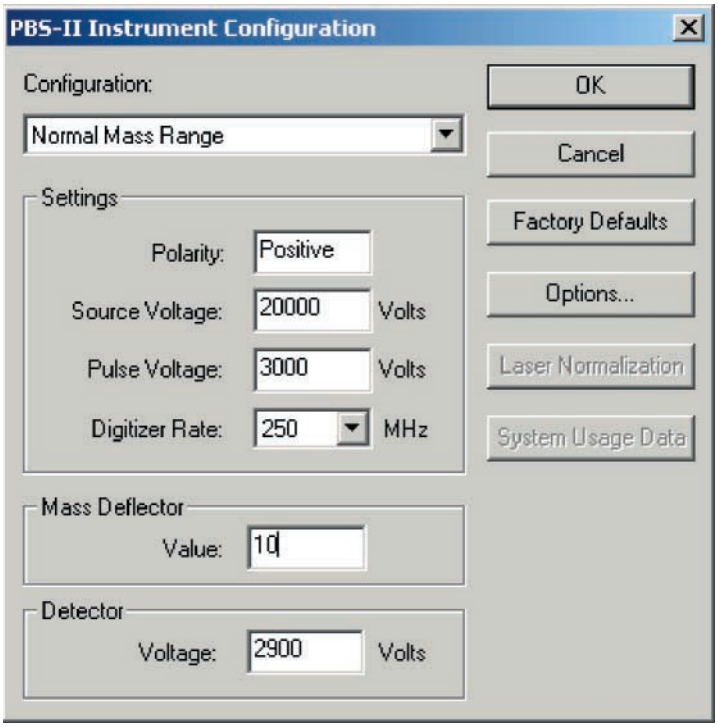

Figure 2-6: The **PBS II Instrument Configuration** dialog.

*IMPORTANT: The **Mass Deflector** setting is not controlled as part of a spot protocol, nor is it saved in the data properties. **You must set and track deflector settings manually!** Also, before you begin collecting data, check the **deflector setting** — someone else may have changed it since you last used it.*

2. It is important not to use an inappropriately high laser intensity, as a saturated detector fails to collect data. Also, the **Mass Deflector** setting will influence the appearance of the data. Try different combinations of laser **Intensity** and **Mass Deflector** settings until you are satisfied with the appearance of the data.

Baseline Fitting

Detector ion saturation may result in an oddly shaped baseline. As a result, the default baseline fitting algorithm may be insufficient.

Analysis Protocol button

To change the baseline fit, open the **Analysis Protocol Properties** dialog by selecting **Analysis Protocol Properties...** from the **Options** menu or by clicking the **Analysis Protocol** button in the **Data Analysis** toolbar. Select the **Baseline** page of the dialog (Figure 2-7).

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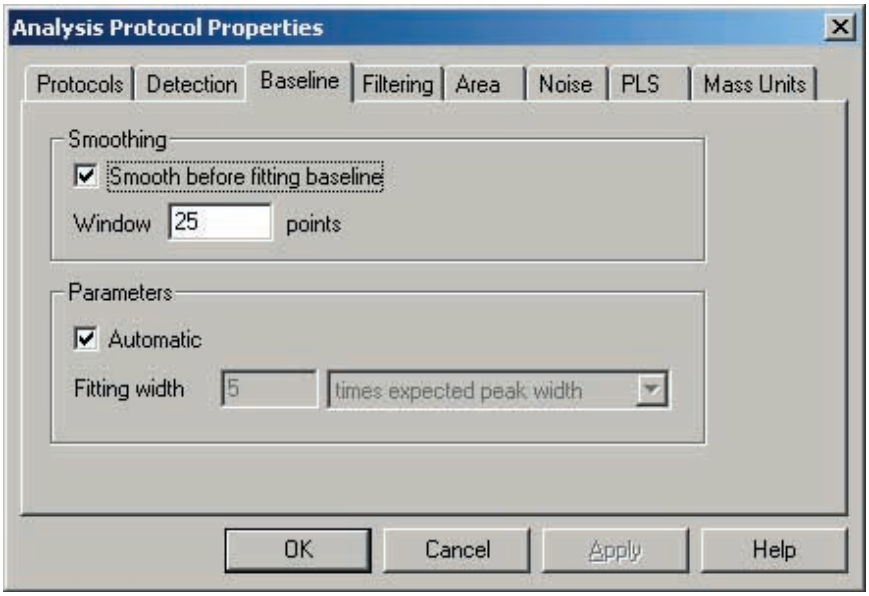

Figure 2-7: The **Baseline** page of the **Analysis Protocol Properties** dialog.

1. Turn off the **Parameters**, **Automatic** setting.
2. Set the **Fitting width** to a lower number, try 3 or 4 times the noise (default is 5).

Figure 2-8 illustrates the effect of the **Fitting width** setting on the baseline curve fit. The green line shows the calculated baseline. As you can see, changing the baseline fit to **4 times expected peak width** results in a much better baseline fit.

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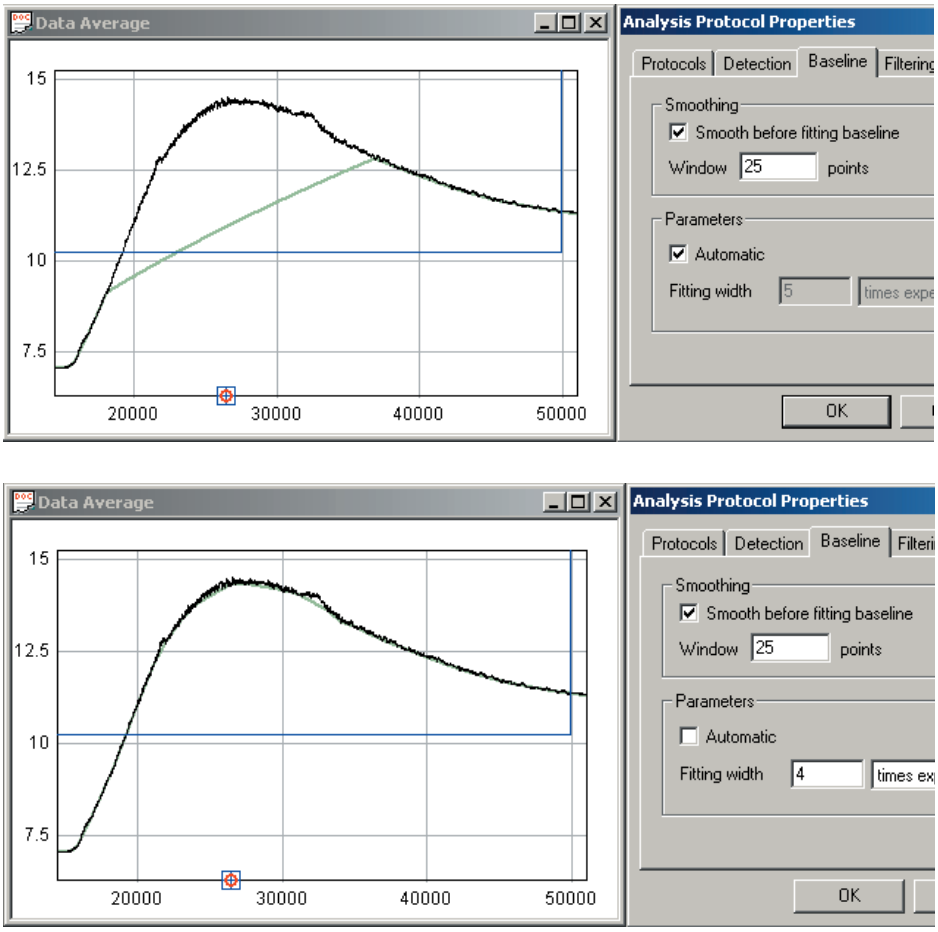

Figure 2-8: Changing the **Fitting width** setting affects the calculated baseline.

Digitizer Rate Options

Available digitizer rates are 125, 250 (default), 500 and 1000 MHz. File sizes will increase accordingly. For high MW range, the lower settings should provide adequate resolution while keeping file sizes smaller. For peptide mapping and other low MW range data, use 500 or 1000 MHz setting.

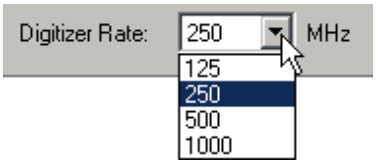

Figure 2-9: Changing the **Digitizer Rate** setting.

IMPORTANT: *The* ***Digitizer rate*** *setting is* ***controlled manually in the instrument configuration. It is not part of a spot protocol.*** *Make sure you check the digitizer rate before you start collecting data.*

Data Collection and Analysis

Following is a useful guide/overview for data collection and analysis using ProteinChip Software 3.0.

This is not intended to be a comprehensive review of ProteinChip Software 3.0. For more information, please refer to the "ProteinChip Software 3.0 Operation Manual".

NOTE: *Check the deflector and digitizer rate settings in the configuration menu immediately before running an automatic data collection.*

Data Collection

Instrument Calibration

1. Open the appropriate calibration file.
2. From **Calibration** pull down menu select **Calibration Equations | Save As**.
3. Instead of entering a new name, select **Instrument Calibration** from the down arrow. This changes the instrument's default calibration to the selected spectrum.
4. When closing the calibration file window ***do not save changes***.
5. To re-calibrate your existing data file, open and select spectra of interest. Then from **Calibration** pull down menu select **Calibration Equations** select appropriate MW and focus matched equation.
6. If you don't have a calibration file, please refer to the ProteinChip Software 3.0 Operation Manual for instructions on how to create one.

Adjust Instrument Configuration Settings for MW Region of Interest

1. Access the configuration dialog box by selecting **Instrument | Configuration...**
2. In most cases, leave the **Configuration** setting at **Normal Mass Range**.
3. For peptide analysis, change the **Digitizer Rate** to 500 or 1000 MHz (this will improve resolution, but also increase file size).
4. Set the **Mass Deflector Value** to
   - 2–4 for peptide analysis
   - 5–15 for small proteins
   - 40–80 for larger proteins
5. Click **OK** to accept changes and close the dialog box

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6. Test the deflector settings to ensure that ions in the MW region of interest are detected.

Manual Data Collection

1. **Laser Intensity**: range of 0–300, typically use settings of 170–240.
2. **Detector Sensitivity**: range of 1–10, typically use settings of 5–10.

- Analytes of higher mass generally require increased laser intensity and detector sensitivity.

3. **Hard Shot**: laser shot used to "warm" the spot. Generally use a value at 5–10 above the intensity chosen in step a. above.
4. **High Mass**: sets the upper limit of data collection.
5. **Optimization Range**: defines the region for time lag focusing if you are using "center mass", see below.
6. **Pulse settings**: **Center** will set a lag time optimized for the center of the specified optimization range. **Mass** will set the lag time for a specific mass. You can also specify a lag time in nanoseconds.

Automatic Data Collection — Spot Protocol

1. To create a new protocol, select **File | New | Spot Protocol...**
2. **Spot Protocol Name**: here's where you name the spot protocol. We recommend using something descriptive, so you will recognize it later.
3. **Data Acquisition Method**: default is **SELDI Quantitation**.
4. **Auto Setup**: sets high mass and optimization range, laser intensity and detector sensitivity (see steps 1–6 of step 3, above).
5. **SELDI Data Acquisition Settings**: define parameters for directing the laser over the spot surface (the **SELDI Quantitation** method is described here):

- **Number of transients** collected (laser shots/location).
- **Starting** and **Ending** positions (spot divided into 100 regions) default settings of 20 and 80 usually exclude spot regions where signal tends to be irregular due to an edge effect.
- **Moving**: this is the increment by which the laser moves. A setting of 5 moves the laser from starting position by increments of 5, i.e. 20 to 25 to 30.....to 80.
- **Warming shots**: shots at laser energy of choice not included in data average.

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6. To edit the spot protocol, double click on the step and make changes. The insert and delete keys can be used to add or delete steps in the spot protocol.
7. Use the **Manage Spot Protocols** option to delete or edit properties of spot protocols

Automatic Data Collection — Chip Protocol

Chip protocols are chip configuration specific.

1. To create a new protocol, select **File | New | Chip Protocol...**
2. **Chip Protocol Name**: enter any name.
3. **Chip Configuration**: select A–H, 1–8, 1–16, or 1–24 spot chip.
4. **Total Runs**: data can be collected on spots multiple times.
5. **Spot Protocol**: use the down arrow to choose from list of spot protocols. A spot protocol must be chosen for each run.
6. **Spots to Process**: the default settings specify all spots on a chip and are generally correct.
7. To edit the chip protocol, click on spot, name or spot protocol and make changes. Use the down arrow to access available spot protocols. The insert and delete keys can be used to add or delete spots to the chip protocol. Data is collected and inserted into an experiment view in the order listed in the spot protocol. Click and drag can be used to rearrange this order.

Data Analysis

Experiments are a powerful way of displaying and analyzing groups of data files.

1. To create a new experiment, select **File | New | Experiment**. To insert data, either go to the **Experiment** menu, select **Insert** and choose files of interest or copy and paste the spectra of interest into the new experiment.
2. Select all spectra (use the **Edit** pull down menu or Ctrl+A keys).
3. Check the calibration applied to the data by right-clicking the spectrum, then select **Show Calibration Information**. Change if necessary (see step I 1. d).
4. Right-click the spectrum, then select **Scale** from the pop-up menu to set spectra X- or Y-axis values, i.e., change spectra intensity to fixed or auto scale values or to specify mass range.
5. **Analysis Protocol Properties**: can be adjusted using the **Analysis Protocol Properties** dialog, accessed from the **Options** menu.

- **Detection** tab: used to auto-identify peaks. Slide the **Sensitivity** bar to increase or decrease detection sensitivity. Click on **Options...** in the **Peak Detection** window to change the sensitivity definition. Click on **Clear Peaks**, then **Detect Peaks** buttons to see the effect of these changes.
- **Baseline** tab: if the baseline fit is not sufficient to remove background (especially 'humps' from high background noise), the baseline fit can be adjusted in this window. This is especially important for data collected at higher laser intensity settings on PBS II+ instruments.
  - » Turn off the **Parameters**, **Automatic** setting.
  - » Set the **Fitting width** to a lower number, try 3 or 4 times the noise (default is 5).
  - » Click **Apply** to see the effect of the new fit
- **Filtering** tab: filtering can significantly improve signal-to-noise ratios by removing high frequency noise from the spectrum. However, excessive filter widths can distort peaks and reduce resolution. Use the **Apply** button to test the results.
  - » **Average** filter: the filter width increases with increase in analyte mass.
  - » **Savitsky-Golay** filter: good for M/Z below 2500, small # is better.
  - » Default settings for both filters are usually sufficient for most data sets.
- **Area** tab: check the **Show Peak Boundarie**s box, then click **Apply** to see the calculated peak area. For any applications utilizing peak area it is absolutely crucial that default settings be checked and modified if necessary.
- Click the **OK** button to apply changes and close the **Analysis Protocol Properties** dialog box.

6. **Presentation Protocol Properties**: adjusted using the **Presentation Protocol Properties** dialog, accessed from the **Options** menu.

- **View** tab: checkmark the **Flatten** box to subtract baseline. Uncheck in the **Fast draw on** box if it is selected. **View type** toggles between trace, gel, and peak views.
- **Text** tab: contains options that let you alter the appearance of text in the experiment (e.g. 90 degree orientation, font size and color, etc.).
- **Scaling** tab: to fit the experiment into one page, choose **Experiment Print Scaling**: **Fit to page** and **Both**.

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- **Experiment** tab: select appropriate spectrum Layout: **Stacked**, **Overlay**, **Offset Overlay**.
  - » Changes to **Spectra Size** are common. Setting width to 7 in. for portrait layout usually works well. **Trace** and **Gel Height** can also be changed. Use **Print preview** to see the changes' effect on a printout.
  - » **Background** color, **Show tags** (next to spectra), and **Mass Labels – Upper and Lower**, are also commonly altered features, particularly for presentation purposes.
- Click the **OK** button to close the **Presentation Protocol Properties** dialog box and apply the changes you've made.

7. Changing the peak label. Click the **Centroid** toolbar button and move the cursor over the peak, then right-click the peak. Select **Peak Label** from the pop-up menu to open the **Peak Label** dialog box. Click the **F** button, then choose the format you wish for the peak label from the **Label Template** drop-box. Click **Apply to All Peaks**, then click **OK** to close the dialog box.

Advanced Data Analysis

Exporting Data

There are now two ways to export data into Excel. Ciphergen recommends the following since it is much faster than using the ProteinChip toolbar in the Excel program.

1. Either manually, by using the **Autoidentify Peaks** option in the **Peaks** pull down menu, or using the Biomarker Wizard, label all peaks of interest for export into Excel.
2. If **Area under the curve** values will be used for analysis, highlight this region by opening the **Analysis Protocol Properties** dialog, then clicking the **Area** tab. To see the area under peaks, be sure the **Show Peak Boundaries** check box is checkmarked. Close the **Analysis Protocol Properties** dialog.
3. Select **File | Export | Export Peak Information (.csv)...**
4. Choose the information to be exported. Select the destination of the saved file
5. Open the .csv file by double clicking or from within Excel
6. Data from all labeled peaks in the active **Experiment** view will be imported into Excel.
7. Plot the graph using Excel, select the appropriate columns for the X and Y axis and then click the **Chart Wizard | select X-Y (Scatter) | Chart** sub-type then click **Finish**.

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Normalizing Peak Intensity for Spectra

1. Choose and label the peak to be used in normalization in all spectra, usually an internal standard.
2. Select all spectra to be normalized using the Shift or Ctrl key.
3. Right-click near the peak to be normalized, then select **Normalize peaks...** from the pop-up menu to open the **Normalize Spectra** dialog.
4. Choose **Intensity, total ion current**, **Intensity, height** or **Intensity, area** as the **Normalize Dimension**. For protein profiling applications **Intensity, total ion current** is recommended.
5. In the M/Z range area, do not choose **Start at beginning**. Instead begin at a MW beyond the EAM signal.
6. Click **Apply** when finished.
7. **Intensity** normalization settings can be adjusted in the **Scaling** tab of the **Presentation Protocol Properties** dialog. Select the spectra to change, open the dialog, then change the scaling factor to 1 and click the **Reset** button.

Normalizing Peak Mass for Spectra

Peak mass can be normalized for a set of selceted spectra if they have all been collected with the same instrument settings.

1. Chose a sharp peak common to all spectra of interest for normalization.
2. Label that peak in all spectra. It does not matter if other peaks are also labeled.
3. Select all of the spectra to be normalized using the Shift or Ctrl key.
4. Right-click near the peak to be normalized, then select **Normalize peaks...** from the pop-up menu to open the **Normalize Spectra** dialog.
5. Select **Mass (add common calibrant)** for the **Normalize Dimension**. Click the **Clear Calibrants** button to remove any previous internal calibration. Check the **Normalize to average** checkbox to use average masses for the masses of the internal calibrants.
6. In the **Select Peak** list, select the first peak to be used for normalization.
7. Click **Apply**.

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8. Repeat steps 6 and 7 for any additional peaks. Note that at least 2 peaks should be used for an accurate calibration.

Creating Peak Maps

1. To create a combination map of two or more spectra, label all the peaks in the spectra, then select **Experiment | Synthetic Maps | Combine Spectra...**, or click the **Combine Spectra** toolbar button.

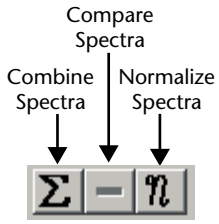

Figure 2-10: The **Combine Spectra**, **Compare Spectra**, and **Normalize Spectra** buttons.

2. To create a comparison map of two or more spectra, label all the peaks in the spectra, then select **Experiment | Synthetic Maps | Compare Spectra...**, or click the **Compare Spectra** toolbar button. This option can be used to subtract spectra.

General Software Notes

Editing Spot Protocols

1. Open the protocol of interest and double click on the step to be edited. Make changes as desired.
2. The insert and delete keys can be used to add or delete steps in the spot protocol.
3. Save changes before using an altered spot protocol as part of a chip protocol.

Editing Chip Protocols

1. Open the protocol of interest.
2. Click on the spot identifier, name or spot protocol to be changed.
3. When editing spot names, the copy and paste functions are accessed with the right mouse button.
4. Use the down arrow to access available spot protocols.
5. The insert and delete keys can be used to add or delete spots to the chip protocol.

6. Data is collected and inserted into an experiment view in the order listed in the spot protocol. Click and drag can be used to rearrange this order.

Accessing Software Functions

All the software options discussed here can be accessed in multiple ways. For simplicity the pull down menus have been described. Toolbar buttons, the keyboard, and the Right Mouse Click button are also common options.

File Extensions

File extensions indicate file type. Data saved within the database does not use these extensions. However, if you want to share this data with someone who does not have access to your database (i.e., your Ciphergen field scientist), the data must be exported into an appropriate file type. This is done by:

1. Open the file of interest.
2. Select **File | Export | Experiment (.exp)**... or choose another file type as appropriate.
   - A spectrum data file (single spot) has the file extension **.spe**.
   - An experiment data file (1 or more spectra) has the file extension **.xpt**.
   - A peak map containing combined or compared data sets has the file extension **.syn**.
   - Spot protocols have file extension **.spot**.
   - Chip protocols have file extension **.chip**.
3. Select a name and destination for the data file. The appropriate file extension will be added when a file is saved — you don't need to add it. The file can now be opened by those without access to your database.

Chapter 3

An Introduction to Using ProteinChip Arrays and Accessories

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Introduction

ProteinChip Arrays have surface chemistries ranging from classic chromatographic moieties to protein-based affinity capture surfaces. Descriptions of the specific chip types and their uses are included to help you understand the principals behind the various types of capture. Basic protocols have also been included to describe the basic steps required for using each particular chip type.

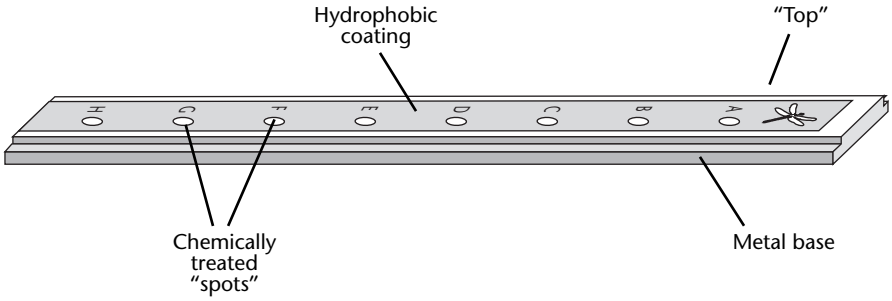

Figure 3-1: A ProteinChip Array.

The ProteinChip Array consists of a metal base with 8 or 24 chemically active sites or "spots" where the actual sample binding occurs. Because molecules bind due to specific chemical interaction with the surface, it is often possible to learn about a protein's chemical properties by using ProteinChip Arrays. For well-studied molecules whose chemical nature is known, the appropriate ProteinChip Array can be chosen quite readily for optimal capture.

ProteinChip Arrays with 8 spots are designed with either 1–8 or A–H labelling. The newer A–H design accommodates 96-well plate formats: twelve ProteinChip Arrays can be aligned to form a 96-well titer plate footprint, thus making them amenable to use with robotics systems and multi-channel pipetting devices.

Many ProteinChip Arrays also have a special hydrophobic coating that enables sample containment. This feature is noted in the particular chip descriptions (see Chapter 5, *"ProteinChip Arrays"*).

Cautions

1. Avoid touching the spot surface and surrounding coating of the ProteinChip Array.
2. Always use powder-free gloves for handling.
3. The active spots of most types of ProteinChip Arrays should be kept moist during the entire binding and capture procedure. Excepting H4 and Normal Phase Arrays, the spots should only be allowed to dry after addition of EAM, when the chip is ready to be analyzed in the ProteinChip Reader.
4. Assay parameters must be optimized for each specific application.
5. Additional information on ProteinChip Arrays can be found in the Product Data Sheets.

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As with any technique, no single protocol can be optimized for every laboratory sample. The ProteinChip System can be used to analyze a diverse array of sample types, including tissue homogenates, cultured cell lysates, extracted proteins, peptides, etc. Therefore, experiments will have to be optimized in order to generate the best data. To get you started, this manual includes a number of basic protocols for using ProteinChip Arrays (see Chapter 5, *"ProteinChip Arrays"*) as well as many detailed protocols for specific applications (see Chapter 7, *"Sample Preparation"*, and Chapters 8–12, which contain application protocols).

Chemicals That Can Interfere With Protein Detection

One of the great advantages of ProteinChip Technology is that samples can be washed quite extensively after application on the chip. Some chemicals that are commonly used in biological assays may nonetheless interfere with a typical ProteinChip Array experiment if they are not washed off the chip surface prior to adding EAM. For instance, the chemicals described below may interfere with co-crystallization or suppress sample ionization during mass analysis in the ProteinChip Reader.

Other chemicals may interfere with binding to the surface of the ProteinChip Array, depending on the specific surface chemistry being used. For example, salts may reduce binding to ionic surfaces but can increase binding through hydrophobic interactions. Therefore, it is very important to read the guidelines for each specific chip type to aid in choosing buffers and wash conditions. Furthermore, a water wash must be performed in most ProteinChip Array experiments prior to EAM addition.

1. **Ionic detergents**. In many cases, ionic detergents will suppress ionization of a protein sample, thus preventing their analysis by mass spectrometry. In particular, proteins that have been boiled in SDS may not be easily detected. If detergents are necessary for sample extraction or sample solubilization, non-ionic detergents, such as Triton X-100, NP40, n-Octyl β-D-glucopyranoside (OGP), or dodecyl maltoside may be present in final concentrations up to 1%.

   Note: SDS can sometimes be removed very effectively from samples boiled in SDS by drying the sample onto the surface of the ProteinChip Array and washing with 80% acetone.

2. **High salt concentrations.** Various salts, including buffers, may interfere with applications on certain chips, as high concentrations of salts may alter binding properties. In particular, salts may interfere with binding to ionic surface chips, including SAX2 and WCX2. By contrast, salts can actually increase binding to hydrophobic-surface chips such as H4.

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3. **Polyethelene glycol** (PEG). PEG is difficult to wash off and gives a strong, very broad peak. Glycerol also interferes with detection of analytes.
4. **DEPC** Diethylpyrocarbonate (DEPC) is often used for RNA preparation and analysis but should be avoided for ProteinChip experiments.
5. **DTT** Dithiothreitol (DTT) is commonly used to reduce disulfide bonds in proteins but residual DTT interferes with analysis when using ProteinChip technology. Weak (millimolar) solutions of beta-mercaptoethanol may often be used in place of DTT for disulfide bond reduction.

The PAP Pen

The PAP pen (Zymed mini-PAP pen, cat. no. 00–8877) is used to outline the spots on arrays that do not have a hydrophobic coating. The PAP pen provides a water-repellent barrier that prevents solutions from bleeding off the chemically active spots of the ProteinChip Array. When using the PAP pen, be careful to avoid touching the chemically active spots with the solution from the pen. This may take some practice, as the liquid from the pen tends to gush, especially when using new pens. Avoid pressing down hard with the pen — doing so can cause the solution to spurt!

Note: acetone dissolves the hydrophobic solution deposited by the pen.

The Humidity Chamber

A humidity chamber provides a humid environment for incubating ProteinChip Array experiments; the humid environment prevents the spots from drying out during binding or washing steps. It is simple to make a humidity chamber for a single chip by using the shipping tube from the ProteinChip Array.

1. Tear off a portion of a laboratory tissue and moisten with water.
2. Place the chip into the tube.
3. Place the moistened tissue into the opening of the tube.
4. Seal with the cap.

For processing several ProteinChip Arrays at one time, a humidity chamber can be assembled from a pipet tip-box.

1. Add water to half-fill an empty tip-box.
2. Place damp tissue paper in the lid.
3. Place the ProteinChip Arrays on the tip-support in the chamber.

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4. Close the lid.
5. For overnight incubations, the chamber may be sealed with tape or parafilm to prevent evaporation.

The Bioprocessor

The bioprocessor is an apparatus that allows larger volumes of sample or wash solution to be applied to individual spots of a ProteinChip Array. Ciphergen's bioprocessors are available in two sizes, 8-well and 96-well. The wells in both bioprocessors have a 500 µL volume. However, for most applications, a maximum of 350 µL solution should be applied. The 8-well bioprocessor comes in two models, one for chips labelled 1–8 and a second design for chips labelled A–H.

The 8-well bioprocessor has four parts: the top, the gasket, the chip holder and the bracket (Figure 3-2).

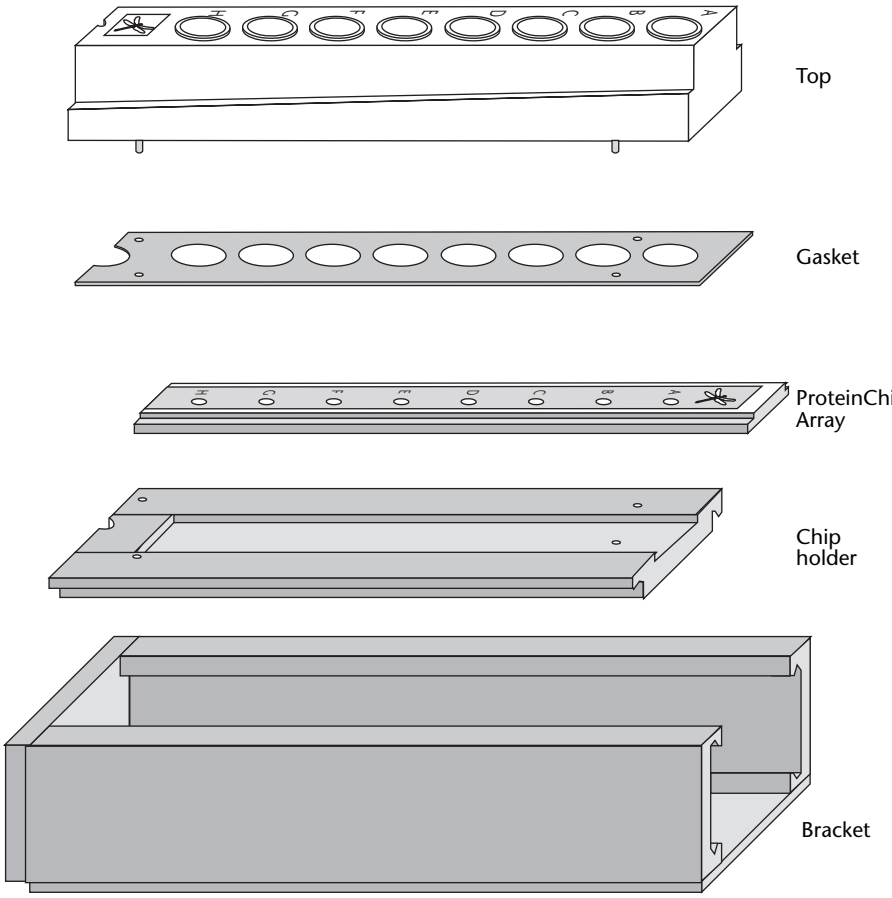

Figure 3-2: An 8-well bioprocessor and ProteinChip array.

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The 96-well bioprocessor has three parts: the top, the gasket, and the bracket (Figure 3-3).

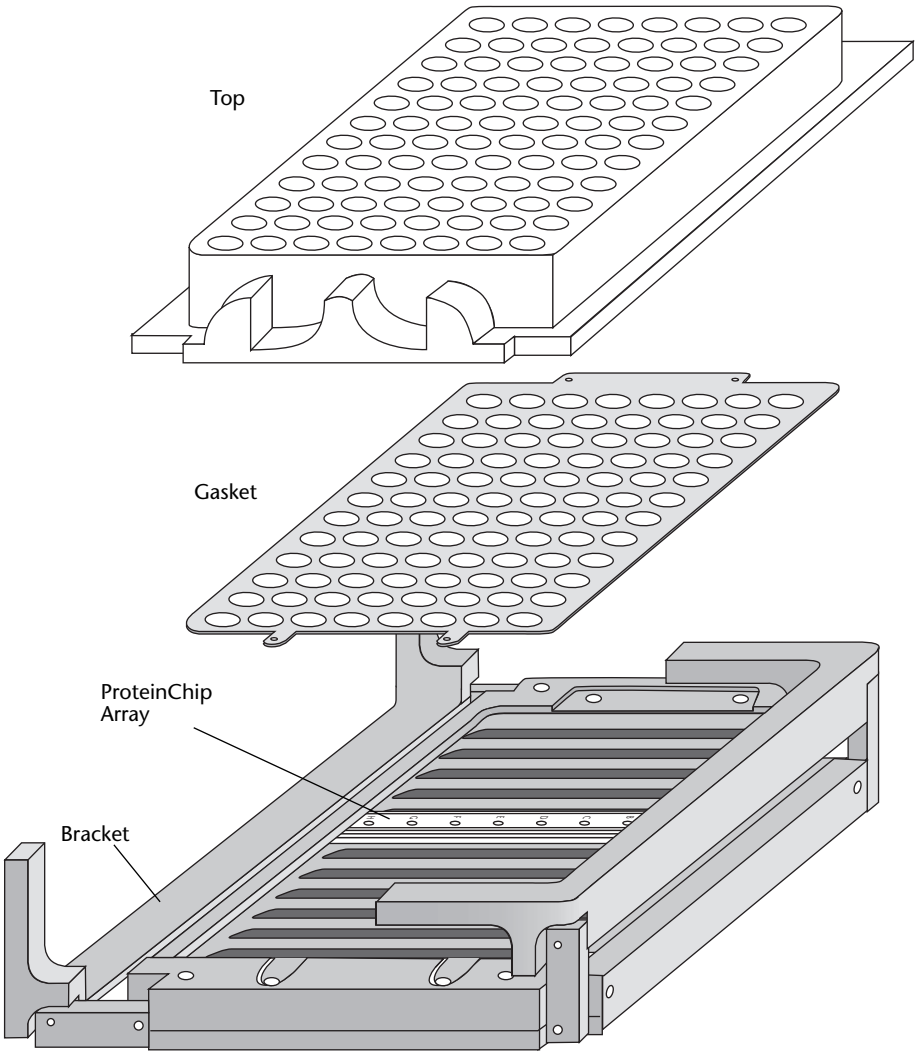

Figure 3-3: A 96-well bioprocessor and ProteinChip Array.

Often you may wish to perform an experiment with fewer than twelve chips. In this case, dummy chips must be used to fill the empty slots in the 96-well bioprocessor.

Using the Bioprocessor

The bioprocessor can be used effectively to capture proteins from samples containing a protein of interest at a low concentration. In many experiments, the use of a bioprocessor may lead to more consistent results by lessening the need for pipetting minute volumes onto the array, thus reducing the amount of experimental error. Most ProteinChip Array experiments can be adapted for use with the bioprocessor.

The 96-well bioprocessor is particularly useful when large numbers of samples must be processed simultaneously — for example, during

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biomarker discovery experiments. In such cases, the 96-well plate format keeps the chips in order and allows the use of multi-channel pipetting devices for applying binding and washing solutions.

The chips must be removed from the bioprocessor prior to applying EAM.

Assembly

The chip(s) to be treated and the bioprocessor unit should be clean and dry. Ensure that you have the correct bioprocessor insert for the chip in use (A-H or 1-8). The chip should be inserted into the chip-holder with the dragonfly logo extending beyond the base. Mount the gasket onto the underside of the bioprocessor top, taking care to align the holes in the gasket with their corresponding pegs. The gasket fits in only one orientation, and the pegs are somewhat fragile, possibly becoming broken if the top is handled improperly. Assemble the bioprocessor by lining up the holes in the metal chip holder with the pegs on the top.

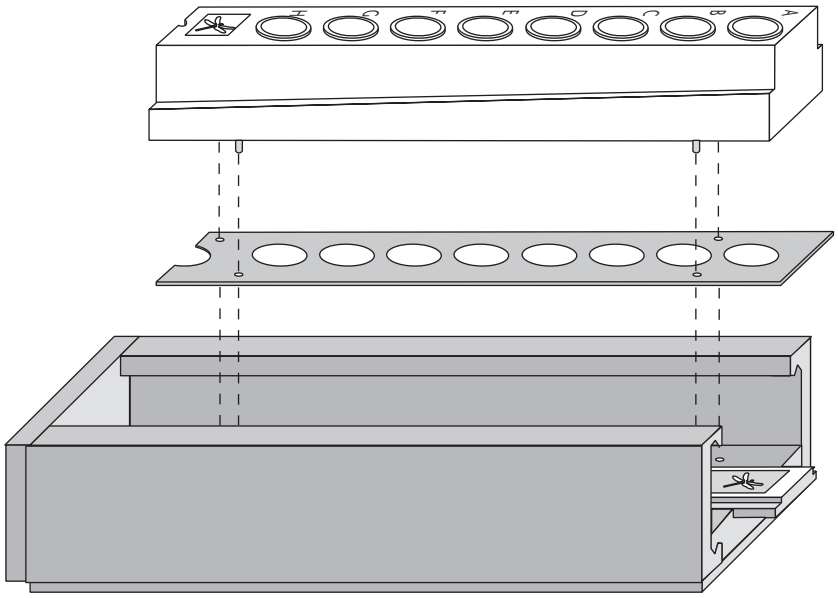

Figure 3-4: Assembling the bioprocessor

This assembly is then inserted into the metal bracket to clamp the unit together snugly, but not too tightly. If the assembly is forced into the bracket, the gasket may stretch or be displaced, causing leakage and/or damage to the gasket.

Checking for Leakage

The bioprocessor wells should be tested for leakage after assembly. Set up the bioprocessor using the chip that will receive the sample. Add 300 µL sample buffer to every other well (e.g. odd-numbered wells), then observe for 10 minutes. The meniscus should not fall during the 10 minutes. The wells should then be drained. The remaining wells (e.g. even numbered wells) should then be tested

for leakage. If there is leakage from any of the wells, the bioprocessor should be dismantled and reassembled. If this fails to stop the leakage, the gasket may need to be replaced.

Sample Loading

The recommended sample volume is 20-350 µL/well to allow for appropriate agitation of the sample. After applying solution to the wells, the top of the bioprocessor should be sealed with cellophane tape or plate sealing tape to prevent sample evaporation. During washing or binding, the bioprocessor must be shaken quite vigorously.

> *Caution: If the wells are filled with too large a volume (>350 µL), splashing may occur, leading to cross-contamination of samples.*

Shaking

During experiments, the bioprocessor should be anchored securely using tape, string or wire and shaken vigorously. A standard 96-well plate shaker can be used at the recommended speed of 400–600 rpm.

Disassembly

The wells should be drained individually with a pipette to prevent cross-contamination of the wells. To separate the well and chip assembly from the bracket, insert a solid cylindrical object, such as the tool provided or the base of a pen, into the hole at the back end of the bioprocessor and tap it gently on a hard surface. The well and chip assembly should loosen itself from the bracket. While holding the chip in place, carefully remove the top and gasket. Immediately dry the chip with a lab wipe, taking care not to touch the sample spots.

Cleaning

The bioprocessor top and gasket should be soaked in a solution of 1% Triton X-100 in 1xPBS, pH 7.2. The top and gasket should be soaked for at least 2 hours before and after use. However, the gasket should not be soaked longer than one day, because it may swell and lose its shape. Immediately before use, both the top and gasket should be rinsed with water to wash off the Triton X-100.

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Chapter 4

Energy Absorbing Molecules (EAMs)

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Introduction

EAMs are an essential component to a successful ProteinChip experiment. The term “EAM” is a generic name for molecules that assist in the desorption and ionization of the analyte. Known as “matrix” in traditional MALDI mass spectrometry, the EAM is applied in organic solvent, solubilizing many proteins on the chip surface. As the EAM solution dries, the proteins co-crystallize with the EAM. These crystals absorb the laser energy and generate the ionized proteins detected by the ProteinChip Reader.

The quality and chemical nature of EAM used has a dramatic effect on the data, as does the method of EAM application. Be sure to ask your Field Scientist about this important technique.

Using EAMs

Selection and Preparation

Ciphergen offers three different EAM compounds that work very well for most applications. In general, the EAM is chosen based on the molecular weight of the analyte of interest.

CHCA

Alpha-cyano-4-hydroxy cinnamic acid (CHCA, MW = 189.2) is especially good for small molecules, <15 kDa. Use a saturated solution for molecules between 5–15 kD, and a 1/5 dilution of the saturated solution for anything smaller (the dilution of CHCA can be adjusted as needed). For small compounds, the saturated CHCA can be diluted up to 1/50 in solvent.

EAM1

EAM1 (MW = 231.21) works very well for proteins 10–50 kDa. Use a saturated solution or a 2-fold dilution (in solvent). EAM1 tends to generate multiply charged species and peaks tend to be broader. However, it can often allow the desorption/ionization of proteins that are difficult to detect.

SPA

Sinapinic acid (SPA, MW = 224.2) is recommended for proteins >15 kDa. Use a saturated solution of SPA. If SPA gives little or no detection of analyte then try EAM1. In general, SPA gives better resolution and fewer multiply charged ions than EAM1. For protein profiling using only a single EAM, SPA is the molecule of choice.

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Protein Description	EAM	Solvent	Concentration
≤15 kDa	CHCA	50% ACN 0.5% TFA	10–50% saturated
≥5 kDa	EAM1, SPA	50% ACN, 0.5% TFA	Saturated
Glycosylated; 15–50 kDa	EAM1, CHCA or SPA	50% ACN, 0.5% TFA	Use protein mass to decide

Notes: EAM1 is a proprietary molecule.
ACN = acetonitrile
TFA = trifluoroacetic acid

Preparing EAM Solutions

Solvent Choice

- For most applications, EAM molecules are prepared in an aqueous solution containing 50% acetonitrile (ACN) and 0.5% TFA.
- As an alternative, a solvent containing 30% ACN, 15% isopropanol, 0.5% TFA and 0.05% NP-40 (or Triton X-100) works well.
- For difficult proteins and peptides, especially the hydrophobic variety, try adding formic acid to the mix. Dissolve the EAM in 150 µL of 50% ethanol (100 proof). Microfuge, then transfer 90 µL of supernatant to a fresh tube. Add 10 µL formic acid.
- For glycoproteins, the addition of a small amount of detergent (0.02–0.1% NP-40) to the 50% acetonitrile and 0.5% trifluroacetic acid solvent may be helpful.

Solution Preparation

The solvent system for EAMs and the EAM solution itself must be prepared fresh every day. Acetonitrile volatilizes rapidly and EAMs break down significantly within 24 hours in solution at room temperature. Freezing EAMs after dissolving them in solvent may preserve them for a week or so; however, Ciphergen strongly recommends preparing fresh solvent and EAM every day.

1. Ciphergen normally supplies EAM as 5 mg of dried powder in a tube. Add 250 µL of the solvent of choice to make a solution of 1 mg EAM/50 µL. Vortex and let stand at room temperature for ~5 minutes.

   Note: the amount of EAM per tube may change. Be sure to check the tube label for information.

2. Microfuge for 2 minutes or more at maximum speed to pellet any particulates. The EAM is now ready to use.

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3. Add 0.5 µL per spot. Some chip surfaces or applications require two additions of 0.5 µL each.

Applying EAM to ProteinChip Arrays

The method of EAM application to the chip surface requires some practice as it can significantly influence the quality of data. A minute volume (0.3–0.5 µL) of EAM in organic solvent must be applied to each sample prior to analysis. EAMs can be applied to the spots while they are slightly moist, to obtain the greatest signal, or after they have completely dried, for the greatest spot-to-spot consistency.

It is important to avoid touching the spots while pipetting EAM onto them. However, it is also beneficial to spread the EAM solution across the spot before it dries; doing so requires some patience and a steady hand as you are manipulating a small volume of EAM in organic solvent, while trying to avoid touching the pipet tip to the chip surface. Ideally, the EAM solution will be spread evenly across the sample area, without bleeding outside of the chemically treated spot.

After applying the EAM and allowing it to dry, it is useful to observe the sample in good light, or under a dissecting microscope. The sample should be distributed fairly uniformly across the spot surface. Sometimes application of EAM leads to a ring of sample around the periphery of the spot. In such cases, analysis of the middle of the spot yields low signal, while analysis of the periphery of the spot yields high signal.

CHCA and SPA are the most frequently used EAMs. SPA is most often used for detecting high molecular weight proteins and therefore is usually applied as a saturated solution in two 0.5 µL aliquots. CHCA is usually used to detect lower molecular weight analytes, so often only a single application is necessary. When analyzing peptides or very small molecules, a diluted solution of CHCA may be best. Start by making a saturated solution of CHCA and dilute this into solvent; dilutions of 1:5 – 1:25 are typical.

- When pipetting EAM onto your samples, be careful to avoid the undissolved EAM at the bottom of the tube.
- Always use new, packaged pipette tips; never use tips that have been autoclaved or re-used.
- EAMs are light-sensitive and should be stored in the dark during the day (for example, in a convenient drawer).

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Chapter 5
ProteinChip Arrays

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Chip Chart

Chip Name		
Surface Chemistry	Functionality	Uses
H4 — Hydrophobic Surface		
Used for capturing proteins through hydrophobic interactions.	The active spots contain chains of 16 methylene groups that bind proteins through reverse phase chemistry. Binds proteins abundant in alanine, valine, leucine, isoleucine, phenylalanine, tryptophan, or tyrosine.	•Ascertaining the purity of a protein preparation •Rapid protein analysis •Biomarker discovery
NP1 & NP2* — Normal Phase		
General protein binding surface. Recommended for hydrophilic proteins.	The active spots contain silicon oxide which allows proteins to bind via serine, threonine or lysine.	•Quickly analyze proteins in a sample •Check for purity •Verify the presence or absence of a molecule •Calibrate using peptides or proteins of known molecular weight
PS1 & PS2* — Preactivated Surface		
Covalently immobilizes biomolecules for the subsequent specific capture of proteins from complex biological samples.	Reactive carbonyl diimidazole moieties on the PS1 chip and epoxy groups on the PS2 chips. PS2 chips also have a hydrophobic coating that allows sample containment on the spots. Due to differences in surface properties, the PS2 surface is especially recommended for sensitive detection, low non-specific binding, and when the target protein is less than 1% of total protein.	•Antibody-antigen •Receptor-ligand •Nucleic acid-binding protein •Any specific protein-protein pair
SAX2 — Strong Anion Exchange		
Used to analyze molecules that have negative charges on the surface.	Active spots contain cationic, quaternary ammonium groups that interact with the negative charges on the surface of target proteins, e.g., aspartic acid or glutamic acid.	•Selective analysis of proteins with low pI's •Biomarker discovery
WCX2 — Weak Cation Exchange		
Used to analyze molecules that have a positive charge on the surface.	The active spots contain weak anionic carboxylate groups that interact with the positive charges on the surface of the analyte, e.g., lysine, arginine or histidine.	•Selective analysis of proteins with high pI's •Biomarker discovery
IMAC3 — Immobilized Metal Affinity Capture		
Used to capture molecules that bind divalent cationic metals such as nickel, copper and zinc.	The active spots contain nitrilotriacetic acid (NTA) groups on the surface, that chelate the metal ions. Proteins applied to the chip surface may bind to the chelated metal ion through histidine, tryptophan, cysteine, and phosphorylated amino acids.	•Analysis of metal-binding proteins •Analysis of phosphorylated proteins •Analysis of histidine tagged proteins •Biomarker discovery

** These chips have a hydrophobic coating for sample containment.*

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H4 Arrays

Hydrophobic surface arrays, called “H4 chips”, are used for capturing proteins through hydrophobic interactions. The active spots chains of 16 methylene groups that can bind proteins through reverse phase chemistry via alanine, valine, leucine, isoleucine, phenylalanine, tryptophan, or tyrosine.

Uses for H4 arrays include:

- ascertaining the purity of a protein preparation
- rapid protein analysis
- biomarker discovery

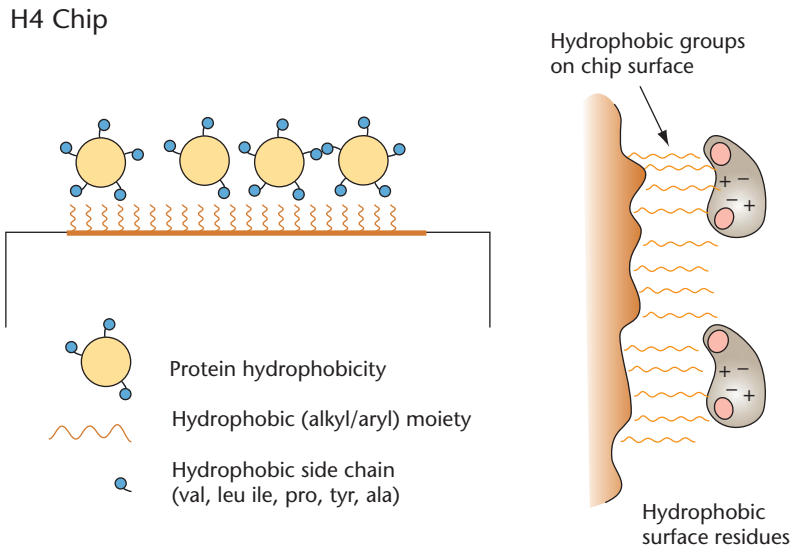

Figure 5-1: H4 chip surface chemistry with proteins.

H4 Protocol

Notes

- Salts will increase hydrophobic interactions and therefore can be included in the binding buffer (50–1000 mM).
- Increasing the concentration of organic solvent will increase the selectivity of the surface.
- Detergents can be used to increase the stringency of the protein/surface interaction.
- Pretreating the H4 surface may enhance binding of certain proteins, particularly those that are less hydrophobic.

Method 1

1. Outline each spot using a (PAP) hydrophobic pen. Allow to air dry.
2. Pretreat the spots with 5 µL acetonitrile (the same percentage solution that will be used for binding and washing steps).

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3. Remove the acetonitrile and replace with 2–3 µL sample (suspended in an aqueous acetonitrile solution). Do not allow the spots to air dry during sample exchange.
4. Incubate in a humidity chamber for 20 minutes.
5. Wash each spot with 5 µL binding buffer three times. Air dry.
6. Apply 0.5 µL saturated EAM solution to each spot.
7. Analyze the chip using the ProteinChip System.

Method 2

1. Outline each spot using a (PAP) hydrophobic pen. Allow to air dry.
2. *Optional:* pretreat each spot with 5 µL acetonitrile. Remove the acetonitrile immediately and continue on to step 3.
3. Add 1–2 µL sample to each spot (the sample can be suspended in an aqueous acetonitrile solution).
4. Allow the sample to air dry on the surface (this approach can force hydrophobic interactions).
5. Wash each spot with 5 µL binding buffer three times. Air dry.
6. Apply 0.5 µL saturated EAM solution to each spot.
7. Analyze the chip using the ProteinChip System.

Method 3: SDS Removal on the Chip Surface

This method has been used to remove SDS from samples containing from 2–10% SDS. The efficiency of SDS removal is unknown, but the expected proteins have been detected. In general the detection sensitivity is reduced even after removing the SDS. Thus, putting more protein on the chip is recommended.

1. Add 1-2 µL sample to each spot (the sample can be diluted with water, if necessary). Allow to air dry. If the protein concentration is low, multiple additions of sample can be dried onto the spot.
2. Apply 1 µL 80% acetone to each spot. Let the acetone sit on the spot ~20 seconds (it will evaporate quickly).
3. Wash the spot with 2 µL 80% acetone, 2 times (pipet the acetone up and down 3–4 times during each wash).
4. Apply 0.5 µL saturated EAM solution to each spot and allow the solution to air dry.
5. Analyze the chip using the ProteinChip System.

Recommended Buffers

0–60% acetonitrile ± 0.1% TFA. Isopropanol or methanol can also be used.

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H4 Protocol Using a Bioprocessor

1. Pre-treat the spots with 5 µL acetonitrile.
2. Assemble the chip in the bioprocessor and add 350 µL binding buffer to each well (recommended sample dilution: 50–2000 µg/mL total protein). Incubate for 5 minutes at room temperature with vigorous shaking (e.g., 250 rpm).
3. Remove the buffer from the wells by pipetting and immediately add 50–350 µL/well sample diluted in binding buffer (see second point in Notes, above). Incubate with vigorous shaking for 30 minutes.
4. Remove the samples from the wells and wash each well with 350 µL binding buffer for 5 minutes.
5. Repeat the wash twice more for a total of three washes, 5 minutes each.
6. Remove the chip from the bioprocessor and rinse the chip briefly with 8 mL of water in a 15 mL conical centrifuge tube. Repeat the water rinse.
7. Air dry the array and apply 0.5 µL EAM solution per spot (sinapinic acid or EAM1 for proteins 10–200 kDa, CHCA for peptides 0–10 kDa). It may be desirable to outline the spots with a PAP pen prior to adding EAM.
8. Analyze the chip using the ProteinChip System.

Recommended Binding Buffers

0–60% ACN +/- 0.1% TFA. A reasonable starting buffer is PBS + 10% ACN + 0.25 M NaCl.

Salts will increase hydrophobic interactions and are recommended for binding buffers at 50–100 mM NaCl.

Detergents will decrease hydrophobic interactions and may be diluted out or eliminated to maximize binding.

IMAC3 Arrays

The Immobilized Metal Affinity Capture array, called “IMAC3” arrays, can be used to capture molecules that bind divalent cationic metals such as nickel, gallium, copper and zinc. The active spots contain nitrilotriacetic acid (NTA) groups on the surface, that chelate the metal ions (Figure 5-2). Proteins applied to the chip surface may bind to the chelated metal ion through histidine, tryptophan, cysteine, and phosphorylated amino acids.

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Uses for IMAC3 arrays include:

- analysis of histidine tagged proteins
- analysis of phosphorylated proteins
- analysis of metal-binding proteins
- biomarker discovery

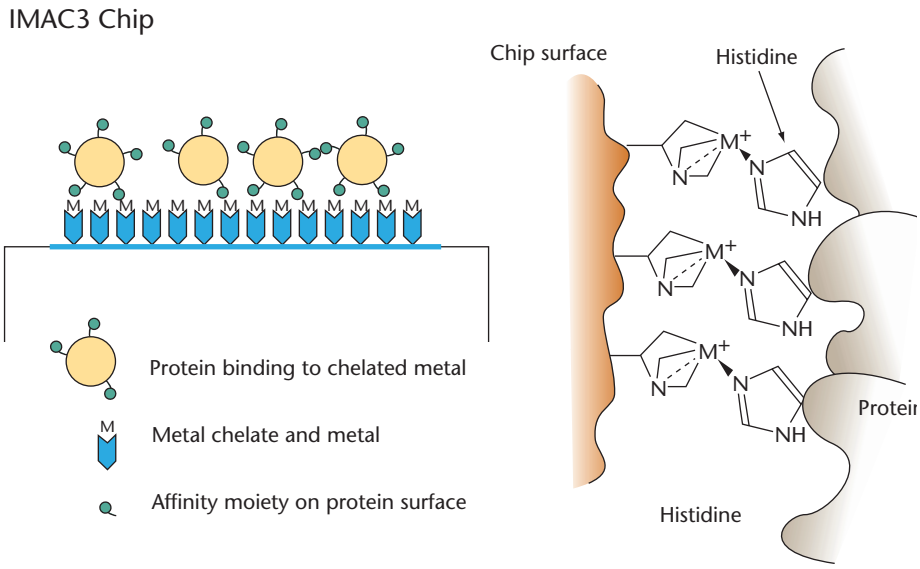

Figure 5-2: IMAC3 chip metal binding surface with metal and protein.

IMAC3 Protocol

Notes

- IMAC3 chips are manufactured in a metal-free form and must be loaded with the metal prior to use. The protocol below is written for use with nickel, but other metals can be used (see *"Copper Protocol"*, below, and *"IMAC3-Gallium Array Phosphopeptide Capture"* starting on page 12-11).
- Complex biological samples can be solubilized in 8 M urea, 1% CHAPS, PBS, pH 7.2, vortexed for 15 minutes at room temperature and further diluted in 0.5 M NaCl/PBS to a final concentration of about 1 M urea. The samples should be spun down after dilution to pellet insoluble material.

Nickel Protocol

1. Outline each spot using a hydrophobic pen. Allow to air dry.
2. Apply 10 µL 100 mM nickel sulfate to each spot and incubate in a humidity chamber for 15 minutes. Do not allow the solution to air dry. Repeat.
3. Rinse chip under running deionized water for about 10 seconds to remove excess nickel.

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4. Apply 5 µL 0.5 M NaCl in PBS or other binding buffer to each spot. Incubate on a shaker for 5 minutes. Wipe the chip dry around the spots and remove excess buffer without touching the active surface.
5. Apply 2–3 µL sample per spot.
6. Incubate the chip in a humidity chamber for 20 to 30 minutes at room temperature.
7. Wash each spot with 5 µL binding buffer five times, followed by two quick washes with water (5 µL per wash).
8. Tap the chip on the benchtop to remove water droplets. Wipe dry around the spots.
9. Apply 0.5 µL saturated EAM solution to each spot while it is still moist. Air dry. Apply a second aliquot of EAM to each spot and air dry.
10. Analyze the chip using the ProteinChip System.

Copper Protocol

Note: copper is somewhat corrosive to the chip surface and should not be left on the spots longer than the recommended time.

1. Outline each spot using a hydrophobic pen. Allow to air dry.
2. Load 10 µL 100 mM copper sulfate to each spot and incubate in a humidity chamber for 15 minutes. Do not allow the solution to air dry. Repeat the loading procedure.
3. Rinse the chip under running deionized water for about 10 seconds to remove excess copper.
4. Rinse spots with an excess of 50 mM sodium acetate, pH 4.0.
5. Rinse the chip again under running deionized water for about 10 seconds.
6. Proceed with the protocol above, starting at step 4.

Recommended Buffers

- A binding buffer containing sodium chloride (at least 150 mM and up to 1 M) and detergent (e.g. 0.1% Triton X-100) is recommended to minimize non-specific ionic and hydrophobic interactions, respectively.
- High salt may cause some proteins to precipitate. Complex biological samples can be solubilized in urea and detergent.
- EDTA and DTT should be avoided in the sample buffer.
- Imidazole in concentrations between 10–250 mM may be used to increase binding selectivity.

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- Growth media containing histidine may weakly compete for binding to the IMAC surface.

IMAC3 Protocol Using a Bioprocessor

1. Outline each spot using a hydrophobic pen. Allow to air dry.
2. Apply 10 µL 100 mM nickel sulfate to each spot and incubate in a humidity chamber for 15 minutes. Do not allow the solution to air dry. Repeat.
3. Rinse chip under running deionized water for about 10 seconds to remove excess nickel.
4. Apply 5 µL 0.5 M NaCl in PBS or other binding buffer to each spot. Incubate on a shaker for 5 minutes. Wipe dry around the spots and remove excess buffer without touching the active surface.
5. Assemble the chip in the bioprocessor and add 200–350 µL binding buffer to each well. Incubate for 5 minutes at room temperature with vigorous shaking (e.g., 250 rpm).
6. Remove the buffer from the wells and immediately add 50–100 µL/well sample diluted in binding buffer (recommended sample dilution 50–2000 µg/mL total protein). Incubate with vigorous shaking (250 rpm) for 30 minutes.
7. Remove the sample from the wells and wash each well twice with 200–350 µL binding buffer, 5 minutes per wash.
8. Remove the chip from the bioprocessor and bulk wash the chip briefly with 8 mL of water in a 15 mL conical centrifuge tube.
9. Air dry the array, then outline its spots with a PAP pen. Allow to air dry again.
10. Apply 0.5 µL EAM to each spot. Repeat the EAM addition. Air dry the chip.
11. Analyze in ProteinChip Reader.

Recommended Binding Buffers

See the list of recommended buffers on page 5-8.

NP1 and NP2 Arrays

Normal phase arrays, called “NP1” and “NP2” chips, are used for general binding of proteins for analysis. The chemistries on the active spots of both arrays contain silicon oxide which allow proteins to bind via serine, threonine or lysine. The NP2 chip has a blue hydrophobic coating that allows sample containment on the spots.

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NP1 and NP2 arrays can be used to:

- quickly analyze proteins in a sample
- check for purity
- verify the presence or absence of a molecule
- calibrate using peptides or proteins of known molecular weight

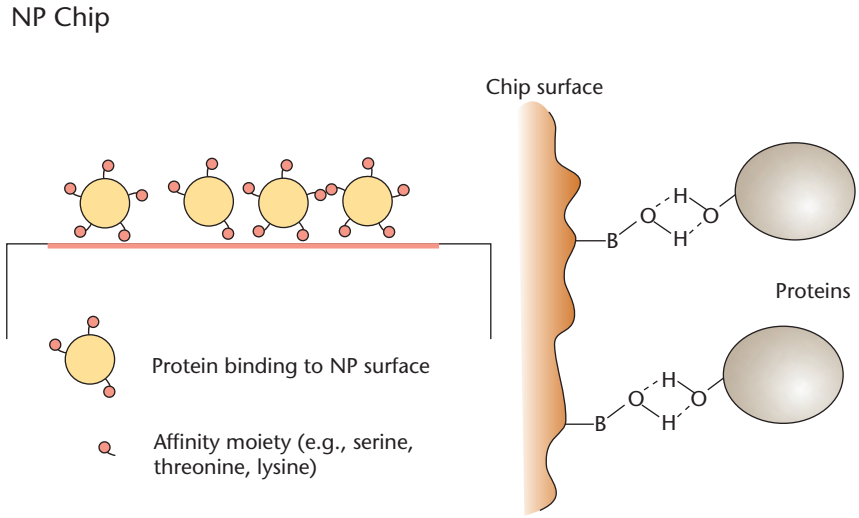

Figure 5-3: NP1/NP2 chip surface chemistry with proteins.

NP1 and NP2 Protocols

Note: to achieve optimal signal, the spots on the NP2 chip should be washed with water and allowed to dry before sample is applied.

Method 1

1. Dilute or dissolve the sample in binding buffer. This surface tolerates most buffers.
2. If using a NP1 chip, outline each spot with a hydrophobic pen. Allow to air dry.
3. Apply 1-3 µL sample to each spot. The ideal protein concentration is 10–200 µg/ml (10-fold less for peptides).
4. Allow the sample to air dry onto the chip surface (5–10 minutes).
5. Wash each spot with 5 µL HPLC grade water. Repeat 1–2 times.
6. Allow the chip to air dry.
7. Apply 0.5 µL saturated EAM solution to each spot. Allow the spots to dry (~1 minute).
8. Analyze the chip using the ProteinChip System.

Method 2

1. Apply 1 µL/spot of appropriately diluted sample (diluted in water) to each spot. Incubate for a few minutes without allowing the

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sample to dry. This incubation can be as long as is convenient (use a humidified chamber if incubating for more than a few minutes).

2. If necessary, the spots can be washed twice with 3–5 µL water per wash.
3. Apply 0.5 µL EAM to each spot after some evaporation has occurred but before the spots dry out completely. Air dry.
4. Analyze the chip using the ProteinChip System.

NP1 Protocol Using a Bioprocessor

Note: this protocol is intended for use with the NP1 array. Although sometimes the bioprocessor can be used with NP2 arrays, proteins may stick to the hydrophobic coating on the NP2 array.

1. Assemble the chip in the bioprocessor and add 350 µL binding buffer to each well (see below for recommended buffers). Incubate for 5 minutes at room temperature with vigorous shaking (e.g., 250 rpm).
2. Remove the buffer from the wells and immediately add 50–350 µL/well sample diluted in binding buffer (recommended sample dilution: 50–2000 µg/mL total protein). Incubate with vigorous shaking for 30 minutes.
3. Remove the samples from the wells and wash each well with 350 µL binding buffer for 5 minutes.
4. Repeat the wash twice more for a total of three washes, 5 minutes per wash.
5. Remove the chip from the bioprocessor and rinse the chip briefly with 8 mL of water in a 15 mL conical centrifuge tube. Repeat the water rinse.
6. Air dry the array, outline the spots with a PAP pen, then apply 0.5 µL of saturated EAM solution per spot (sinapinic acid or EAM1 for proteins 10–200 kDa, CHCA for peptides 0–10 kDa).
7. Analyze the chip using the ProteinChip System.

Recommended Binding Buffers

Increasing the salt 50–500 mM, the detergent 0.01–0.1%, or the organic solvent 0–50% will increase selectivity. Decreasing the pH will increase selectivity.

PS1 and PS2 Arrays

Preactivated surface chip arrays, called “PS1” and “PS2” chips, are used to covalently immobilize biomolecules for the subsequent capture of proteins from complex biological samples. The surface

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chemistries of the two chips differ, with carbonyl diimidazole moieties on the PS1 chip and epoxy groups on the PS2 chips (Figure 5-4). Additionally, the PS2 chips have a hydrophobic coating that allows sample containment on the spots. Due to differences in surface properties, the PS2 surface is especially recommended for sensitive detection, low non-specific binding, and when the target protein is less than 1% of total protein. In general, both chip types must be tested to determine which is optimal for a particular experimental system.

Uses for PS1 and PS2 arrays include:

- antibody-antigen
- receptor-ligand
- nucleic acid-binding protein
- any specific protein-protein interaction

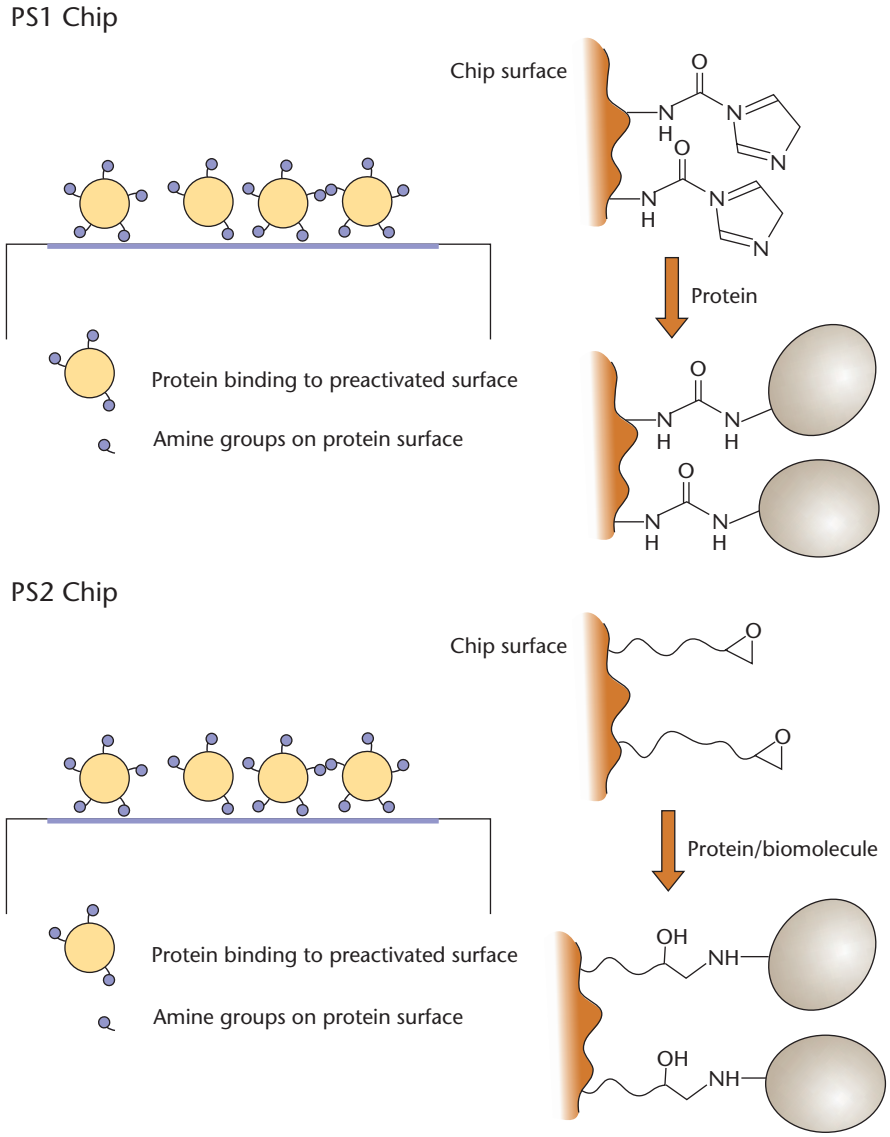

Figure 5-4: PS1 and PS2 chip surface chemistry with proteins.

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PS1 and PS2 Protocol

Note: binding/washing buffer is usually PBS supplemented with detergent (0.1–0.5% Triton X-100) and/or NaCl (0.1–0.5 M). The choice of buffer is determined empirically.

Ethanolamine Preparation

This protocol requires 1 M ethanolamine (FW 97.54, Sigma catalog no. E-6133) in 1x PBS. 100 mL 1 M ethanolamine can be prepared as follows:

1. Combine:

10x PBS	10 mL
Milli-Q water	70 mL
ethanolamine HCl	9.75 g

2. Adjust the pH to ~8.0 with 1 N NaOH.
3. Add Milli-Q water to bring the final volume to 100 mL.

Array Preparation

1. If using a PS1 chip, outline each spot with a PAP pen. Allow to air dry.
2. Place the preactivated chip in a humidity chamber. Pre-equilibrate the chemical surfaces by applying 3 µL PBS to each spot.
3. Apply 1 µL of a solution containing 0.2–0.5 mg/mL (approximately 1 picomole) of the protein of interest to each spot. For a negative control use a similar concentration of a different protein on another spot.
4. Incubate for 1 hour in the humidity chamber at room temperature or at 4 °C overnight to allow the protein to covalently bind to the spots.
5. Block residual active sites by adding 3 µL 1 M ethanolamine (made up in PBS and adjusted to pH 8). Incubate for 30 minutes at room temperature in the humidity chamber.
6. Remove unbound proteins by washing the chip in a 15 mL conical tube containing 7 mL PBS + 0.5% Triton X-100. Place the tube containing the chip and the wash buffer on a shaking platform for 15 minutes at room temperature. Repeat the wash.
7. Wash with PBS three times, 5 minutes each. This wash is to eliminate the Triton X-100, since residual detergent can cause samples to spread in the next step, especially on PS2 chips.
8. Wipe off excess buffer around the spots. At this point, it is acceptable if the spots dry out slightly. After the next step, however, it is imperative to keep the spots moist until after the final wash step, just prior to adding EAM.

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Capture of Specifically Binding Protein

Note: in general, when a range of concentrations is given for buffer preparation, the less stringent wash conditions (i.e., less salt and less detergent) are recommended for the PS2 chips. Similarly, vigorous, bulk washes are often necessary to remove non-specifically binding proteins from PS1 chips, while spot washes are generally recommended for PS2 chips.

1. Prepare the protein extract in the usual way for the tissue to be examined. The solution should contain 0.1–0.5% Triton X-100 or other detergent to prevent non-specific binding to the chip.
2. Apply 5 µL protein extract to each spot and incubate in a humidity chamber at 4 °C overnight or for 4 hours at room temperature.
3. Wash the chip with 3 x 5 µL binding buffer. The final wash can be performed in bulk in a 15 mL tube for 10 minutes with vigorous shaking. For PS2 chips, a final 1 minute bulk wash may be useful; however, PS2 chips have a lower binding capacity and bulk washes may be too harsh for some protein-protein interactions.
4. Rinse the chip in bulk with binding buffer that does not contain detergent.
5. Rinse the chip in bulk with HPLC-grade or Milli-Q water.
6. Dry the edges of the chip with a lab wipe and allow the spots to air dry.
7. Apply 0.5 µL saturated EAM solution to each spot. If using PS2 chips, two additions of 0.5 µL EAM are best.
8. Analyze the chip using the ProteinChip Reader.

PS1 and PS2 Protocol Using a Bioprocessor

For an example using PS1 chips with a bioprocessor, please see *"Using the Bioprocessor in ProteinChip Immunoassays"* on page 8-14.

SAX2 Arrays

The Strong Anion Exchange array, called the “SAX2” chip, can be used to analyze molecules with a negative charge on the surface. The active spots contain cationic, quaternary ammonium groups that interact with the negative charges on the surface of target proteins, e.g., aspartic acid or glutamic acid.

Uses for SAX2 arrays include:

- selective analysis of proteins with low pI’s
- biomarker discovery

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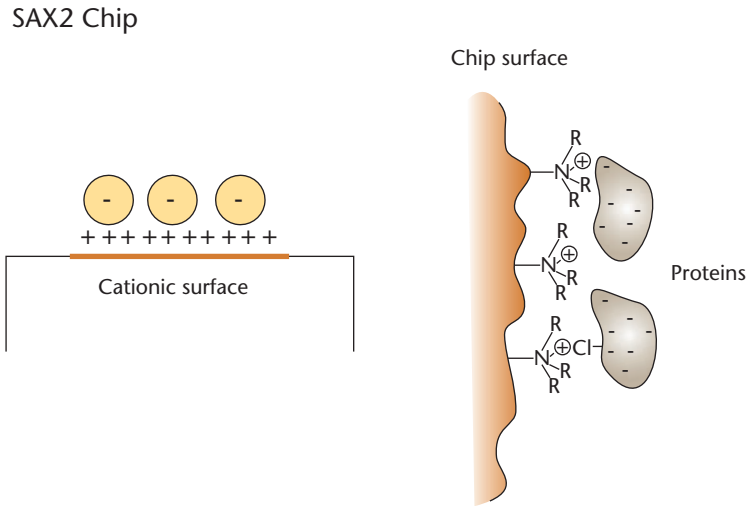

Figure 5-5: SAX2 chip surface chemistry with protein.

Notes

- Analyze the SAX2 chips within 2 hours of placing the EAM on the surface.
- Salts and ionic detergents in high concentrations can disrupt binding of some proteins on the SAX2 surface.
- The pH of the binding and/or wash buffers can be lowered to increase binding selectivity.
- The salt concentration of the binding and/or wash buffers can be increased to increase binding selectivity.

SAX2 Protocol

1. Outline each spot using a hydrophobic pen. Allow to air dry.
2. Apply 10 µL binding buffer to each spot and incubate in a humidity chamber at room temperature for 5 minutes. Do not allow the spots to become dry.
3. Remove excess buffer from the spots without touching the active surface. Repeat steps 2 and 3 one more time.
4. Load 2–3 µL sample per spot. The samples should be prepared in binding buffer. A non-ionic detergent can be included in the binding and washing buffers (e.g. 0.1% OGP or Triton X-100) to reduce non-specific binding. Varying the pH and ionic strength of the binding and/or washing buffer can also modify ionic binding.
5. Incubate in a humidity chamber for 20 to 30 minutes.
6. Wash each spot with 5 µL binding buffer five times, followed by two quick washes with water (5 µL per wash).

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7. Wipe dry around the spots. Apply 0.5 µL saturated EAM solution to each spot while it is still moist, but not wet. Air dry. Apply a second aliquot of EAM and air dry.

8. Analyze the chip using the ProteinChip System.

Recommended Buffers

20 to 100 mM sodium or ammonium acetate (for pH 4–6), Tris HCl or 50 mM Tris base buffers containing a non-ionic detergent, e.g. 0.1% Triton X-100 (for pH 6.85–9.5).

SAX2 Protocol Using a Bioprocessor

1. Assemble the chip in the bioprocessor and add 350 µL binding buffer to each well (see below for recommended buffers). Incubate for 5 minutes at room temperature with vigorous shaking (e.g., 250 rpm).

2. Remove the buffer from the wells and immediately add 50–350 µL/well sample diluted in binding buffer (recommended sample dilution: 50–2000 µg/mL total protein). Incubate with vigorous shaking for 30 minutes.

3. Remove the samples from the wells and wash each well with 350 µL binding buffer for 5 minutes.

4. Repeat the wash twice more for a total of three washes, 5 minutes each.

5. Remove the chip from the bioprocessor and rinse the chip briefly with 8 mL of water in a 15 mL conical centrifuge tube. Repeat the water rinse.

6. Air dry the array, outline the spots with a PAP pen, then apply 0.5 µL of saturated EAM solution per spot (sinapinic acid or EAM1 for proteins 10–200 kDa, CHCA for peptides 0–10 kDa).

7. Analyze the chip using the ProteinChip System.

Recommended Buffers

20 to 100 mM sodium or ammonium acetate (for pH 4–6), Tris HCl or 50 mM Tris base buffers containing a non-ionic detergent, e.g. 0.1% Triton X-100 (for pH 6.85–9.5).

WCX2 Arrays

The Weak Cation Exchange array, called “WCX2” arrays, can be used to analyze molecules with a positive charge on the surface. The active spots contain weak anionic carboxylate groups that interact with the positive charges on the surface of the analyte, e.g., lysine, arginine or histidine (Figure 5-6).

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Uses for WCX2 arrays include:

- selective analysis of proteins with low pIs
- biomarker discovery

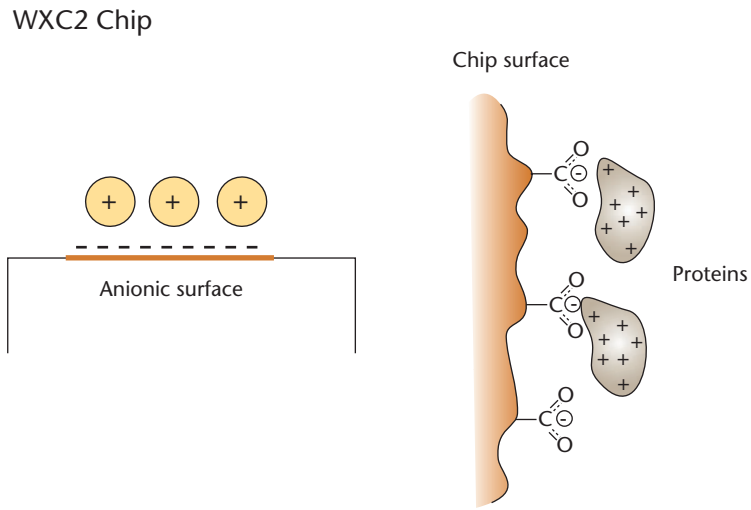

Figure 5-6: WCX2 chip surface chemistry with protein.

Notes

- The WCX2 chip is shipped in the salt form with sodium as the counter-ion. In order to minimize the sodium adduct peaks in the mass spectra, it is recommended that the chip be pretreated with hydrochloric acid as described below before loading the sample.
- Target protein binding is reduced by increasing the concentration of salt or by increasing the pH of the washing buffers.

WCX2 Protocol

Pretreatment of the WCX2 Chip

1. Outline each spot using a hydrophobic pen. Allow to air dry.
2. Wash the chip in a 15 mL conical tube with 10 mL 10 mM hydrochloric acid on a rocker for 5 minutes, or perform spot washes using 5 µL 10 mM HCl per spot for 5 minutes.
3. Rinse the chip with 10 mL water three times.
4. Wipe dry around the spots.

Protocol

1. Apply 10 µL binding buffer (e.g., 100 mM ammonium acetate, pH 6.5) to each spot and incubate in a humidity chamber at room temperature for 5 minutes.
2. Remove excess buffer from the spots without touching the active surface.

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3. Repeat steps 1 and 2.
4. Apply 1-3 µL sample (in binding buffer) per spot and incubate the chip in a humidity chamber for 20 to 30 minutes.
5. Wash each spot with 5 µL binding buffer five times, followed by a quick wash with water (5 µL, two times). Wipe dry around the spots.
6. Apply 0.5 µL saturated EAM solution to each spot while it is still moist. Air dry.
7. Apply a second aliquot of EAM and air dry.
8. Analyze the chip using the ProteinChip System.

Recommended Buffers

20 to 100 mM ammonium acetate and phosphate buffers containing low concentration of a non-ionic detergent (e.g., 0.01–0.1% Triton X-100).

Note: all procedures are performed at room temperature.

WCX2 Protocol Using a Bioprocessor

1. Pre-treat the spots for 10 minutes with 10 mM HCl.
2. Rinse the chip with 10 mL water three times in a conical tube.
3. Assemble the chip in the bioprocessor and add 350 µL binding buffer to each well (recommended sample dilution: 50–2000 µg/mL total protein). Incubate for 5 minutes at room temperature with vigorous shaking (e.g., 250 rpm).
4. Remove the buffer from the wells and immediately add 50–350 µL/well sample diluted in binding buffer (see second point in *"Notes"* on page 5-17). Incubate with vigorous shaking for 30 minutes.
5. Remove the samples from the wells and wash each well twice with 350 µL binding buffer, 5 minutes per wash.
6. Remove the chip from the bioprocessor and briefly rinse it with 8 mL of deionized water in a 15 mL conical centrifuge tube.
7. Air dry the array and apply 0.5 µL of EAM solution per spot. It may be desirable to outline the spots with a PAP pen prior to adding EAM.
8. Apply 0.5 µL EAM to each spot. Air dry the chip. Repeat the EAM addition and air dry the chip.
9. Analyze the chip in the ProteinChip Reader.

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Recommended Binding Buffers

50 mM ammonium acetate or sodium acetate or phosphate, pH 4.0 –7.0 (the pH can be raised to increase selectivity). Triton X-100 (0.1%) should be included to reduce hydrophobic binding to the spots.

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Chapter 6

Protein Biochemistry for the ProteinChip System

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Basic Protein Biochemistry

The following section contains basic information on protein biochemistry and explains how certain biochemical properties of proteins affect ProteinChip Array analysis.

General Information on Proteins, Including Modifications

Proteins are comprised of numerous amino acids, as dictated by the specific gene sequence. The number and type of amino acids give each protein its distinctive biochemical nature. In addition to amino acids, many proteins also have other types of structures attached to them, either covalently or non-covalently. These structures may include prosthetic groups, such as lipids (lipoproteins), sugars (glycoproteins), metals (metalloproteins), heme groups and vitamins (which may act as coenzymes). Amino acids may also be directly modified by the addition of phosphate to serine, tyrosine or threonine.

The mass of a protein, as determined by the ProteinChip Reader, may be affected by prosthetic groups. Covalently attached prosthetic groups (lipids, sugars, phosphates) will increase the observed mass of a protein in ProteinChip Array analysis. Non-covalently attached prosthetic groups (metals, heme groups, vitamins) may dissociate from the protein either during sample preparation or during the process of EAM addition and crystallization, when organic solvent is typically added to the sample. However, non-covalently attached prosthetic groups may be bound so tightly to the protein that they remain bound, even during ProteinChip Array analysis.

Prosthetic groups also affect the chemical nature of the protein and thus may affect the protein's binding activity to particular ProteinChip Array chemical surfaces.

Isoelectric Points

The isoelectric point of a protein (pI) is simply the pH value at which the protein has no net charge. The pI of a protein results from the charges of all the amino acid groups on the protein, plus the N-terminus and C-terminus. Acidic amino acid residues (e.g., aspartic acid, glutamic acid) decrease the pI of a protein. Basic amino acid residues (lysine, arginine, histidine) increase the pI of a protein.

The isoelectric point of a protein will strongly influence how well it binds to the ionic ProteinChip Arrays. Proteins with a low pI bind strongly to the anion exchange surface (SAX2). If the buffer solution is lowered below the pI of the protein, the protein will begin to bear a net positive charge and will bind more weakly to the surface of the array. Proteins with a high pI bind strongly to the cation exchange surface (WCX2). If the buffer solution is increased above the pI of the protein, the protein will begin to bear a net negative charge and will bind more weakly to the surface of the array. Proteins with

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extremely high or extremely low pI's may be purified away from the majority of other proteins by binding to an ionic ProteinChip Array in a very high or very low pH buffer.

Bear in mind that other properties of the protein influence binding to ProteinChip Array surfaces.

Native *vs.* Denatured Proteins

A protein in its native state is folded properly, i.e., the protein is folded as it would be when fully functional inside the cell. The tertiary structure of a protein is stabilized by four forces: hydrogen bonding, ionic attraction, hydrophobic interaction, and covalent bonds. Some proteins, such as proteins that occur in complexes, may need other proteins to allow them to adopt their native conformation.

ProteinChip Array analysis may investigate the behavior of either native or denatured proteins, depending on the specific application. Protein-protein interaction studies, using a binding protein covalently attached to a preactivated surface array, are often used to capture a protein in its native state. For example, an antibody or receptor that recognizes a ligand in its native conformation may be used to capture that protein onto the ProteinChip Array.

For all ProteinChip Array analyses, the mass determination occurs independently of the protein's conformation on the chip surface. Covalent bonds are not disturbed during the ionization/desorption process. However, the overwhelming majority of non-covalent bonds are disrupted upon EAM addition, due to high concentrations of organic solvent. Therefore, SELDI analysis typically shows the molecular weight of monomeric species, although dimers and trimers are occasionally observed.

Proteins That "Fly" or Don't

ProteinChip Array analysis requires that proteins "fly" through a vacuum tube in order to obtain time-of-flight data. Technically, "flying" refers to the fact that the protein must be able to become ionized and undergo a transition from the solid, crystalline phase on the chip surface into the gas phase following irradiation with the laser.

Ionization of proteins is a complex process which is not completely understood. For ionization to occur, it is imperative that the analyte actually co-crystallize with the small molecule that serves as EAM. All EAMs are acidic, i.e., strong proton donors with low pKs. Ionization may occur either during crystal formation and/or upon irradiation with the laser and is influenced by the exact chemical nature of the protein — some proteins easily accept an added charge while other proteins resist accepting the extra charge.

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Glycosylated Proteins: Biochemistry Overview and Analysis

The information below provides guidance in using ProteinChip Array technology to analyze glycoproteins. It provides the researcher with experimental approaches rather than precise protocols.

Background Information

Most glycoproteins contain several different sugar chains in one molecule. Oligosaccharides are commonly attached to proteins through N glycosidic linkages to asparagine residues or through O glycosidic linkages to serine and threonine residues. The isolation and purification of an oligosaccharide bound to a protein can be a tedious task because more than one oligosaccharide may be linked to different amino acid sites. In addition, even a protein with a single N linked glycosylation site will normally contain a group of related but structurally different oligosaccharides (known as microheterogeneity). In contrast to protein biosynthesis, no template is used in the synthesis of oligosaccharides. Therefore, microheterogeneity is an inherent characteristic of the sugar chains in glycoproteins.

Carbohydrate groups on glycoproteins are thought to be important in cell adhesion. Cell-cell interactions probably involve an interaction between the carbohydrate group and a carbohydrate binding protein (lectin). A lectin specifically recognizes a particular carbohydrate structure and can therefore be utilized in the laboratory to deduce some structural information. Monoclonal antibodies raised against specific carbohydrate structures can be used in the same way.

Identification of Structural Motifs Using Lectins

The molecular recognition of certain carbohydrate structures by particular lectin molecules can be used to classify the glycan chains. A glycan differentiation kit can be purchased from Boehringer Mannheim which contains a number of these different lectins and proper controls. The lectins can be bound to a preactivated chip using the standard protocol. If the glycoprotein is retained on the chip surface by a particular lectin, the protein will be visualized upon analysis in the ProteinChip Reader. This would indicate the presence of a particular glycan structural motif.

Determining the Site of Carbohydrate Attachment

If the glycoprotein has been purified and is relatively abundant, the site of attachment of the glycan chain could be determined. The general method would be to digest the glycoprotein with trypsin in solution and expose the resulting peptide fragments to a lectin coated ProteinChip array (and controls). The peptide attached to the carbohydrate group should be retained on the chip surface after washing and visualized in the ProteinChip reader. The observed mass will correspond to the peptide+carbohydrate chain. It may then be

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possible to extend the experiment by adding a deglycosylating enzyme and reading the ProteinChip Array *without* washing the spot. The captured molecule should then be cleaved into the peptide and carbohydrate moieties, and the mass of the peptide could be determined. Alternatively, the cleavage step could be used to elute the peptide from the lectin, and the 'supernatant' could be removed and spotted onto a reverse phase ProteinChip Array. In both cases the proper controls must be performed as some interfering peaks will arise from the enzymes used (trypsin and deglycosylase).

This information is useful if the sequence of the protein is known. Sequence matching programs such as PAWS (software available free from http://prowl.rockefeller.edu) can be used to match the observed peptide mass to the protein sequence in order to reveal the glycosylation site (see Chapter 10, *"Protein Identification with the ProteinChip System"* for more information).

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Chapter 7
Sample Preparation

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Cell Lysates

Tissues and cells must be lysed to extract the proteins before they can be assayed using the ProteinChip System.

Lysis Using Detergents

Tissue Lysates

Tissue lysates can be prepared in an IEF (isoelectric focusing) lysis buffer. This buffer contains 9 M Urea, 2% CHAPS, 1% DTT, and ampholites. The levels of DTT must be lowered before the extract can be used for protein profiling. This can be achieved by diluting the sample in an appropriate buffer (~5 fold dilution), or passing the sample through a K3 buffer exchange spin column (~4 fold dilution).

Soluble Cytoplasmic Protein Lysates

Note: this method is not applicable to nuclear or microsomal proteins.

Add buffer to the cells (25 mM HEPES-KOH, pH 7.4, 100 mM KCl, 5 mM EDTA, 1 mM DTT, 0.1 mM PMSF) and perform dounce homogenization (100 strokes). For a better yield, add 50 µg/mL digitonin, which is a very mild detergent.

Lysis Without Detergents

Method 1: Sonication

Notes

- Cells are ruptured by sonication with a sonicator probe. (Bath sonication does not produce good disruption of cells).
- The probe must be large enough so that when inserted into the sample, a large proportion of the cell suspension is in contact with the probe— this ensures sufficient transfer of energy. The optimal sonicator settings will have to be determined empirically.
- Keeping the sample cold in between sonication times will minimize the proteolytic degradation that results from heating the sample.
- Either one of the buffers below may be used successfully for lysis by sonication. The amount of detergent in Buffer B may be increased. Again, optimization must be done empirically.
- The amount of cell lysis may be inferred by spinning the sample on high speed in a microcentrifuge. The pellet will be smaller as lysis is more complete.

Buffers

Buffer A: 0.1 x PBS
Buffer B: 50 mM NaCl, 0.1% dodecyl maltoside, 25 mM HEPES

1. Dilute cells in a Buffer A or B to a final concentration of ~10^7 cells/mL.

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Note: 4 mM $MgCl_2$ will stabilize nuclei during hypotonic lysis. However, if nuclear rupture is required, ensure that $MgCl_2$ is not present.

2. Sonicate the cell suspension in short bursts, keeping the sample cold between sonication times. For example, sonicate 5 seconds on a low setting, then keep sample on ice for 1 minute. Repeat until most or all cells are lysed.

Method 2: Lysis Using a Needle

1. Wash the cells in PBS.
2. Add a small volume of H_2O. Use a pipette (or a needle) to draw the sample up and down to break up the cells and DNA.

   Optional: waterbath sonication can be used to aid in cell disruption
3. Spin the cell lysates at high speed to pellet the membranes and other insoluble materials.

   Optional: add a small volume of acetonitrile or detergent to lyse the membranes left in pellet. Microcentrifuge again and use the supernatant for ProteinChip Array analysis.
4. Analyze the supernatant directly on ProteinChip Arrays.

Method 3: Plant Material

These techniques have been used successfully for leaf and seed tissues:

1. Homogenize sample in liquid nitrogen.
2. Resuspend in PBS *or*

   extract sample in PBS:methanol:chloroform to remove lipids.

Some proteins may fractionate into both of the phases.

Specialized Lysis Methods

Purification of Nuclei from Mammalian Tissues

Notes

- This protocol describes nuclei purification and extraction from cultured cells and most mammalian tissues, excluding heart and muscle tissues.
- All operations should be performed on ice or in a cold room.

1. Mince the tissue with scissors and homogenize in a glass homogenizer with a motor driven teflon pestle (or in an Eppendorf tube with a blue Eppendorf plastic pestle) in a solution of 0.15 M NaCl, 10 mM HEPES or Tris-HCl, pH 7.4–7.5. Cultured cells should be collected by centrifugation at 3000 rpm in a microfuge and washed with the same solution.

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2. Filter through 2 layers of cheesecloth.
3. Centrifuge at 3000 rpm in a microfuge for 5 minutes.
4. Resuspend the pellet in: 0.33 M sucrose, 10 mM HEPES (or Tris-HCl), pH 7.4, 1 mM $MgCl_2$, 0.1% Triton X100 in 5:1 v/v (solution/cells).
5. Leave on ice for 15 minutes, resuspending gently with a thin glass or plastic rod (use a yellow loop for bacterial cultures) at 5 minute intervals. Try to disperse any aggregates.
6. Spin at 3000 rpm in a microfuge for 5 minutes at 4 °C. Collect the supernatant, which is the cytosol extract. Store aliquots at -70 °C.
7. Repeat steps 4–6 with the pellet. Discard the supernatant. The pellet will contain a pure preparation of nuclei, without the nuclear outer membrane.
8. To the nuclear pellet add 0.45 M NaCl, 10 mM HEPES, pH 7.4 (protease inhibitors may be added to all of the solutions) and resuspend gently on ice.
9. Stir the suspension at 5 minute intervals for 15 minutes. Under these conditions most nuclear proteins (except histones and nuclear matrix proteins) will be extracted. Do not exceed 0.45 M NaCl as you will extract lots of histone.
10. Spin for 5 minutes at maximum speed in a microfuge.
11. Collect the supernatant and store in aliquots at –70 °C.

Protein Extraction Using "Trizol"

"Trizol" is a TRI reagent available from Sigma Chemical Co.

Sample Preparation

Note: be sure to record the total amount of Trizol reagent used in the initial homogenization as this volume will be used in subsequent steps.

- Tissue — homogenize tissue samples in Trizol (1 mL per 50–100 mg of tissue) using a Polytron or another appropriate homogenizer. The volume of the tissue should not exceed 10% of the volume of the Trizol.
- Monolayer cells — lyse cells directly on the culture dish. Use 1 mL of Trizol per 10 cm^2 of culture plate surface area. The cell lysate should be passed several times through a pipette to form a homogenous lysate.
- Suspension cells — isolate cells by centrifugation and then lyse in Trizol by repeated pipetting. 1 mL reagent is sufficient to lyse 5–10 x 10^6 animal, plant or yeast cells or 10^7 bacterial cells (some yeast and bacterial cells may require a homogenizer).

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Note: after lysis in Trizol, samples can be stored at -70 °C for up to 1 month. If samples contain a high content of polysaccharides or extracellular material then centrifuge the homogenate at 12,000g for 10 minutes at 4 °C. This should remove insoluble material (membranes, polysaccharides, high MW DNA). If there is a layer of fatty material on the aqueous phase then this should be removed.

Lysis

1. Allow samples to stand in Trizol for 5 minutes at room temperature to ensure complete dissociation of nucleoprotein complexes.
2. Add 0.2 mL of chloroform per mL of Trizol. Cover the sample tightly and shake vigorously for 15 seconds, then incubate for 2–15 minutes at room temperature.
3. Centrifuge at 12,000*g* for 15 minutes at 4 °C. Mixture will partition into 3 phases: a red organic phase (containing protein and DNA), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA).

Removing DNA from the Organic Phase

1. Remove the colorless (upper) aqueous phase and discard.
2. To precipitate the DNA, add 0.3 mL 100% ethanol per 1 mL of Trizol initially used. Mix by inversion and incubate at room temperature for 2–3 minutes.
3. Centrifuge at 2000*g* for 5 minutes at 4 °C.
4. Remove the supernatant and keep at 4 °C for protein isolation.

Precipitating Proteins from the Phenol-ethanol Supernatant

1. Add 1.5 mL isopropanol per 1 mL of Trizol initially used. Incubate for at least 10 minutes at room temperature
2. Centrifuge at 12,000*g* for 10 minutes at 4 °C.
3. Discard the supernatant and wash the pellet 3 times in 0.3 M guanidine hydrochloride/95% ethanol, using 2 mL per 1 mL of Trizol originally used, washing as follows:
   a. Incubate the samples in the wash solution for 20 minutes at room temperature.
   b. Centrifuge the samples at 7500*g* for 5 minutes at 4 °C to pellet the protein.
   c. Resuspend the pellet in 0.3 M guanidine hydrochloride/95% ethanol, using 2 mL per 1 mL of Trizol originally used.
4. After completing the 3 washes, discard the supernatant and add 2 mL 100% ethanol and vortex the protein pellet. Incubate for

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30 minutes at room temperature. Centrifuge at 7500*g* for 5 minutes at 4 °C.

5. Dry the protein pellet under vacuum for 5–10 minutes.
6. Dissolve the pellet in 1% detergent (Triton X-100, OGP or dodecyl maltoside) aided by repeated pipetting of the solution. Alternatively, if the samples are to be applied to a reversed-phase (H4) ProteinChip Array, resuspend in 10% acetonitrile.
7. Remove any insoluble material by centrifugation at 10,000*g* for 10 minutes at 4 °C. Transfer supernatant to a new tube and use immediately.

Protein samples suspended in either of the wash solutions can be stored for 1 month at 4 °C or 1 year at -20 °C.

Mammalian Tissue Lysate Preparation Using the Ribolyzer

This protocol describes how to use the Ribolyzer for preparing lysates of mammalian tissue. This method has been used successfully to generate excellent data using ProteinChip technology. This method is highly recommended for preparing protein extracts for ProteinChip Array applications.

Prepare the Lysis Buffer

Component	Final Concentration	For 50 mL
Urea	9.5 M	30.0 g
CHAPS	2% (w/v)	1.0 g
DTT	1% (w/v)	0.5 g

Note: do not heat urea above 37 °C.

1. Add deionized water to 30.0 g urea for a final volume of 50 mL. Stir until dissolved.

   Note: urea swells as it dissolves so first add ~40 mL deionized water, allow the urea to dissolve and then make the final volume 50 mL with deionized water. Filter before use.

2. To 48 mL of the urea solution, add 1.0 g CHAPS and 0.5 g DTT. Aliquot and freeze at -80 °C.

   Important: powder-free gloves should be used at all stages.

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Prepare the Tissue Lysate(s)

Materials

- Dewar flask of liquid nitrogen
- Ice bucket of dry ice
- Ice bucket of “wet” ice
- Defrosted lysis buffer aliquots, or fresh lysis buffer. The buffer contains urea; therefore do not heat it above 37 °C (to prevent carbamylation), or let its temperature remain below 15 °C (to prevent crystallization).

Method

1. Remove the tissue(s) from a -80 °C freezer and place on dry ice.
2. Approximately 1 mL lysis buffer is required for 100 mg of tissue. Place 0.75–1.0 mL lysis buffer into a separate ribolyzer tube for each sample to be prepared and note the volume of lysis buffer in each tube.
3. Line a ceramic pestle and mortar with aluminium foil and carefully pour in a small amount of liquid nitrogen.
4. Weigh out approximately 100 mg of tissue and note the weight of the tissue.
5. Place the tissue in the mortar with the liquid nitrogen and crush the tissue with the pestle.
6. When all of the liquid nitrogen has evaporated, but before the tissue defrosts, place the powdered tissue in a ribolyzer tube of lysis buffer. Leave the sample at room temperature while you prepare the rest of the samples.
7. When all of the tissues have been prepared, place the tubes in the ribolyzer. Check that the tube lids are tightly sealed to avoid spillage.
8. Set the ribolyzer running for two 10-second bursts at speed 6. Place the tubes on ice between homogenizations to cool the samples.
9. Spin the ribolyzer tubes in a microfuge for 5 minutes at 13,000 rpm to reduce air bubbles.
10. Pipette the lysate into appropriate tubes and spin at 42,000*g* for 1 hour at 15 °C.
11. Remove the supernatant from the tubes, aliquot it into 50 µL samples. Store the samples at -80 °C.

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12. Check the protein concentration of the samples using a Bradford Quantification Assay.
13. Analyze the protein content of the samples using the ProteinChip System.

Sera and Plasma

There are two methods for processing blood to derive serum or plasma. The difference between serum and plasma lies in the removal of fibrinogen and clotting factors during the process of coagulation.

1. Plasma — in order to see clotting factors and wound healing proteins/peptides, it is necessary to use an anti-coagulant. Neither heparin nor EDTA is a problem in ProteinChip analysis so either can be used. The blood can then be centrifuged to remove the red blood cells (RBCs).
2. Serum — in order to look at circulating proteins, clotting is a good way of removing RBCs and simplifying the sample. Serum is usually the better choice for profiling if you're not interested in profiling clotting factors or fibrinogen.

Storing Sera and Plasma

RBCs must be removed before storing blood samples. Serum and plasma should be flash frozen in liquid nitrogen and stored at -20 °C for the short term or -70 °C for the long term. It is best to avoid freeze-thaw cycles; therefore samples should be aliquoted before freezing. Most importantly, samples to be compared should have the same sample handling history whenever possible.

Removing Hemoglobin

If serum/plasma is prepared correctly, limited RBC lysis should occur. However, if handled incorrectly, samples can become reddish in color, showing that they have suffered significant RBC lysis, leading to the presence of hemoglobin in the samples. Hemoglobin will dominate further ProteinChip analysis and must be removed.

Hemoglobin has an affinity for nickel and cobalt. Therefore, Ni^{2+} beads (commonly used for histidine-tagged protein purification), or Co^{2+} beads can be used to deplete hemoglobin from the serum samples. Following the incubation the lysate should be clear and the beads should have turned red, indicating that the hemoglobin has been captured onto the beads. The beads are then removed from the sample by centrifuging.

Note: although the method described below will deplete hemoglobin, it may also deplete other proteins in the sample. Therefore, it is preferable to minimize red blood cell lysis and concomitant hemoglobin contamination when preparing samples.

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Method

1. Wash the Ni^{2+} beads with PBS/0.3 M NaCl.
2. Add 100 µL 50% Ni^{2+} bead suspension (~50 µL beads in 50 µL PBS/0.3 M NaCl) to 100 µL lysate in a 1.5 mL microfuge tube.
3. Mix gently on a rotating platform for 20 minutes at 4 °C.
4. To remove the beads, centrifuge the tube at the highest speed of a microcentrifuge for 2 minutes at 4 °C.

Delipidation

Human serum may contain large amounts of lipids, chilomicrons, free fatty acids and lipid-carrying proteins. Some lipids can be removed by placing the serum on ice, then microfuging the sample at 4 °C, isolating most of the lipid in an upper layer. This approach can be inefficient and variable, but helpful nonetheless.

Lipids can be removed by extraction with ether/methanol or $CHCl_3$/methanol. A generic, starting protocol for lipid extraction is outlined below. It has been modified from a protocol for apolipoprotein extraction and the volumes can be scaled down for smaller samples. (A chromatographic resin for removing lipids, called Lipidex [hydroxy-alkoxypropyl Dextran, Sigma cat. no. H6258], may also be of use.)

> *Note: avoid using solvents such as isoamylalcohol in the delipidation as they may extract peptides from the serum.*

Method

1. Dialyze the serum samples against 0.15 M NaCl, 1 mM EDTA, 5 mM NH_4HCO_3.
2. Add 2 mL or less of the protein solution dropwise to 15 mL vortexing methanol at 0 °C in a 45–50 mL conical tube.
3. Fill the tube immediately with diethylether, invert several times and place in wet ice for 10 minutes.
4. Sediment the protein by low speed centrifugation (e.g., 2,000 rpm in a clinical centrifuge with a swinging bucket rotor) for 2 minutes.
5. Remove the organic solvent by aspiration or decanting, then resuspended the protein in methanol. Refill the tube with diethylether and place in wet ice for 10 minutes.
6. Pellet the protein by centrifugation (this time 3,000 rpm for 4 minutes) and discard the organic solvent.

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7. Rinse the protein twice with diethylether and dry in a thin film on the tube surface with a stream of nitrogen. Remove residual traces of ether by vacuum suction.

8. Resolubilize the protein with a detergent solution (use a non-ionic detergent compatible with ProteinChip analysis such as Triton X-100 or NP-40). Alternatively, aqueous solutions containing 6–8 M urea, 4–6 M guanidine HC1, 50% acetic acid or 0.1 M NH_4OH can be used. Solubilization in simple Tris buffers at pH 8.0 can be tried, but resolubilization in such buffers is sometimes slow and incomplete.

The most frequent reasons for inadequate delipidation, non quantitative recovery of protein, or recovery of protein with poor solubility characteristics include: (1) failure to dialyze excess KBr from the lipoprotein solution; (2) dialysis of the lipoprotein solution against distilled water rather than 0.15 M NaCl; (3) failure to keep organic solvents at 0 °C or less; (4) use of prolonged or high speed centrifugation to initially sediment the protein when the delipidation mixture contains the original volume of water (the conditions of centrifugation are much less important after the initial protein sedimentation); and (5) drying the protein in a pellet rather than a thin film. Methanol is the critical component of this organic solvent mixture.

Laser Capture Microdissection

Laser capture microdissection (LCM) is useful to prepare cells for protein profiling. Two lysis methods are described. You may want to test both methods to decide which works best for you.

Particularly when using a LCM lysis protocol for the first time, it is wise to use as many cells as possible, e.g., 5,000–10,000 cells. It might be worthwhile to run the protocol at first using test samples, i.e., samples that are not particularly precious.

Tips for LCM Protein Profiling

- Using high quality material is critical to the success of the assay. Before actually microdissecting, examine the proteins in the frozen section via a 1D gel or ProteinChip analysis to check for sample integrity.
- Consistency of sample preparation is also critical, including the person preparing the samples.
- Frozen sections are significantly better than any other method of sample preparation; paraffin samples are extremely difficult to work with.
- Ethanol fixation works best.

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- Staining by H&E can diminish the yield of proteins; some people suggest using the most diluted stains possible.
- Use fresh stain every time. Some people use protease inhibitors in the staining solution.
- Some people suggest microdissecting cells for only a short time (e.g., 10–15 minutes) to avoid proteolysis.
- T-per, a bicine-based proprietary detergent from Pierce, has been used successfully to solubilize proteins from procured cells for immunoassay.
- GITC can also be used for lysis, but dilution is necessary prior to ProteinChip analysis, which leads to lower peak intensities.

Urea Lysis Method

Urea Lysis Buffer

8 M urea, 1% CHAPS, in PBS.

Lysis Procedure

1. Add 2 µL urea buffer to a cap containing at least 2000 to 5000 cells and pipette up and down 3–5 times.
2. Leave the lysis buffer on the cap for ~5 minutes.
3. Drop the cap with the lysis buffer into a 0.5 mL eppendorf tube containing 6 µL PBS. The final buffer concentration before applying the sample to a chip is 2 M urea, 0.25% CHAPS.

Guanidinium Lysis Method

Guanidinium Lysis Buffer

Mix 6 M guanidine thiocyanate in 50 mM HEPES, pH 7.5, with 1% deoxycholate or Triton X-100 in equal portions. Final concentrations in lysis buffer are 3 M guanidine thiocyanate, 25 mM HEPES, 0.5% deoxycholate.

Lysis Procedure

1. Add 2 µL guanidinium buffer to a cap containing at least 2000 to 5000 cells and pipette up and down 3–5 times.
2. Leave the lysis buffer on the cap for ~5 minutes.

   Caution: the buffer may dry up in low humidity, so it might be best to keep the cap in a humid chamber during this step.
3. Drop the cap with the lysis buffer into an 0.5 mL eppendorf tube containing 8 µL 50 mM HEPES. The final concentration before applying the sample to a chip is 0.6 M guanidine thiocyanate, 5 mM HEPES, 0.1% deoxycholate.

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Cytoplasmic and Membrane Isolations

Isolating Fractions for ProteinChip Proteomics

The following procedure enables you to separate membrane proteins from cytosolic proteins. In addition, the cytosolic proteins are extracted without any detergent. The cells need to be in the active log phase of growth, and you will need at least $4x10^7$ cells. As this procedure involves treating different components of the cells with separate sets of steps before analyzing them with the ProteinChip System, a protocol flow chart is included (Figure 7-1).

Flow Chart

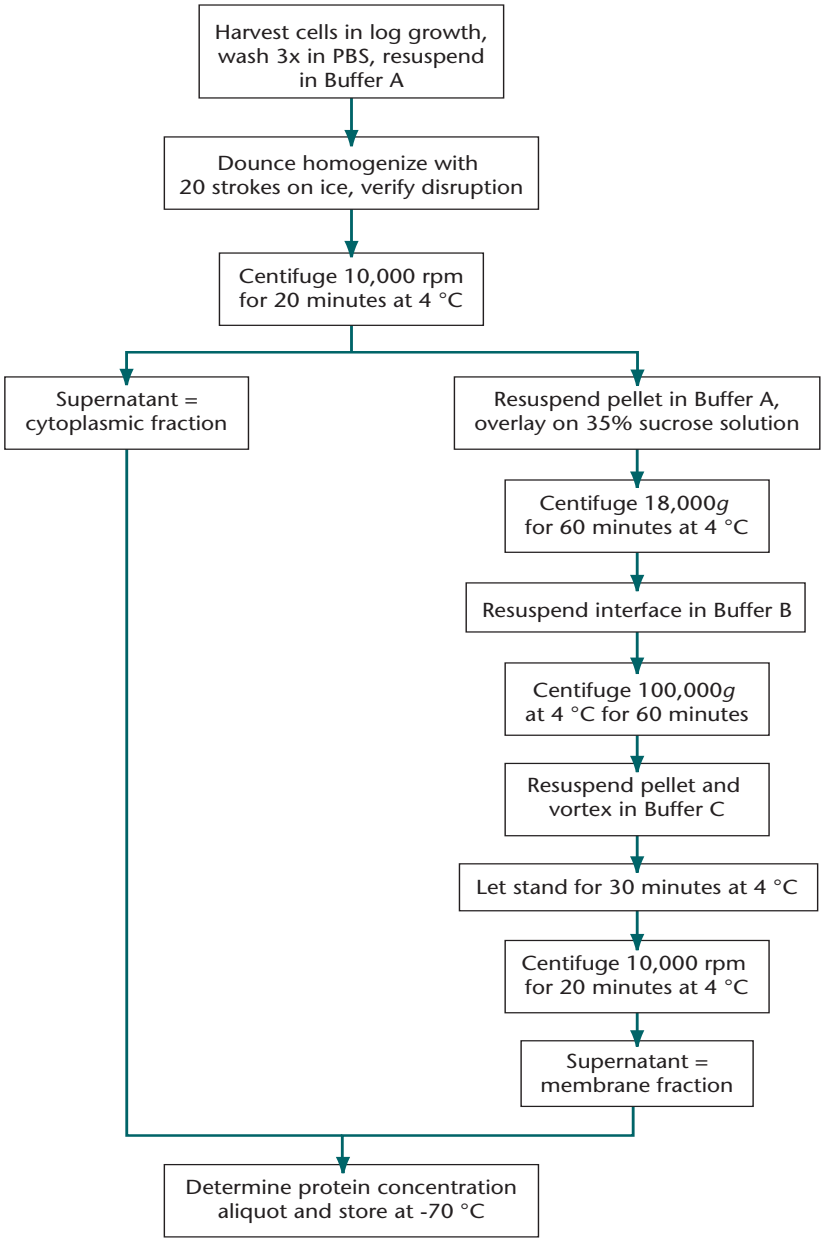

Figure 7-1: Flow chart for cytoplasmic and membrane isolations.

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Protocol

Buffers

Buffer A: 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$

Buffer B: 10 mM Tris-HCl, pH 7.4, 250 mM sucrose with fresh protease inhibitors (protease inhibitors at 1x concentration).

Buffer C: 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.5% Triton X-100, 1.0% NP-40

Method

1. Harvest mammalian cells in the log growth phase. Wash the cells by centrifuging and resuspending 2 times in PBS. Resuspend once more in 0.5 mL PBS and transfer the suspension to an 1.5-mL eppendorf tube. Centrifuge again.
2. Resuspend cells in Buffer A at ~4 x 10^7 cells/mL.
3. Add protease inhibitors to the cell suspensions. Protease inhibitors are usually sold in 10x or 20x stocks, and need to be added in volumes such that they are at 1x in the cell suspension. For example, add 50 µL 20x protease to a 1-mL cell suspension.
4. Dounce homogenize with 20 strokes on ice. Verify cellular disruption using a light microscope.
5. Centrifuge at 10,000 rpm for 20 minutes at 4 °C (i.e., at full speed on a table top centrifuge).

   Caution: if the cells are centrifuged too hard, some of the mitochondria and microsomes might be lost, depending on the speed.
6. Separate the contents of the tube into cytoplasmic and membrane fractions:

   a. Transfer the supernatant (the cytoplasmic fraction) to a fresh eppendorf tube. It contains the cytoplasmic fraction, at approximately 2–10 mg protein/mL. Store on ice until ready to determine the protein concentration.

   b. Resuspend the pellet (the membrane fraction) in 1 mL Buffer A, then overlay it on a 35% (w/v) sucrose solution.
7. Centrifuge the pellet at 18,000*g* for 60 minutes at 4 °C
8. Remove the interface. Resuspend interface in 1 mL Buffer B, then centrifuge at 100,000*g* for 60 minutes at 4 °C.
9. Resuspend the pellet thoroughly in 1 mL Buffer C containing 1x protease inhibitors, then vortex.
10. Incubate for 30 minutes at 4 °C. Centrifuge at 10,000 rpm for 20 minutes at 4 °C

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11. Transfer the supernatant into a fresh tube. It contains the membrane fraction, typically at a protein concentration ranging from 1–5 mg/mL, depending on the cell type and the homogenization.

12. Determine protein concentration of both fractions (the BioRad technique is an acceptable method). Aliquot both fractions into freezing vials and store at -70 °C until use.

Using Spin Columns for Protein Separation

Two types of spin columns are available for separating proteins prior to analysis on ProteinChip Arrays. As described below, the two classes of spin columns separate proteins according to either size or charge. Size-selection spin columns and anion-exchange spin columns may be used sequentially to maximize protein separation for ProteinChip Array analysis. Most biological samples, including serum, urine, and most cell extracts, are amenable to spin column preparation. See *"Sample Fractionation Using Spin Columns and the ProteinChip System"* on page 7-20 for an example of protein separation that combines use of both columns.

Please check with your Field Scientist regarding purchasing size-selection and anion-exchange spin columns.

Size-selection Spin Columns

Introduction

Size-selection spin columns are used to fractionate proteins from complex samples for improved ProteinChip Array analysis. Small sample volumes (20–30 µL) may be efficiently separated with the aid of a microcentrifuge. The spin columns are designed to accommodate small sample volumes while causing minimal sample dilution. The three size-selection resins, K-3, K-30 and K-70, selectively retard molecules based on size so that larger proteins or protein complexes to elute off the column first. Keep in mind that the MW cutoffs are guidelines — oddly shaped proteins, multimers and aggregates may not run through size-selection resins according to their monomeric MW.

Uses for the three types of spin columns include:

- Desalting and buffer exchanging peptide (>3 kDa) or protein samples (K-3 spin columns)
- Separating molecules and proteins smaller than 15 kDa from larger proteins (K-30 spin columns)
- Separating proteins larger than 70 kDa from smaller proteins and other low-mass molecules (K-70 spin columns)

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Important: as of August 2000, Ciphergen is planning to supply size-selection spin columns. Contact Ciphergen for current information.

Using Size-selection Spin Columns

Capacity of the Size-selection Spin Columns

Small molecules (e.g., sodium ions, buffering molecules such as HEPES) retained in the pores of the column's matrix are effectively retained at concentrations up to ~1 M. Larger molecules (500–<3000 daltons for a K-30 spin column and 5000–7000 daltons for a K-70 spin column) can be retained at concentrations in the millimolar range. Larger proteins do not enter the pores of the gel matrix; therefore capacity is not a problem since they flow around the column resin.

Special Handling of Highly Concentrated Biological Samples

At high protein concentrations, many proteins aggregate to form very large complexes. These complexes may not flow through the column easily and may become trapped between the gel beads. They typically elute in multiple fractions.

Some small molecules bind tightly to larger proteins or protein complexes. They may elute together with the protein complexes in the early fractions. the number of small molecules associated with larger proteins or protein complexes should be minimized before spin column purification.

The solution to both of these problems is to add a small amount of detergent (0.01% Triton X-100) and ~0.3 M NaCl to the samples before protein purification to decrease non-covalent molecular association. The relatively small detergent and salt molecules will be removed by the column resin if they are not present in the column's equilibration buffer.

Column Hydration

If the column has been stored dry, the resin will need to be hydrated in binding buffer as described below before the sample can be applied.

1. Gently tap the column to ensure that the dry gel has settled to the bottom of the spin column.
2. Remove the top column cap and reconstitute the column by adding 0.8 mL binding buffer. Replace the column cap and vortex vigorously for ~5 seconds. Remove any air bubbles by sharply tapping the bottom of the column. It is important to hydrate all of the dry gel.
3. Allow at least 2 hours of hydration time at room temperature prior to use. It is best to equilibrate overnight. Reconstituted columns may be stored refrigerated at 4 °C for several days. For longer periods of storage include 10 mM sodium azide (NaN_3) in the binding buffer. Allow refrigerated columns to warm to room

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temperature before use. Note that if sodium azide is present in the hydration buffer it must be washed out of the column prior to use (see *"Buffer Exchange Protocol"* on page 7-17).

Column Packing

If the resin is not uniformly packed at the bottom of the spin column, or if there are bubbles trapped in the resin, invert the column several times to resuspend the resin, then place the column upright in a rack to allow the resin to settle to the bottom of the column.

Buffer Exchange Protocol

If the column has been stored in a different buffer than the binding buffer for your assay, you will need to buffer-exchange it. Otherwise, you can immediately proceed with sample preparation for the protein fractionation/purification protocol.

1. Open the outlet cap of the spin column. Insert the column into a 1.5 mL tube.
2. Open the spin column's top cap. Centrifuge the column and tube at ~700*g* (~3000 rpm) for a few seconds.
3. Add ~750 µL of the desired equilibration buffer to the column and let it flow through the column matrix by gravity. Repeat this step two more times so that three column volumes of the new equilibration buffer have passed through the column.

Protein Purification Protocol

1. Open the outlet cap of the spin column. Insert the column into a 1.5 mL tube. Open the top cap of the spin column.
2. Centrifuge the spin column at ~700*g* (~3000 rpm) for 3 minutes in a tabletop microcentrifuge. If the eluted buffer in the tube touches the outlet tip of the spin column, empty the tube and centrifuge again. The column matrix should be packed down and semi-dry but the surface of the packed bed should not be cracked.
3. Transfer the spin column to a new 1.5 mL tube (or empty the storage buffer completely from the original tube). Slowly apply 20 to 30 µL protein sample to the center of the packed column matrix. Do not allow the sample to run down the side of the column matrix.
4. Centrifuge at ~700*g* (~3000 rpm) for 3 minutes. The purified proteins will be in the collection tube.
5. *Optional:* To collect progressively smaller proteins in subsequent fractions, repeat steps 3 and 4 of the protein purification protocol using 30 µL aliquots of buffer.

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Anion-exchange Spin Columns

Introduction

Ion-exchange chromatography (IEC) is a separation method for proteins based on their net charge. Anion-exchange spin columns are designed for fractionation of proteins so that proteins having similar pI or binding affinity to the ion-exchangers elute together. Proteins will bind to an anion-exchanger if they have a net negative charge (buffered at a pH higher than their pI) and are in a buffer having a low salt concentration. Proteins can then be eluted from the spin column using elution buffers that have either a decreasing pH or an increasing salt concentration (see page 20 for a table of suggested buffers for use with anion-exchange spin columns).

Important: as of August 2000, Ciphergen is planning to supply anion-exchange spin columns. If you would like to make your own, we suggest Pharmacia's QAE Sephadex A-50, cat. no. 17-0200-01 (100 grams). Contact Ciphergen for current information.

Note: please see Chapter 6, "Protein Biochemistry for the ProteinChip System" for introductory information regarding the isoelectric point (pI) of proteins.

Column Hydration

If the column has been stored dry, the resin will need to be hydrated in binding buffer as described below before the sample can be applied.

1. Gently tap the column to ensure that the dry gel has settled to the bottom of the spin column.
2. Remove the top column cap and reconstitute the column by adding 0.8 mL binding buffer. Replace the column cap and vortex vigorously for ~5 seconds. Remove any air bubbles by sharply tapping the bottom of the column. It is important to hydrate all of the dry gel.
3. Allow at least 2 hours of hydration time at room temperature prior to use. It is best to equilibrate overnight. Reconstituted columns may be stored refrigerated at 4 °C for several days. For longer periods of storage include 10 mM sodium azide (NaN_3) in the binding buffer. Allow refrigerated columns to warm to room temperature before use. Note that if sodium azide is present in the hydration buffer it will be washed out of the column prior to use (see below).

If the column has been stored in a different buffer than the binding buffer for your assay, you will need to buffer-exchange it. Otherwise, you can immediately proceed with sample preparation for the protein fractionation/purification protocol.

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Buffer Exchange Protocol

1. Remove the outlet cap of the spin column. Insert the column into a 1.5–2 mL tube.
2. Open the top cap of the spin column. Let the storage buffer drain into the tube by gravity until no more drops come out of the column. If the storage buffer does not come down easily, tap the column and tube unit on a hard surface several times, or centrifuge at 1000 rpm for ~20 seconds. Pour the buffer out of the tube.
3. Apply ~0.5 mL of the desired binding buffer to the column and let it flow through column matrix by gravity. Repeat this step two more times so that at least ten column volumes of new buffer have passed through the resin.

Protein Sample Preparation

1. Protein samples should be prepared in the same buffer used to equilibrate the anion-exchanger spin column. Thus, samples should have a salt concentration of 0.05 M or less and the desired pH — typically a relatively high pH (9.0) is used to allow most proteins to bind initially.
2. If the samples contain high salt concentrations, or if the pH of the sample is significantly different from that of the binding buffer, they should be first buffer-exchanged on a size-selection spin column (K-3 or K-30) equilibrated with the binding buffer.
3. Once the samples are in the correct binding buffer, they can be diluted further with binding buffer as appropriate for application to the spin column.

Protein Fractionation Protocol

1. Remove the outlet cap of the spin column. Insert the column into 1.5- or 2 mL tube. Open the top cap of the spin column.
2. Centrifuge the spin column at 1000 rpm for 20 seconds to 1 minute in a tabletop centrifuge. The column matrix should be packed down and semi-dry but should not be cracked.
3. Transfer the spin column to a new 1.5 mL tube (or empty the storage buffer completely from the first tube). Apply 20 to 500 µL protein sample in binding buffer to the top center of the packed column matrix. Allow the sample to run through the anion-exchanger resin by gravity for a few minutes or until no more drops come out of the column.
4. Centrifuge at 1000 rpm for 1 minute. The proteins collected in this first collection tube (fraction #1) do not bind to the column

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because they have neutral or positive net charges in this binding buffer, or the capacity of the column has been exceeded.

5. To maximize the capture of proteins on the anion-exchange resin, steps 3 and 4 can be repeated (i.e, reapply the sample to the column).
6. Transfer the column to a second tube. Wash the column with 100 µL binding buffer. Centrifuge at 1000 rpm for 1 minute. Save fraction #2.
7. Transfer the column to a third tube. Apply 100–200 µL of elution buffer A and wait for ~1 minute. Centrifuge at 1000 rpm for 1 minute. Save fraction #3.
8. Continue this process with the subsequent elution buffers.
9. Analyze the fractions in the ProteinChip reader, using the instructions for H4 and Normal Phase ProteinChip Arrays.

Suggested Buffers

Buffer	Buffers
Binding buffer	20 mM Tris-HCl + 5 mM NaCl, pH 9.0
Elution buffer A	20 mM Tris-HCl + 5 mM NaCl, pH 8.0
Elution buffer B	20 mM sodium phosphate, pH 7.0
Elution buffer C	20 mM sodium phosphate, pH 6.0
Elution buffer D	50 mM sodium acetate, pH 5.0
Elution buffer E	50 mM sodium acetate, pH 4.0
Elution buffer F	50 mM sodium phosphate and citrate, pH 3.4
Elution buffer G	50 mM sodium phosphate and citrate + 0.2 M NaCl, pH 3.4
Elution buffer H	50 mM sodium phosphate and citrate + 1.0 M NaCl, pH 3.4

Sample Fractionation Using Spin Columns and the ProteinChip System

This protocol describes how to fractionate biological samples using size selection and anion exchange spin columns designed for use with small sample volumes. It has been successfully applied to numerous sample types, including

- Serum
- Mammalian cell lysates
- Bacterial lysates
- Seminal plasma

A 30 µL sample containing ~5 mg/mL protein is used for a complete fractionation. Proteins are first separated by size using size-selection spin columns. The size-selection spin column also simultaneously buffer-

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exchanges the sample so that it is ready for fractionation based on the proteins' isoelectric points (pI) in anion-exchange spin columns.

It is reasonable to use elution buffers in steps of a single pH unit for this fractionation procedure, and one fraction should be collected for each change in pH. For example, collect one fraction each using buffers of pH 9.0, 8.0, 7.0, 6.0, 5.0 and 4.0.

Note
K-3, K-30 and K-70 size selection spin columns are available for sale from Ciphergen. As of August 2000, Ciphergen is planning to supply anion-exchange spin columns. If you would like to make your own, we suggest Pharmacia's QAE Sephadex A-50, cat. no. 17-0200-01 (100 grams). Contact Ciphergen for current information.

Flow Chart

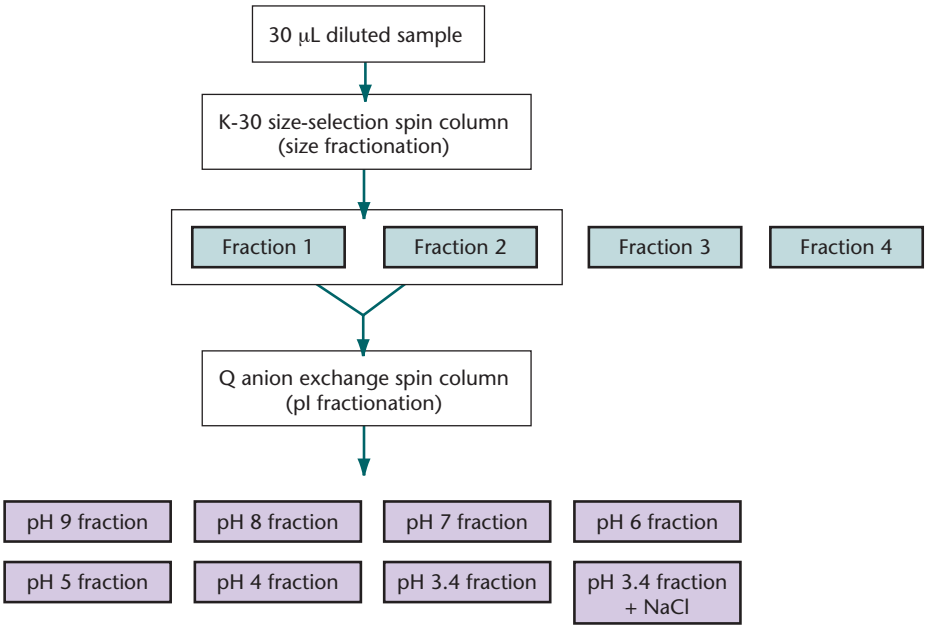

Figure 7-2: Protein fractionation using spin columns.

Protocol

Protein Fractionation by K-30 Size-Selection Spin Columns

1. Starting samples should have ~5 mg/mL protein or higher. Adjust the sample to contain 0.4 M NaCl and 0.01% (v/v) Triton X-100 using 5 M NaCl and 1% Triton stocks, so that the original sample is minimally diluted. Mix well and incubate on ice for 20 minutes.

2. Equilibrate a Ciphergen K-30 spin column in 20 mM Tris-HCl, pH 9.0, following the basic protocol for equilibrating spin columns (see *"Using Size-selection Spin Columns"* on page 7-16).

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3. Spin the column in a microcentrifuge at 700*g* (3000 rpm) for 3 minutes.

4. Apply a 30 µL aliquot of the sample to the equilibrated K-30 spin column's resin. Place the spin column in a microcentrifuge tube.

5. Spin the column in a microcentrifuge at 700*g* (3000 rpm) for 3 minutes. Transfer the column to a new tube and apply 30 µL 20 mM Tris-HCl, pH 9.0 to the column resin. Centrifuge again at 700*g* for 3 minutes. Repeat twice more to collect a total of four fractions, 30 µL per fraction.

Protein Fractionation by Q Anion-exchange Spin Columns

1. Combine fractions 1 and 2 from the K-30 column. Adjust the volume to 100 µL using 20 mM Tris-HCl, pH 9.0. Mix well and incubate on ice for 5 minutes.

2. Equilibrate a Q anion-exchange spin column with 20 mM Tris-HCl, pH 9.0.

3. Apply 100 µL sample to the column resin of the equilibrated Q anion-exchange spin column. Follow the protocol for Q anion-exchange spin columns (see *"Anion-exchange Spin Columns"* on page 7-18 for instructions).

4. Centrifuge the column at 80*g* (1000 rpm) for 1 minute. Reapply the flow-through to the same column to maximize the protein binding. Again centrifuge the column at 1000 rpm for 1 minute. The resulting fraction is the pH 9 fraction.

5. Transfer the column to a new tube. Apply 100 µL 20 mM Tris-HCl, pH 9.0 to the column resin. Centrifuge the column at 1000 rpm for 1 minute. The resulting fraction is the pH 9 wash fraction. It can be combined with the pH 9 fraction.

6. To create the pH 8 fraction, transfer the column to a new tube, apply 100 µL 20 mM Tris-HCl, pH 8.0 to the column resin. Apply a little pressure at the column top to allow buffer to move through the resin slowly. Incubate 5 minutes at room temperature. Centrifuge the column at 1000 rpm for 1 minute. The resulting fraction is the pH 8 fraction.

7. Repeat step 6 with the remaining, lower-pH buffer solutions.

8. A final fractionation using 1 M NaCl or 50% acetonitrile in low pH buffer may be desirable.

ProteinChip Analysis of Fractionated Proteins

Normal Phase chips can be used for profiling proteins from the fractions generated by spin columns. H4 hydrophobic chips are particularly suited for fractions containing a high concentration of NaCl.

1. Deposit a 1 µL aliquot of each fraction to a spot on the normal phase chip. Let the chip dry at room temperature for about 5 minutes. Additional volume of the same sample can be applied to the same spot and dried. Samples deposited on H4 chips should be washed twice with 5 µL water before letting it dry.
2. Apply EAM, either CHCA, SPA or both, to the spots as desired, following the instructions for each chip type.
3. Analyze the proteins using the PBS II ProteinChip Reader. Collect 10–15 shots per area and collect approximately 100 shots per spot in total. Also, collect two sets of data, one using low laser intensity and the other using high laser intensity.

Thanks to Thang Pham, Ph.D. for contributing this protocol.

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Chapter 8
ProteinChip Immunoassays

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This chapter contains protocols for capturing molecules with antibodies covalently coupled to PS1 or PS2 ProteinChip Arrays. For an example of a more general protein-protein interaction protocol and technical information, please read Chapter 11, *"Protein-Protein Interactions Using Preactivated Surface ProteinChip Arrays"*.

Antibodies

When using antibodies in ProteinChip Array applications, there are many aspects of the antibody preparation that will determine the relative success or failure of an assay. This section describes some important antibody preparation considerations and should be used as a guide alongside the protocols for ProteinChip Array Immunoassays. The more you know about your antibody, the better you'll be able to design an effective ProteinChip Immunoassay.

Background on Antibodies

Antibodies are used as reagents in many laboratory applications due to both their specificity and strong binding affinities. Typically, antibodies used in assays are of the IgG class, largely because they are easy to produce abundantly in mice and other animals. Like all antibodies, IgG antibodies have two identical binding sites for the antigen. The antibody typically binds to a specific series of ~3–6 amino acids on the antigen, although a much larger area can be recognized in some cases. The region to which the antibody binds is known as the epitope. The epitope may either be continuous, consisting of a linear sequence of amino acid residues, or it may be discontinuous, consisting of amino acid residues that are brought into close proximity through protein folding. Some antibodies recognize an epitope on a native protein; other antibodies recognize an epitope found only on a denatured protein. Some epitopes can be found on both the native and the denatured protein.

When performing a ProteinChip Array immunoassay, the antibody can be covalently bound to a preactivated surface ProteinChip Array to perform antigen capture. Alternatively, Protein A or Protein G can be bound to the array first, then the antibody is tethered non-covalently on the surface. In the latter case, the antibody will be seen during analysis with the ProteinChip Reader.

Monoclonal vs. Polyclonal Antibodies

Antibody preparations come in two basic categories: polyclonal and monoclonal. As the name implies, a polyclonal antibody preparation consists of many different antibodies that recognize different parts of the same target protein, i.e., different epitopes. The antibody preparation is generated by harvesting total IgG from an animal that has been injected with the target antigen to generate an immune response. Thus, polyclonal antibodies are typically 1–10%

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antigen specific and contain 90–99% contaminating antibodies. Depending on the affinity and the percentage of target-specific antibodies in the preparation, polyclonal antibodies may or may not work in ProteinChip Array applications. Polyclonal antibodies will either be supplied in crude serum or as a protein A/protein G purified fraction; for ProteinChip Array applications, polyclonals must be purified over protein A/protein G before use to eliminate serum proteins.

In contrast, a monoclonal antibody preparation is derived from a cell line which has been isolated based on its ability to produce that one specific antibody. This cell line is grown in the peritoneal cavity of an animal. When the antibodies from the peritoneal cavity are harvested, they are comprised mostly of target-specific antibodies but are invariably contaminated with some of the host's serum antibodies. Monoclonal antibody harvests contain typically ~90% antigen-specific and ~10% contaminating antibodies. Due to their purity and specificity, monoclonal antibodies are generally preferred for ProteinChip Array studies. However, the affinity and specificity of monoclonal antibodies can be highly variable from one reagent to another, and some monoclonal antibodies recognize only the denatured form of a protein, limiting their use in this context. In general, if the antibody is recommended for immunoprecipitation, it is likely to be a good reagent in the ProteinChip Array Immunoassay.

The most pure antibody preparations are antigen-purified. This means that an affinity column with the target protein has been made, the crude antibody preparation has been passed through the column, and thus the non-specific antibodies have been removed. Note that antibody preparations labelled as "affinity purified" may simply have been Protein A or Protein G purified, and therefore, the preparation may contain a high concentration of unwanted antibodies.

Antibody Preparation Purity

Many commercially available antibodies are supplied with an abundance of carrier protein such as BSA or gelatin. This information is usually, but not always, listed on the data sheet supplied with the antibody solution. Always check with the supplier if you are unsure of the composition of your antibody solution. In addition, the presence of BSA in your antibody solution can be rapidly checked by spotting a small amount on a normal phase ProteinChip Array (see *"H4 Protocol"* on page 5-4).

If your antibody contains one or more carrier proteins you will need to purify the IgG fraction using a Protein A or G coupled affinity column. The immunized animal type and antibody class will determine whether you should use Protein A or G (see table). Note

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that Protein G binds most IgG as well as or better than Protein A, but Protein G is more expensive.

Species	Protein G	Protein A
Human		
IgG_1	++	++
IgG_2	++	++
IgG_3	++	–
IgG_4	++	++
Rabbit	++	++
Cow	++	+
Horse	++	–
Goat	++	+
Guinea Pig	+	++
Sheep	++	–
Dog	+	++
Pig	++	++
Rat	+	-
Mouse	+	+
Chicken	–	–

Antibody binding preferences for Protein A and Protein G. ++ = strong binding, + = medium binding, – = weak or no binding

Alternatively, you can couple the Protein A or G to the surface of a pre-activated ProteinChip Array and purify the antibody on the chip before capturing the antigen. Attaching the antibody to the chip via Protein A/G has the advantage of orienting the antibody correctly for antigen capture. The disadvantage is that the antibody protein(s) will "fly" during data analysis and may interfere with the detection of your antigen. Typically, antibodies generate peaks at ~22 kDa (light chain), ~48 kDa (heavy chain), ~74 kDa (half antibody) and ~148 kDa (intact IgG). Antibodies coupled directly to the chip will not 'fly', because the laser will not break the covalent bond to the surface of the ProteinChip Array.

Native vs. Denatured Antigens

Some antibodies will only react with a protein that is denatured, exposing an epitope (antibody binding site) that is normally masked by the protein's secondary structure. These antibodies are suitable for Western blotting, but will not work on an ELISA or in an immunoprecipitation. In ProteinChip immunoassays, the applied analyte solution may contain native proteins. Therefore, antibodies that only work on Western blots may not work in a ProteinChip immunoassay. Check to see whether your antibody reagent can be

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used effectively in an ELISA and/or an immunoprecipitation before proceeding with your ProteinChip assay.

Buffers for Antibodies

Antibodies (or indeed any protein) that will be coupled directly to the surface of a pre-activated ProteinChip array need to be free of nucleophilic agents (e.g. Trizma base, glycine, etc.). The recommended buffer for covalent coupling is standard PBS. *Attempting to couple the antibody in Tris buffer will not work*. Many antibodies also are stored in solutions containing sodium azide as a preservative, which will inhibit antibody coupling to PS1 or PS2 chip surfaces.

Important
If in doubt, dialyze the antibody solution into PBS!

Profiling Soluble Amyloid β Peptides from Cultured Cell Media

This protocol details a method for analyzing cell culture fluid (CCF) for amyloid β peptides (Aβ) by incubating a few milliliters of CCF with the ProteinChip Array in bulk. As an alternative, it is possible to use the bioprocessor as described in the second protocol.

Antibodies

The 6E10 monoclonal antibody appears to be better than 4G8 for these experiments. 6E10 can be used to look at C-terminally truncated fragments. (4G8 recognizes an internal sequence comprising amino acids 17–24.) The 6E10 monoclonal antibody is specific for the human form of Aβ, recognizing an epitope site between amino acids 4–13. Anti-Aβ 6E10 can be obtained from Senetek (Maryland Heights, MO). Ensure that the antibody is free of carrier protein and is suspended at a concentration of 0.5 mg/mL in PBS before coupling it to the ProteinChip Array.

Cell Culture Supernatants

Mouse neuroblastoma (N2a) cells stably transfected with the human amyloid precursor protein (APP) Swedish mutant cDNA can be used effectively to produce high levels of soluble amyloid peptides easily detected by this assay. This assay has also been used to capture detectable levels of amyloid β (1–40) from cells expressing human wild-type APP.

Protocol Without a Bioprocessor (PS2 Array)

Preparation of ProteinChip Array with 6E10 Antibody

1. Place an 8 spot PS2 ProteinChip Array on a flat, clean surface.
2. Apply 1 µL PBS followed by 1 µL 6E10 monoclonal antibody, 0.5 mg/mL in PBS, to one or more spots on the 8 spot array.

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Ensure that one of the spots on the array is coupled with a control monoclonal antibody to monitor non-specific binding.

3. Immediately transfer the chip to a humidity chamber and incubate for 1–2 hours at room temperature or at 4 °C overnight.

4. Block residual active sites on the chip by placing the whole array in a 15 mL conical tube with 8 mL blocking buffer (0.5 M ethanolamine in PBS, pH 8.0). Mix gently on a rocking platform for 15 minutes at room temperature.

   Note: blocking is done in bulk here for convenience but can also be accomplished by applying 5 µL ethanolamine per spot. ***Important:*** *PS1 bulk incubations, the* ***entire*** *chip must be blocked with ethanolamine because the* ***entire*** *chip surface, both within the spots and outside the spots, is activated.*

5. Pour off the blocking buffer and add 8 mL PBS + 0.5% Triton X-100, then agitate vigorously on a shaker for 15 minutes. Pour off the wash buffer and repeat.

Capture and Purification of Amyloid β Peptides from Cell Culture Supernatant

1. Mix 2.5 mL cell culture supernatant (see notes above) with 5 mL PBS + 0.5% Triton X-100.

2. Pour off the wash buffer from Step 5 and add the diluted cell culture supernatant to the tube. Agitate vigorously for 2 hours at 4 °C.

3. Pour off the cell supernatant, add 8 mL PBS + 0.5% Triton X-100, then agitate vigorously on a shaker for 15 minutes. Pour off the wash buffer and repeat, then wash the chip 3 times with 10 mL PBS, allowing 5 minutes for each wash, followed by 2–6 brief washes with HPLC grade water. These washes eliminate salts and residual Triton X-100 from the chip.

4. Remove the chip from the tube, flick off any water and allow to air dry.

5. Carefully outline the spots with a PAP pen and allow to air dry.

Addition of EAM

1. Dissolve Ciphergen's CHCA powder in 1 mL 50% acetonitrile, 0.5% TFA. Make up a final EAM solution as follows: 400 µL acetonitrile, 150 µL 1% TFA, 250 µL water and 200 µL CHCA solution.

2. Add 0.4 µL EAM to each spot, ensuring that the solution does not flow outside the spot area.

3. Dry the chip and insert it into the ProteinChip Reader. Collect 50–100 averaged laser shots per spot using low laser intensity.

   • Collecting data with a large number of shots at low laser

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power will produce good peptide resolution.

- Insulin can be used as an external standard.
- The expected masses of human amyloid peptides can be derived from the sequence using PAWS (software available free from http://prowl.rockefeller.edu).

Protocol with a Bioprocessor (PS1 Array)

Preparation of Preactivated Surface ProteinChip Array with 6E10 Antibody

1. Covalently couple the 6E10 antibody to a PS1 ProteinChip Array and block with ethanolamine as described in the PS2 protocol that doesn't use a bioprocessor. Include a negative control antibody, such as another mouse IgG. Two spots should be used for every sample, including one spot with the 6E10 antibody and one spot with the negative control antibody.

Capture and Purification of Amyloid β Peptides from Cell Culture Supernatant

1. Apply 300 µL homogenate to each well of the bioprocessor. Rotate slowly overnight at 4 °C.
2. Wash in bulk using 9 mL per wash, with vigorous shaking for 5 minutes at room temperature per wash. Wash using the following solutions in the order given:
   - Twice with PBS/0.5% Triton X-100.
   - Once with PBS/0.5% Triton X-100/0.5 M NaCl.
   - Twice with PBS.
3. Quickly rinse the array twice in bulk in distilled water.

Addition of EAM (Energy Absorbing Molecules)

1. Apply EAM and read the chip as described above in the PS2 protocol that doesn't use a bioprocessor.

Special thanks to Huw Davies, Ph.D. and Vanitha Thulasiraman, Ph.D. for contributing to this protocol.

Epitope Mapping

The protocol below describes how to identify the peptide that contains an epitope recognized by an antibody of interest. Two methods can be used. In the first method, the antibody on the ProteinChip Array captures the intact antigen, and the antigen is then digested with a protease. Alternatively, the antigen may be

digested with protease in solution and then the epitope-containing peptide is captured from the proteolytic mixture onto an antibody on a ProteinChip Array.

Detecting Whole Antigen

It may be of interest to first determine if you can detect the antigen (if the pure protein is available) using the PBS II ProteinChip Reader, especially for a large glycoprotein. However, even if the intact protein is not visible, the fragments generated by proteolysis will more than likely be detectable, making this procedure broadly applicable.

Continuous vs. Discontinuous Epitopes

The on-chip digest is helpful in cases where you suspect a discontinuous conformational epitope, which cannot be detected via digestion in solution. Solution digests only work for continuous epitopes with limited conformational requirements.

Trypsin

Trypsin must be stored properly or it will lose its activity. Storing small aliquots at -70 °C is ideal, because repeated freezing and thawing destroys the enzyme activity. Follow the manufacturer's instructions for storage. For detailed information about storing trypsin, please see *"Important Information on Trypsin Preparation"* on page 10-15.

Flow Chart

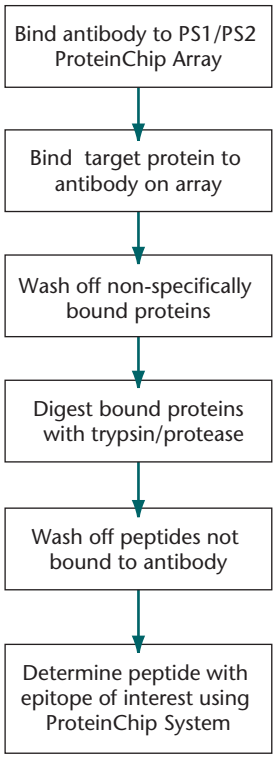

Figure 8-1: Epitope mapping ProteinChip assay protocol.

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Protocol

1. Follow the protocol for binding an antibody to a PS1 or PS2 ProteinChip Array and block with ethanolamine (see *"PS1 and PS2 Protocol"* starting on page 5-13 for details of blocking with ethanolamine). Keep the array moist until you are ready to add the antigen.
2. Dilute the target protein to 0.1 mg/mL in PBS.
3. Wipe off excess solution around the spots. Apply 2–5 µL of the target protein to each of the still-damp spots. Incubate in a humidity chamber for 2 hours at room temperature with shaking, or overnight at 4 °C.
4. Wash the chip with 7 mL of the following buffers in the order given, washing each time for 5 minutes at room temperature with shaking:
   - Twice in 0.5% Triton X-100 in PBS
   - Once in 0.5 M NaCl in PBS
   - Once in PBS (leave the chip soaking in the PBS)
5. Dilute sequencing grade trypsin in PBS to a concentration between 5 mg/mL to 50 mg/mL. Add 2 mL trypsin to the PBS the chips are soaking in, then incubate for one hour at room temperature. (For an alternate, on-chip digestion method, see below.)

   Note: the ideal amount of trypsin must be determined empirically.
6. Wash the spots three times with PBS.
7. Quickly rinse twice with water.
8. Prepare a 20% dilution of saturated CHCA in 50% acetonitrile and 0.5% TFA. Add 0.4 µL CHCA to each spot. Allow to air dry.
9. Analyze the chip in the ProteinChip Reader.

Alternative Digestion Method

In Step 5 above, you can perform an on-chip tryptic digestion. This allows trypsin to be added to the spots in smaller volumes, as outlined below:

1. Apply 5 µL of trypsin, in concentrations ranging from 1–50 µg/mL, to the sample spot. A native, glycosylated protein will require much more trypsin than a denatured protein.
2. The reaction should be carried at 37 ^{o}C for 30 minutes to 1 hour in a humidity chamber to optimize the digestion time.
3. Perform a quick PBS bulk wash and a water wash to remove peptides not bound to the antibody.

   Note: to detect peptides not bound to the antibody, run the protocol with no washes (the washes may reduce or eliminate some peptides).

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4. Prepare a 20% dilution of saturated CHCA in 50% acetonitrile and 0.5% TFA. In some cases, EAM1 at a 1:25 dilution may be added instead of CHCA to improve the signal produced by glycosylated proteins/peptides. Add 0.5 µL CHCA to each spot. Allow to air dry.

5. Analyze the chip in the ProteinChip Reader.

   Note: you may be able to get a stronger signal by immunoprecipitation with antibody-conjugated beads, since the capacity of spots is lower than the capacity of beads. Immunoprecipitate using your favorite method. Wash the beads with PBS 3 times followed by a quick water wash. Digest the captured antigen using a range of trypsin concentrations/times as suggested above. Elute the peptide(s) with 20 µL of 10% AcCN/0.5% TFA, then load 5 µL onto the H4 chip and let dry. Add EAM and analyze with the ProteinChip Reader.

Alternative Approach

Instead of binding the protein to the chip, then digesting with trypsin, it is possible to treat the pure denatured or native antigen with protease in solution and then bind the protein fragments to an antibody-coupled PS1 or PS2 chip. Again, various incubation times and/or varied concentrations of trypsin must be tested to determine the optimal conditions for your protein.

Thanks to Vanitha Thulasiraman, Ph.D. for developing this protocol.

ProteinChip Immunoassays

The following protocol describes how to couple an antibody to a PS1 or PS2 ProteinChip Array and how to use this array to capture the target protein from a solution. Be sure to read *"Antibodies"* on page 8-3 before using this protocol. Also, for a detailed protocol using specific reagents, red through Chapter 3, *"Advanced Tutorial: Antibody-Antigen Capture on PS1 and PS2 ProteinChip Arrays"*.

Both the PS1 and PS2 chips should be used for initial experiments to determine which chip is best for your specific application. It is not possible to predict this outcome beforehand — rather, the best chip to use must be determined empirically for each antibody/target pair.

Prior to performing the immunoassay, it is recommended to analyze the antibody on a normal phase ProteinChip Array to check for degradation and purity. The same can be done for purified antigens.

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Flow Chart

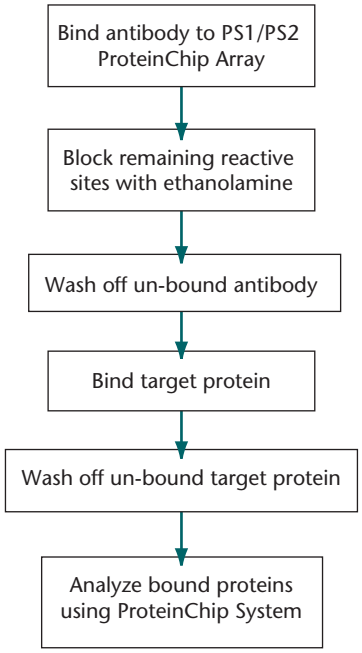

Figure 8-2: Flow chart for the ProteinChip immunoassay.

Protocol

Note: binding/washing buffer is usually PBS supplemented with detergent (0.1–0.5% Triton X-100) and/or NaCl (0.1–0.5 M). The choice of buffer is determined empirically.

Note: this protocol is the same as the basic PS1 and PS2 protocol given in Chapter 5.

Ethanolamine Preparation

This protocol requires 1 M ethanolamine (FW 97.54, Sigma catalog no. E-6133) in 1x PBS. 100 mL 1 M ethanolamine can be prepared as follows:

1. Combine:

10x PBS	10 mL
Milli-Q water	70 mL
ethanolamine HCl	9.75 g

2. Adjust the pH to ~8.0 with 1 N NaOH.
3. Add Milli-Q water to bring the final volume to 100 mL.

Array Preparation

1. If using a PS1 chip, outline each spot with a PAP pen. Allow to air dry.
2. Place the preactivated chip in a humidity chamber. Pre-equilibrate the chemical surfaces by applying 3 µL PBS to each spot.

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3. Apply 1 µL of a solution containing 0.2–0.5 mg/mL (approximately 1 picomole) of the protein of interest to each spot. For a negative control use a similar concentration of a different protein on another spot.

4. Incubate for 1 hour in the humidity chamber at room temperature or at 4 °C overnight to allow the protein to covalently bind to the spots.

5. Block residual active sites by adding 3 µL 1 M ethanolamine (made up in PBS and adjusted to pH 8). Incubate for 30 minutes at room temperature in the humidity chamber.

6. Remove unbound proteins by washing the chip in a 15 mL conical tube containing 7 mL PBS + 0.5% Triton X-100. Place the tube containing the chip and the wash buffer on a shaking platform for 15 minutes at room temperature. Repeat the wash.

7. Wash with PBS three times, 5 minutes each. This wash is to eliminate the Triton X-100, since residual detergent can cause samples to spread in the next step, especially on PS2 chips.

8. Wipe off excess buffer around the spots. At this point, it is acceptable if the spots dry out slightly. After the next step, however, it is imperative to keep the spots moist until after the final wash step, just prior to adding EAM.

Capture of Specifically Binding Protein

Note: in general, when a range of concentrations is given for buffer preparation, the less stringent wash conditions (i.e., less salt and less detergent) are recommended for the PS2 chips. Similarly, vigorous, bulk washes are often necessary to remove non-specifically binding proteins from PS1 chips, while spot washes are generally recommended for PS2 chips.

1. Prepare the protein extract in the usual way for the tissue to be examined. The solution should contain 0.1–0.5% Triton X-100 or other detergent to prevent non-specific binding to the chip.

2. Apply 5 µL protein extract to each spot and incubate in a humidity chamber at 4 °C overnight or for 4 hours at room temperature.

3. Wash the chip with 3 x 5 µL binding buffer. The final wash can be performed in bulk in a 15 mL tube for 10 minutes with vigorous shaking. For PS2 chips, a final 1 minute bulk wash may be useful; however, PS2 chips have a lower binding capacity and bulk washes may be too harsh for some protein-protein interactions.

4. Rinse the chip in bulk with binding buffer that does not contain detergent.

5. Rinse the chip in bulk with HPLC-grade or Milli-Q water.

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6. Dry the edges of the chip with a lab wipe and allow the spots to air dry.
7. Apply 0.5 µL saturated EAM solution to each spot. If using PS2 chips, two additions of 0.5 µL EAM are best.
8. Analyze the chip using the ProteinChip Reader.
9. Add 0.5 µL saturated EAM solution to each spot and analyze the chip using the ProteinChip Reader.

Notes

Chip Handling

Only remove the chip from its shipping tube when you are ready to add the antibody solution.

Never allow the spots to dry until the experiment is completed.

Wash Buffers

The binding and wash buffer is usually PBS supplemented with detergent (Triton X-100 0.05–0.5%) and/or NaCl (0.1–0.5 M). The choice of buffer is determined empirically. The same wash buffer should be used for all steps in the procedure — including washing to remove unbound antibody

Analyte Concentration

Always dilute analyte solutions containing antigen at least 1:1 with wash buffer. Higher dilutions of the sample into wash buffer can reduce non-specific binding but may also reduce the concentration of antigen too much. The optimal ratio of sample to wash buffer will need to be determined empirically. If adding purified antigen, dilute the protein into wash buffer containing 0.5 mg/mL BSA as a blocking agent. The final optimal antigen concentration should be 100–300 nM for proteins >15 kDa and 20–200 nM for proteins and peptides <15 kDa.

Using the Bioprocessor in ProteinChip Immunoassays

1. Remove an 8 spot PS1 ProteinChip array from its storage tube and outline the spots with a PAP pen. Do not let the solution get onto the spots. Allow to air dry for 5 minutes.

   Note: the bioprocessor can also be used successfully with PS2 ProteinChip immunoassays. However, this protocol has been designed to work with PS1 arrays. Contact Ciphergen for guidance in adapting this protocol to work with PS2 arrays.

2. Apply 1–2 µL antibody (0.2–0.5 mg/mL) to each spot on the chip. Place the chip in humidity chamber and incubate for two hours at room temperature or overnight at 4 °C. Do not let the antibody solution dry out on the spot.

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3. Place the chip in a 15 mL tube and add 8 mL blocking buffer (1 M ethanolamine in PBS, pH 8.0 — you will have to adjust the pH to 8 with concentrated HCl before use; store stock of blocking buffer at 4 °C). Place the tube on a rocking platform for 15 minutes. Allow to incubate for 30 minutes at room temperature in a humidity chamber.

4. Pour off the blocking buffer and add 8 mL wash buffer (PBS + 0.5% Triton X-100) to the same tube and place it on a rocking platform at the highest speed setting for 15 minutes. Pour off the wash buffer and repeat the wash twice more.

5. Remove the chip from the tube. Do not allow the buffer to dry on the surface of the chip. Assemble the chip in the bioprocessor (see *"Using the Bioprocessor"* on page 3-7 for instructions). Add 25–350 µL per well analyte solution containing antigen diluted at least 1:1 (v/v) with wash buffer (PBS + 0.5% Triton X-100) (see *"Analyte Concentration"* on page 8-14 for more information on diluting analyte).

6. Cover the bioprocessor wells with plastic wrap, then agitate vigorously on a shaker for 2 hours at room temperature or overnight at 4 °C.

7. Pour off the analyte solution. The bioprocessor can be tapped gently upside down on a paper towel to remove the last of the binding and wash solutions.

8. Add 350 µL wash buffer (PBS + 0.5% Triton X-100) to each well and agitate vigorously for 15 minutes. Pour off the wash buffer and repeat twice more for a total of three washes.

9. Disassemble the bioprocessor and place the chip in a 15 mL tube. Rinse briefly with 8 mL PBS followed by two rinses with 8 mL filtered deionized water.

10. Remove the chip from the tube and flick off excess water. While the spots are still slightly damp, add 0.4 µL EAM to each spot, ensuring that EAM does not flow outside the area of the spot. If necessary, outline the spots with the PAP pen and let the chip air dry prior to adding EAM. Allow EAM to air-dry.

11. Analyze in the ProteinChip Reader.

Bulk Incubations for ProteinChip Immunoassays

Antibody coupling and blocking steps are performed as described for using the bioprocessor.

1. Prepare the protein extract in the usual way for the cells or tissue to be examined. The solution should contain detergent to prevent non-specific binding to the chip

2. Place the chip in a 15 mL tube and add 2–5 mL protein extract. Ensure that enough extract is added to cover the chip. Incubate the chip with vigorous shaking at 4 °C overnight (or for a few hours at room temperature).
3. Pour off the analyte solution and wash with 10 mL wash buffer. Wash 3 times, 15 minutes per wash, with vigorous shaking.
4. Rinse the chip with wash buffer (without detergent).
5. Finally, rinse the chip with HPLC grade or Milli-Q water.
6. Dry the edges of the chip and add 0.5 µL EAM, ensuring that EAM does not flow outside the spot area.

Thanks to Huw Davies, Ph.D., Lee Lomas, Ph.D. and Kathryn Vardy, Ph.D. for contributing to the development of these methods.

Quantitation of Analyte Captured by Antibody

This protocol may be used to quantitate the amount of a particular protein in a solution by capturing the protein from solution onto an antibody coupled to either a PS1 or PS2 ProteinChip Array. A purified source of the analyte of interest must be available for this protocol and this protocol is recommended for analytes of MW 25 kDa or less. First, a standard curve is generated by capturing the molecule of interest from solutions of known concentration, using an antibody-coupled ProteinChip Array. Next, the sample with the unknown concentration of analyte is assayed and the concentration of the unknown is extrapolated from the standard curve.

Flow Chart

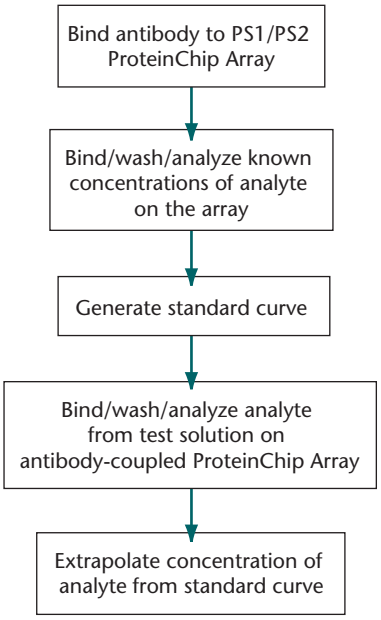

Figure 8-3: Protocol for quantitating captured analyte by using a standard curve.

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Protocol

Preparing a Standard Curve Using Purified Capture Molecule of Interest

1. Covalently bind the antibody to either the PS1 or PS2 chip and block with ethanolamine.
2. Make a 5-fold dilution series of the capture molecule in the solution of choice (see note below). Prepare each dilution in triplicate to allow the assay to be repeated. Also, make enough solution to use the bioprocessor, e.g., 300 µL for three samples of 100 µL each.

   Note: the solution of choice depends on the type of sample being analyzed in the final experiment. If the final experiment of interest entails using a lysate, then use a comparable lysate to dilute the purified target protein. Obviously, one must use a lysate that lacks any endogenous target protein so that the only source of target protein is added exogenously.
3. Attach the bioprocessor to the ProteinChip Array.
4. Apply 100 µL of the various concentrations of the protein solution to individual wells of the bioprocessor.
5. Allow the protein to bind for 4 hours at room temperature or overnight at 4 °C.
6. Wash the chip 3 times, using 100 µL PBS/0.1% Triton X-100 per wash. For each wash, shake the bioprocessor on a rotator for 15 minutes at high speed.
7. Rinse the chip with deionized water.
8. Remove the bioprocessor and rinse the chip again with deionized water.
9. Add EAM solution to the spots, being careful to keep the solution within the spot area. Read the chip in the ProteinChip Reader.
10. Export the peak intensities to a spreadsheet program and create a standard curve by plotting peak intensity *vs.* concentration.

Analyzing the Experimental Sample to Determine Analyte Concentration

1. Covalently bind the antibody to either the PS1 or PS2 chip and block with ethanolamine (see *"PS1 and PS2 Protocol"* on page 5-13 for instructions).
2. Make a two-fold dilution series of the solution that contains the capture molecule of interest. The dilutions should be made in the solution of choice (see notes above), and the peak intensities should vary from 1–50. It may take some practice to gain an intuitive feel for which dilutions will yield peak intensities in the desired range.

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3. Follow steps 3–9 of the protocol above.
4. The peak intensities obtained from the experimental sample should be directly extrapolated onto the standard curve to determine the concentration in the sample. Remember to take the dilution factors into account when calculating the concentration of the original sample.

Special thanks to Vanitha Thulasiraman, Ph.D. and Tai-Tung Yip, Ph.D. for developing this protocol.

Chapter 9

Protein Profiling with the ProteinChip System

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LCM Protein Profiling

This protocol describes how to perform basic protein profiling using cells derived from laser capture microdissection (LCM). Two lysis methods are described in *"Laser Capture Microdissection"* on page 7-11. You may want to test both methods in order to decide which method works best for you. Before using this protocol, please read the *"Tips for LCM Protein Profiling"* on page 7-11 for valuable information on sample preparation for LCM.

Particularly when using this protocol for the first time, it is wise to use as many cells as possible, e.g., 5,000–10,000 cells. It might be worthwhile to run the protocol at first using test samples, i.e., samples which are not particularly precious.

This protocol uses four chip types (SAX2, H4, IMAC3 and NP) but other chip types can also be used.

Flow Chart

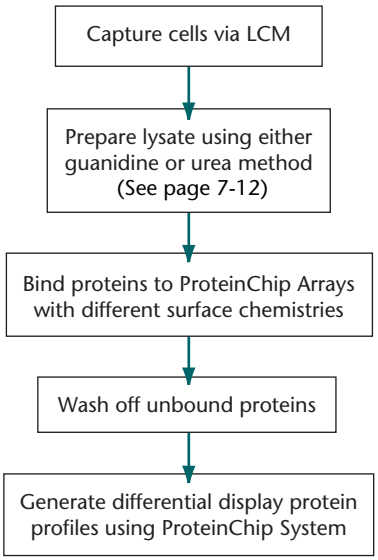

Figure 9-1: Protocol for protein profiling of LCM lysis products using the ProteinChip System.

Notes

- If GITC was used for lysis, dilution will be necessary prior to ProteinChip analysis, which may lead to lower peak intensities.
- 0.1% Triton X-100 or NP-40 can be included in the wash buffer, especially if bulk washes will be performed. Perform two final washes with buffer alone to remove Triton polymers from the low mass region.

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Protocol

Preparing the Chips

1. Prepare the SAX2 chip:

   a. Remove excess buffer with tissue immediately before applying samples to spots.

   b. Apply 5 µL 20 mM Tris, pH 7.0, to each spot.

   c. Incubate for 5 minutes at room temperature.

2. Prepare the H4 chip:

   a. Pre-wet each spot with 2 µL 50% acetonitrile.

3. Prepare the IMAC3 chip:

   a. Load 5 µL 50 mM $CuSO_4$ per spot and incubate for 5 minutes at room temperature. Repeat.

   b. Rinse off excess Cu(II) with distilled water.

   c. Load each spot with 5 µL 0.1 M sodium acetate buffer, pH 4.0, to remove unbound copper.

   d. Rinse the chip with water, then load 5 µL PBS per spot and incubate for 5 minutes at room temperature.

   e. Tap the PBS off of the chip.

4. Prepare the NP chip:

   a. Pre-wet each spot with 2 µL 50% acetonitrile.

Sample Loading

1. Gently vortex the samples, then load them onto the chips:

   a. Load 2 µL sample onto each spot of the SAX2 chip.

   b. Load 2 µL sample onto each spot of the H4 chip (remove the 50% acetonitrile immediately before this step).

   c. Load 3 µL PBS (or 3 µL PBS + 0.5 M NaCl) onto each spot of the IMAC3 chip, then add 2 µL sample to each spot. Mix together on the chip.

   d. Load 2 µL sample onto each spot of the NP chip (remove the 50% acetonitrile immediately before this step).

2. Incubate the chips in a humidity chamber for 2 hours at room temperature.

   Note: steps 1–2 may be repeated as desired, in order to use up all of the sample. After incubation, pipette off the unbound material and transfer to a normal phase chip, then load the additional sample as in step 1.

3. Transfer the unbound material to a normal phase chip and incubate in a humidity chamber for 1 hour at room temperature.

Washing

1. Different washes are necessary for different chip types.

   a. *SAX2 chip:* wash each spot of the SAX2 chip 5 times with 5 µL PBS per wash. Follow with 2 washes of 5 µL water per wash per spot. Add 2 x 0.5 µL saturated SPA in 50% ACN, 0.5% TFA per spot.

   b. *H4 chip:* wash each spot 3 times with 5 µL 50% acetonitrile. Follow with 2 washes of 5 µL water per wash per spot. Add 1 x 0.5 µL SPA per spot.

   c. *IMAC3 chip:* wash 3 times with PBS, 5 minutes per wash at room temperature, either in bulk or adding 3 µL PBS to each spot. Apply 2 x 0.5 µL SPA per spot.

   d. *NP chip:* wash 3 times in succession with 5 µL water per spot (or wash 3 times with 5 µL 0.1 M NaCl, then 2 times with 5 µL water. Apply 1 x 0.5 µL SPA per spot.

2. Analyze the chips using the ProteinChip Reader.

   Note: CHCA may be used as the EAM to enhance detection of lower molecular weight proteins.

Alternative Wash Solutions

Other washes (listed below) may be substituted for the washes in the protocol above, for instance when examining a single pair of samples.

SAX2:

PBS
PBS+1 M NaCl,
0.1 M sodium acetate pH 4 plus up to 0.5 M NaCl

H4:

water
50% acetonitrile

IMAC-copper:

PBS+0.5 M NaCl,
PBS+25 mM imidazole

NP:

water
0.1 M sodium acetate, pH 4.0, plus up to 0.5 M NaCl, 25% acetonitrile

Special thanks to Sau-Mei Leung, Ph.D., Vanitha Thulasiraman, Ph.D., James LeBlanc, Ph.D., Tai-Tung Yip, Ph.D., Eric Fung, M.D., Ph.D., and Deb Diamond, Ph.D. for developing this protocol.

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Serum Protein Profiling Using the 96-well Bioprocessor

Figure 9-2 depicts the analysis of proteins from four different serum samples (A, B, C, D) on ProteinChip Arrays having four different surface chemistries (SAX2, IMAC-Cu(II), normal phase and WCX2). Each sample was washed with two different washes per chip type (wash 1 and wash 2); the washes varied with the surface chemistry of the chip. Each sample was then analyzed using two EAMs (SPA and CHCA). Thus, each sample was analyzed under a total of 16 different conditions in order to maximize the diversity of proteins detected. Samples were analyzed in triplicate to minimize the impact of experimental variability. An experiment of this type is useful for initial studies of a small set of samples. The results from such a pilot study can be used to determine which chips provide the largest number of differentially displayed proteins for your particular sample.

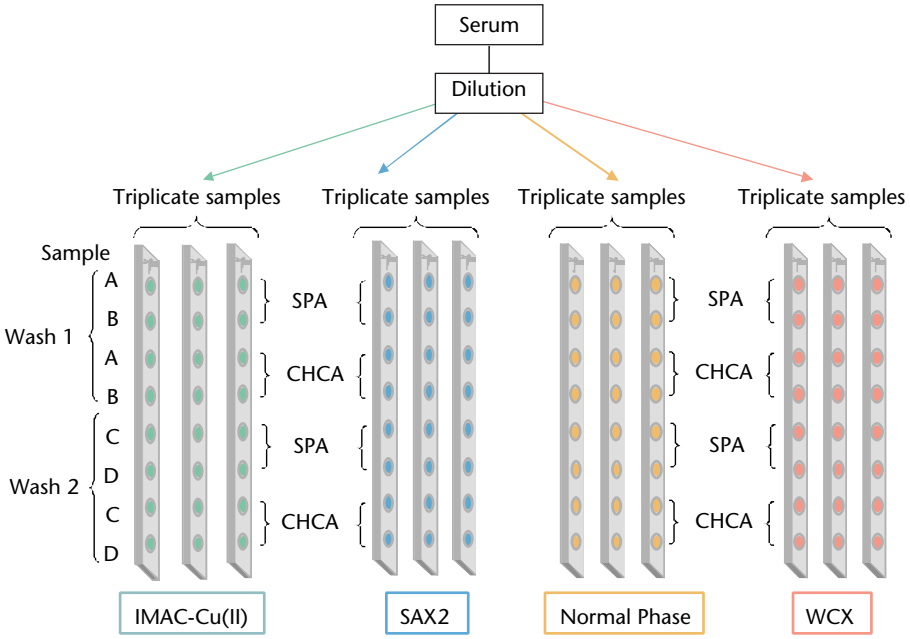

Figure 9-2: Serum protein profiling with four chip types.

After choosing one or two preferred chip types, the protocols below can be used to analyze numerous samples on the chosen chip type. Specifically, the protocols below describe how to analyze eight different serum samples on a single chip type under sixteen different bind/wash/EAM conditions. This setup is illustrated in Figure 9-3. While this protocol has been developed using human serum samples, the same general experimental setup can be used for protein profiling any sample of interest.

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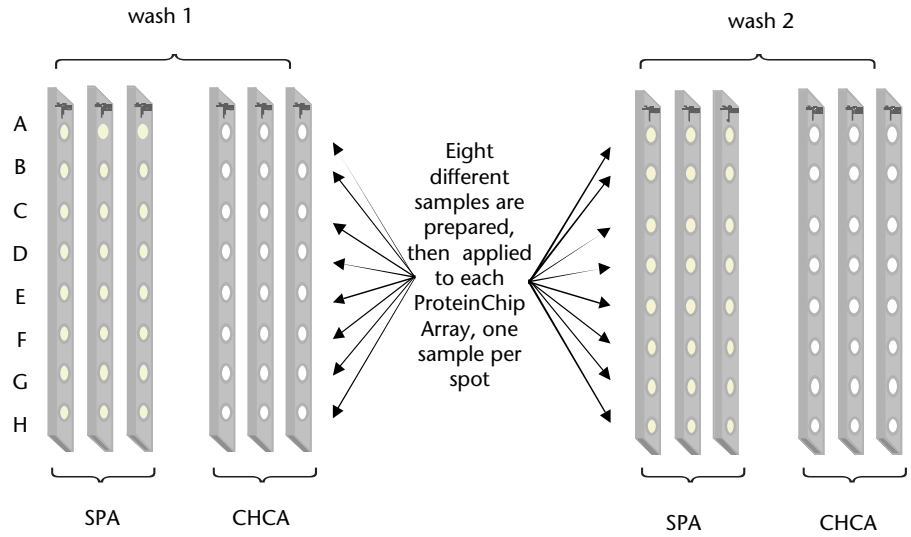

Figure 9-3: Serum protein profiling with a single surface chemistry.

Notes

- Before performing this protocol, please read *"Using the Bioprocessor"* on page 3-7 to 3-9.
- Please read Chapter 2, *"Basic Tutorial: Calibrating the PBS II ProteinChip Reader"* before using this protocol. When collecting data under different time lag focusing settings, it is important to calibrate the instrument first for that focus mass before collecting data.

Flow Chart

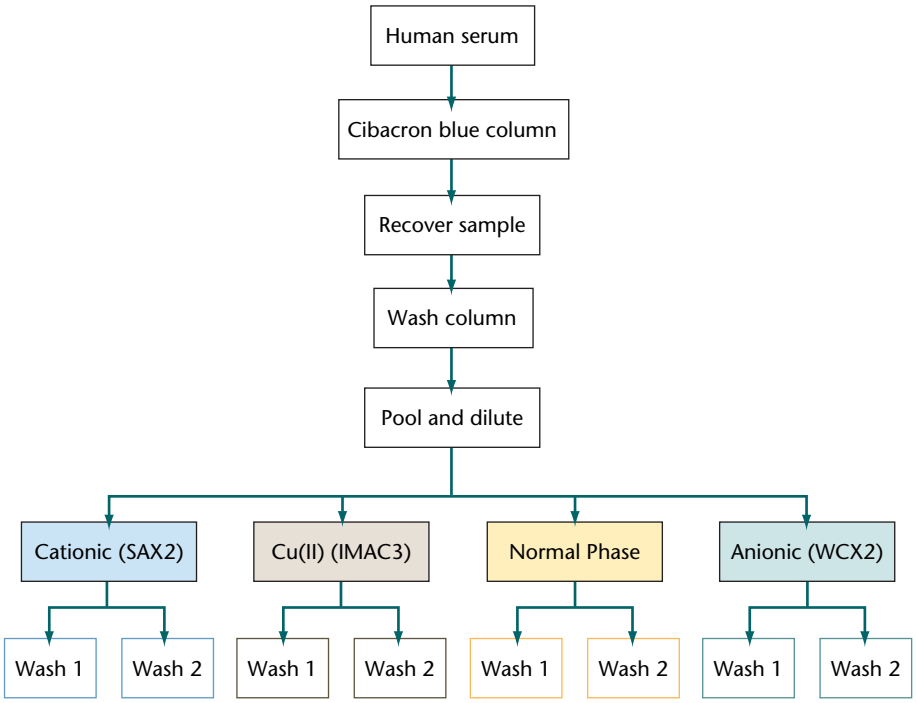

Figure 9-4: Flow chart for serum protein profiling on four chip types.

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Protocol

Serum Preparation for Protein Profiling.

Buffers

- Prepare a buffer of 8 M urea (Sigma electrophoresis grade), 1% CHAPS, 1x PBS.
- Prepare a second buffer of 1 M urea, 0.125% CHAPS, 1x PBS.

Note: when following the protocol, be careful to use the correct buffer!

Serum Preparation

1. To 40 µL human serum add 60 µL 8 M urea buffer.
2. Vortex at 4 °C for 10 minutes (an automatic vortexer is suited to this purpose).
3. Prepare a Cibacron Blue spin column (Sigma cat. no. C-1285, Cibacron Blue 3GA immobilized on 4% beaded agarose). Alternatively, anti-HSA antibody coupled beads can be used.
   a. Start with 150 µL of a 50% (v/v) bead suspension.
   b. Equilibrate with 3 x 300 µL of the 1 M urea, 0.125% CHAPS, 1x PBS buffer. Spin the column dry at 1000*g*, 30 seconds per time.
4. Add 100 µL of the diluted serum sample to the column.
5. Wash the original serum tube with 100 µL of the 1 M urea, 0.125% CHAPS, 1x PBS buffer. Add the wash buffer to the column.
6. Put the column into a fresh collection tube. Vortex at 4 °C for 15 minutes.
7. Centrifuge the column at 1000*g* for 30 seconds. Save the filtrate in the collection tube.
8. Add 100 µL of the 1 M urea, 0.125% CHAPS, 1x PBS buffer to the column.
9. Put the column into a fresh collection tube. Vortex at 4 °C for 15 minutes.
10. Put column back into the first collection tube. Spin the column at 1000*g* for 30 seconds.
11. Pool the 2 filtrates. The volume should be approximately 300 µL.

Protein Profiling on SAX2 ProteinChip Arrays

Wash Solutions

Approximately 30 mL of each wash solution will be needed.

Wash 1: 0.25 M NaCl, 50 mM HEPES, pH 7.4

Wash 2: 1 M urea 0.125% CHAPS, 0.25 M NaCI, 50 mM HEPES, pH7.4

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Protein Profiling

1. Put 12 SAX2 chips in the bioprocessor. Attach the top securely.
2. Add 200 µL 50 mM HEPES, pH 7.4 to each well. Mix at 250 rpm on a platform shaker for 5 minutes at room temperature.
3. Discard the buffer and repeat the wash with fresh HEPES, pH 7.4.
4. Dilute the serum albumin-depleted serum samples 10-fold in 50 mM HEPES, pH 7.4.
5. Apply 50 µL diluted serum sample to each spot.
6. Mix at 250 rpm on a platform shaker for 15 minutes at room temperature.
7. Remove the samples from the SAX2 chips.
8. Wash the chips:
   a. To chips 1–6, add 200 µL of 0.25 M NaCl, 50 mM HEPES, pH 7.4 (Wash 1).
   b. To chips 7–12, add 200 µL of 1 M urea 0.125% CHAPS, 0.25 M NaCI, 50 mM HEPES, pH7.4 (Wash 2).
   c. Mix at 250 rpm on a platform shaker for 5 minutes at room temperature. Remove the washes from the spots.
9. Wash the chips again:
   a. To chips 1–6, add a fresh 200 µL of 0.25 M NaCl, 50 mM HEPES, pH 7.4 (Wash 1).
   b. To chips 7–12, add a fresh 200 µL of 1 M urea 0.125% CHAPS, 0.25 M NaCI, 50 mM HEPES, pH7.4 (Wash 2).
   c. Mix at 250 rpm on a platform shaker for 5 minutes at room temperature. Remove the washes from the spots.
10. Wash the chips a third time:
    a. To chips 1–6, add a fresh 200 µL of 0.25 M NaCl, 50 mM HEPES, pH 7.4 (Wash 1).
    b. To chips 7–12, add a fresh 200 µL of 1 M urea 0.125% CHAPS, 0.25 M NaCI, 50 mM HEPES, pH7.4 (Wash 2).
    c. Mix at 250 rpm on a platform shaker for 5 minutes at room temperature. Remove the washes from the spots.
11. Wash the chips 3 times with water by filling and emptying the bioprocessor wells. Remove the chips from the bioprocessor and rinse them well with deionized H_2O.
12. Air dry the chips. Outline the spots with a PAP pen if it has washed off during the procedure.

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13. Apply EAM:

    a. Add 0.5 µL SPA to chips 1–3 and 7–9 two times (air dry the spots between additions).

    b. Add 0.5 µL CHCA to chips 4–6 and 10–12.

Protein Profiling on IMAC3 ProteinChip Arrays

Wash Solutions

Approximately 30 mL of each wash solution will be needed.

Wash 1: PBS buffer, pH 7.2

Wash 2: 1 M urea 0.125% CHAPS, 0.5 M NaCI, 100 mM acetate, pH 4.5

Protein Profiling

1. Add 10 µL of 100 mM $CuSO_4$ to each spot on 12 IMAC3 chips.
2. Mix on a shaker for 5 minutes at room temperature.
3. Rinse the chips with water.
4. Wash each spot with 10 µL 100 mM sodium acetate, pH 4.0 on a shaker for 5 minutes at room temperature.
5. Rinse the chips with water.
6. Put the chips in the bioprocessor and attach the top securely.
7. Add 200 µL PBS buffer, pH 7.2, to each well of the bioprocessor.
8. Mix at 250 rpm on a platform shaker at room temperature for 5 minutes. Discard the buffer.
9. Repeat the wash with fresh PBS.
10. Dilute the serum albumin-depleted serum sample 10-fold in PBS.
11. Apply 50 µL diluted serum to each spot.
12. Mix at 250 rpm on a platform shaker at room temperature for 15 minutes.
13. Remove the samples from the chips.
14. Wash the chips:

    a. To chips 1–6, add 200 µL PBS (Wash 1).

    b. To chips 7–12, add 200 µL 1 M urea 0.125% CHAPS, 0.5 M NaCI, 100 mM acetate, pH4.5 (Wash 2).

    c. Mix at 250 rpm on a platform shaker at room temperature for 5 minutes. Remove the washes from the spots.

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15. Wash the chips again:

    a. To chips 1–6, add a fresh 200 µL PBS (Wash 1).

    b. To chips 7–12, add a fresh 200 µL 1 M urea 0.125% CHAPS, 0.5 M NaCI, 100 mM acetate, pH4.5 (Wash 2).

    c. Mix at 250 rpm on a platform shaker at room temperature for 5 minutes. Remove the washes from the spots.

16. Wash the chips a third time:

    a. To chips 1–6, add a fresh 200 µL PBS (Wash 1).

    b. To chips 7–12, add a fresh 200 µL 1 M urea 0.125% CHAPS, 0.5 M NaCI, 100 mM acetate, pH4.5 (Wash 2).

    c. Mix at 250 rpm on a platform shaker at room temperature for 5 minutes. Remove the washes from the spots.

17. Wash the chips 3 times with water by filling and emptying the bioprocessor wells. Remove the chips from the bioprocessor and rinse them well with deionized H_2O.

18. Air dry the chips. Outline the spots with a PAP pen if it has washed off during the procedure.

19. Apply EAM:

    a. Add 0.5 µL SPA to chips 1–3 and 7–9 two times (air dry the spots between additions).

    b. Add 0.5 µL CHCA to chips 4–6 and 10–12.

Protein Profiling on Normal Phase (NP) ProteinChip Arrays

Wash Solutions

Approximately 30 mL of each wash solution will be needed.

Wash 1: 50 mM HEPES, pH 7.4

Wash 2: 50% acetonitrile in water

Protein Profiling

1. Put 12 NP chips in the bioprocessor. Attach the top securely.
2. Add 200 µL 50 mM HEPES, pH 7.4, to each well. Mix at 250 rpm on a platform shaker at room temperature for 5 minutes.
3. Remove the HEPES from the spots and repeat the wash with fresh HEPES.
4. Dilute the serum albumin-depleted serum samples 10-fold in 50 mM HEPES, pH 7.4.
5. Apply 50 µL diluted serum sample to each spot.

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6. Mix at 250 rpm on a platform shaker at room temperature for 15 minutes. Remove the samples from the spots.
7. Wash the chips:
   a. To chips 1–6, add 200 µL 50 mM HEPES, pH 7.4 (Wash 1).
   b. To chips 7–12, add 200 µL 50% acetonitrile in water (Wash 2).
   c. Mix at 250 rpm on a platform shaker at room temperature for 5 minutes. Remove the washes from the spots.
8. Wash the chips again:
   a. To chips 1–6, add a fresh 200 µL 50 mM HEPES, pH 7.4 (Wash 1).
   b. To chips 7–12, add a fresh 200 µL 50% acetonitrile in water (Wash 2).
   c. Mix at 250 rpm on a platform shaker at room temperature for 5 minutes. Remove the washes from the spots.
9. Wash the chips a third time:
   a. To chips 1–6, add a fresh 200 µL 50 mM HEPES, pH 7.4 (Wash 1).
   b. To chips 7–12, add a fresh 200 µL 50% acetonitrile in water (Wash 2).
   c. Mix at 250 rpm on a platform shaker at room temperature for 5 minutes. Remove the washes from the spots.
10. Air dry the chips. Outline the spots with a PAP pen if it has washed off during the procedure.
11. Apply EAM:
    a. Add 0.5 µL SPA to chips 1–3 and 7–9.
    b. Add 0.5 µL CHCA to chips 4–6 and 10–12.

Protein Profiling on WCX2 ProteinChip Arrays

Wash Solutions

Approximately 30 mL of each wash solution will be needed.

Wash 1: 100 mM ammonium acetate, pH 6.0

Wash 2: 1 M urea 0.125% CHAPS, 0.25 M NaCl, 100 mM acetate, pH 6.0

Protein Profiling

1. Place 12 WCX2 chips in the bioprocessor. Attach the top securely.
2. Apply 200 µL 100 mM ammonium acetate, pH 6.0, to each spot. Mix at 250 rpm on a platform shaker at room temperature for 5 minutes.

3. Discard the buffer and repeat the wash with fresh ammonium acetate.
4. Dilute the albumin-depleted serum sample 10-fold in 100 mM acetate buffer, pH 6.0.
5. Apply 50 µL diluted serum sample to each spot.
6. Mix at 250 rpm on a platform shaker at room temperature for 15 minutes. Remove the samples from the spots.
7. Wash the chips:
   a. To chips 1–6, add 200 µL 100 mM ammonium acetate, pH 6.0 (Wash 1).
   b. To chips 7–12, add 200 µL 1 M urea 0.125% CHAPS, 0.25 M NaCI, 100 mM acetate, pH 6.0 (Wash 2).
   c. Mix at 250 rpm on a platform shaker at room temperature for 5 minutes. Remove the washes from the spots.
8. Wash the chips again:
   a. To chips 1–6, add a fresh 200 µL 100 mM ammonium acetate, pH 6.0 (Wash 1).
   b. To chips 7–12, add a fresh 200 µL 1 M urea 0.125% CHAPS, 0.25 M NaCl, 100 mM acetate, pH 6.0 (Wash 2).
   c. Mix at 250 rpm on a platform shaker at room temperature for 5 minutes. Remove the washes from the spots.
9. Wash the chips a third time:
   a. To chips 1–6, add a fresh 200 µL 100 mM ammonium acetate, pH 6.0 (Wash 1).
   b. To chips 7–12, add a fresh 200 µL 1 M urea 0.125% CHAPS, 0.25 M NaCI, 100 mM acetate, pH 6.0 (Wash 2).
   c. Mix at 250 rpm on a platform shaker at room temperature for 5 minutes. Remove the washes from the spots.
10. Wash the chips 3 times with water by filling and emptying the bioprocessor wells. Remove the chips from the bioprocessor and rinse them well with deionized H_2O.
11. Air dry the chips. Outline the spots with a PAP pen if it has washed off during the procedure.
12. Apply EAM:
    a. Add 0.5 µL SPA to chips 1–3 and 7–9 two times (air dry the spots between additions).
    b. Add 0.5 µL CHCA to chips 4–6 and 10–12.

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Sequential ProteinChip Array Analysis of Human Serum Proteins

The following data are from a human serum experiment in which a 28 kDa protein was purified away from HSA using SAX2 capture with increasing salt washes. The buffers were composed of 25 mM Tris, pH 7.5 containing 100 mM NaCl, and 25 mM Tris, pH 7.5 containing 500 mM NaCl. Note the sequential elution of first HSA, then of HSA and transferrin as the salt concentration increases.

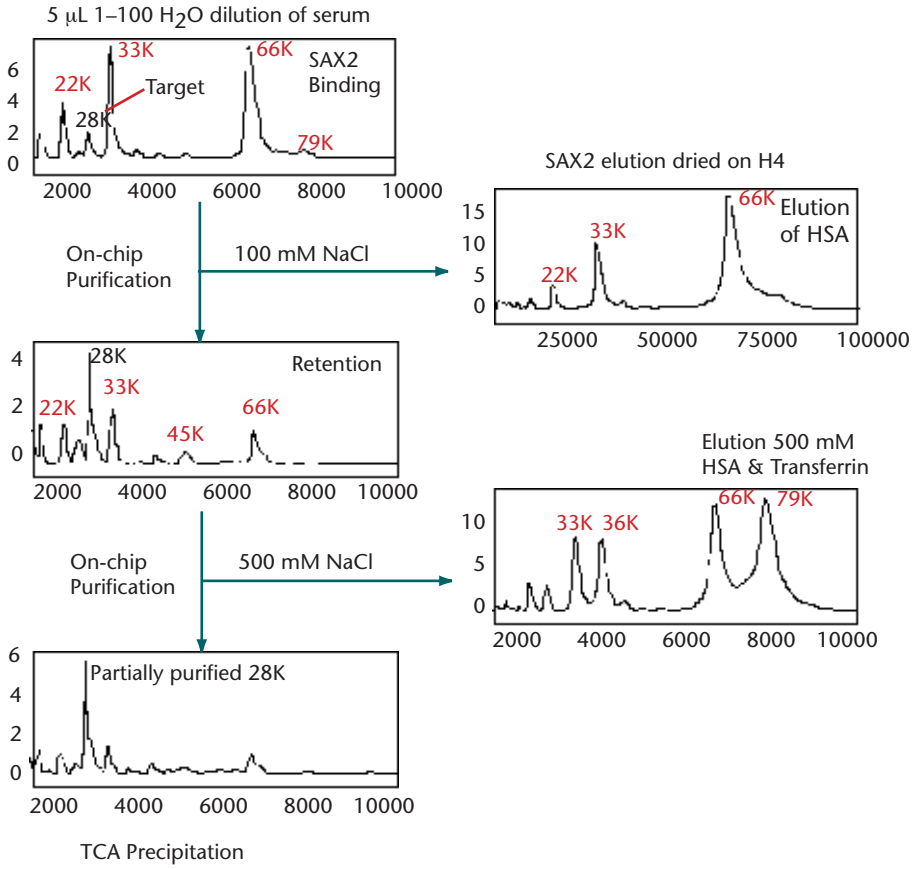

Figure 9-5: Data from sequential ProteinChip Array analysis of human serum proteins.

This approach can sometimes generate the greatest number of protein peaks on a single chip. This method could be also applied to more intractable lysates (membrane fractions, lipids, SDS, high glycerol, dye and other interfering compounds).

Thanks to Ma Sha, Ph.D. for contributing this information.

Urine Profiling

The ProteinChip System vs. 2D Gels

The urine 2D gel shown in Figure 9-6 is from a well known 2D database on the web. A total of 485 spots can be seen and 195 spots have been identified. Among them are 45 albumin spots, 30 fibrinogen spots, and many other multiple spots from single proteins. This phenomenon is due in some part to the sample processing methods for 2D gels, which result in multiple observed pIs for a single

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proteins. Therefore, although 485 spots appear on the gel, only 30 unique proteins are identified via a 2D-gel approach.

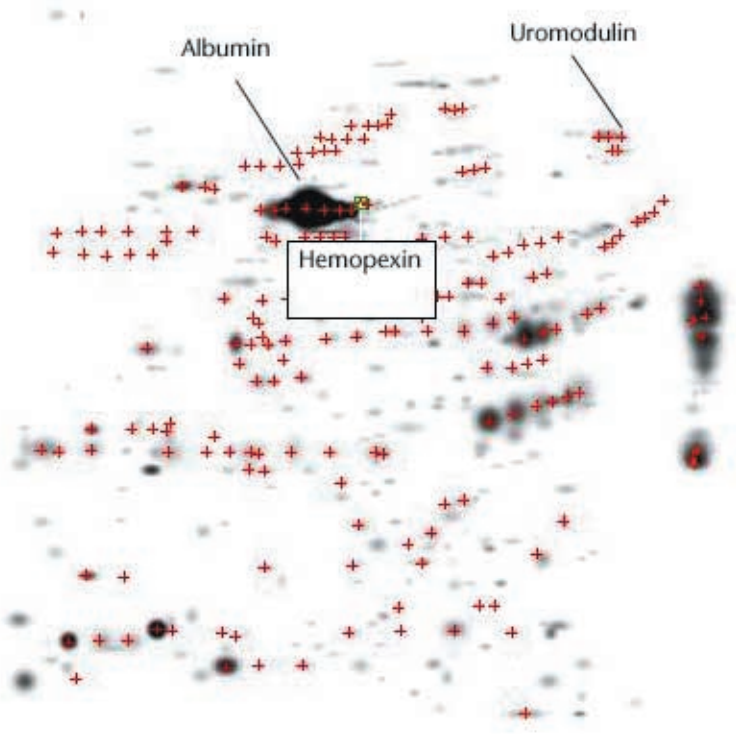

Figure 9-6: A 2D gel of urine proteins.

In addition, 2D gels require proteins to be loaded in nanomolar quantities, much more than needed to perform ProteinChip analysis. This presents problems when performing functional studies or validation — too much material is required, and it is difficult to meet the 200+ sample requirement for validation.

2D gels also do not detect most proteins under 10 kDa, and are therefore not suitable for detecting small cytokines and antibiotic peptides, such as defensins, frequently found in urine.

Albumin

If viewed as a simplified and diluted version of serum, urine has a similar percentage of albumin, about 70% of the total protein content. In many cases the albumin will add a “blind spot” at 33 kDa, 22 kDa, and even 11 kDa, due to its multiply-charged state.

A comparison of the relative amounts of human serum albumin (HSA) captured from urine using the different types of ProteinChip Arrays gives a rough indication of which chips capture less albumin in urine.

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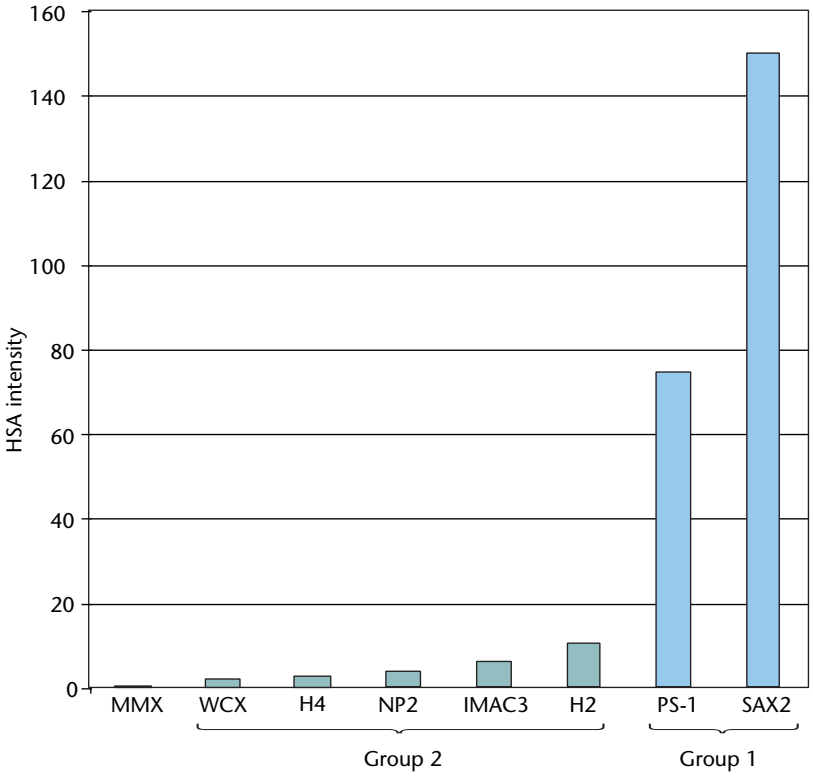

Figure 9-7: Comparison of albumin binding by various ProteinChip Arrays.

The assay was performed using raw urine directly bound to the chips (using the wet method) for 30 minutes at room temperature, then EAM1 was applied to the spots. Although these results are averages from samples run in triplicate under the same conditions, no internal standards were used and they are not intended to rank the chips quantitatively— the 2- to 3-fold differences observed are not dramatic enough to be used as a sole factor in choosing the chip for a urine assay.

The data in the chart shows the SAX2 and PS1 chips (Group 1) bind the most HSA; WCX, H4, NP2, IMAC3 and H2 chips (Group 2) bind a moderate amount of HSA; and the MMX chip binds the least amount of HSA.

Note

Pre-binding the urine samples to the SAX2 chip surface does allow large amounts of HSA to bind to the surface. However, this procedure does not deplete the urine of sufficient amounts of HSA to improve subsequent protein profiling on other types of chips. If desired, HSA may be removed using anti-HSA coupled beads or another method.

Special thanks to Ma Sha, Ph.D. for contributing this information.

Rapid Profiling of Urine Samples

This protocol will allow you to analyze urine samples very rapidly on a normal phase (NP1) ProteinChip Array.

1. Carefully outline every spot of a 24 spot NP1 ProteinChip array with a thin-tip PAP pen.
2. Apply 4 µL urine to each spot and incubate for 5 minutes on the benchtop.
3. Draw the samples off the chip with the folded edge of a lab wipe.
4. Rinse the chip briefly with 10 mL purified water in a 15 mL conical tube.
5. Flick off the excess water and allow the chip to dry.
6. Apply 0.5 mL SPA (saturated solution in 50% acetonitrile/0.5% TFA) to each spot.
7. Collect data from all 24 spots using a ProteinChip quantitation protocol.

Thanks to Huw Davies, Ph.D. for contributing this protocol.

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Chapter 10

Protein Identification with the ProteinChip System

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This application protocol provides ProteinChip® technology users with sufficient background knowledge and technical instruction to fractionate complex samples from eukaryotic cell extracts and cell culture supernatants for:

- Protein profiling experiments aimed at biomarker discovery
- Biomarker purification and identification

Principles of Ion-Exchange Chromatography

The amino acids that make up peptides and proteins are ionizable in aqueous solution. The α-carboxyl and amino groups, as well as any acidic and basic side chains, will become ionized as a function of pH. As a simple guide, both the acidic (i.e. carboxyl) groups and basic (i.e. amino) groups tend to be protonated (COOH, NH_3^+) at low pH (e.g., pH 2.0). As the pH is raised the carboxyl groups become deprotonated (COO^-, NH_3^+). Further increases in the pH eventually result in deprotonation of the basic groups (COO^-, NH_2). Thus, the net charge on a protein will vary with pH.

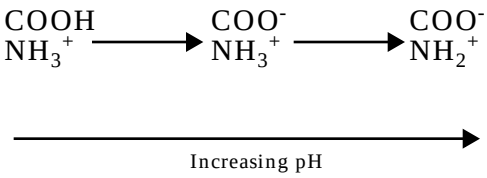

Figure 10-1: pH affects protonation.

Every protein, at a particular pH called its isoelectric pH, or isoelectric point (pI), will contain an equal number of positive and negative charges such that the protein is electrically neutral (i.e., its net charge = 0). The pI of a protein depends on the number of acidic and basic functional groups in the protein. The pI decreases with increasing quantity of acidic groups (aspartate, glutamate). By contrast, the pI increases with increasing quantity of basic groups (lysine, arginine, histidine). For example, human serum albumin is a fairly acidic protein with a pI of 4.9; this protein would have a net *negative* charge on it in a solution at pH 7.0. By contrast, bovine histone is a fairly basic protein with a pI of 10.8; this protein would have a net *positive* charge on it in a solution at pH 7.0.

When the pH of a buffer solution is equal to the pI of a protein the net charge on that protein is zero ($[COO^-] = [NH_3^+]$). At pH values below the pI, proteins are positively charged ($[COO^-] < [NH_3^+]$). By contrast, at pH values above the pI, proteins are negatively charged ($[COO^-] > [NH_3^+]$). *These pH-dependent variations in net charge form the basis of ion-exchange chromatography.* There are two types of ion-exchange chromatography:

Anion-exchange chromatography: negatively-charged peptides or proteins bind to positive groups coupled to the column matrix.

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Cation-exchange chromatography: positively-charged peptides of proteins bind to negative groups coupled to the column matrix.

Various proteins in a mixture will bind an ion-exchange column with different affinities, depending on their relative abundance and net charge. Alterations in pH are used to change the net charge on the protein, thus altering its binding affinity. Due to their ionic properties, salts can effectively disrupt the electrostatic interaction between the column and the protein, thus displacing the protein from the column. The proteins are therefore eluted from the column by washing with buffers of altered pH and/or increasing salt concentration. Proteins with lower binding affinity will elute from the column during lower stringency washes. Proteins with higher binding affinity will require more stringent wash conditions (i.e., increasing salt and more radical changes in pH) to elute.

The choice of anion- or cation-exchange will depend on the goal of the experiment. This protocol encompasses two applications:

- Purification of a known target
- Fractionation of a complex sample for protein profiling on ProteinChip Arrays

Purification of a Known Target

If the pI of the target protein is known, the appropriate anion-exchange or cation-exchange column is easily chosen. Anion-exchange columns are typically used for acidic proteins (pI < 6). Cation-exchange columns are typically used for basic proteins (pI > 8). For proteins having a near-neutral pI, either type of column may be used, in conjunction with the appropriate pH solution.

Consider a specific example:
Given a mixture of human serum albumin (pI = 4.9) and bovine histone (pI = 10.8) in 20 mM sodium phosphate, pH 7.0, how could the albumin be purified? We know that pH 7.0 is well above the pI for albumin; therefore, the protein will carry a net negative charge. By contrast, pH 7.0 is well below the pI for histone, so this protein will carry a net positive charge.

If the sample were applied to an anion-exchange column, the negatively charged albumin would interact with the positively charged functional groups on the column. In addition, charge repulsion should preclude bovine histone from binding. The albumin could be eluted from the column by washing with a buffer of pH < 4.9, in which the albumin will have a net positive charge.

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Sample Fractionation and Profiling

The techniques below have been developed using fractionation and analysis of cultured neuroblastoma cells. We expect that a similar procedure could be followed successfully for tissue extracts and bacterial cells prepared in a hypotonic extraction buffer. If using this procedure with serum, please see specific notes included below.

Small-scale protein fractionation reduces the complexity of a protein sample (i.e., whole cell extracts, cell culture supernatants containing large amounts of albumin and transferrin) for subsequent ProteinChip profiling and biomarker discovery. Fractionation confers an obvious advantage in that it can be combined with additional on-chip purification or electrophoretic separation (SDS-PAGE) to facilitate the protein ID process. Because the pI for the majority of proteins is around pH 7, it is reasonable to use an anion-exchange column with a binding buffer of pH 9.0 or higher for fractionation.

Sample Preparation

Prior to fractionation, the sample must be buffer-exchanged into "binding buffer" (see below). The following method was developed for samples containing approximately 2–2.5 mg total protein as starting material (concentration = 10–20 mg/mL). In this example, good protein profiles can be generated by ProteinChip analysis and on Coomassie-stained gels after the fractionation.

Note: to avoid the need for buffer exchange, a hypotonic lysate of cultured cells can be prepared by sonicating cell pellets in 50 mM Tris, pH 9.0, 5 mM NaCl (see "Cell Lysates" and "Specialized Lysis Methods" in Chapter 7, "Sample Preparation" for more information).

1. Prepare the binding buffer: 20 mM Tris, pH 9.0, 5 mM NaCl. You may add 0.01% non-ionic detergent (Triton X-100 or OGP [β-octyl-glucopyranoside]) to reduce non-specific binding to column resin. If you are going to be looking at the low mass range (<2000 Da), use OGP instead of Triton X-100.

   Note: the choice of binding buffer will depend on the goal of the experiment (see the preceding discussion on the principles of anion-exchange fractionation). In this example protocol, the sample was buffer exchanged into either 20 mM Tris, 5 mM NaCl, pH 9.0, ***or*** *in 50 mM Tris, pH 9.5.*

Bacterial or Eukaryotic Cell Lysates

1. Remove insoluble proteins/aggregates from lysate by thawing the lysates on ice, then spinning the lysates at full speed in a tabletop centrifuge for 20 minutes, 4 °C.

2. Carefully remove the supernatant without disturbing any of the pelleted material.

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3. Buffer exchange the supernatant into binding buffer using one of the following methods:

   a. *Dialysis.* Use a relatively large volume (1 liter) of binding buffer to change the solution in which the sample was prepared. Four hours of constant stirring at 4 °C should be sufficient to achieve equilibrium. The process should be repeated several times (at least 3) to ensure complete buffer exchange (Slide-a-lyzer® dialysis products from Pierce are excellent for dialyzing small amounts of sample).

   b. *Ultrafiltration.* The sample can be diluted in binding buffer and concentrated using a Centricon50. Although the membrane has a 50 kDa MW cutoff, we have observed that the filtrate consists primarily of low MW species below 10 kDa. Thus this device is a good choice for both buffer exchange and sample concentration. For ~ 300 µL sample in 10 mM Tris, pH 8.0, dilute with 1.5 mL binding buffer and concentrate to ~ 300 µL final volume. Repeat the dilution/concentration steps at least once more to ensure adequate buffer exchange.

   c. *Readjust the pH to 9.0–9.5.* About 20 µL 1 M Tris base, pH 10.0 should be sufficient for 100 µL sample in 10 mM Tris, pH 8.0. Check the pH using pH paper. The final salt concentration is ~166 mM which is somewhat high and likely to interfere with protein binding to the column (the salt ions will compete with the proteins for binding). Therefore, the sample should be diluted with an equal volume of MilliQ or HPLC grade water so that the final salt concentration is 80 mM prior to loading on the column.

   d. *K-30 size selection column.* Size selection columns are not recommended for eukaryotic lysate buffer exchange or for samples of protein concentration below 5 mg/mL, as a significant loss of protein may occur due to non-specific binding to the column resin. However, 0.01% non-ionic detergent, such as Triton X-100 or OGP, may be added to reduce the non-specific binding. The K-30 column can be used effectively for buffer exchange and fractionation of proteins in serum samples and most lysates, but be aware that some extracts may contain very sticky proteins that will interfere with fractionation.

Serum Samples

1. Add 198 µL binding buffer to 22 µL of neat serum, preferably in a 500 µL capacity microcentrifuge tube.

2. Vortex briefly to mix.

3. Spin at full speed in a table top centrifuge for 20 minutes at 4 °C to remove aggregated proteins.

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4. After microcentrifugation, if a pellet is visible, 200 µL of the supernatant can be loaded on the anion exchange column. Be careful not to disturb the pellet when removing the supernatant.

   If no pellet is visible after microcentrifugation, only 150 µL of the supernatant should be loaded on the column. When no pellet is visible, the protein concentration in the supernatant is usually very high; if you load 200 µL onto the spin column, the column will be overloaded.

Anion-exchange Column Preparation

Column Hydration

If the column has been stored dry, the resin will need to be hydrated in binding buffer as described below before the sample can be applied.

1. Gently tap the column to ensure that the dry gel has settled to the bottom of the spin column.
2. Remove the top column cap and reconstitute the column by adding 0.8 mL binding buffer. Replace the column cap and vortex vigorously for ~5 seconds. Remove any air bubbles by sharply tapping the bottom of the column. It is important to hydrate all of the dry gel.
3. Allow at least 2 hours of hydration time at room temperature prior to use. It is best to equilibrate overnight. Reconstituted columns may be stored refrigerated at 4 °C for several days. For longer periods of storage include 10 mM sodium azide (NaN_3) in the binding buffer. Allow refrigerated columns to warm to room temperature before use. Note that if sodium azide is present in the hydration buffer it will be washed out of the column prior to use (see below).

Buffer Exchange Protocol

1. Just before applying the sample, remove the top cap from the column, then remove the stopper from the bottom of the column.
2. Place the column in a conical tube and allow the buffer to gravity drain. Do not allow the resin to dry excessively.

   Note: if the buffer doesn't gravity drain very well you can replace the top cap on the column in order to produce enough pressure for the buffer to begin draining. Gently remove the cap as the buffer begins to drain.
3. Discard the eluant and wash the column with 0.8 mL binding buffer, allowing the column to gravity drain.
4. Discard the eluant and perform a second wash with 0.8 mL binding buffer, allowing the column to gravity drain. Then spin

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the column at 1000 rpm for 1 minute at room temperature to remove any remaining excess fluid. Discard the eluant.

Sample Fractionation

Sample Application and Column Elution

1. Prior to fractionation, label sufficient 1.5 mL eppendorf tubes for the fractions to be collected. To process 2 samples using this protocol you will need a total of 16 tubes, 8 for each sample.

2. Prepare the elution buffers. The elution of sample proteins from the column will be accomplished by washing the column with buffers having progressively decreasing pH and/or increasing the salt concentration. The proteins with the lowest binding affinity will be washed off the column in the earlier, milder washes, while those with higher binding affinities will be eluted in the later, more stringent washes. Since the bed volume of the column is ~ 75 µL, 150 µL (i.e., 2 column volumes) of each elution buffer should be sufficient for elution. The following scheme is recommended as an initial attempt at elution:

   Binding buffer: 20 mM Tris, 5 mM NaCl, pH 9.0 *or* 50 mM Tris, pH 9.5. Washing with the initial mobile phase will cause many of the unbound species to be eluted.

   Elution buffer A: 20 mM Tris, 5 mM NaCl, pH 8.0

   Elution buffer B: 20 mM sodium phosphate, pH 7.0

   Elution buffer C: 20 mM sodium phosphate, pH 6.0

   Elution buffer D: 20 mM sodium phosphate and citrate, pH 5.0

   Elution buffer E: 20 mM sodium phosphate and citrate, pH 4.0

   Elution buffer F: 20 mM sodium phosphate and citrate, pH 3.4

   Elution buffer G: 30% acetonitrile in elution buffer F

   Note: elution buffer G is intended to elute everything possible from the column resin. Some proteins will become denatured in this buffer.

   This scheme is only a suggested starting point and the fractionation can be optimized for your application by changing the pH and salt gradient used for elution. Recent experiments with cell lysates have indicated that a narrowly-stepped gradient (i.e., increments of 0.5 pH unit) provides better separation resolution of proteins in such complex samples. Thus, a gradient of washes from pH 9.5, 9.0, 8.5, 8.0, etcetera, can be used for more rigorous fractionation. For serum samples, a gradient of washes from pH 9.0, 8.0, 7.0, 6.0, 3.4 and 30% acetonitrile in pH 3.4 buffer has worked well.

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3. Apply the sample (up to ~1 mL total volume) to the column. If bubbles become trapped in the column resin, gently disperse them with a gel loading pipette tip. Allow the sample to gravity drain, then reapply the flow-through to the column one or more times to improve the interaction efficiency of the protein mixture in the sample with the column matrix.

4. Spin the column at 1000 rpm at room temperature for 1 minute and collect the flow-through. Remove the flow-through from the conical tube and place it in one of the 1.5 mL eppendorf tubes. Place the eppendorf tube on ice.

5. Elute the proteins from the column:

   a. Apply 75 µL binding buffer to the column, then spin it at 1000 rpm at room temperature for 1 minute.

   b. Apply another 75 µL binding buffer to the column and spin again at 1000 rpm at room temperature for 1 minute.

   c. Remove the 150 µL eluant from the conical tube and place it in a (labeled) eppendorf tube. Place the tube on ice.

   d. Repeat steps a–c with each elution buffer, using the buffers in order from elution buffer A to elution buffer G (i.e., from least stringent to most stringent).

Special Considerations When Protein Profiling Cell Culture Supernatants

The anion-exchange column can accommodate a maximum of ~200 µL sample containing 10% FCS. Therefore, if the sample contains 1% FCS, up to 2 mL total volume can be fractionated using a single spin column. Ciphergen has had good success by first dialyzing a sample containing 1% FCS against 50 mM Tris, 5 mM NaCl, pH 9.5 prior to applying 1 mL total volume to the column. The column can then be eluted as described above.

Working with Dilute Fractions

It's possible that the fractions may be too dilute to produce good results. Analyze 2 µL each from a few of the fractions; if the ProteinChip analysis indicates numerous proteins, you can proceed with protein profiling. If the fractions are too dilute you can either use the bioprocessor to analyze up to 350 µL per spot (read *"Using the Bioprocessor"* on page 3-7to 3-9 for detailed information on using the bioprocessor).

Alternatively, you can concentrate the samples in a SpeedVac. Dry the samples down to approximately the same final volume (20–30 µL) You may need to add a few µL to some of the samples so that all tubes contain the same volume.

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Note: do not allow the samples to dry completely. Doing so can result in significant protein loss due to adsorption of proteins to the walls of the eppendorf tube.

The fractions collected are now ready for protein profiling on NP1/ NP2 or H4 ProteinChip Arrays.

Protein Profiling of Fractions

Note: samples should be applied to spots on the ProteinChip array in duplicate to allow analysis with both CHCA and SPA (see step 3, below).

1. Apply 5 µL of the samples (or 1–2 µL of the concentrated samples) to the spots of a normal phase (NP1 or NP2) or hydrophobic (H4) ProteinChip Array and allow to dry. Optionally, samples can also be analyzed on IMAC3 copper chips (see below for details).

2. Wash each spot with three 5 µL washes. Use HPLC grade water for NP chips and 10% acetonitrile for H4 chips.

   Note: protein profiles may be different with and without washes; try both if possible.

3. Apply EAM to all spots, using CHCA for detecting proteins in the low mass range (<15 kDa) and SPA for proteins in the higher mass range (>15 kDa). If you only want to use a single EAM, use SPA. To detect more low-mass proteins, duplicate samples must be analyzed using CHCA. (For more information on selecting EAMs, see Chapter 4, *"Energy Absorbing Molecules (EAMs)"*).

   CHCA: Apply 0.5 µL CHCA saturated solution in 50% acetonitrile:0.5%TFA. Allow to air dry.

   SPA: Apply 0.5 µL SPA (saturated solution in 50% acetonitrile, 0.5% TFA), then allow the chip to air dry. Add a second application of SPA and allow the chip to air dry again.

4. Analyze using the ProteinChip System.

Optional IMAC3 Profiling

1. Apply 40–80 µL of each fraction up to pH 5.0 to an IMAC3 copper chip using a bioprocessor. See the *"Copper Protocol"* for IMAC3 chips on page 5-8 for more information.

2. Incubate the samples on the chip for 30 minutes, then remove the samples from the bioprocessor wells.

3. Wash three times with PBS, then once with water.

4. Dry the chip. Apply 0.5 µL SPA (saturated solution in 50% acetonitrile, 0.5% TFA), then allow the chip to air dry. Add a second application of SPA and allow the chip to air dry again.

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5. Analyze using the ProteinChip System.

Notes on Data Collection Settings

The time lag focusing setting (TLF) and laser intensity settings will have a strong impact on the quality of data generated from samples on ProteinChip Arrays. (Please read *"Adjusting the Laser Intensity and Detector Sensitivity"* on page 14-19 and *"Time Lag Focus/Pulse Settings"* on page 14-24 for detailed information).

Briefly, time lag focusing allows the ProteinChip System to sharpen the peaks at a specific mass. It is not currently possible to optimize data collection parameters over the entire mass range used for protein profiling (typically, 0–200 kDa). Instead, it is necessary to focus on a narrower mass range and collect data from the same chip several times. Typical TLF and Laser Intensity settings are given in the table below. Note that the values given in the table are starting points only; in particular the Laser Intensity should always be set such that the signals from proteins in the mass range of interest are on-scale, i.e., the peak intensities are below 100. If the peaks have flat tops, decrease the laser intensity during data collection.

Mass Range	TLF (Mass)	Laser Intensity
Low Mass (CHCA or SPA)	10 kDa	200–220
Mid Mass (CHCA or SPA)	40 kDa	240–260
High Mass (SPA Only)	100 kDa	280–300

Biomarker Purification and Identification (Detailed Protocol)

Protein profiling with the ProteinChip System allows the investigator to pinpoint a number of biomarker candidates of known size; these candidates are easily traced back to specific anion-exchange fractions. The next step is to identify the biomarker candidates, a process known as “protein ID.”

The protein ID process may require additional protein purification, particularly for proteins in low abundance, to generate a protein sample of sufficient quantity and purity. For example, a small but consistent biomarker peak of interest may be easy to detect using ProteinChip analysis, but may not be sufficiently abundant in the anion-exchange fraction to allow further identification.

Some biomarkers have unusual properties, such as an extreme pI, that allow on-chip purification, proteolysis and identification. Usually, however, biomarkers of interest must be purified prior to proteolysis, typically via SDS-PAGE. SDS-PAGE both resolves and

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isolates sufficient amounts of the biomarker and also denatures the protein, facilitating subsequent proteolysis.

Fractions containing a biomarker candidate of interest are concentrated and run on an SDS-PAGE gel to obtain 1–10 picomoles of purified protein candidates. The 4–20% acrylamide gradient gel is popular since it provides good separation for a wide range of protein sizes (5–120 kDa); however, the resolution provided by these gels may be inferior for the mass range of interest compared to a fixed percent acrylamide gel. For example, 7–9% gels are a good choice for proteins above 70 kDa, 10–12% gels are a good choice for protein between 70–40 kDa, 12–16% gels are good for proteins between 40–20 kDa proteins, and 16–20% gels are good for proteins between 20–2 kDa proteins. (4–20% gradient gels are of use in seeing proteins in all molecular weight ranges, but bands of similar molecular weight will be poorly resolved). Tricine gels are very good for low molecular weight proteins. The length of the gel should be chosen to optimize resolution — this will be an important consideration if you want to cut out and ID closely-migrating gel bands.

Recommended Reagents

- Novex 4–20% Tris-glycine gel, cat. no. EC6025.
- Novex 16% tricine gel, cat. no. EC6695.
- Novex buffers, protein molecular weight standards, and staining solutions.

Preparing Samples for Gels

Fractions containing acetonitrile will need to be completely dried in a SpeedVac. The dry proteins can then be resuspended in 10–40 µL 1x sample buffer for loading on the gel; do not exceed the largest volume accommodated by the wells in your gel. Rinse the wall of the microcentrifuge tube with the sample buffer to maximize the recovery of proteins from the tube.

All other fractions should be brought to 1x using 4x SDS-PAGE buffer.

If any of the samples turn yellow when sample buffer is added, the pH has become acidic, which can lead to aberrant migration, poor resolution and poor solubilization of proteins. The pH can be restored by adding a small amount of 1 M Tris, pH 8.0 or higher. Start by adding 1 µL, vortex gently after each addition, and stop adding Tris when the sample turns blue.

Once a candidate protein band has been selected for identification the following protocol may be used for protein ID.

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In-gel Sample Purification and Proteolysis

Notes

When using this protocol for the first time, we strongly recommend practicing on a known protein. The proteins in typical molecular weight standards are a good source for this, or you can order a wide variety of purified proteins and peptides from Ciphergen.

For biomarker ID, ≥5 picomoles protein is ideal for generating a large peptide signal; however, protein ID can also be accomplished using as little as 0.5 picomoles.

A number of proteases can be used for protein ID, including trypsin, V8 (also called GluC), and LysC. For in-gel digestion with trypsin, it is best to allow the reaction to incubate overnight. Prepare trypsin according to the instructions on page 10-15.

Digestions with V8 (Glu C) or Lys C usually generate fewer peptides. These enzymes can be used in addition to trypsin digestion for protein ID. Prepare GluC at a stock concentration of 0.1 µg/µL in 50 mM ammonium bicarbonate buffer, pH 7.8. Store aliquots at -20 °C and discard after one month. Prepare LysC at a stock concentration of 1 mg/mL in 25 mM Tris, pH 8.0. Store aliquots at -20 °C and discard after 2 years.

Enzymes Recommended for Protein Digestion

- *Trypsin:* clips at the C-terminal side of lysine and arginine residues. It will cut most proteins.
- *Asp-N:* cuts at the N-terminal side of aspartate and cysteic acid residues depending on pH, buffers, and concentration. See the instructions from the supplier.
- *Glu-C (V-8):* cuts on the C-terminal side of aspartate and glutamine residues depending on pH, buffers, and concentration. See instructions from the supplier.
- *Lys-C:* cuts on the C-terminal side of lysine and occasionally arginine, depending on pH, buffers, and concentration. See instructions from the supplier.

Gel-purify Proteins

1. Run the proteins of interest on an appropriate SDS-PAGE gel.
2. Stain the gel with Coomassie blue R-250 (0.1% w/v) in 40% methanol, 10% acetic acid.
3. Destain with a large excess volume of 40% methanol, 10% acetic acid for several hours with 1 or 2 changes of fresh solution. Follow the instructions from the suppliers if other dye staining kits are used (e.g., Novex).

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4. Cut out the gel piece containing the protein of interest (~1 mm gel). Try to minimize the amount of gel surrounding the protein, but if the protein band is faint cut out the gel pieces covering the entire band and transfer to the same tube.Transfer the gel piece to a 0.5 mL microfuge tube.

   Alternative method: use a Pasteur pipette to punch out circular pieces of gel containing the protein of interest. Insert the narrow end of the Pasteur pipette into the protein band of interest and remove a small gel piece. Gently exert pressure from a pipetting bulb to eject the gel into a 0.5 mL microfuge tube.

5. Cut out an equivalent portion of protein-free gel to use as a "no protein" control. This control will be processed separately but in the same way as the gel containing protein, to establish which peaks are of interest, and to determine which peptides are derived from the protease itself.

Destain and Remove SDS

1. If the gel has already been destained using 50% methanol, 10% acetic acid, proceed to step 2.

   If the gel was destained using water (e.g., if using Novex's Colloidal Blue staining kit) then incubate the gel piece(s) in 0.4 to 1 mL 50% methanol, 10% acetic acid. Leave the tubes on a shaker for 1 hour. Replace with fresh destaining solution and incubate for another hour.

2. Remove the solution from the gel piece(s). Incubate the gel piece(s) in 0.4 mL 0.1 M ammonium bicarbonate, pH 8.0 on a shaker at room temperate for 10 minutes. Repeat once more, using fresh buffer.

3. Remove the buffer. Wash the gel piece(s) with 0.5 to 1 mL 50% acetonitrile, 0.1 M ammonium bicarbonate, pH 8.0 on a shaker at room temperate for 1 hour.

4. Remove the buffer. If a Pasteur pipette was not used to cut the gel pieces, cut the gel piece into 2–3 smaller pieces and return all of the pieces to the tubes.

5. Add 50 µL 100% acetonitrile. Incubate at room temperature for ~15 minutes. Remove all solvent. Dry the gel pieces in a SpeedVac for ~15 minutes.

Important Information on Trypsin Preparation

For protein ID we recommend Roche Molecular Biochemicals sequencing grade modified trypsin (see table of catalog numbers below). If you plan to perform several protein ID experiments, you may want to order the larger sizes.

Quantity	Catalog No.
1 x 25 µg	1 520 423
4 x 25 µg	1 418 025
4 x 100 µg	1 418 033

Lyophilized bovine pancreatic trypsin is stable when stored dry at 4 °C. However, trypsin in solution will lose substantial activity with repeated freeze-thaw cycles. Dissolve the enzyme immediately prior to use and store unused aliquots adequate for a single use, as described below.

Dissolve 25 µg trypsin in 125 µL 10 mM HCl to produce a 0.2 µg/µL trypsin solution (10 x the concentration used for protein ID). Store 5 µL aliquots at -20 °C or preferably at -80 °C, for no longer than 6 months. To make a working solution of trypsin, just before use thaw a 5 µL aliquot tube and add 45 µL 25 mM ammonium bicarbonate, pH 8.0. Apply 10 µL of this diluted trypsin (0.02 µg/µL) to the dried gel piece.

Each aliquot of trypsin should be subjected to only one freeze-thaw cycle: distinctly mark the tube after the first thawing; throw it out after the next use. Repeated freeze-thaw cycles will degrade the protease and will compromise your experiments.

In-gel Protease Digestion

Note: for additional information on in-gel protease digestion, refer to the flow chart on page 10-24, as well as the "Notes" on page 10-23, and the introductory paragraph of "Biomarker Protein ID (Condensed Protocol)" on page 10-23.

In-gel Digestion

1. Add 10 µL 25 mM ammonium bicarbonate, pH 8.0, containing ~ 0.02 µg/µL trypsin to the gel pieces. The amount of enzyme should be sufficient for 1 to 10 pmoles of protein. However, the amount of enzyme should be empirically optimized for the amount of protein in the gel pieces. Incubate at room temperature for 15 minutes.

2. If necessary, add 5–40 µL 25 mM ammonium bicarbonate, pH 8.0 to cover the gel pieces. Incubate the closed tube at 37 °C for ~16 hours. A closed, dry air incubator is preferable to a waterbath because it will keep the temperature more even.

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Peptide Mapping Protocol

1. Use 1 µL aliquots of the ammonium bicarbonate solution surrounding the gel pieces for peptide profiling on ProteinChip Arrays (either NP1, NP2 or H4). Most peptides are detected without washing. However, some peptides are detected better after drying and water washing on H4 arrays.

   Note: if there is no buffer outside the gel pieces after the overnight incubation, add 15 µL 25 mM ammonium bicarbonate, pH 8.0 and vortex, then allow the mixture to equilibrate for 30 minutes prior to analysis. Use 2-5 µL of solution per spot for analysis.

2. Include a spot with EAM alone and a spot with the “no protein” gel slice control to control for background signals.

3. Add 0.5 µL CHCA (10% saturated solution in 50% acetonitrile, 0.25% TFA) to each spot. Allow to air dry, then analyze in the ProteinChip Reader.

Calibrating the ProteinChip Reader

Before beginning data collection, it is absolutely imperative to calibrate the ProteinChip Reader in the appropriate peptide mass range, to ensure a reasonable mass accuracy.

Follow the calibration instructions given in Chapter 2, *"Basic Tutorial: Calibrating the PBS II ProteinChip Reader"* to calibrate the ProteinChip Reader to a particular peptide mass range, using the peptides given below. Additional information can be found in Chapter 14, in the section titled *"Calibration"* starting on page 14-20.

External Calibration

External calibration is performed for two reasons: 1) determine the ratio of the peptide calibrants necessary to give good signals and sharp (well resolved) peaks for each peptide in the mixture, and 2) calibrate the sample-containing spots to within ~0.1% mass accuracy. The following protocol is recommended:

1. Prepare the following peptide calibrants from a Ciphergen peptide standard kit according to the kit instructions:

Peptide Standard	Mass (Da)
angiotensin 1 (human)	1296.5
Glu1 fibrinopeptide B	1570.6
beta-endorphin (human, 61–91)	3465.0

2. Prepare the following mixture of the peptide calibrants:

angiotensin 1	2 µL
Glu1 fibrinopeptide B	2 µL
beta-endorphin	4 µL
Water	42 µL

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3. Apply 1 µL of the peptide calibrants solution to an unused spot and allow to air dry.

4. Add 0.5 µL 10% saturated CHCA to the spot. Allow to air-dry, then read the calibrants on the spot (refer to Chapter 2, *"Basic Tutorial: Calibrating the PBS II ProteinChip Reader"* for specific information on collecting data).

5. If the heights of the calibrant peaks differ by more than 2-fold, you may want to adjust the relative amounts of the calibrants.

Data Collection Parameters for Protein ID

In other ProteinChip applications it is generally advisable to collect a large number of spectra from many locations within a spot to generate the averaged data. However, a different strategy is needed when collecting data for peptide mapping. The spectra used for averaging in peptide mapping practically have to be "hand-picked," meaning that only the transients with the best peak resolution and signal-to-noise ratio should be added to the averaged spectra.

1. To ensure the correct spectra are included, it is first necessary to determine the optimal parameters.

   Time Lag Focusing: focus on mass ~2 kDa (the middle of the calibrants' mass range).

   Sensitivity: set to 5–8.

   Laser Intensity: fire a few shots to determine the minimum laser intensity required to produce a signal. Increase the laser intensity slowly until the major peaks of interest have intensities of ~5–50 (y-axis). Stop collection.

   Optional: click the **Peak Information** button, then select a peak from the spectrum. A table will automatically appear, containing the resolution of the peak (toward the bottom of the table). Given a peak of 2 kDa, for which the time lag focusing is optimized, you should expect a resolution of at least 300. For a pure peptide, the resolution should be close to 600. If the resolution is substantially less than 200, review the calibration and data collection steps described above and make sure the time lag focusing has been set properly.

   Note: if the laser intensity and time lag focusing settings have changed, it is best to re-calibrate using the chip prepared above, then collect the sample data. This method ensures the highest mass accuracy.

2. Using the settings determined above, move to a fresh position on the spot, click the **Fire Continuously** button, then click the **Average** button after about 5 shots. Be sure to keep the **Transients** window open; as soon as the data begins to look poor (signal has decreased ~2-fold), click the **Stop** button. Move to a new position on the spot and repeat the data collection procedure.

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3. Continue this process until ~100 transients have been averaged.

   Note: it is crucial that none of the peak intensities exceed 95, because mass accuracy is extremely important. During averaging, even a few transients with signal intensities over 100 will decrease the mass accuracy.

Internal Calibration

The goal of internal calibration is to generate calibrant peaks with intensities similar to those of the experimental peptides. The same mixture of peptide calibrants used for external calibration is used to internally calibrate the sample-containing spot to obtain the best mass accuracy possible (~ 0.02%). As mentioned in the Calibration Tutorial (Chapter 2), internal calibration requires careful adjustment, so allow extra time for this section.

You can always add more peptide calibrants solution (i.e., the internal standard) to a spot if the signal is too low, but you can't remove it if the signal goes off-scale. If you accidentally add too much internal standard, many of the peptide signals from your protease-digested biomarker will be overwhelmed by the signals from the calibrants. Nonetheless, it may still be possible to calibrate using the highest intensity peaks, and then use these known masses to calibrate the sample data that have been externally calibrated. In other words, you'll only be able to use the main peaks from your first set of peptide data to calibrate.

1. To the first spot on the sample chip, add a small amount of the peptide calibrants solution in 10% saturated CHCA (0.1–1 µL). Pipette the solution up and down while applying it, to make sure the peptides from the unknown sample already on the chip become mixed with the calibrants. It's best to pipette using a 0.5–1 µL volume; if necessary, dilute the peptide calibrants solution, then add a larger volume (up to 1 µL) to each spot.

2. Collect data using the settings and technique described above in *"Data Collection Parameters for Protein ID"*.

3. Determine the exact mass of at least two peptides from the biomarker of interest or from trypsin autolysis. You can use the CHCA as one of the peaks of known mass. In most cases the digested trypsin peak will appear at 2163.3 Da in your sample spot, and you can use this 2163.3 peak as one of your internal standards.

   Note: if you can't find the peak at 2163.3 Da, it is likely that the activity of your trypsin is low.

   Identify the analogous peaks in the data that was externally calibrated. Internally calibrate, using the peaks with the newly assigned masses.

   Note that the mass accuracy of data collected in the above manner will be sub-optimal. It is best to analyze a fresh aliquot of your original proteolytic digest.

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4. The data from the trypsin control spot should contain only peaks from CHCA and trypsin autolysis. Use the **Select Peaks** button to remove the labels from any peaks that appear in *both* the experimental sample spot and the trypsin control spot, leaving an experimental spectrum in which only peptides of interest are labelled (see the table below for a list of peptides expected from trypsin autolysis).

Database Searches for Protein ID

Public databases are available to help identify a protein of interest using the mass values of its internally calibrated peptides. If the protein of interest is not in the database, the search will not work (of course, in most cases, you won't know at this point whether your biomarker is in the database or not). To increase the chances of protein identification, it is important to enter only the masses of peptides derived from the protein of interest (the quality of the protein purification obtained via the 1D gel will determine whether extraneous peptides are included).

The following sites provide tools for protein identification using their public databases:

ProFound
http://prowl.rockefeller.edu/cgi-bin/ProFound

Expasy
http://www.expasy.ch/tools/peptident.html

EMBL
http://www.embl-heidelberg.de/Services/index.html

UCSF MS-Fit
http://prospector.ucsf.edu/

Analyzing Mass Data via a Database Search

1. Select **List Substances** from the **Peaks** menu. A table should appear, listing various characteristics relating to the *labeled* peaks in your spectrum.
2. Select the information in the **Mass** column (not the **Mass/z** column). Copy the information in this column using the **ctrl-C** keyboard command.
3. Open one of the websites listed above and paste in the list of peptides, using the **ctrl-V** command. The website should produce a list of peptides of known mass that are derived from your biomarker of interest.

Parameters Used for Database Searches

- Use more than 7 peptide fragments if possible, choosing the strongest peaks.

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- Specify the species that the protein is from (not the host cell for recombinant protein).
- Specify the molecular weight range as close to the protein-of-interest as possible (at first). In some case, e.g. if the protein-of-interest is a fragment of a larger protein or the protein is modified (i.e. glycosylated), you must specify a wider MW range.
- If your protein contains cysteine residues, they have most likely been modified by acrylamide during migration through the gel. We recommend you search for masses assuming that the cysteines have been thus modified.
- Peptide mass tolerance: ±1.0 Da (for internally calibrated spectra).
- Maximum missed cleavages: 1–4. Ideally, a protein will be completely digested by the protease; however, incomplete digestions are common, leading to peptides with "missed cleavages."

Notes on Bovine Pancreas Trypsin

Sequence of Bovine Trypsin

1: VDDDDKIVGGYTCGANTVPYQVSLNSGYHFCGGSLINSQWVVSAAHCYKS
51: GIQVRLGEDNINVVEGNEQFISASKSIVHPSYNSNTLNNDIMLIKLKSAA
101: SLNSRVASISLPTSCASAGTQCLISGWGNTKSSGTSYPDVLKCLKAPILS
151: DSSCKSAYPGQITSNMFCAGYLEGGKDSCQGDSGGPVVCSGKLQGIVSWG
201: SGCAQKNKPGVYTKVCNYVSWIKQTIASN

A peak at 2163.3 Da is commonly observed from trypsin autolysis.

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Partial and Complete Trypsin Autolysis Products

Fragment	Residues	Mass	Fragment	Residues	Mass
6	96–97	259.349	4–5	56–95	4418.908
15	207–208	260.293	7–9	98–142	4474.996
10	143–145	362.494	2	7–49	4553.141
18	224–229	632.672	4–6	56–97	4660.241
3	50–55	658.756	11–13	146–192	4674.228
16	209–214	663.772	9–12	132–176	4676.349
1	1–6	705.677	6–9	96–142	4716.330
7	98–105	804.859	7–10	98–145	4819.475
15–16	207–214	906.050	13–17	177–223	4892.598
11	146–155	1020.172	10–13	143–192	5018.706
6–7	96–105	1046.192	8–11	106–155	5034.788
17	215–223	1111.330	3–5	50–95	5059.648
9	132–142	1153.255	6–10	96–145	5060.808
10–11	143–155	1364.651	12–14	156–206	5087.708
14	193–206	1433.652	2–3	7–55	5193.882
13	177–192	1495.612	1–2	1–49	5240.802
9–10	132–145	1497.733	3–6	50–97	5300.982
14–15	193–208	1675.930	12–15	156–208	5329.986
17–18	215–229	1725.987	4–7	56–105	5447.085
16–17	209–223	1757.087	13–18	177–229	5507.254
15–17	207–223	1999.365	7–11	98–155	5821.632
4	56–75	2163.327	5–8	76–131	5836.671
12	156–176	2194.475	1–3	1–55	5881.543
5	76–95	2273.596	12–16	156–214	5975.742
14–16	193–214	2321.687	6–11	96–155	6062.965
16–18	209–229	2371.743	3–7	50–105	6087.825
9–11	132–155	2499.890	11–14	146–206	6089.864
5–6	76–97	2514.930	9–13	132–192	6153.946
8	106–131	2552.913	11–15	146–208	6332.143
15–18	207–229	2614.021	10–14	143–206	6434.343
3–4	50–75	2804.067	10–15	143–208	6676.621
13–14	177–206	2911.248	5–9	76–142	6971.911
13–15	177–208	3153.526	11–16	146–214	6977.899
11–12	146–176	3196.631	12–17	156–223	7069.057
5–7	76–105	3301.773	8–12	106–176	7211.247
7–8	98–131	3339.757	5–10	76–145	7316.389
14–17	193–223	3415.002	10–16	143–214	7322.378
10–12	143–176	3541.110	2–4	7–75	7339.193
6–8	96–131	3581.090	9–14	132–206	7569.582
12–13	156–192	3672.071	12–18	156–229	7683.714
8–9	106–142	3688.153	9–15	132–208	7811.860

Commonly observed →

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Fragment	Residues	Mass	Fragment	Residues	Mass
13–16	177–214	3799.283	4–8	56–131	7981.983
14–18	193–229	4029.658	7–12	98–176	7998.091
8–10	106–145	4032.631	1–4	1–75	8026.854
11–17	146–223	8071.214	2–8	7–131	13157.849
6–12	96–176	8239.424	3–12	50–176	13281.057
5–11	76–155	8318.546	5–14	76–206	13388.238
10–17	143–223	8415.693	7–18	98–229	13487.330
9–16	132–214	8457.617	5–15	76–208	13630.516
3–8	50–131	8622.723	6–18	96–229	13728.664
11–18	146–229	8685.871	1–8	1–131	13845.510
8–13	106–192	8688.844	4–13	56–192	14117.913
10–18	143–229	9030.349	5–16	76–214	14276.273
4–9	56–142	9117.222	2–9	7–142	14293.088
4–10	56–145	9461.701	2–10	7–145	14637.567
7–13	98–192	9475.687	3–13	50–192	14758.654
9–17	132–223	9550.932	1–9	1–142	14980.750
2–5	7–95	9594.774	1–10	1–145	15325.228
6–13	96–192	9717.021	5–17	76–223	15369.588
3–9	50–142	9757.963	4–14	56–206	15533.550
2–6	7–97	9836.108	2–11	7–155	15639.724
3–10	50–145	10102.441	4–15	56–208	15775.828
8–14	106–206	10104.480	5–18	76–229	15984.244
9–18	132–229	10165.588	3–14	50–206	16174.290
1–5	1–95	10282.435	1–11	1–155	16327.385
8–15	106–208	10346.758	3–15	50–208	16416.568
4–11	56–155	10463.857	4–16	56–214	16421.584
5–12	76–176	10495.005	3–16	50–214	17062.325
1–6	1–97	10523.769	4–17	56–223	17514.899
2–7	7–105	10622.951	2–12	7–176	17816.183
7–14	98–206	10891.324	4–18	56–229	18129.556
8–16	106–214	10992.515	3–17	50–223	18155.640
3–11	50–155	11104.598	1–12	1–176	18503.844
6–14	96–206	11132.657	3–18	50–229	18770.297
7–15	98–208	11133.602	2–13	7–192	19293.779
1–7	1–105	11310.612	1–13	1–192	19981.441
6–15	96–208	11374.936	2–14	7–206	20709.416
7–16	98–214	11779.358	2–15	7–208	20951.694
5–13	76–192	11972.602	1–14	1–206	21397.077
6–16	96–214	12020.692	2–16	7–214	21597.451
8–17	106–223	12085.830	1–15	1–208	21639.355
4–12	56–176	12640.317	1–16	1–214	22285.112

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Fragment	Residues	Mass	Fragment	Residues	Mass
8–18	106–229	12700.487	2–17	7–223	22690.766
7–17	98–223	12872.674	2–18	7–229	23305.422
6–17	96–223	13114.007	1–17	1–223	23378.427
			1–18	1–229	23993.084

Thanks to Deb Diamond, Ph.D., Thang Pham, Ph.D., and Vanitha Thulasiraman, Ph.D.

Biomarker Protein ID (Condensed Protocol)

Note: if you haven't performed a biomarker protein ID before, please read the detailed protocol, starting on page 10-11.

This protocol will allow you to enzymatically cleave a protein of interest which has been identified with a ProteinChip assay and isolated by running it out on an SDS-PAGE gel. The protein is excised from the gel, de-stained, dried and rehydrated with a solution containing protease. The protease enters the gel during rehydration and subsequently cleaves the protein. The small peptides that result are small enough to diffuse out of the gel matrix and into the surrounding solution. Finally, the precise mass of peptides in solution are determined using the ProteinChip System. A database search is used to identify the protein of interest.

Notes

Note: these notes are expanded upon in the previous protocol.

- About 5 picomoles of protein should be sufficient to obtain a good signal from the resultant peptides. If a protein band is strongly visible by Coomassie staining, that is in general a sign that a sufficient amount of protein is available in the band to allow protein ID.
- Smaller proteins are often more easily digested to completion than larger proteins. However, because smaller proteins generate fewer fragments, they may not provide the definitive identification that digestion of a larger protein might provide.
- Proteases may be contaminated with non-specific proteases. Preparations from different suppliers vary; therefore it is a good idea to determine the best one for your digestion before you start.
- Different peptides may fly better on different chip surfaces.
- Calibration of the instrument is very important for obtaining highly accurate peptide masses. The instrument should be calibrated using 3 peptide standards in the mass range of interest, using the same time lag focus (TLF) setting that will be used for collecting data. If the TLF setting is changed for collecting data, re-calibrate the instrument using that setting.

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Enzymes for Protein Digestion

Use only sequencing grade enzymes or other very clean preparations. Contaminants are normally closely related proteins, and will interfere with the results. Purchase enzymes from the recommended sources of sequencing reagents or from quality suppliers of protein chemistry reagents.

Flow Chart

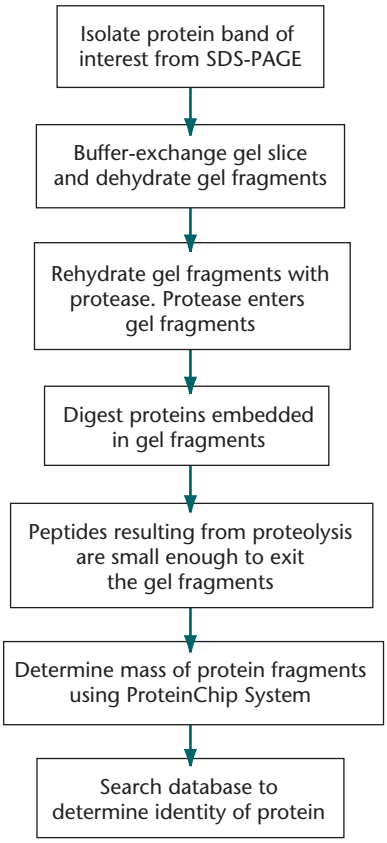

Figure 10-2: Biomarker protein identification protocol.

Protocol

SDS/Acrylamide Gel

1. Run the proteins of interest on an appropriate SDS-PAGE gel.
2. Stain the gel with Novex Colloidal Blue (Novex, cat. no. LC6025).
3. Destain the gel with deionized water for approximately 1 hour.
4. Cut out the gel piece containing the protein of interest (~1 mm gel). Try to minimize the amount of gel surrounding the protein.Transfer the gel piece to a 0.5 mL microfuge tube.

   Alternative method: use a Pasteur pipette to punch out circular pieces of gel containing the protein of interest. Insert the narrow end of the Pasteur pipette into the protein band of interest and

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remove a small gel piece. Gently exert pressure from a pipetting bulb to eject the gel into a 0.5 mL microfuge tube.

5. Cut out a portion of the gel that does not have any protein in it to use as a "no protein" control. This control will be used to determine which peptides are derived from the protease itself.

Destain and Remove SDS

1. Incubate the gel piece(s) in 0.4 mL 50% methanol, 10% acetic acid on a shaker at room temperature for 1 hour.
2. Remove the solution from the gel piece(s). Replace with fresh solution and incubate for another hour.
3. Remove the solution from the gel piece(s). Incubate the gel piece(s) in 0.4 mL 0.1 M ammonium bicarbonate, pH 8.0 on a shaker at room temperate for 1 hour.
4. Remove the buffer. Wash the gel piece(s) with 0.5 mL 50% acetonitrile, 0.1 M ammonium bicarbonate, pH 8.0 on a shaker at room temperate for 1 hour.
5. Remove the buffer. If a Pasteur pipette was not used to cut the gel pieces, cut the gel piece into 2–3 smaller pieces and return all of the pieces to the tube.
6. Add 50 µL 100% acetonitrile. Incubate at room temperature for ~15 minutes. Remove all solvent. Dry the gel pieces in a SpeedVac for ~15 minutes.

In-gel Protease Digestion

Note: see additional information on trypsin handling on page 10-15. Trypsin will lose substantial activity with repeated freeze-thaw cycles. Resuspend lyophilized preparations of trypsin according to the manufacturer's instructions, and freeze in aliquots adequate for a single use.

Trypsin can be stored for several months at -20 °C, but is probably best stored at -70 °C. We recommend Roche Molecular Biochemicals sequencing grade, modified trypsin, cat. no. 1418 025 (4 x 25 micrograms) or 1418 033 (4 x 100 micrograms).

1. Add 10 µL 25 mM ammonium bicarbonate, pH 8.0 containing ~ 0.02 µg/µL trypsin to the gel pieces. This amount of enzyme should be sufficient for 1 to 10 pmoles of protein. However, the amount of enzyme should be empirically optimized for the amount of protein in the gel pieces. Incubate at room temperature for 15 minutes.
2. If necessary, add 5–40 µL 25 mM ammonium bicarbonate, pH 8.0 to cover the gel pieces. Incubate the closed tube at 37 °C for ~16 hours. A closed, dry air incubator is preferable to a waterbath because it will keep the temperature more even.

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Peptide Mapping

1. Use 0.5 to 1 µL aliquots of the ammonium bicarbonate solution surrounding the gel pieces for peptide profiling on ProteinChip Arrays (either NP1/NP2 or H4). Most peptides are detected without washing. However, some peptides are detected better after drying and water washing on H4 arrays.

   Note: if there is no buffer outside the gel pieces after the overnight incubation, add 15 µL 25 mM ammonium bicarbonate, pH 8.0 and vortex, then allow the mixture to equilibrate for 30 minutes prior to analysis. Use 2-5 µL of solution per spot for analysis.

2. Include a spot with EAM alone and a spot with the "no protein" gel slice control to control for background signals.

3. Add 0.5 µL CHCA (saturated solution diluted 5-fold into 50% acetonitrile, 0.25% TFA) to each spot. Allow to air dry, then analyze in the ProteinChip Reader.

4. After initially collecting data, it may be useful to add a mixture of 2–5 internal standards and collect data again. This method will ensure that you obtain the most accurate mass readings possible. However, adding internal standards may change the spectrum profile of the peptide map, due to high signals from internal standards and/or ion suppression! See *"Internal Calibration"* on page 2-3 for details of using internal standards.

   Recommended standards include:

Peptide Standard	Mass (Da)
angiotensin 1 (human)	1296.5
Glu1 fibrinopeptide B	1570.6
big endothelin 1 (human, 19–37)	2182.5
ACTH (human, 1–24)	2933.5
beta-endorphin (human, 61–91)	3465.4

Data Collection

Collect data using a large number of shots at low laser power to get good peptide resolution. The peaks from control spots (i.e., from digests using blank gel slices) should be subtracted from the spots containing target protein digests. See *"Comparing Spectra"* on page 14-49 for detailed information on subtracting peaks.

Searching for Protein Identity

The program ProFound can be used to search for the protein(s) that might produce peptides with the specific masses determined from the experiment. Database-searching software is available free from http://prowl.rockefeller.edu. Most peptides are modified by

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acrylamide during SDS-PAGE, making it important to include that information in your search.

Thanks to Thang Pham, Ph.D. for developing this protocol.

Biomarker Protein ID (Alternative Protocol)

This protocol works well with Coomassie, silver or negative metal staining protein bands. It has been used successfully in protein ID of silver stained proteins in relatively large abundance. However, in cases where silver staining is necessary to detect a faint protein band of interest, there may not be sufficient protein in the sample to allow successful protein ID using this protocol.

Reagents

- 25 mM ammonium bicarbonate/50% acetonitrile.
- 25 mM ammonium bicarbonate pH 8.
- 0.1 µg/µL sequencing grade trypsin (Promega) in 25 mM ammonium bicarbonate, pH 8.
- 50% acetonitrile/5% trifluoroacetic acid.

Protocol

Destain and Remove SDS from Proteins in Acrylamide Gel

1. Use a 24-gage or thinner needle to punch several holes in the bottom of a 0.5 mL microcentrifuge tube.
2. Cut the band containing the protein of interest from an SDS gel using a razor blade, then place the band in the 0.5 mL tube. Also include a sample containing protein-free gel pieces to be carried through the process as a negative control.
3. Put the 0.5 mL tube in a 1.5 mL tube and centrifuge at 14000 rpm briefly until the gel pieces are minced and collected into the 1.5 mL tube.
4. Destain and dehydrate the gel pieces with ~3 washes: cover the pieces with enough 25 mM ammonium bicarbonate/50% acetonitrile to immerse them, then place on a shaker for ~10 minutes. Use small-bore pipette tips to remove and discard the supernatant after each wash. Perform a sufficient number of washes so that the gel pieces shrink and turn an opaque white, and the removed supernatant is colorless.
5. Dry the destained gel particles for ~30 minutes in a SpeedVac® system or comparable device.

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In-gel Protease Digestion

1. Rehydrate the gel pieces in 25 mM ammonium bicarbonate buffer containing 0.1 µg/µL trypsin. Add only enough solution so that it is all absorbed by the gel (use ~5 µL aliquots).

   Note: the concentration of enzyme results in enzyme:substrate ratios >1:10 for in-gel digestions. Also, when trying this protocol for the first time, it is very important to use a variety of trypsin concentrations in side-by-side digests to determine the optimum concentration.

2. Overlay the gel with 25 mM ammonium bicarbonate buffer pH 8. Add just enough buffer to ensure the gel pieces remain immersed throughout digestion.

3. Incubate 12–16 hours (overnight) at 37 °C (it may be possible to incubate for a shorter time).

ProteinChip Analysis

1. Use a small-bore pipet tip to remove 2 µL digest solution from between the gel pieces. Aliquot 0.5 to 1 µL of the digest solution on the spots of a Normal Phase (NP) Chip Array for analysis without a water wash.

2. Add 0.5 µL CHCA (saturated solution diluted 5-fold into 50% acetonitrile, 0.25% TFA) to each spot. Allow to air dry, then analyze in the ProteinChip Reader.

3. Be sure to analyze a spot with EAM alone and a spot with the "no protein" gel slice control to control for background signals.

4. It may be useful to add a mixture of 2–5 internal standards and collect data again. This method will ensure that you obtain the most accurate mass readings possible. However, adding internal standards may change the spectrum profile of the peptide map, due to high signals from internal standards and/or ion suppression! See *"Internal Calibration"* on page 2-3 for details of using internal standards.

   Note: if there is not enough signal, you will need to recover all peptides from the gel pieces. Perform 2–3 extractions in 50% acetonitrile/5% TFA: cover the digest mixture with approximately twice the volume necessary to immerse the gel pieces, then place on a shaker for ~10 minutes per extraction). Pool the supernatants from all of the extraction.

   Concentrate the recovered peptides by reducing the final volume of the extracts to < 5 µL in a SpeedVac, then adding enough 50% acetonitrile/5% TFA to bring the volume to ~25 µL. The amount of volatile salts can be decreased by a few cycles of adding water and reducing the volume in a SpeedVac.

Thanks to Ma Sha, Ph.D. for contributing this protocol.

Chapter 11

Protein-Protein Interactions Using Preactivated Surface ProteinChip Arrays

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Protein-Protein Interaction

Note: this protocol contains a great deal of technical information that may help in troubleshooting a variety of ProteinChip experiments.

This protocol describes how to capture specifically binding proteins from a whole cell lysate by using a GST-fusion protein covalently bound to a Preactivated Surface (PS) ProteinChip Array. The overall process is similar to that of the ProteinChip Immunoassay and the protocol can be adapted for analysis of a variety of protein-protein interaction experiments using the ProteinChip System. Please read through the technical notes at the end of this protocol before you begin.

Flow Chart

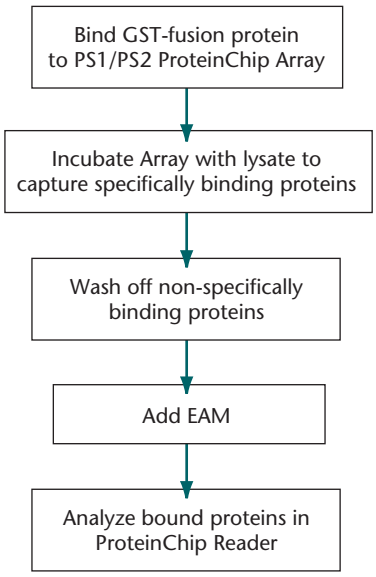

Figure 11-1: Using GST-fusion protein to capture specifically-binding proteins for a ProteinChip assay.

Protocol

Coupling GST-fusion Protein to the PS1 ProteinChip Array

Note: see notes at the end of the protocol for using a PS2 array.

1. Outline each spot with the hydrophobic pen. Air dry for at least 3 minutes.
2. Add 50% acetonitrile to each spot. Incubate ~2 minutes in a humidity chamber.
3. Dilute the GST-fusion protein[1] to ~0.5 mg/mL in a Tris-free solution at pH 7.4–8.2. PBS and carbonate buffer work well; reagents to be avoided include glutathione, Tris, azide, and glycine. Include GST as a negative control. If necessary, the concentration of GST-fusion protein may be as low as 0.1 mg/mL.

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4. Remove the ACN from the chip with a lab wipe and replace with a drop of PBS immediately before adding the GST proteins. Do not leave the PBS on the spots more than a few minutes.
5. Remove the PBS, 1 or 2 spots at a time, to avoid letting the spots dry out, and immediately add 2 µL/spot GST-fusion protein.
6. Incubate the chip in a humidity chamber 1–2 hours at room temperature.
7. Block residual active sites on the chip by adding 3–5 µL 1 M ethanolamine (pH 8, in PBS) or 1 M Tris, pH 8. Incubate in a humidity chamber at room temperature for 15 minutes.
8. Remove the sample from the spots with the edge of a lab wipe.
9. Bulk wash the chip with 4–10 mL 0.5% NP-40 or Triton X-100 in PBS, for 10–30 minutes at room temperature. The detergent concentration and/or wash times can be adjusted for maximal fusion protein stability. The minimum detergent concentration for this wash should be at least two times the detergent concentration used in the binding and washing steps below.
10. Rinse the chip with PBS or TBS[2]. Leave the ProteinChip Array in the buffer until ready to use. Same-day use is recommended.

Binding of Interacting Proteins

1. Remove the chip from the buffer. Very gently blot the remaining liquid off the chip with a lab wipe.
2. Immediately apply a few µL binding buffer[2] to each spot to keep them wet until sample addition.
3. Prepare 4–15 mL lysate per chip[3]. Dilute the lysate to a concentration between 0.2–2.0 mg total protein/mL in TBST (TBS + 0.05% Tween 20) + 0.05% BSA. Place the diluted lysate into a ProteinChip Array shipping tube (4 mL) or a larger tube.
4. Place the chip in the tube containing the lysate, then seal the tube. Incubate with shaking overnight at 4 °C.

   Note: rapid shaking and end-over-end rotation work quite well. Slow rocking is not recommended.
5. Prepare a saturated solution of the EAM of choice prior to washing the chip (see EAM use and preparation protocol, page 4-4).
6. Bulk wash the ProteinChip Array 2 times, 2–5 minutes per wash, in TBST[4] (use a shorter wash time for lower affinity interactions).
7. Bulk wash the ProteinChip Array once for 1 minute, in 5 mM Tris, pH 7.6 or in 5 mM HEPES.
8. Shake the excess buffer off the chip.

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9. Add 0.5 µL EAM to each spot before they completely dry out, but after most of the liquid has evaporated. Make sure there is no liquid between the spots before adding the EAM — blot between the spots with a lab wipe if necessary. If SPA is the EAM of choice, use two 0.5 µL aliquots.
10. Allow the chip to air dry, then analyze it in the ProteinChip Reader.

Technical Considerations

There are many important considerations for protein-protein interaction applications that will determine the relative success or failure of an assay. The section below outlines some of these considerations and should be used as a guide.

[1]GST-fusion Protein Sample

How pure is the capture protein, in this case the GST-fusion protein?

GST-fusion proteins purified on glutathione beads often contain free GST. The free GST is generally between 26–29 kDa, depending on the expression system used. It is recommended that fusion protein samples be analyzed prior to coupling (see separate protocol for analyzing protein purity using Normal Phase or H4 chips). If there is significant contamination by free GST or other proteins, a sample cleanup step is suggested (e.g., size separation). The fusion protein linker, usually containing a cleavage site, seems to be somewhat labile and free GST can appear as a degradation product, often having some size heterogeneity. Additionally, any endogenously produced GST will co-purify with the GST-fusion protein.

Does the GST-fusion protein sample contain glutathione or other problematic components?

GST-fusion proteins are generally eluted from glutathione beads with free glutathione. Once eluted, the protein sample will contain high molar concentrations of the glutathione. The coupling chemistry of the PS1 and PS2 chips reacts with free amino groups in the sample. Glutathione (and other buffer components with free amino groups) will compete for coupling to the preactivated chip surface, thus it is necessary to remove it. Removal can be accomplished by dialysis or a low molecular weight cutoff spin column. Microcon filters are easy to use and will also concentrate the protein if desired. Glycerol at 5% or less does not appear to interfere with coupling.

Should the GST be cleaved from the fusion protein?

The GST portion of these fusion proteins can usually be removed by a thrombin cleavage of the linker sequence, although this will depend on the choice of expression vector. Thrombin cleavage can also be used after binding the fusion protein to glutathione beads. If the GST domain interferes with the binding of interacting proteins, its removal

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is recommended. Complete information regarding proteolytic cleavage is usually included with the expression system information.

GST dimerization will be apparent in ProteinChip experiments.
GST can dimerize. As a result, some of the GST on the ProteinChip Array will *not* be covalently attached. This is important to consider when analyzing the data, as GST-fusion protein peaks will be seen in the data set. These are frequently the most abundant signal; use the **Reference Lines** function of the software to aid in identifying multiply-charged or multimer peaks. If these GST-fusion protein peaks overlap or mask peaks of interest, it may be best to remove the GST domain (see above). However, keep in mind that the dimerization may increase the avidity of the desired protein-protein interaction. If this avidity increase is required for stable binding, removing the GST will weaken the binding interaction and thus increase complex dissociation.

[2] Binding and Washing Buffer Choice — What is Best for ProteinChip Experiments?

Have other protein-protein interaction methods been used with the same target proteins?
If previous experiments with the same capture protein (GST-fusion protein in this case) have been successful with a particular set of binding and washing conditions, it is reasonable to assume these conditions will also work for ProteinChip analysis. The GST protocol above was designed based on the successful precipitation of the target proteins with GST-fusion proteins bound to glutathione beads and might not be optimal for your experimental system. Optimize the protocol for your system as appropriate.

Effect of buffer composition.
Buffer pH, detergent type and concentration, concentration of salts, presence of denaturing agents, metal ion concentration, etc., can significantly alter the stability of the interaction of interest. Again, use previous experience as a guide. In this protocol TBST was used because it had been successfully used in a precipitation of the target proteins with GST-fusion proteins bound to glutathione beads. If no other experimental information is available, it is reasonable to mimic predicted physiologic conditions or successful experiments using similar types of proteins. We suggest adding 0.05% BSA to reduce non-specific binding.

[3] Lysate Preparation

Protein conformation and availability.
It is crucial that the proteins of interest maintain whatever conformation is required for the desired interaction. For example, denatured proteins frequently will not interact with other proteins. On the other hand, if the protein of interest is already interacting with a partner in the lysate (or other sample type), it might not be available

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for binding the GST-fusion protein. It might be necessary to dissociate preformed complexes prior to incubation with GST-fusion protein on the chip surface. The lysate-protein complexes may be dissociated with high salt, detergent and/or denaturing agents, then diluted into a buffer that supports protein-protein interaction.

Lysate sample source

The cell or tissue lysate should be prepared to maximize the concentration of expected target proteins relative to other proteins in the sample. Often a cytoplasmic or nuclear extract or a membrane preparation rather then a whole cell lysate is most successful, although not required. Additionally, high total protein concentrations in the lysate (i.e., >10 mg/mL) allow the user to dilute the sample into an appropriate buffer. In this example whole cell lysates were used. Be aware that the target protein may not be available for capture due to interaction with another protein in the lysate (or other sample, see comments above).

The effect of lysate buffer composition on protein-protein interaction.

Since lysates often contain 1% detergent, denaturing agents, and/or other components intended to disrupt protein-protein interaction, dilution of the lysate sample is often crucial to the experiment's success. In this example the lysate contained 1% Triton X-100, 10% glycerol, NaCl and EDTA, so dilution was necessary for the desired binding reaction.

Lysate volumes and binding time — high or low?

Although most ProteinChip applications require only a few microliters sample per spot, protein-protein interaction experiments are more likely to succeed if larger volumes are used. If the target or targets are expected to be at low concentration (especially after required dilution), the lysate volume should be increased. This can be done 3 ways: 1) incubate a large volume of lysate (4–15 mL) in a tube with a single ProteinChip Array; 2) use a bioprocessor for volumes of 0.025–0.4 mL/spot; or 3) multiple additions of a smaller volume can be used, i.e., three additions of 5 µL/spot with enough time between additions for equilibrium to be attained. Larger volume incubations take longer to reach equilibrium; adjust binding times accordingly.

[4] Washing Steps — Time and Number

How do washing times and number relate to affinity and concentration?

The total amount of washing time and volume depends on the affinity of the interaction, (specifically the off-rate) and the amount of non-specific binding expected. If the affinity is low or the off-rate is relatively fast, short wash times are recommended. This protocol assumes a moderately rapid off-rate. However, wash times can be as low as 1 minute each. Additionally, since femtomolar amounts of target protein are captured on the chip surface, extended high volume washes will significantly decrease the amount even of high

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affinity protein-protein interactions. It is important to use consistent wash volumes and times.

PS2 ProteinChip Array Suggestions

PS2 ProteinChip arrays are specifically designed for reduced non-specific background binding. Thus, shorter wash times and less detergent are required for a successful experiment. PS1 chips tend to have some hydrophobic background binding, so the inclusion of a low concentration of detergent (0.05–0.2%)is highly recommended unless it will eliminate the specific binding.

Many thanks to Lisa Bradbury, Ph.D. for this protocol and extensive technical notes.

Chapter 12
Additional ProteinChip Applications

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DNA- or RNA-Binding Protein Assay

This experimental protocol allows you to capture proteins that bind to nucleic acids. The DNA/RNA binding experiments can be performed two ways. The first protocol (starting on page 12-4) describes the on-chip binding method. The second protocol (starting on page 12-6) describes binding in solution followed by analysis on a streptavidin coated ProteinChip Array, since binding in solution may be more efficient in some cases.

Both the PS1 and PS2 ProteinChip Arrays can be used successfully for this application. We recommend trying both chips initially and noting which chip type works best for your experimental system.

Notes on Reagents

Streptavidin, Avidin, and NeutrAvidin

Streptavidin, Avidin, and NeutrAvidin are tetrameric, containing 4 identical, noncovalently associated 12–15 kDa subunits. Not all subunits will couple to the chip surface; thus, a monomer peak between 12–15 kDa results.

Streptavidin has been used successfully in ProteinChip applications.

Avidin has carbohydrate moieties and tends to have higher non-specific background than streptavidin.

While *NeutrAvidin* has been used successfully, it is not recommended for ProteinChip Array studies. NeutrAvidin is designed for reduced non-specific binding. However, it gives rise to a significant number of multimeric peaks, ranging from dimers up to hexamers, which can interfere with data interpretation.

Target DNA or RNA

Free biotin or biotinylated PCR primers need to be removed from target nucleic acid prior to use to maximize capture of the target on the Streptavidin chip surface.

The target sequence should have only 1 biotin per DNA or RNA molecule.

The target sequence is more effective when multiple copies of the protein binding site are present.

The target DNA or RNA should be at least 30 bases long, and can be up to thousands of bases long.

Protein binding site should be at least 10 bp and preferably at least 20–30 bp from the biotinylated end of the target for maximal accessibility.

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Double-stranded DNA with Biotin Attached to One End of One Strand

Double-stranded DNA with biotin attached to one end of one strand can be created by annealing synthetic overlapping oligonucleotides, with one oligo having a biotin on the 5´ end.

Alternatively, PCR can be used to create the necessary DNA or RNA fragment. Primers, one biotinylated and the other unlabeled, can be used for the PCR. The unreacted oligos must be removed from the sample prior to using it in the ProteinChip Array experiment.

Binding Buffers for the DNA- or RNA-Protein interaction

The chosen binding buffer should support optimal DNA-protein interaction. $MgCl_2$, KCl, DTT, EDTA may be required for binding. Often, what works for a gel shift assay will work for this application.

Poly d(I-C)•d(I-C), salmon sperm DNA, or some other nucleic acid should be present in the binding buffer to reduce non-specific interactions. Important: The notation for d(I-C)•d(I-C) DNA is a bit tricky. Be sure to use DNA containing a mixture of (alternating) dI and dC on each strand— you want to avoid DNA with all dI on one strand and all dC on the other strand.

BSA at 100 µg/mL can be added to reduce non-specific interactions.

The following binding buffer has been used successfully:

> 30 mM Tris, pH 8.0, 3.0 mM $MgCl_2$, 0.1 M KCl, 0.2 mM DTT, 1–5 mM EDTA, 2 mg/50 mL d(I-C)•d(I-C)

In Vitro Translated Proteins

It may be desirable to test *in vitro* translated proteins for their DNA- or RNA-binding capacity using this protocol. In that case, you must use wheat germ extract for the *in vitro* translation; avoid reticulocyte lysate because it contains many proteins (including an overwhelming amount of hemoglobin) that will interfere with the binding reaction and subsequent protein profiling.

Protocol Using Biotinylated DNA/RNA Bound to Chip

Coupling Streptavidin to a Preactivated Surface ProteinChip Array (PS1 or PS2):

1. If using a PS1 chip, outline each spot with a hydrophobic pen. Air dry the chip for ~5 minutes.
2. Place the ProteinChip Array (PS1 or PS2) in the humidity chamber.
3. Optional: if using a PS1 chip, apply 5 µL 50% acetonitrile to each spot. Incubate ~2 minutes.

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4. Dilute the streptavidin to ~0.1 mg/mL (PS1 chip) or 0.5 mg/mL (PS2 chip) (= ~1.6 or 8.0 pmol/ml) in a Tris-free buffer solution at pH 7.4–8.2.

   Note: PBS and carbonate buffers work well. Avoid azide and glycine in the buffer.

5. If the spots have been treated with acetonitrile, wash them with PBS, either in bulk or using 5 µL per spot.

6. Working on 1 or 2 spots at a time, remove the PBS and immediately add 2 µL streptavidin per spot.

7. Incubate in a humidity chamber 1–3 hours at 37 °C or room temperature, or overnight at 4 °C.

8. To each spot, add 4 µL 1 M ethanolamine, pH 8, in PBS to block residual active sites on the chip. Incubate in a humidity chamber for 30 minutes at room temperature.

9. Wash the chip in a 15 mL conical tube with 9 mL 0.5% Triton X-100 or NP-40 in PBS, for 5–10 minutes at room temperature.

10. Rinse the chip with PBS. The chip can be stored in 50 mM HEPES, pH 8.0, for up to 48 hours (storage in PBS tends to lead to precipitates forming on the chip).

Binding of Biotinylated Nucleotides

Note: target DNA or RNA should have no free biotin or biotinylated oligomers present in the sample. These will compete for binding to the streptavidin surface. It may be necessary to purify the target sequence with a spin column or agarose gel before applying it to the chip.

1. Remove the chip from the PBS. Gently blot the remaining liquid off the chip with lab wipe.

2. Immediately add 4 µL PBS or binding buffer to each spot to keep them wet until sample addition.

3. Dilute biotinylated DNA or RNA in Tris, PBS, or other buffer (see reagent notes above) to 1–5 pmol DNA/spot. Dilute non-biotinylated control DNA or RNA to the same concentration.

4. With the corner of a lab wipe, draw buffer off a spot, and immediately add 2–4 µL biotinylated DNA or RNA solution. Leave at least 1 spot as a negative control, using either no DNA or non-biotinylated DNA.

5. Incubate the chip in a humidity chamber for 15–60 minutes at room temperature.

6. Wash twice in bulk with 7 mL binding buffer, 5–10 minutes at room temperature for each wash.

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On-Chip Capture of DNA/RNA Binding Proteins

Note: when executing this experiment for the first time, it can be advantageous to set up chips to test binding at both room temperature and 4 °C. Both temperature and salt concentrations can affect binding.

1. Very gently blot any remaining liquid off the chip with a lab wipe. Add 4 µL binding buffer to each spot to prevent drying.
2. Dilute the DNA binding protein to between 10–2000 femtomole/µL in buffer that will support strong DNA/protein interaction. Be sure to include cofactors and non-specific binding inhibitors — please read the notes above on binding buffers for the DNA- or RNA-protein interaction.
3. Add 3 µL of the protein solution to each spot. Incubate the chip in a humidity chamber ~1 hour at room temperature or overnight at 4 °C.

   If desired, multiple additions of sample can be used to increase the amount of protein captured:

   a. Remove the protein solution from the chip with the corner of a folded lab wipe.

   b. Apply 3 µL protein solution to each spot.

   c. Incubate the chip for 1 hour at room temperature.
4. Prepare the appropriate EAM.
5. Remove the sample from the spots using a lab wipe.
6. Wash the chip twice in 9 mL binding buffer for 5 minutes at room temperature.

   Note: additional or longer washes may be necessary if non-specific binding is a problem. Increasing the salt concentration may also decrease non-specific binding.
7. Quickly rinse the chip 2 times in bulk with deionized H_2O.
8. Wipe off any excess water around the spots. While the spots are still moist, add 0.5 µL EAM per spot. If SPA is used, apply a second 0.5 µL per spot for maximal signal.
9. Allow the chip to air dry.
10. Analyze in the ProteinChip Reader.

Protocol for Capture of DNA-/RNA-Protein Complexes Formed in Solution

Note: when executing this experiment for the first time, it may be advantageous to set up chips to test binding at both room temperature and 4 °C. Both temperature and salt concentrations can affect binding.

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1. Prepare streptavidin chip as described in Step 1 of the Protocol for ProteinChip Array capture of biotinylated DNA/RNA on page 30. Prepare target DNA or RNA as described for that protocol, i.e., remove free biotin or biotinylated oligomers.
2. Add between a 10-fold and 1000-fold excess of competitor DNA to the sample containing binding proteins to block non-specific binding sites.
3. Add biotinylated target DNA or RNA to the sample. Prepare 10–12 µL binding reaction per spot. Use ~0.2–0.8 pmol DNA/10 µL binding reaction. If a nuclear extract is used as a source of binding proteins, maximize the total protein concentration for best results. The binding proteins comprise only a small percent of the total protein. Incubate these reactions for 1 hour or more, with mixing, at room temperature or 4 °C. Optimal time and temperature are affinity- and concentration-dependent.

   In addition, create a negative control tube using non-biotinylated DNA or RNA.
4. Add 3 µL of the binding reaction to each spot on the streptavidin chip. Incubate for 20 minutes at room temperature. Equilibrium is quickly reached due to the high affinity of biotin-streptavidin interaction (K_d ~10^{-15}).
5. Repeat step 4 above 2 more times for a total of 9 µL/spot. Larger or smaller sample volumes can be used, depending on the conditions and the affinity of the interaction.

Using the Bioprocessor

The bioprocessor can be used to add larger sample volumes, 25–400 µL/spot. Diluting the sample often reduces the non-specific binding. However, larger volumes may result in the capture of fewer target proteins when they are limited in the sample. The quantity captured depends on the binding affinity between these proteins and the nucleic acid target and their concentration in the solution. If the bioprocessor is used, the incubation time should be increased since it will take longer for equilibrium to be reached.

Special thanks to Lisa Bradbury, Ph.D. for this protocol.

Ligand Binding Assays for Receptors Embedded in Membrane

This is a general set of protocol guidelines for using Ciphergen's ProteinChip® technology to measure the binding of ligands (peptides, proteins, compounds) to receptors embedded in a membrane. This approach is particularly useful for multi-transmembrane receptors (i.e., GPCRs) whose conformation is disrupted when the membrane is removed. These experiments are expected to require some optimization (see notes at end). The ligand binding is done in a tube and after washing away unbound ligand, and the membranes are directly analyzed on a Normal Phase ProteinChip array.

Membrane Preparation and Characterization

The quality of the membranes used for the binding experiment is very important for experimental success. In general, the membranes should be prepared at the same time from cells at about the same stage of cell cycle (i.e., log phase growth or confluence) and cell density. Avoid using trypsin to isolate adherence cells from a tissue culture plate as extracellular domains could be digested. Test and control membrane preps should have similar number of freeze-thaw cycles (if any), isolation procedure, etc.

Prior to the ligand binding experiment, the membranes must be characterized to generate a membrane "baseline" data set representing endogenous proteins and peptides tightly associated with or integral to the membranes. Also, if there are endogenous proteins that might interfere with ligand detection (i.e., same MW) these should be stripped with additional washing steps prior to the ligand binding step. It is strongly recommended that negative controls be included to aid in data interpretation.

Protocol

Part I: Preliminary Ligand and Membrane Characterization Experiments

1. Determine the best conditions for generating a good ligand signal. Test different EAMs and solvent composition if necessary (see EAM notes). Note best instrument settings for ligand detection for step 4.

2. Wash the cell membranes (5–10 µg) with the same buffers that will be used for the ligand binding and washing steps (i.e., mimic the ligand binding assay).

3. Titrate the amount of washed membrane applied to the spots of the ProteinChip Array to determine the optimal amount to use in the experiment. The optimal amount is generally between 0.6–1.0 µg/spot, but test a broad range initially.

4. Using approximately the same instrument settings that will be required for ligand detection, collect data from the washed

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membranes to generate a "baseline" data set for this particular membrane preparation.

5. If any of the peaks in the "baseline" data set might interfere with ligand detection, test additional washing steps to be used prior to the ligand binding step. Specifically, high salt/EDTA, or longer wash times can help remove interfering peripheral membrane proteins.

Part II: Binding Assay

1. To set up a 50 µL binding reaction, place ~5 µg cell membranes into a microfuge tube. Set up additional tube(s) as negative (and positive) control.
2. Wash the membranes in each tube with 400 µL/tube PBS + 1 M NaCl once (or use PBS + EDTA if necessary). Microfuge the tubes briefly to pellet the membranes, then remove the wash buffer.
3. Briefly wash the pellets once with 400 µL/tube binding buffer.
4. Resuspend the cell membranes in one tube in 50 µL binding buffer ± ligand (and ± competitors, if using). Ligand concentration will depend on the affinity, therefore for the first experiment test multiple ligand concentrations — 0.01 to 1 µM is suggested. Resuspend the cell membranes in the other tube in 50 µL binding buffer without ligand, to create a negative control.
5. Incubate the tubes at 37 °C for 10 minutes, or at 4 °C for 30 minutes, assuming a rapid on-rate. Increase binding times as on-rates decrease and as total volume increases.
6. Wash the contents of each tube twice with PBS/0.5 M NaCl. You can alter the buffer composition as necessary for strong specific ligand-receptor interaction with minimal non-specific binding.
7. Wash once at 4 °C with PBS (the time will depend on the off-rate and non-specific binding levels). Pellet the membranes, then remove as much of the PBS as possible.
8. Resuspend the cell membranes in each tube using an appropriate volume of EAM such that 1 µL will deposit the optimal amount for data collection (as determined above) on the spot.
9. Apply 1 µL/spot of the membrane+ligand+EAM mix onto a Normal Phase chip. Also apply 1 µL of the membrane+EAM mix on 1–2 spots to serve as a negative control. Additional spots with 0.5 and 2 µL/spot can also be prepared. If the ligand is less then 1200 Da, it is recommended that an EAM-only control spot be included to help distinguish between ligand and EAM signal.
10. Analyze the chip using instrument settings optimized for the ligand. Compare negative and test samples to assess specific ligand binding.

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Other Experimental Variables

Although this protocol uses a 50 µL reaction volume, it is reasonable to use any convenient volume. Consider the effect of volume changes on equilibrium binding.

Assays to measure inhibition of ligand binding can be performed in multiple ways. The easiest methods are to add competitors prior to adding ligand (before step 4 of the binding protocol) or to add competitors with ligand (during step 4 of the binding protocol).

It may be possible to elute bound ligands from the membrane samples with high salt, acid, or other reagents. The eluate can then be analyzed on a Normal Phase or H4 ProteinChip Array.

After ligand binding, the membranes can be solubilized with detergent or other methods. Use standard protein profiling methods to look at ligand binding.

Binding/Washing Stringency

The binding and washing stringency both pre- and post-ligand binding depend on the membranes themselves and the ligand-receptor affinity. In general:

- Pre-binding washes can be fairly stringent but detergents should be avoided.
- Binding and post-binding washes should not significantly reduce the specific binding while minimizing non-specific binding. Changes in buffer salt concentration and pH might be useful if nonspecific binding is a problem.
- Washing and binding temperatures will alter on and off rates and nonspecific binding. Lower temperatures will slow both on and off rates and reduce non-specific binding.
- Binding and washing time changes can improve signal to noise ratios. If the off-rate is slow, wash steps can be fairly long.

Special thanks to Lisa Bradbury, Ph.D. for this protocol.

IMAC3-Gallium Array Phosphopeptide Capture

Flow Chart

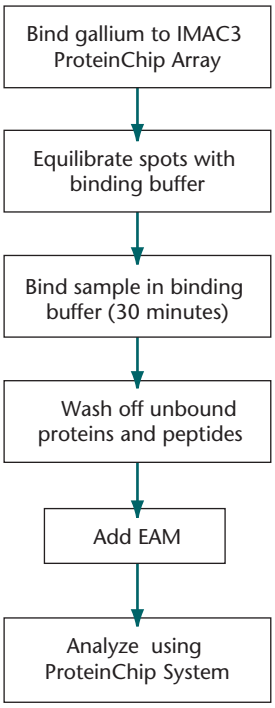

Figure 12-1: Flow chart for phosphopeptide capture on IMAC3 ProteinChip Arrays.

Protocol

Binding Buffer

50 mM sodium acetate, pH 5.4, 2.5 M NaCl, 0.1% OGP (n-acetyl-B-D-glucopyranoside), 5 mM sodium malate.

Note: iron is not recommended for use with IMAC3 chips. For very complex samples or samples with high lipid content, urea can be added (<2 M) into the binding buffer to improve selectivity.

As with other ProteinChip array protocols, all of the binding and washing steps should be carried out in a humidity chamber to keep the active spots of the array moist.

1. Apply 5 µL 50 mM $Ga(NO_3)_3$ to each spot of an IMAC3 ProteinChip array.
2. Incubate at room temperature for 5 minutes, pipetting occasionally to mix. Tap the chip on the benchtop to remove excess metal solution.
3. Apply another 5 µL 50 mM $Ga(NO_3)_3$ to each spot. Incubate at room temperature for 5 minutes, pipetting occasionally to mix.
4. Wash the chip in bulk by placing it in a 15 mL centrifuge tube with 8 mL deionized water, then shaking vigorously for 1 minute. Flick extra water off the chip by tapping the side of the chip on the benchtop.

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5. Equilibrate the spots with binding buffer (50 mM sodium acetate, pH 5.4, 2.5 M NaCl, 0.1% OGP (n-acetyl-B-D-glucopyranoside), 5 mM sodium malate). Apply 5 µL of the equilibration solution to each spot, allow to stand for 5 minutes, then remove. Repeat twice more for a total of 3 equilibrations.
6. Apply sample to the spots (5 µL phosphopeptide in peptide mix, dissolved in 50 mM sodium acetate, pH 5.4, 2.5 M NaCl, 0.1% OGP, 5 mM sodium malate). Allow to stand at room temperature for 30 minutes to mix.
7. Use a pipettor or the corner of a lab wipe to remove the samples from the spots, then wash the spots with 50 mM sodium acetate, pH 5.4, 2.5 M NaCl, 0.1% OGP, 5 mM sodium malate. Apply 5 µL of the wash solution to each spot, allow to stand for 5 minutes, then remove. Repeat twice more for a total of 3 washes.
8. Wash the chip in bulk by placing it in a 15 mL centrifuge tube with 8 mL deionized water, then shaking vigorously for 1 minute. Flick extra water off the chip by tapping the side of the chip on the counter. If necessary, dry around the spots with the corner of a lab wipe.
9. Apply 0.5 µL SPA twice (for proteins) or 0.5 µL of a 10% saturated solution of CHCA once (for peptides) to each spot.
10. Analyze in the ProteinChip Reader.

Thanks to Tai-Tung Yip, Ph.D., and Vanitha Thulasiraman, Ph.D. for developing this protocol.

Analysis of Intact Glycoproteins

Due to the microheterogeneity of glycoproteins, when a protein contains groups of related but structurally different oligosaccharides, the peaks produced in ProteinChip Array analysis are characteristically broad, giving the impression of poor peak resolution. It has been observed that removing the carbohydrate moeity from glycoproteins greatly improves the resolution of the peak (see Figure 12-2).

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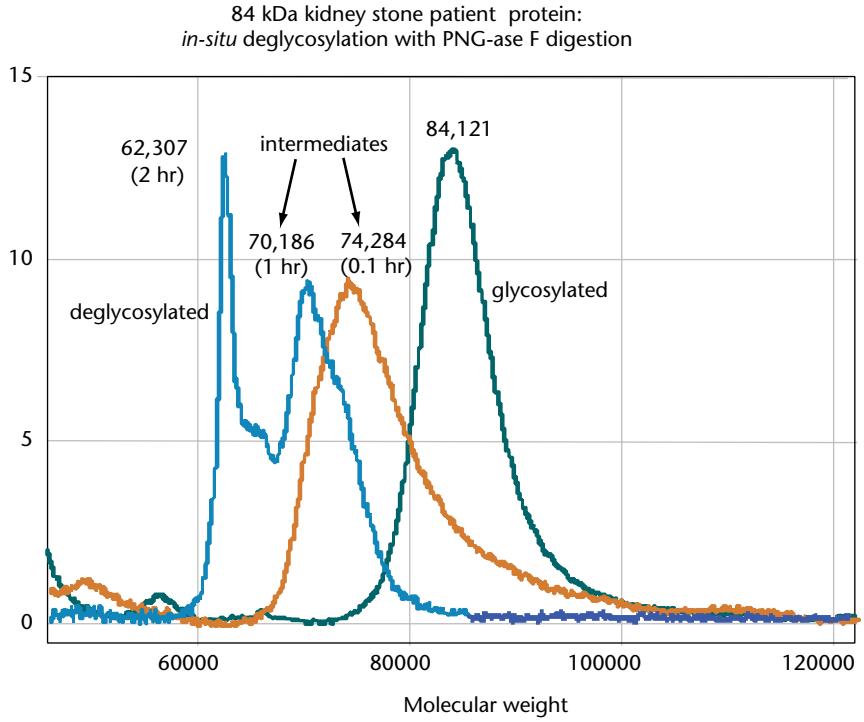

Figure 12-2: Deglycosylation improves peak resolution.

Glycoproteins can prove difficult to ionize, so it may be necessary to examine a variety of matrices and solvents. For large proteins SPA and EAM1 have both been used with success. Ferrulic acid is another possibility. For smaller proteins and peptides, DHBA can give good results. Sometimes adding a small amount of detergent to the EAM can be helpful. Try either Triton X-100 or NP-40 at 0.01–0.1% in 50% ACN, 0.5% TFA. Another alternative matrix solvent is 30% ACN, 15% isopropanol, 0.5% TFA and 0.05% Triton X-100 (or NP-40).

Deglycosylation of Glycoproteins

N-linked (and to a lesser extent O-linked) carbohydrates can be removed either chemically or enzymatically. A class of enzymes called N-glycanases, the most common of which is PNGase, can be used to remove N-linked carbohydrates. These enzymes can be purchased from a number of sources, but are difficult to purify and thus may contain a number of other proteins, peptides and contaminants. Pure preparations of N-glycanases are very unstable. O-glycanase is also commercially available but this enzyme is far less effective than PNGase and may not remove all of the O-linked carbohydrate. It is worth trying if an O-linked carbohydrate is suspected. Preparations of this enzyme also tend to contain numerous contaminants. Both enzymes should be examined prior to use by spotting a small amount on a normal phase chip and analyzing the components.

Chemical cleavage of carbohydrates should only be attempted as a last resort since it is tricky and uses particularly nasty chemicals.

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Enzymes for Protein Digestion and Deglycosylation

Use sequencing grade enzymes only or other very clean preparations. Contaminants are normally closely related proteins, which will interfere with the results. Purchase these enzymes from the recommended sources of sequencing reagents or from quality suppliers of protein chemistry reagents.

- *N-glycosidase:* cuts carbohydrate from N-linked sites. There are a number of types, *N-glycosides-F* (*PNGase*) are the most common and the first choice, depending on pH, buffers, and concentration. See instructions from the supplier.
- *Neuraminidase:* removes sialyic acid groups from carbohydrate chains.
- *O-glycosidase:* sometimes removes O linked carbohydrate from S or T residues, depending on pH, buffers, and concentration. See instructions from the supplier.

Preparing Deglycosylation Reagents

It is advantageous to denature the protein but this is not absolutely necessary. Glycosylation is normally on the surface of a protein, but the enzymes get the best access to the protein when the structure is removed and the protein is relatively linear.

N-glycanase needs to be in solution at 1 to 2 units per mL in order to keep activity of the enzyme and to clip the carbohydrate properly. Salt at concentrations >150 mM (including buffer) will inactivate the enzyme.

Notes on Deglycosylation

Deglycosylation in Solution

If you have a reasonable amount of your protein, you can perform the deglycosylation reaction in solution according to the manufacturer's instructions. The data sheet should provide guidance on resuspension buffers and enzyme:protein ratios. The deglycosylating enzyme may also be seen in subsequent analysis and so should be examined alone first to determine its mass and the presence of any contaminating proteins.

On-chip Deglycosylation

Deglycosylation enzymes can be added in a cocktail containing neuraminidase and N-glycosidase-F and O-glycosidase since they will work in the same buffer and do not interfere with each other. It is also important to understand where the N-glycosidase cuts the carbohydrate (there are several types) since some leave residues attached to the protein and some cut off all of the carbohydrate. Check the manufacturer's data sheet.

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Some proteins may require more time to remove the carbohydrate but in general, carbohydrate can be removed from the protein in two hours.

If only small quantities of your protein are available, it can be first immobilized on a ProteinChip Array and then deglycosylated. As explained below, the deglycosylating enzyme may also be detected in the ProteinChip analysis.

Protocol

1. Apply the protein to the chip surface in a mixture of 50/50 acetonitrile/water and let the spot dry. If you are using a reverse phase chip apply the sample in 6 M guanidine HCl. Warming the chip to 50 °C for 5 to 10 minutes in the presence of 6 M Guanidine HCl will increase binding to the surface and aid evaporation.
2. Wash the chip well with 5% acetonitrile/water
3. Place the chip in a bioprocessor and add 10 µL 50 mM Tris, pH 8.0.
4. Add the enzyme or mixtures of enzymes to the buffer. PNGase should be added to a final enzyme concentration of 1 to 2 units/mL.
5. Seal the well with parafilm or adhesive tape.
6. Incubate the sample at 37 °C for at least 2 hours.
7. Wash the chip with 5% acetonitrile in water.
8. Let the chip dry and analyze with the EAM of choice.

Examining Glycan Structure

At this point, the researcher could go in many different directions, some of which may not yet have been attempted using ProteinChip technology. As has been discussed, structural determination is problematic due to the heterogeneity of sugar chains. It may only be realistic to broadly classify the structure of the glycan.

Sugar Composition

Deciphering the sugar components of a carbohydrate is complicated by the fact that many monosaccharides have the same molecular mass and can only be distinguished by their molecular structure. ProteinChip Array analysis alone will only the reveal the mass of the sugar molecules and therefore may not be sufficient to identify them. For example, glucose, galactose and fructose all have a MW of 180 Da (MW is 162 Da after condensation), but differ in their structure (Figure 12-3). These sugars would not be distinguished by ProteinChip Array analysis. In addition, more complex sugar chains are defined by the way in which the monosaccharides are linked

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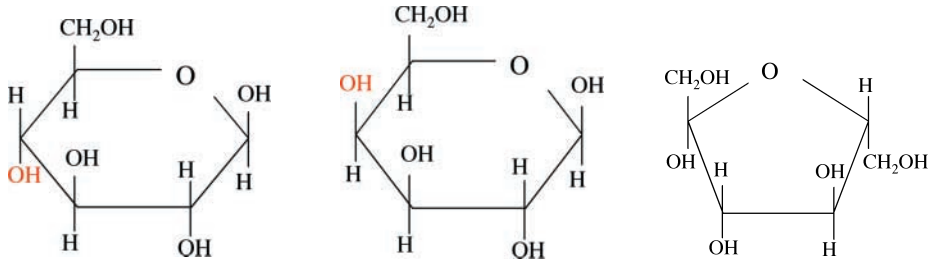
together (α1–4 linkages indicates carbon 1 of the first sugar is covalently linked to carbon 4 of the adjacent sugar). Again, ProteinChip Array analysis alone would not indicate how the sugars were linked. However, there are various enzymes available that only cleave carbohydrates at specific sugar structures. These enzymes could be used in combination with ProteinChip Array analysis to deduce the types of sugar structures that are present.



Glucose ($C_6O_6H_{12}$) Galactose ($C_6O_6H_{12}$) Fructose ($C_6O_6H_{12}$)

Figure 12-3: Molecular structures of three monosaccharides

The glycan moiety could be cleaved and digested into its constituent monosaccharides. This would indicate the simple sugars used in generating the glycan, as far as they can be identified by their mass alone. Sugars often ionize as Na^+ or K^+ adducts, producing peaks at m+22.9 and m+39.1. Some sugars will exclusively ionize with these metal adducts, and not produce a parent ion peak ($m+H^+$). This must be remembered when observing the mass of carbohydrates. As noted previously, it may be necessary to try a variety of EAMs to successfully ionize glycans. THAP, HPA, CHCA and DHBA have all been successfully used. It may be necessary to dilute (e.g., 10-fold or more) the saturated EAM solution in order to visualize molecules that appear within the mass range of the EAM itself.

Alternatively, simply analyzing the intact sugar chain may give some structural indications. Figure 12-4 shows the analysis of a purified synthetic carbohydrate (pullulan) composed of a repeating structure of maltose units (glucose triplets).

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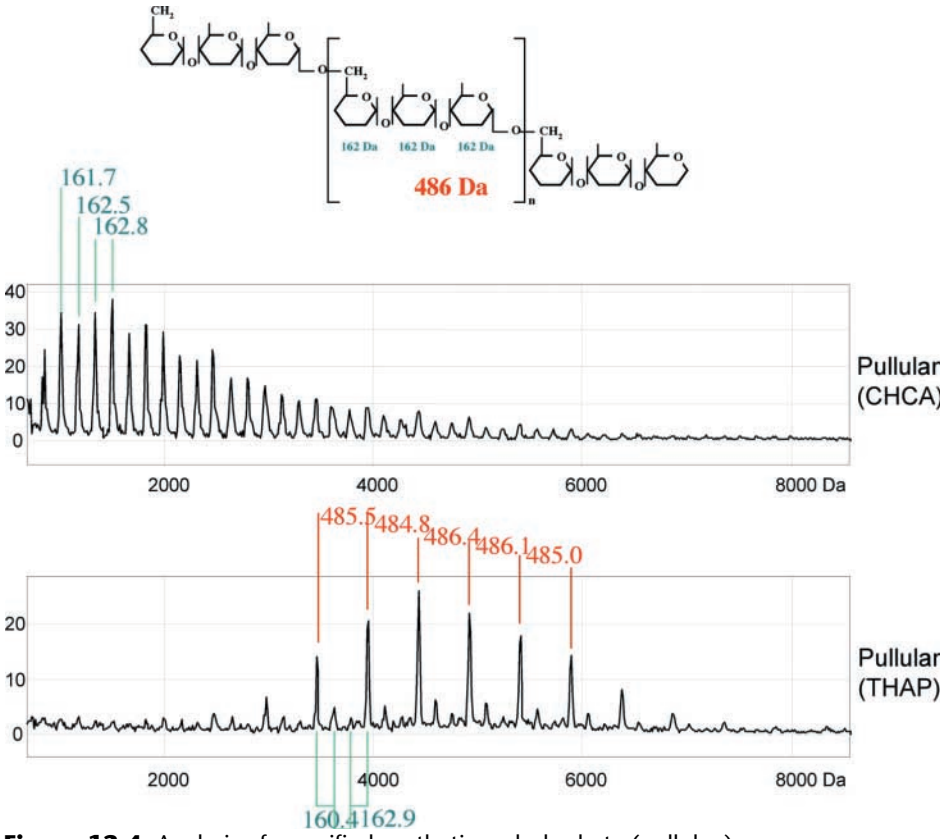

Figure 12-4: Analysis of a purified synthetic carbohydrate (pullulan).

ProteinChip analysis was performed using 2 different EAMs, and the resulting spectra are markedly different. The individual glucose molecules can be deduced from the repeating sequence of peaks of Δ162 Da seen in the CHCA spectrum, while the maltose units are indicated by the major peak series of m+486 Da in the THAP spectrum. However, as noted above, the mass shift of 162 Da could only be assigned as glucose because the expected structure of this carbohydrate was previously known.

Special thanks to Brad Thatcher, Ph.D., Kathryn Vardy, Ph.D. and Ma Sha, Ph.D. for their contributions to this section.

Quantitation on a ProteinChip Array

This protocol can be used to quantitate the amount of a particular protein in a solution by extrapolating a peak intensity measurement to a standard curve. This protocol has been developed specifically to address the issue of deducing the original concentration of a molecule in solution based on a ProteinChip Array experiment that includes washing steps. Analytes with molecular weights of 25 kDa or less are best suited for this type of analysis and a purified source of the analyte of interest must be available.

In any system, quantitative methods are more demanding than qualitative methods, and thus this protocol involves a sequence of

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several important steps (see the flow chart on page 12-19). The protocol differs from most in that an internal standard is added onto the same spot that already contains the molecule of interest. In other words, after an initial ProteinChip analysis has taken place, an internal standard is added to the spot and the chip is analyzed again in the ProteinChip Reader. This procedure is followed because, for accurate quantitation, is imperative to have the control protein on the same spot as the experimental sample protein.

Quantitating molecules which are not available in known concentrations is not possible since different molecules ionize and desorb differently. For example, an unknown protein might ionize extremely well relative to a standard, leading to concentration determinations that are falsely high.

Choosing Internal Standards

Internal standards are required in this protocol. The internal standards must be chosen carefully to ensure accurate quantitation. Specifically, the internal standards must be of a molecular weight similar to the analyte of interest, but the peaks from the standards must not overlap with peaks from the analyte. Generally, we recommend choosing standards of a molecular weight within a mass range of about ± 25% of the mass of the analyte. For example, for a protein of mass 10 kDa, choose internal standards of masses between 7.5 kDa and 12.5 kDa. Ideally, the internal standards should ionize and desorb with an efficiency similar to that of the analyte, to facilitate obtaining similar peak heights. The best internal standards would have biochemical properties (e.g., pI, amino acid composition and mass) that are very similar to those of the analyte of interest.

Notes

The peak intensities from all compounds of interest in the sample (i.e., from the analyte itself and from internal standards in a given spectrum) should be very similar for accurate quantitation. If the peak intensities vary by more than 5-fold, the compounds should be re-mixed and the assay should be re-run (see *"Obtaining Similar Peak Heights"*, below).

As with any quantitation procedure, the protocol should be run in duplicate or triplicate.

Obtaining Similar Peak Heights

First, make an equimolar solution of the Molecule Of Interest (MOI) and one of the Internal Standard (IS) in EAM/solvent. For example, make a solution that is 1 µM MOI, 1 µM IS. Spot 1 µL onto a Normal Phase chip and allow the solution to dry. Read the chip in the ProteinChip System. Alternatively, use a ProteinChip Array and protocol that are appropriate for your particular analysis.

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- If the peak height of the MOI is much greater than the peak height of the IS, make a new solution with 10-fold lower concentration of MOI or 10-fold higher concentration of IS.
- If the peak height of the IS is much greater than the peak height of the MOI, make a new solution with 10-fold lower concentration IS or 10-fold higher concentration MOI.

Flow Chart

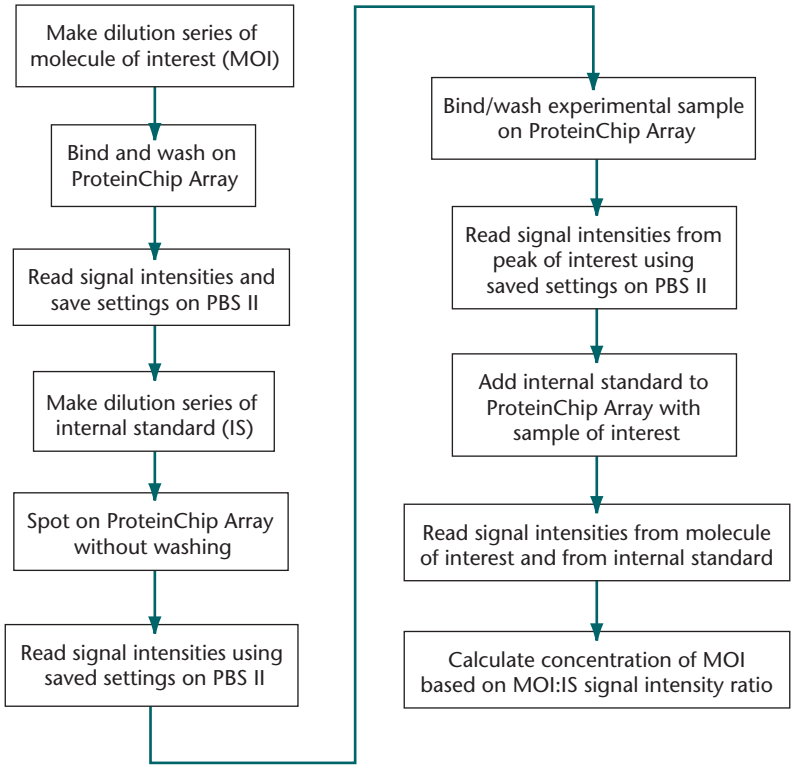

Figure 12-5: ProteinChip assay protocol for quantitation of an analyte in solution.

Protocol

Establish a Standard Curve for the MOI Using an IS

1. Prepare 10 µL EAM solution having an appropriate concentration of IS to yield a peak height intensity of ~10 from 0.5 µL.
2. Make a 5-fold or 10-fold dilution series of the EAM solution. The goal is to create a series of IS solutions such that, when 0.5 µL of each is spotted on a chip, a wide range of signal intensities is achieved.
3. Prepare a dilution series of the MOI solution in binding buffer. Make 10 µL of each solution, with concentrations ranging from 30 µM to 1 nM MOI.
4. Bind 1 µL of each solution to the array of choice, and follow the appropriate bind/wash/read procedure outlined below. The type

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of surface and protocol used here should be the same as that used in the analysis of a sample having an unknown concentration of the molecule of interest (see page 12-21).

a. Apply 1 µL MOI from the dilution series to each spot.

b. Wash the ProteinChip Array as appropriate for your protocol.

c. Read the ProteinChip Array in the ProteinChip Reader.

d. Save the ProteinChip Reader settings used for analysis.

Note: the goal is to have the MOI in various concentrations so that 1 µL analyzed on a ProteinChip Array yields a peak height intensity between 1 and 60 upon analysis, after following the appropriate binding and washing steps for the sample of interest.

5. Remove the array from the Reader.
6. Apply 0.5 µl EAM solution containing IS to a single sample spot. The concentration of EAM should be chosen so that the peak height of the IS will be close to the peak height of the MOI.

   Remember, it is best to err on the side of adding too little IS than adding too much IS! You can always add more IS if the signal is too low.
7. Read the ProteinChip Array in the ProteinChip Reader, using the saved settings.
8. Based on the information now available regarding the IS peak intensity, apply IS to each sample spot in various concentrations, as prepared in step 1 above, trying to match the peak intensity of the IS to the peak intensity of the IS.
9. Read the ProteinChip Array in the ProteinChip Reader, using the saved settings.
10. Export the peak intensity data into Excel and create a logarithmic scale plot showing the ratio of

$$\frac{100 \times \text{MOI peak height}}{\text{IS peak height} \times \text{dilution factor}} \quad \textit{vs. MOI concentration (nM)}$$

    Apply linear regression to draw the best straight line.

Data Acquisition Notes

1. At low concentrations, where the signal is weak, increase the sensitivity as high as possible to detect weak signal.
2. Data for the entire experiment should be collected from the same instrument.

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Analysis of a Sample Having Unknown Concentration of the Molecule of Interest

1. Prepare two-fold dilutions of the sample of interest in binding buffer. Make enough of each dilution to perform the experiment in triplicate.
2. Apply 2 µL of each solution to 4 or more spots on the ProteinChip Array. Follow the subsequent steps in the appropriate ProteinChip Array protocol for binding and washing.
3. Apply 0.5 µl EAM with IS solution to each sample spot.
4. Read the ProteinChip Array in the ProteinChip Reader.
5. Export the peak height data and plot the value of [100 x MOI peak height]/[IS peak height x dilution factor] on the standard curve.
6. Calculate the original concentration of analyte by extrapolating from the standard curve. Remember to take into consideration any dilutions that were performed on the samples.

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Chapter 13

The ProteinChip System Hardware

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Installing the ProteinChip System

Location and Mounting Requirements

Electrical Requirements

For safety, the ProteinChip System must be connected with an IEC-approved power cord rated to at least 10 Amps to a grounded outlet. Power supply voltage fluctuations must not exceed ±10% of the nominal voltage.

Environmental Requirements During Operation

The ProteinChip System is intended for indoor use. Its acceptable temperature range is from 5 °C to 40 °C, with maximum relative humidity of 80% for temperatures up to 31 °C, decreasing linearly to 50% relative humidity at 40 °C. The System can be used at altitudes up to 2000 m above sea level.

Environmental Conditions During Storage or Transport

The temperature can range from 0 °C to 60 °C, with maximum relative humidity 100% non-condensing, altitude up to 10,000 m above sea level.

Ventilation

The ProteinChip System requires 12 inches clearance at the rear of the instrument (at the vented door) and on at least one side for adequate ventilation. The area around the instrument should be kept free of dust.

Mounting

The ProteinChip System has no special mounting requirements. If your location is subject to earthquakes, chocks should be used on at least two of the castors to prevent the System from moving.

Setting Up the System

1. Position the System's shipping crate at least 6 feet from the wall.
2. Remove the crate's side panel and attach it to the crate as a ramp. Carefully roll the System down the ramp.
3. Inspect the system for physical damage. If the system appears to be damaged contact Ciphergen Customer Service.
4. Verify the selected operating voltage at the power entry module (located on the recessed surface below the louvered door). The selected operating voltage is indicated by aligned arrows.

   If the selected voltage is incorrect, change it by removing and reinserting the fuse assembly with the arrows aligned to the proper operating voltage.
5. Verify that the oil level in the mechanical vacuum pump is within the proper range. Unlock and open the solid (not

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louvered) door panel. The oil level in the pump should be between the two horizontal lines 2/3 up the sight glass. If necessary, add high quality rotary-vane pump oil of the same type already in the pump. Close and lock the door panel.

6. Check that the power switches for the PBS II and the computer are in the OFF (0) position, and that all panels are secured in place and the cabinet doors are closed and locked.

   Connect the PBS II to the power outlet with the supplied power cord or a 10A IEC320 equivalent. Connect the computer to the PBS II using the supplied GPIB cable or any shielded GPIB cable up to 9 feet long.

7. Verify that the sample port lid (the uppermost black square on top of the cabinet) is closed. If necessary, close the lid by centering it over the opening.

8. Switch the PBS II's power on. You should hear the mechanical vacuum pump begin to operate, followed by a click as the "backing valve" actuates, and then the spin-up of the turbomolecular pump.

9. Switch the computer on. After it has finished booting up, start the "SELDIUtl.exe" software application and monitor the pressure. The inverted magnetron pressure gauge is not energized until the turbomolecular pump has reached its nominal speed. This should take less than 10 minutes.

10. When the utility indicates Turbo Speed is "OK", enter the **Open** command. The sample port lid should open. Next, enter the **Close** command. The lid should close, and the sample carrier should move to the "lower limit".

11. When the pressure is less than 10^{-6} torr, the PBS II is ready to use. If the pressure is not less than 10^{-6} torr within 4 hours, contact Ciphergen for technical assistance.

Optional Accessories

A printer can be attached to the computer according to the printer and computer manufacturers' instructions.

The computer can be networked according to the interface card and computer manufacturers' instructions.

Reading ProteinChip Arrays

ProteinChip Arrays are inserted into the sample port slot. Prepare the ProteinChip Reader for sample introduction or removal by clicking the **Sample Exchange** button in the ProteinChip Software and clicking the **Open Lid** button in the **Sample Exchange** window.

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The instrument will move the sample carrier into the exchange port, release the vacuum to the sample port, and open the lid.

If a ProteinChip Array is already in the slot, remove it by grasping it with your fingers and pulling it straight up.

The ProteinChip Array fits into the sample port such that the dragonfly logo is at the top and all spots face toward the back of the instrument. Push the chip into the slot until it is flush with the top of the sample port.

> *Important:* ***do not*** *force the lid open when the green LED adjacent to the sample entry port lid is not lit; doing so will vent the analysis chamber.*

If the sample port O-ring looks dusty or dirty in any way, clean it using compressed air or "Dust Off." For severe contamination, wipe the O-ring with a cloth dampened with methanol, ethanol or isopropanol (never use acetone).

Click the **Close Lid** button in the ProteinChip Software. The instrument will close the lid automatically, draw the sample into the instrument, and establish the correct vacuum.

Shutting Down the ProteinChip System

The instrument is designed to remain powered at all times. If it is necessary to interrupt power for more than a few minutes, use the **Shutdown** software utility. The utility will guide you through the steps required to keep the analysis chamber under vacuum while bringing the pump lines to ambient pressure in order to prevent oil from entering the pump lines. The unit will then be ready for shipment or storage.

Cleaning and Maintenance

Cleaning

If the instrument cabinet needs cleaning, wipe it with a damp cloth. Be careful not to let liquid enter any opening in the instrument.

The sample port O-ring should be cleaned periodically with a lint-free cloth moistened with ethanol or isopropanol.

The cabinet interior should be kept free of dust. Clean with a vacuum cleaner with a soft brush attachment. Do not defeat the safety interlocks before cleaning.

Maintenance

The following maintenance procedures should be performed only by qualified personnel who understand how to avoid contact with potentially hazardous components or emissions inside the cabinet.

For safety, all power and internal interconnection cables should be maintained in good condition. If a power cord appears worn, replace it immediately with a properly rated cord. If the interconnection cables appear to be worn or damaged, contact Ciphergen Customer Service.

Changing the Pump Oil

The pump oil level should be checked monthly.

1. Unlock and open the solid (not louvered) door panel.
2. View the oil level in the pump through the sight glass. It should be between the two horizontal lines 2/3 up the sight glass.
3. If necessary, add high quality rotary-vane pump oil of the same type already in the pump.
4. Close and lock the door panel.

The pump oil should be replaced every 12 months (or more often if it becomes contaminated). It should be replaced with Varian oil type GP or equivalent. See the pump manufacturer's instructions for details.

Sample Translator

The sample translator lead screw should be lubricated with a molybdenum-disulfide based bearing grease every 500 cycles or as needed. Using a swab, apply a small amount of grease (0.25 mL) to the threads when a sample is at the upper limit switch (in the sample port).

The sample translator shaft should be lubricated with high vacuum grease every 500 cycles or as needed. Use only Krytox LVP grease. Use a swab to apply a very small amount of grease (0.1 mL) around the top of the polished shaft when the sample translator is at the lower limit switch.

The sample translator requires lubricating every 500 cycles, usually about every 3–6 months. Follow the procedure below. The sample translator is accessible when the instrument's front door is open.

1. Close the **PEAKS** program and open the **SELDI Utilities** program, then press any key.
2. Type **SAMP MIN** into the **SELDI Utilities** dialog. The sample translator will move to its lowest position.
3. Use a cotton swab to smear about 0.1 mL of high quality high vacuum grease around the top of the polished translator shaft (Figure 13-1, right side).
4. Type **SAMP MAX** into the **SELDI Utilities** dialog. The sample translator will move to its highest position (Figure 13-1, left side).
5. Use a cotton swab to smear about 0.25 mL bearing grease (molybdenum-disulfide) on the threads of the translator shaft.

6. Type **SAMP MIN**, wait for the translator to stop moving, type **SAMP MIN** and wait again, then repeat this step twice more.
7. Close **SELDI Utilities** and return to the **ProteinChip Software**.

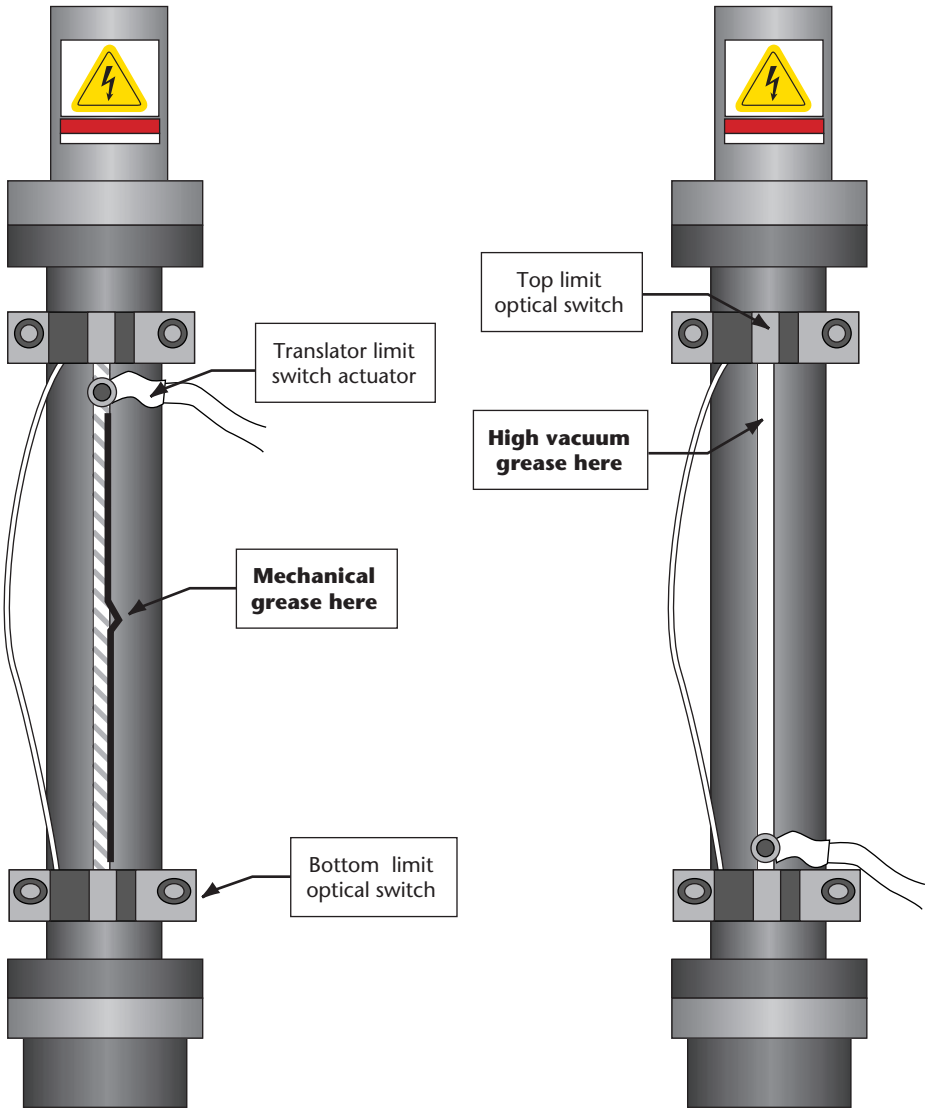

Figure 13-1: Lubricating the sample translator.

Fuses

There are two fuses in the power entry module.

Ciphergen Part Number	Use For	Description
0-00553	115VAC operation	1/4″x1.25″ 10A 250V time delay fuse
0-00554	230VAC operation	5 x 20 mm, 6.3A 250V time delay fuse

Moving the ProteinChip System

The ProteinChip Reader is equipped with casters and is not intended to be lifted or carried. Carrying the Reader is not recommended because the weight of the instrument poses a significant risk of physical harm. In the event that it is necessary to do so, use the following guidelines. If a lift or dolly is to be used, the unit should first be placed in its original shipping crate. If the original shipping crate is not available, the unit may be strapped to a pallet capable of supporting its weight. **Do not** use any device that will support the instrument by only one edge; the frame may bend.

If it is necessary to lift the instrument by hand, it can be held by the darker-colored bezels at the ends of the instrument. This is best accomplished with 4 individuals — 2 per end. The weight of the instrument can be reduced by temporarily removing the side panels and doors. Reinstall the panels before powering the instrument.

Instrument Service

Aside from the periodic maintenance described above, there are no user-serviceable parts inside the cabinet. Contact Ciphergen for technical assistance. Unauthorized modification or replacement of parts inside the enclosure may void the warranty or service contract and may result in extra fees for service.

Warranty and Service Agreements

The ProteinChip System that is delivered as part of the ProteinChip Partnership Program has three components: The ProteinChip Reader, ProteinChip Software and ProteinChip Arrays. As of the writing of this document, the Warranty Period is 90 days following System installation at the customer site and covers instrument servicing and software upgrades. As of June, 2000, Ciphergen offers a number of warranty options for purchase to cover instrument servicing and upgrades of ProteinChip Software beyond the first 90 days. Please ask your Ciphergen Field Scientist or Program Manager for complete information on our warranties.

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Appendix A

ProteinChip® Technology Laboratory Setup Guide

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Introduction

This appendix contains recommendations for preparing your laboratory for the Ciphergen ProteinChip® System.

System Requirements

The PBS II ProteinChip System (Figure A-1) is approximately 28.5″ high x 22.5″ wide x 40″ deep (72 cm h x 56 cm w x 99 cm d). The system will fit under, or beside, a normal height laboratory bench. The interface cable from the system to its computer is 6.5 feet (2 meters) long, consequently the system and computer must be located near each other. Only the computer and printer occupy space on the benchtop, but an additional 6 feet (2 meters) of bench space near the system is recommended for sample preparation, etc.

The system will be delivered in a wooden packing case and weighs about 397 lb (180 kg). The packing case is fitted with runners and it **must** be unloaded and moved with a forklift or pallet jack.

The packing case has a removable front panel that is used as a ramp for removing the system from the case (after the packing case is out of the delivery vehicle). Once unpacked, the system is easily moved as it is fitted with castors. The system cannot negotiate steps or stairs but will fit in most elevators.

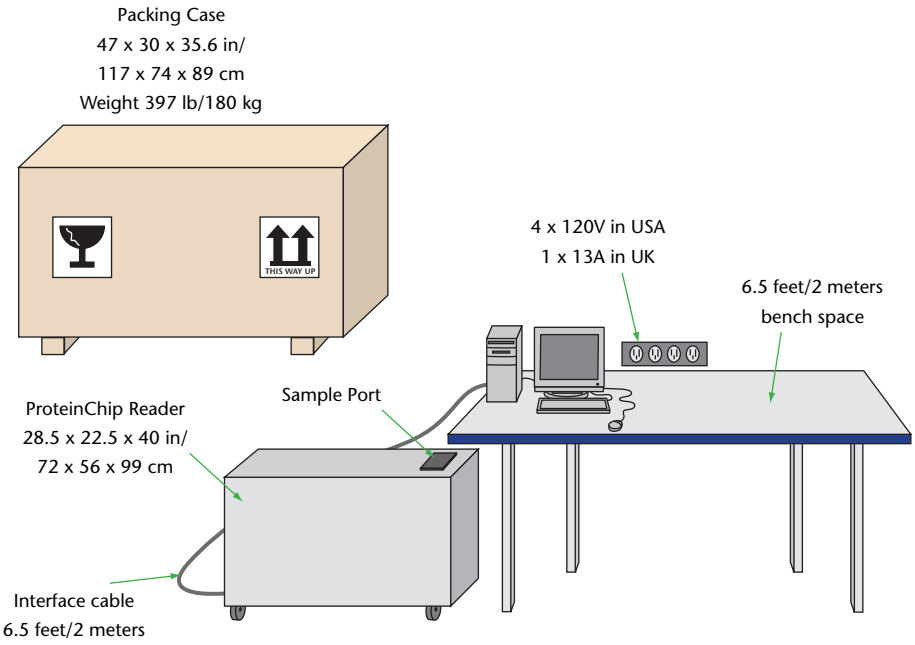

Figure A-1: Shipping case and system dimensions, with space requirements.

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Setup Time

It usually takes about a day to get the system "up and running", including unpacking and moving the system to the lab, connecting it to the power supply and installing the computer supplied with the system.

Equipment and Reagents

The checklist (below) identifies equipment that is frequently used in conjunction with the ProteinChip System.

Equipment

- ☐ Adjustable pipettors (2 µl, 10 µl, 200 µl, and 1000 µl) and pipette tips.
- ☐ Vortexing mixer.
- ☐ Empty pipette tip box with lid (used to make a simple humidity chamber).
- ☐ Variable speed microcentrifuge.
- ☐ Rocking platform mixer.
- ☐ pH meter.
- ☐ Balance (precision to 0.1 mg).
- ☐ Disposable gloves. **Note:** Do not use latex gloves when handling ProteinChip Arrays. (See latex glove warning in Chapter 2).
- ☐ Equipment for temperature-controlled incubations (i.e. ice, heating block, etc.).

Reagents

Some commonly used reagents are:

- ☐ Ethanolamine.
- ☐ Phosphate buffered saline (PBS).
- ☐ Variety of detergents, i.e. Triton X-100, OGP, Tween-20, etc.
- ☐ HPLC-quality water, used in washing steps and making up solutions.
- ☐ Sequencing grade endoproteases (e.g. Trypsin, Lys-C, Glu-C; for peptide mapping and/or epitope mapping).
- ☐ 10x PBS stock is useful for preparation of PBS buffers containing detergent or salt. Triton is added at 0.1-1.0%.
- ☐ Ethanolamine is used to block the chip surfaces during capture experiments. A 1 M solution (in PBS at pH8) is commonly used.

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☐ $CuSO_4$ and other metal-sulfate solutions, typically 0.5 M concentration, used in IMAC experiments.

☐ Sequencing quality acetonitrile and trifluoroacetic acid (TFA) are used to make up the EAM (energy absorbing molecule) solutions. Acetonitrile is also used in some washes. No more than 50 mL of acetonitrile, and only 1 mL of TFA are required.

☐ Other useful buffers/stock solutions:

- 0.1 M sodium acetate, pH 4.5 (~100–200 mL to start).
- 0.1 M sodium acetate, 0.5 M NaCl, pH 4.5 (~100–200 mL to start).
- 0.1 M sodium acetate, 0.5 M NaCl, pH 4.5, 1% Triton X100 (~100–200 mL to start).
- 0.1 M Tris HCl, 0.1% Triton X100, pH 6.5 (~50 mL).
- 0.1 M Tris, pH 8.0 (~50 mL).
- 50 mM Tris base, 0.1% Tx100, pH 9.5 (~50 mL).
- 1 M stock of imidazole (~25 mL).
- 4 M NaCl stock (~10 mL).
- 8 M urea, 1% CHAPS, 50 mM Tris, pH 9.5 (~10 mL).
- 8 M urea, 1% CHAPS, PBS, pH 7.2 (~10 mL).
- PBS supplemented to 0.5 M NaCl (~100-200 mL).
- PBS supplemented to 0.5 M NaCl, 1% Triton X100 (~100-200 mL).
- 10% acetonitrile in water (~50 mL).
- 50% acetonitrile in water (~50 mL).

Sample Preparation

Biological Capture Molecules

Biological capture molecules (e.g. antibodies, receptors, etc.) should be purified and resuspended in a phosphate based solution (PBS works well for antibodies) or some other non-nucleophilic buffer for covalent attachment to ProteinChip Arrays. Buffers containing primary and secondary amines should be avoided. Antibodies ideally should be monoclonal and must be free of carrier proteins such as BSA or gelatin. The concentration of the purified capture molecule should be between 0.1 and 0.5 mg/mL for optimal covalent coupling. Only one microliter is used per spot (8 spots per chip) so the total amount of capture molecule used is very small. A non-specific antibody or receptor, etc., should also be available to serve as a negative control in capture experiments.

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For more information on using antibodies with the ProteinChip System, please see "Antibodies" on page 8-3.

Analyte Solutions

Analyte solutions containing protein, peptide, or small molecule of interest (which do not require covalent coupling) do not limit the choice of buffer or the complexity of the sample mixture. However, wherever possible, avoid the use of strong ionic detergents such as SDS in sample preparation since they are notoriously difficult to wash away and can interfere with the analysis. Non-ionic detergents such as Triton X-100, NP-40, or OGP are preferred.

Special Sample Types

LCM samples: caps containing ~4000–5000 cells should be prepared to begin a profiling experiment. Several caps may be necessary to develop an optimal profiling protocol for subsequent validation. Flash-frozen tissue sections are used for ProteinChip Array analysis. Formaldehyde fixation or similar treatments will cross-link the proteins, making these types of preparations less than optimal for protein profiling experiments.

Tissue samples: Homogenates from approximately 100 mg tissue are usually sufficient. We recommend approximately 200 µg total protein per sample for initiating a fairly extensive protein profiling experiment.

Cultured Cells: A single plate of cells is usually sufficient for protein profiling.

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Appendix B
Frequently Asked Questions

ProteinChip Arrays

How long does it take to perform an on-chip incubation?
On-chip incubations can be as short as a few minutes, but usually the goal is equilibrium binding. However, as in any protein binding experiment, incubation time must be extended for proteins with very slow on-rates or for proteins in low abundance, or if the binding is carried out in the cold.

Are the chips stable once proteins have been added? If so, under what conditions?
Ciphergen has not performed formal studies on storing chips with proteins attached, but we do have some information about storage conditions that have worked for many of our customers. After proteins are bound to the chip, the chip may be stored dry for several days before EAM addition and analysis. It is important that the chip be kept away from moisture or the spots may corrode. In this case, hydrate the spots with 0.5 µL/spot H_2O before the addition of EAM for better detection. PS1 or PS2 chips with antibodies attached have been successfully stored at 4 °C for up to a week in 10 mM HEPES buffer. PS1 and PS2 chips may also be stable with protein G covalently attached to the surface, if the chips are stored dry, but Ciphergen has not formally tested this.

Are the chips reusable?
No. The NP chips may be washed and re-used but doing so isn't recommended since a small amount of contaminating protein can be very troublesome.

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How many samples can you put on the chip?

Each array contains 8 active spots for sample application. H4 and Normal Phase chips are also available in 24-spot configurations.

How does the chip work?

The chip works via affinity capture of proteins or other molecules via a chemical or biological surface. ProteinChip Arrays consist of an aluminum chip that contains 8 chemically treated spots for sample application/protein capture. Each type of array has a specific chemical surface so that proteins with a particular biochemical property will be captured. See Chapter 5, *"ProteinChip Arrays"* for more information on the individual chip types and their surface properties.

Does it really matter if I have TFA present to prewet my hydrophobic chip? Does it really matter if I use 50 or 100% acetonitrile to prewet my chips (i.e., Normal Phase vs Preactivated Surface)?

Pre-wetting can change the binding characteristics of the chip. However, Ciphergen hasn't done any formal experiments to quantitate the results.

What is the binding capacity of the spots?

The binding capacity of the spots varies with the type of array. The table below is based on information from Pil-je Um Ph.D. as of 28 April, 2000.

Chip	SAX2	WCX2	IMAC3	SCX1	H4	IMAC2	PS1	PS2
Capacity (BSA)	10–50 pmol	10–50 pmol			≤1 pmol			
Capacity (transferrin)			10–50 pmol			≤1 pmol		
Capacity (lysozyme)		1 nmol		≤1 pmol				
Capacity (IgG)							200 fmol	?

Details regarding the capacity of spots on various chips were provided by Pil-je Um, Ph.D. and further details regarding the methods used to generate these numbers can be obtained from Chipware/Ciphergen R&D.

The comment below regarding chip capacity comes from Chris Pohl, Ph.D.:

In general, it can be quite misleading to place a great deal of emphasis on chip capacity in terms of a specific numerical value. While static capacity of a retention surface can in principle be measured by a variety of methods, it is only effective capacity with real samples that is relevant in making analytical decisions about chip protocols. Unfortunately, there is only a very weak relationship between static capacity and effective capacity.

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The effective capacity is a function of a variety of experimental parameters including pH, ionic strength, ion selectivity, molecular weight of analyte, analyte valency, temperature, analyte charge distribution and hydrophobicity distribution, along with the analogous characteristics of all other proteins and peptides that may be present in the sample. Given the observable range of all these experimental parameters it is generally not possible to quote a specific capacity for a given chip (especially in picomoles, since capacity should be expressed in binding equivalents rather than in moles).

Nonetheless, … the static capacity is in fact a legitimate basis for evaluating and comparing chips in product development. Unfortunately, measurement of chip capacity via a SELDI experiment is unlikely to provide a true measure of chip capacity, due to the fact that second generation chips (SAX2, IMAC3 and WCX2) have capacities that are in excess of the EAM formulations' tolerance. A good estimate of chip capacity for second generation chips is probably in the 100 picomole range (for a 50,000 molecular weight protein).

What is the difference between a hydrophobic interaction and a reverse phase surface?

Hydrophobic usually refers to binding via hydrophobic residues/areas on the surface of the protein, while reverse phase implies that the molecule has been denatured, thus exposing hydrophobic regions of the protein that are usually internally sequestered.

Will ProteinChip Arrays work on a standard MALDI?

No. Ciphergen's ProteinChip Arrays are specifically designed for use with the ProteinChip System. The chips, software and instrumentation have been optimized to work well as an ensemble.

Can ProteinChip Arrays be used in a Biacore?

No (see above).

Sample Type/ Preparation

In what affinity range can the ProteinChip System work?

Ciphergen hasn't formally studied this question, but probably in the micromolar range. Low affinity interactions can be monitored if there is a sufficient specific protein concentration to compensate.

Can membranes be put on the chips?

Yes. There are a number of options for the analysis of membranes — direct, solubilization, and detection of peptides or proteins bound to the membranes, including NP and H4 chips.

How are samples loaded on the chips?

By pipetting a small volume (1–5 µL) onto the array surface. Larger samples (up to ~300 µL) can be applied by using the bioprocessor, or the entire chip can be incubated in a tube with a few milliliters of sample.

Can glycosylated proteins be used on the ProteinChip System?

IgG and BSA are commonly used proteins, and both are glycosylated. For an example of on-chip deglycosylation, see Ma Sha's case study showing deglycosylation of a large protein isolated from urine.

Can beads be put on the chip?

No. Chromatography resin beads can fall off the ProteinChip Array inside the ProteinChip Reader and damage the sample translator vacuum seals. For analysis of samples captured by beads, Ciphergen recommends eluting the sample from the beads and analyzing the eluant on a ProteinChip Array.

Can I bind any protein I want to a chip?

Yes. Any protein can be covalently bound to the PS1 or PS2 chip surface, either via lysine residues or by the N-terminus.

Can proteins be bound to chips through thiol groups?

Yes, it is possible to couple a protein via thiol groups to the Preactivated Surface (PS1 or PS2) ProteinChip Arrays. However, primary amines will compete very effectively with the cysteine so multiple linkages may ensue. Also, Ciphergen recommends using a small molecule linker for effective thiol coupling. Talk to the Ciphergen Chipware team for further information.

Can hydrophobic proteins be used on the ProteinChip System?

Of course. The H4 chip would typically be chosen for capturing hydrophobic proteins. (This subject is usually of interest 2D gel scientists because hydrophobic proteins don't enter the IEF gel.)

Can the ProteinChip System fish for DNA binding proteins?

Absolutely. The ProteinChip System has been used to fish for transcription factors and has distinguished mutated DNA binding sites from wildtype, functional binding sites by using an on-chip DNA-binding assay. See *"DNA- or RNA-Binding Protein Assay"* on page 12-3 and related case studies by James LeBlanc and Lisa Bradbury.

How large a sample volume can I apply?

Technically up to 500 µL using the bioprocessor. However, Ciphergen recommends using only up to 350 µL to maximize binding. Binding in a tube allows for much larger volumes, but all spots on the array are exposed to the same sample. In some cases this much larger volume is necessary (see Lisa Bradbury's SH2 Domain Case Study from WWM, April, 2000). See also *"The Bioprocessor"* on page 3-6.

What must I do to my sample before applying it to the chip?

Often, nothing at all. Typically, a few microliters are loaded on the chip, the spot is washed with buffer and then water, EAM is applied, then the chip is read. Some samples may need to be adjusted for pH or salt concentration.

Can detergent be used in sample preparations?

Yes, because you can wash it away after the sample has been applied to the chip. Non-ionic detergents, including dodecyl maltoside, octylglucopyranoside (OGP) and Triton X-100 are tolerated in low concentrations during analysis. Ionic detergents, such as SDS, interfere with collecting data. However, thanks to the affinity capture capabilities of the ProteinChip Arrays, samples that have detergents are often just fine since the detergent may be washed away prior to analysis.

Can trypsin be used in sample preparations?

Yes, trypsin and other proteases can be used to digest protein either directly on-chip, or prior to applying samples to the chip.

When I use a spin column, my sample doesn't elute where I expect it. Why?

Keep in mind that the MW cutoffs are guidelines — oddly shaped proteins, multimers and aggregates may not run through size-selection resins according to their monomeric MW. See *"Using Spin Columns for Protein Separation"* in Chapter 7 for more information.

Antibody Capture

Does Ciphergen sell Protein A chips?

You can make your own Protein A or Protein G chip by covalently binding Protein A to preactivated surface chips, PS1 and PS2. See *"Antibodies"* in Chapter 8 for details.

Why use Protein A or G for an immunoaffinity capture?

Protein A or G can be used to capture an antibody from solution. This procedure can have two advantages. First, if the antibody solution is contaminated with other proteins or if the protein is degraded, Protein A or G can improve the purity of intact antibody. Second, using Protein A or Protein G to capture an antibody and then capture an antigen sometimes improves the signal from the analyte. Presumably, the Protein A/G puts the antibody in an orientation that favors capture of the analyte, thus increasing the amount of target molecule captured onto the array.

One potential drawback of using Protein A/G is that the antibody is then non-covalently linked to the chip, and the antibody will be detected during SELDI analysis. The second drawback is that during an extended incubation, the IgG will establish a new equilibrium based

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on the off-rate, and much of the antigen can be lost. Please refer to the section titled *"Antibodies"* in Chapter 8 for further information.

What if all I have is Protein A or G sepharose?

Antibodies can be purified via Protein A/G sepharose and then used on the PS1 or PS2 ProteinChip Array. Please refer to the section titled *"Antibodies"* in Chapter 8 for further information.

How long does it take to covalently bind an antibody to a chip?

See the ProteinChip Immunoassay protocol starting on page 8-11. The actual binding of antibody to the chip takes about an hour. Binding can be allowed to continue for up to 24 hours.

How can you epitope map *in situ* — wouldn't the antibody be digested as well?

Antibodies are surprisingly protease resistant, thanks to their existence at many *in vivo* sites containing active proteases. However, some antibody fragments may be seen, but control experiments allow you to distinguish the peaks derived from the target analyte *vs* from antibody, thus allowing epitope mapping of captured proteins.

Do ProteinChip Arrays work with antibody-antigen pairs that have fast off-rates?

From Lisa Bradbury, Ph.D.:

Yes, the ProteinChip System works with fast off-rates. However, this fact needs to be considered when designing the experiment. Fast off-rates require shorter time and lower volume washes. Background increases can be reduced by higher stringency binding and washing conditions - if the interaction is stable under these conditions. Specifically, post-binding washes can be truncated to as little as two 1-minute washes (4 mL per wash) in wash buffer followed by a quick rinse with 5 mM HEPES or Tris pH 7.4-7.6 or H_2O. Alternatively, on-spot washes of ~5 µL/spot can be used to further minimize protein loss.

The background will increase, so it is crucial to have an appropriate negative control for comparison purposes. If necessary, a very high stringency wash buffer (i.e., up to 1% detergent) can be used as long as the interaction is stable in the buffer of choice. Depending on the requirements for binding, it might be possible to use a high stringency binding buffer to reduce the background as well.

Since the loss of antigen post binding is expected to be high, it is best to maximize the capture if possible. Depending on the experimental system, this might be accomplished by increasing the antigen concentration, increasing the volume (multiple loadings or bioprocessor), or choosing a sample with higher levels of the antigen.

Detection

Can the ProteinChip System differentiate phosphorylated from non phosphorylated proteins?

Yes, there are multiple ways to do so. First, each phosphate addition results in an increase of 80 Da. For proteins below 20 kDa that have minimal heterogeneity the resulting MW shifts can be detected. However, since the level of phosphorylation is frequently low (>10%) and since phosphorylation-site usage is often heterogeneous, phosphorylation can be difficult to detect directly. Phosphatases or kinases can be used to induce a mass shift, and the mass difference will indicate the number of phosphate groups per molecule. Alternatively, phosphorylation specific antibodies or Ga-IMAC can be used to capture phosphorylated proteins. See *"IMAC3-Gallium Array Phosphopeptide Capture"* on page 12-11 for a sample protocol.

> *Note: while it is possible to use iron with the IMAC3 chips, we recommend Gallium for phosphopeptide capture. See the above-referenced protocol for more details.*

Can the ProteinChip System differentiate methylation or acetylation?

A mass shift of 14 Da is expected for addition of a methyl group to replace a hydrogen; acetylation should cause a mass increase of 29 Da. Probably the best way to approach this issue would be to generate peptides and look for the expected mass differences.

Can the ProteinChip System detect molecules other than proteins?

Yes, the System can detect small molecules, peptides and nucleic acids (DNA and RNA).

Does the ProteinChip System detect all of the proteins in a sample?

Most, but not all, proteins are detectable in the ProteinChip System. In fact, due to the chemical nature of the ProteinChip Array surfaces, the ProteinChip System is often able to analyze proteins that are invisible via traditional MALDI-TOF spectroscopy. That said, it can still be difficult to analyze purified proteins of mass greater than 200,000 kDa. Additionally, proteins present in a mixture can be more difficult to analyze than purified proteins. Nonetheless, the ProteinChip System can routinely detect dozens of proteins in a single spot on a ProteinChip Array. Also, the system's utility lies in part in its ability to separate out proteins on various ProteinChip Array surfaces, thus obviating the need to detect all proteins simultaneously in a single spot.

How many proteins can the ProteinChip System detect?

It can detect hundreds of proteins via ProteinChip protein profiling.

Why isn't the most abundant band on my gel in the spectrum?

Some proteins don't desorb/ionize well and thus go undetected in the ProteinChip Reader. The situation is similar for staining with gels, in that different proteins are more or less amenable to staining. Ciphergen has obtained data showing that most bands on the gel can be correlated to peaks detected with ProteinChip technology.

A size bias exists in detecting molecules via both methods. In SELDI, a single molecule gives only 1 detector signal, no matter what the size. Larger molecules tend to be harder to detect than smaller molecules. For most gel staining methods, larger proteins bind more moles of stain per mole of protein, thus facilitating the detection of large molecules via gel; however, small molecules (<10,000 Da) are usually run off into the buffer chamber.

Is ion suppression produced when other proteins are present?

"Ion suppression" refers to the situation in which the presence of some ions reduces or obliterates the detection of another ion. Typically, small proteins and peptides readily desorb and ionize, with the resulting signal reducing the signal from larger proteins in a sample on the same spot of the ProteinChip Array.

Reducing sample complexity can improve the detection of any single protein or other analyte. Two protocols in this Users Guide address this issue: *"Serum Protein Profiling Using the 96-well Bioprocessor"* on page 9-6 and *"Sample Fractionation Using Spin Columns and the ProteinChip System"* on page 7-20.

Can the ProteinChip System detect multimers?

Non-covalent multimers in general fall apart into individual subunits during the detection process. However, high concentrations of protein on the chip surface may lead to multimer formation independent of normal protein higher order structure. SPA permits multimer formation more readily than CHCA. A carefully designed experiment might allow detection of native multimer structure.

Can I elute my protein from the chip surface and recover it?

It is usually possible to elute the protein from the chip surface. However, keep in mind that the beauty of ProteinChip Technology lies in part in the tiny sample volumes that are applied to the array. The total capacity of each spot on the array is limited to tens of picomoles, and so only a small amount of protein will be eluted from the surface.

ProteinChip Reader/General ProteinChip Technology

What is the limit of detection, or the lower limit of sensitivity?
Generally, you can anticipate that a protein can be detected at levels as low as 1 femtomole captured on the spot. However, the lower limit of detection varies depending on the molecular weight and desorption and ionization efficiency. Some proteins cannot be detected at all.

What is the molecular weight range with which the ProteinChip System can work?
The detection range appears to be 100–400000 daltons, based on our experience. Huw Davies and others have generated data analyzing small molecules of a couple hundred Da. At the other end of the scale, molecules as large as 300 kDa can be detected by the PBS II. However, a molecule this big generally requires a large amount of protein and not all molecules of this size will be detectable in the System.

How can I work with dynamic ranges that exceed the ProteinChip System's linear range?
The dynamic range of any system is dependent on numerous factors. In this system, the dynamic range can be extended by sample dilution or changes in data collection parameters, or by the use of larger volumes.

Can ProteinChip technology be used for quantitation?
Absolutely. Quantitation can be performed for protein-protein capture (i.e., antibody) or capture on a chromatography surface.

What is the resolution of the current ProteinChip System (PBS II)?
It depends on the analyte and the sample itself, but generally in the range of 0.1%. However, the affinity capture, which allows the separation of proteins based on biochemical or biological properties, creates the ability to resolve proteins of the exact same MW. The resolution in general decreases with increasing mass of analyte or if the analyte is a glycosylated protein.

How reproducible are the signals from analytes bound to ProteinChip Arrays?
The CVs generated from protein profiling of complex samples on chromatography surfaces are typically 10–30%. For example, see Huw Davies' study (WW Meeting, April 2000).

What does SELDI stand for?
Surface-Enhanced Laser Desorption/Ionization.

Isn't SELDI the same as MALDI?
ProteinChip Technology is derived from SELDI, and SELDI technology was successfully patented because it represents a substantial technological innovation over MALDI. The ProteinChip System consists of interacting components: hardware, chipware and software.The ProteinChip Reader is a SELDI-TOF mass spectrometer.

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However, the affinity capture component of this system is its strength. It enables a researcher to look at protein directly from a complex biological sample, which is impossible with MALDI. On-chip purification provides a substantial technical advantage in sample preparation for the biologist.

How does SELDI compare to MALDI?

SELDI is a unique technology encompassing micro-scale affinity capture of proteins with time of flight mass spectrometry. Traditional mass spectrometry is limited by the requirement for extensive sample prep prior to analysis. ProteinChip Technology allows on-chip purification of proteins from minute sample volumes – typically a few microliters.

Is the ProteinChip Reader a mass spectrophotometer?

Yes, the ProteinChip Reader is a time-of-flight mass spectrometer (see above).

How does ProteinChip analysis compare to 2D gels?

ProteinChip Technology provides protein and proteomics analysis which is complementary to that provided by 2D gels. ProteinChip Technology excels in detecting and quantitating proteins of 25 kDa and less. The technology also allows purification of proteins with very high or very low pI's. It can also detect hydrophobic proteins, which don't enter 2D gels. ProteinChip technology provides 2 distinct advantages: tiny amounts of sample are sufficient for ProteinChip Analysis, with 2D gels consuming 5–10 times more sample; it is also very easy to run multiple samples.

What's an EAM?

EAM stands for Energy Absorbing Molecule. It is a small molecule added to the sample spot for the detection of molecules captured on the chip surface. EAM is synonymous with "matrix," the term used for the analogous molecule in traditional MALDI mass spectrometry.

Is EAM required for a ProteinChip assay?

Currently, the answer to this question is "yes", with the exception of some small molecules that have the ability to desorb and ionize when the laser strikes the chip surface. However, please see papers on SEND and DIOS.

How fast can the ProteinChip System read a chip?

Reading a chip takes about 5-10 minutes for 8 spots. Analyzing the data takes longer (however, the computer can read faster when you close or minimize windows where possible).

When I do retentate mapping and find a difference or many differences how do I find out what these proteins are?

It's possible to determine the identity of specific biomarkers in a variety of ways by searching the genome for potential matches by

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MW, peptide mapping and other methods. Ciphergen has numerous Case Studies showing identification of protein biomarkers discovered by ProteinChip differential protein profiling.

Can I get sequence info? How much sample do I need?
Yes, there are several options for obtaining sequence information from a protein. For example, you can try an on-chip digestion with carboxypeptidase to generate an amino acid sequence ladder, which is tricky but does work.

Software Applications & Data Analysis

When comparing 2 spectra how do I determine if a difference in relative abundance is significant?
Ciphergen recommends that questions regarding statistical significance of data be handled by a qualified statistician. ProteinChip Arrays can be used to generate data with CVs of 10–30%, so certainly, differences in expression that vary by only a few percent may not be statistically significant. Determining statistical significance may require the analysis of numerous controls.

Do you have any bioinformatics software to create a data base for your protein profiling experiments?
Currently, no.

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Appendix C

Troubleshooting the ProteinChip System

This appendix contains possible problems you may encounter using your ProteinChip System, and lists the possible causes for each, as well as the corrective action(s) to take. If none of the suggested solutions solves the problem, contact your Field Scientist or Ciphergen Technical Support.

Problem	Possible Cause	Action
The instrument takes a long time to reach vacuum or won't reach vacuum at all.	This problem can occur if the instrument lid has been left open for a long time, e.g., overnight.	Try closing the lid and letting the instrument re-equilibrate.
The laser won't turn on or won't fire, and either of the following error messages "Digitizer failed to trigger" or "High voltage control error" is displayed.		Re-initialize the instrument via the software controls. Turn the power off, wait a few seconds, then turn the power on.
No peaks and no EAM signals in a spectrum when attempting to collect data	Incorrect chip format setting (e.g., chip is A-H format but instrument is set up to read 24-spot format) Laser is focused on spot which lacks sample Chip is upside-down No EAM was added EAM is "bad" or TFA is "bad" Problem with electronics or laser bench	Choose the correct chip format Move to spot with sample Insert chip into instrument with dragonfly logo up. Add EAM Use fresh EAM and fresh, HPLC grade TFA Contact Ciphergen Technical Support
"Time out" error message upon inserting new chip into the ProteinChip Reader	Dust or hair trapped between the O-ring and the lid	Wipe the O-ring in the sample port with a finger or 70% alcohol. Do not use lab wipes.

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Problem	Possible Cause	Action
No peaks. EAM signals are present when collecting data.	Laser energy too low	Increase laser energy
	Signals too low	Use filtering to sharpen the peaks
	Incorrect instrument settings	Choose the correct settings
	Hit "cold" spot	Move to a new position
		Use only the clear supernatant of the EAM solution
	Sample is not retained on the chip surface	Try ProteinChip Arrays with different surface chemistries
		Try different wash conditions
	Sample does not ionize with the EAM being used	Use different EAMs or solvent systems
		Try different sample concentrations
	Sample concentration is low	Increase sample concentrations
Peaks disappear below baseline after 500-600 Da mass.	Often indicates a detector problem.	Contact Ciphergen.
Mass assignments appear to be wrong.	Incorrect calibration	Check calibration equations; re-calibrate the spectrum and/or instrument in the mass range of interest
Masses are increased by 23 or 39 Da.	Na+ or K+ adducts	Use HPLC grade reagents, including HPLC grade water
TFA turns brown.	Photodegradation	Use fresh, HPLC grade TFA and store in brown bottle at 4 °C
Chip does not perform as expected.	Various causes are possible.	Verify that the instrument is working correctly by reading a calibration chip or other "positive control" chip. Contact Ciphergen technical support. Remember to save the chip and record the problem.
EAM stability (does the EAM need to be prepared daily?)	EAMs are photosensitive.	Prepare new EAM daily. Store EAM in the dark.

Getting Technical Assistance

Telephone support is always available at no charge. If you have problems with your instrument, call your Field Scientist or contact Ciphergen's Technical Support:

Main Office: 510-505-2100
Toll Free: 888-864-3770
Fax: 510-505-2101
Email: support@ciphergen.com
For a complete and up-to-date telephone and email contact information, please visit our web site at

www.ciphergen.com.

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Appendix D
Technical Specifications

The ProteinChip Reader is a linear laser desorption/ionization time of flight mass spectrometer.

Enclosure

- Floor-standing unit with castors.
- Dimensions: 98 cm (38.5″) wide x 56 cm (22″) deep x 71 cm (28″) high.
- Mass: 145 kg (320 pounds).

Mass Analysis Range

- From less than 0.1 kDa to greater than 500 kDa.

Sensitivity

- Better than 3:1 average signal to noise ratio on the analysis of 50 femtomole of bovine IgG when digitally smoothed with a (0.2 times peak width) sliding window average.

Mass Resolving Power

Automatic Mode

- Better than 600 average resolution for 10 “spot protocol” automated 25-shot averages of 5 picomole of human recombinant insulin.

Manual Mode

- Single shot resolution of better than 1000 for the analysis of 5 picomole of human recombinant insulin.

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Mass Accuracy

Internal Calibration, Absolute

- Better than 0.02% absolute error for eight measurements of 1 picomole of bovine insulin B chain calibrated against somatostatin and human recombinant insulin on an 8-position, "A–H," normal phase ProteinChip.

Internal Calibration, Averaged[1]

- Better than ± 0.5 Da 0.5 to 5 kDa
- Better than ± 200 ppm (0.02%) 5 kDa to 10 kDa
- Better than ± 500 ppm, (0.05%) 10 kDa to 300 kDa

External Calibration, Absolute

- Better than 0.1% average absolute error for seven analyses of human angiotensin I, somatostatin, bovine insulin B chain, and recombinant human insulin (~1 picomole each) as analyzed on an 8-position, "A–H," normal phase ProteinChip in which spot D is used as a calibration run.

External Calibration, Averaged[2]

- Better than ± 1 Da 0.5 to 1 kDa
- Better than ± 1000 ppm (0.1%) 1 kDa to 10 kDa
- Better than ± 2000 ppm (0.2%) 10 kDa to 300 kDa

Ion Source

- Laser desorption ionization
- Radial-focusing electrostatic optics
- Operating voltage: 0- ±20 kV (standard software selectable settings: +20 kV, +6 kV, -6 kV)
- Factory burn-in: stable to ±30 kV at termination of conditioning procedure

Flight Tube

- 0.8 m

Ion Detector

- Minimum peak width <8 ns
- Linear dynamic range: linear output to 10^9 electrons (500 mV for a 10 ns pulse)
- Large-pulse recovery <30 ns
- Noise <2 mV rms 0–125 MHz

Transient Digitizer

- Trigger jitter: < 2 ns
- Aperture jitter: <10 ps

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1. average of centroid measurements from ten mass spectra, each with signal/noise >25, given adequate sample preparation and operating parameters
2. average of centroid measurements from ten mass spectra, each with signal/noise >25, given adequate sample preparation and operating parameters

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- Sampling rate: 250 MS/s or 500 MS/s
- Record length: >2 ms
- Input amplifiers: multi-gain linear and logarithmic

Dynamic Range

- 8 bits (256 signal intensity levels per scan on board; 32 bit signal averaging on computer)
- Linear dynamic range software controlled in 9 steps (3–4 dB/step) from -0.020 V to -1 V full-scale
- Logarithmic dynamic range, 0 to 1 V

High Voltage Pulser

- Trigger jitter: <1 ns
- Lag time: 0.17–5 seconds
- Pulse height: 0–5 kV
- Polarity: selectable under computer control
- Risetime: 100 V/ns
- Pulse width: 0.1-25 _seconds

Laser

- Pulsed nitrogen laser (337 nm) 4 ns pulse width
- Intensity adjustment: 300 steps full-scaled 0–150 mJ logarithmic.
- Spot size: 200 mm x 1000 mm
- Maximum pulse rate 10 Hz

Sample Handling

- Linear sample arrays on 9 mm x 78.5 mm x 2.4 mm (72 mm accessible for analysis)
- Vacuum load-lock for rapid sample introduction (<3 minutes from insertion to data acquisition)

Vacuum System

- Base pressure <2 x 10^{-7} torr (no baking).
- Pressure less than 1 x 10^{-6} immediately after sample exchange.
- 250 L/second high compression turbomolecular pump with two-stage rotary-vane backing pump.
- Gauges: active inverted magnetron (high vacuum) and active pirani (low vacuum)

Control System

- Windows NT-based software for automated instrument operation and data analysis
- High end personal computer and monitor
- GPIB interface with >750 MB/s peak data transfer rate
- Data rate: 1–4 spectra per second; Analysis of one “A–H” ProteinChip Array with eight 50-spectrum averages takes approximately 10 minutes.

Supply Voltage

- 110–120 VAC, 10A or 220–240 VAC, 5A 50–60 Hz; 100 VAC available as option.

Appendix E

Safety Information

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Safety Information

No safety symbols are used on the user-accessible part of the instrument. Internal symbols used are

ISO 3864, No. B.3.6 Caution, risk of electric shock
ISO 3864, No. B.3.1 Caution (refer to accompanying documents).

There is a risk of electric shock from the high voltage cables and connections. Caution is required when working with the components that require periodic maintenance.

Symbols inside the cabinet (accessible with a key)

All high-voltage connections are labeled with the “caution, risk of electric shock” symbol (symbol 12, Table 1 of EN 61010-1) and the words “Danger: High Voltage.”

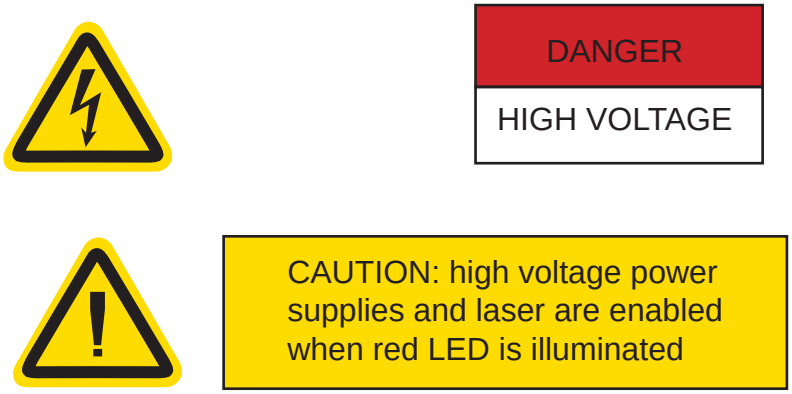

Figure 13-2: Safety warning labels inside the PBS II cabinet.

Safety Interlocks

Warning: If the equipment is used in a manner other than specified, the protection provided by the equipment may be impaired. No contact with hazardous parts or emissions is possible if the instrument panels are kept in place and the doors are closed. It is the user’s responsibility to ensure that untrained persons do not access the interior of the cabinet. Serious injury or death may result from contact with components inside the cabinet under single-fault conditions.

The cabinet is an integral part of the safety of this unit. Each door and side-panel has two safety interlock switches to interrupt power to the high voltage power supplies and laser in the event that a panel is removed. Trained service personnel may defeat the interlock circuit using an interlock defeat plug which fits the labeled port inside the vented door of the cabinet. This plug will prevent closure and locking of the cabinet door. It must be removed to allow the door to be locked for ensured safe operation.

Only personnel aware of the hazards of high voltage and Class 3B laser radiation should operate the instrument with the interlocks defeated. The following label should be present near the safety

interlock defeat port and near the safety interlock switches associated with panels protecting the user from laser radiation:

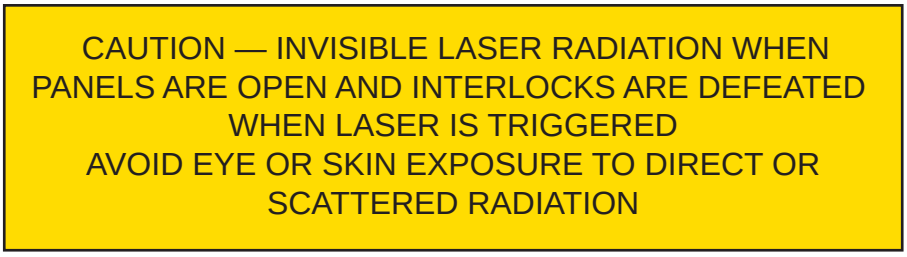

Figure 13-3: Laser safety warning label inside the PBS II cabinet.

Laser Radiation

Warning: This instrument contains a UV emitting pulsed nitrogen laser (337 nm, 175 microJoule maximum energy / 4 ns pulse, 20 Hz maximum pulse rate). The Mass Analyzer is a CLASS 1 LASER PRODUCT according to EN 60825: 1993. This means it is safe under reasonably foreseeable conditions of operation. However, normal operation does not include maintenance or service. When the panels are removed from the cabinet and the safety interlocks defeated, human access to Class 3B laser radiation becomes possible. Safety goggles should be worn to protect the eyes from ultraviolet light whenever the equipment is operated with the panels removed and the safety interlocks defeated. Care should be taken to avoid specular reflection of the beam.

High Voltage

Warning: The unit contains high voltage power supplies. While the components are fully insulated under normal conditions, electro-static charge can accumulate on wires and feedthroughs carrying high voltage as well as nearby components. Contact with these components should be avoided when high voltage may be present.

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Declaration of Conformance

CE

Application of Council Directives:	LVD (Low Voltage Directive) 73/23/EEC EMC (Electromagnetic Compatibility) Directive, 89/336/EEC
Standards to which conformity is declared:	EN-61010-1:1993 Safety requirements for electrical equipment for measurement, control, and laboratory use EN-60825-1:1993 Safety of laser products, Equipment classification, requirements & users guide EN-55011:1996 Limits and methods of measurement of radio disturbance characteristics of industrial, scientific and medical (ISM) radio-frequency equipment EN 50081-1:1992: Electromagnetic compatibility generic emission standard, Part 1: Residential, commercial and light industry EN 50082-1:1992 Electromagnetic compatibility generic immunity standard, Part 1: Residential, commercial and light industry
Manufacturer's Name	Ciphergen Biosystems, Inc.
Manufacturer's Address	6611 Dumbarton Circle Fremont, CA 94555 Phone: 888–364–3770 Fax: 650-424-4303
Importer's Name	Ciphergen Biosystems, Ltd.
Importer's Address	Surrey Technology Centre The Surrey Research Park Guildford, Surrey, GU2 7YG, UK. Phone: +44 (0) 1483 84 5224 Fax: +44 (0) 1483 84 5220
Type of Equipment	ProteinChip System
Model	PBS II
Year of manufacture	2000

I, the undersigned, hereby declare that the equipment specified above conforms to the above Directives and Standards.

Place	Signature
Date	Full Name
	Position

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