ProteinChip® SELDI System

Applications Guide

Volume 2

BIO-RAD

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Chapter 1: Introduction

Introduction

Differential expression profiling refers to the process of comparing the protein profiles from two or more classes of samples (e.g., normal vs. diseased, and patients who respond to a drug treatment vs. nonresponders) in order to find proteins which can discriminate between the classes. These proteins are often referred to as biomarkers. Since, at the start of the experiment, one does not know which proteins are differentially present in the sample classes, the experimental protocols are designed to capture as many proteins as possible in the profiles in order to maximize the probability of finding statistically significant biomarker proteins. The standard differential expression protocol which we recommend prefractionates the sample on an anion-exchange chromatography resin prior to analysis on a range of ProteinChip array chemistries, in order to see as many proteins as possible in the resulting spectra.

Due to the natural biological variability present in real samples, useful discrimination between classes can often only be obtained by simultaneously analyzing the variation of several biomarker proteins. These patterns of biomarkers are found by performing multivariate statistical analysis of the quantitative protein profiling data. Hierarchical clustering and principle components analysis (PCA), in ProteinChip® data manager software are unsupervised multivariate statistical analyses (i.e., the data are clustered based on their similarity to each other, regardless of which class the samples belong to, and then one analyzes how closely the resulting clusters correspond to the sample classes). ProteinChip pattern analysis software is a supervised learning package, in which all samples are identified by class, and the software determines the pattern of markers which gives the greatest differentiation between the two classes. In order to obtain statistically robust results, multivariate statistics generally require more input data points than univariate analyses.

Study Design

A complete discussion of rigorous differential expression study design, especially with clinical samples, is well beyond the scope of this guide. Several of the most important considerations in designing and interpreting the results of differential expression studies are sample stratification, sample annotation, preanalytical variability, and sample types and preparation. These considerations are discussed in the following sections.

Sample Stratification

Although it may seem trivial, rigorous and robust stratification of all samples into the appropriate classes is imperative, since all analysis of the resulting data is based on the class to which a sample is assigned. For example, in a clinical study, the class will likely be based on a physician's diagnosis. How precise are

the diagnostic criteria? If patients are recruited from multiple sites, are the diagnostic criteria consistent between sites? How are patients with potentially confounding copresenting syndromes classified *(*e.g.*,* in a study of prostate cancer, is a patient with benign prostatic hyperplasia "normal"*)*?

Sample Annotation

Sample annotation refers to all the descriptive information associated with a given sample(s). Correct and complete sample annotation prior to the start of experimental work is crucial. Just as in sample stratification, all data analysis will rely on the information contained in the sample annotations, so it is vital that all information is available, correct, and unambiguous. In addition to any unique identifiers, a clear, complete description of the sample and any prior sample treatment procedures are necessary, at a minimum. If you have a version of ProteinChip data manager software which includes the Virtual Notebook functionality, importing the sample annotation information into ProteinChip data manager software without having to reenter the data is quick and easy. See the ProteinChip Data Manager Software Operation Manual for details.

Preanalytical Variability

Preanalytical variability refers to any experimental variability introduced into the analysis prior to the actual assay. Even seemingly unimportant, innocuous experimental parameters in the sample preparation can introduce significant preanalytical bias if uncontrolled. Robust results require that all sample pretreatments be very carefully and consistently applied to every sample, in every sample class. It is especially important if samples are collected at multiple sites, or by multiple people, that a precise sample collection protocol is designed and rigorously adhered to. Even a slight variation in sample collection procedure (e.g.*,* time before freezing, centrifugal speed, etc.) can generate inconsistent results.

Chapter 2: Sample Preparation From Body Fluids

Introduction

Because they can be obtained relatively noninvasively and nondestructively, body fluids are often chosen for differential expression experiments, especially for clinical research. Blood serum and plasma are by far the most common sample types and will be discussed below. However, other clinically accessible fluids such as cerebrospinal fluid, tissue lavage fluid, and tissue aspirate fluid have all been successfully profiled.

Blood

Blood is one of the most easily obtained patient specimens, and because it is hypothesized to contain many of the molecules that might indicate systemic function, it is the sample type that is most often profiled in the hopes of identifying sets of biomarkers for clinical use. In order to avoid interference from the proteins found in erythrocytes (mostly hemoglobin) and leukocytes, the acellular fraction of blood is analyzed.

Serum vs. Plasma

Serum is the fluid remaining from blood after coagulation has occurred and the resulting cellular clot is removed. The tube into which the blood is collected may or may not have a gel separator to make it easier to remove the serum from the top of the clot. If the blood is collected in the presence of a coagulation inhibitor, the liquid left after the cells are removed by centrifugation is referred to as plasma. There are a number of coagulation inhibitors commonly used to generate plasma (e.g., heparin, sodium citrate, EDTA). The septum of blood collection tubes are color coded to indicate which coagulation inhibitor, if any, is present in the tube. Hence, one will often see reference to collection in a “green-top tube” (Na^+/Li^+) -heparin or a “lavender-top tube” (EDTA), etc.

The coagulation inhibitor used to produce plasma may have a significant impact on the resulting ProteinChip® SELDI profiles, either by sequestering proteins in the sample (heparin) or by interfering with binding to the ProteinChip® array downstream (EDTA). Obviously, when using plasma, it is very important to insure that the same coagulation inhibitor at the same concentration is used in all samples.

There is no preference for either serum or plasma in differential expression profiling. Whichever is used, it is crucial that exactly the same collection protocol be used for all samples and controls. Most clinical laboratories have a standard collection protocol which should be adhered to rigorously. If multiple clinics are involved in collecting the samples, it is important to ensure that their respective protocols are identical. Details such as the time, temperature, speed of centrifugation to remove the cells or clot, freezing protocol, etc. must be exactly replicated for all samples to avoid preanalytical bias.

Freezing, Transport, and Storage

Serum and plasma should be immediately frozen, preferably at -70˚C, but at least -20°C. While transporting frozen samples, care must be taken to avoid thawing. During storage, samples must be frozen at -70°C.

Serum and Plasma Pretreatments

Serum and plasma may need to be pretreated to remove either hemoglobin contamination or excess lipid, which naturally occurs. If any of the samples are to be pretreated, it is important that all samples in the study are pretreated identically. Failure to do so will generate unreliable results.

Hemoglobin Removal Protocol

If serum and plasma are prepared correctly, limited hemolysis (lysis of the erythrocytes) should occur. However, if handled incorrectly, there may be sufficient lysis that the samples become reddish in color, due to the presence of hemoglobin in the samples. Hemoglobin will dominate subsequent ProteinChip SELDI analysis and must be removed. Although the method described below will remove most hemoglobin from the sample, it may also sequester other proteins as well. It is always preferable to minimize hemolysis, rather than relying on subsequent hemoglobin removal.

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

Materials

- Ni^{2+} support
- Phosphate-buffered saline (PBS)
- 0.3 M NaCl
- Profinity IMAC Ni^{2+} charged resin

Protocol

1. Wash the Ni^{2+} support with PBS/0.3 M NaCl.
2. Add 100 µl 50% Ni^{2+} support (~50 µl beads in 50 µl PBS/0.3 M NaCl) to 100 µl sample in a 1.5 ml microcentrifuge tube.
3. Mix gently on a rotating platform for 20 minutes at 4°C.

4. To remove the support, centrifuge the tube at the highest speed of a tabletop microcentrifuge for 2 minutes at 4°C.

Delipidation

Depending on the length of time between the last feeding and sample collection, mammalian serum can vary from being clear, amber colored to opaque, and milky white. The opacity is due to large amounts of lipids, chylomicrons, free fatty acids, and lipid-carrying proteins. Some lipids can be removed by placing the serum on ice, then microcentrifuging 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 also be removed by extraction with ether and methanol or $CHCl_3$ (Chloroform) and 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.

NOTE

Avoid using solvents such as isoamylalcohol in the delipidation as they may extract peptides from the serum.

Delipidation Protocol 1

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.

NOTE

The following step is especially crucial and should be performed exactly as described.

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 resuspend 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.

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 nonionic detergent compatible with ProteinChip SELDI analysis, such as Triton X-100 or NP-40). Alternatively, aqueous solutions containing 6–8 M urea, 4–6 M guanidine HCl, 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, nonquantitative recovery of protein, or recovery of protein with poor solubility characteristics include:

- Failure to dialyze excess KBr from the lipoprotein solution
- Dialysis of the lipoprotein solution against distilled water rather than 0.15 M NaCl
- Failure to keep organic solvents at 0°C or less
- 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
- Drying the protein in a pellet rather than a thin film. Methanol is the critical component of this organic solvent mixture

Delipidation Protocol 2

Human serum can be delipidated using the commercial reagent, PHM-L LIPOSORB absorbent, available from Calbiochem.

1. Reconstitute PHM-L LIPOSORB absorbent according to manufacturer's instructions.
2. Use 1.5 volume sample to 1 volume of reconstituted PHM-L LIPOSORB absorbent.
3. Mix 30–60 seconds by vortexing.
4. Spin 10 minutes at 3,000 rpm.
5. Lipids will remain at the bottom of the tube. Remove the top layer — this is the clarified, delipidated sample.
6. Delipidated serum can be diluted in the appropriate ProteinChip array binding buffer or column binding buffer as appropriate before analysis.

Urine

Protein biomarkers for genitourinary conditions are often found in urine, which is an easily obtainable, noninvasive sample source. Since the protein concentration of normal urine is quite low, interfering abundant proteins are less of a problem than with serum or plasma. Most of the protein in urine is due to only two species: albumin and Tamm-Horsfall mucoprotein. When proteinuria exists, the majority of the protein is albumin, although some diseases such as multiple myeloma leak predominantly Bence -Jones protein. Also, normal urine will contain a small number of epithelial cells from the lining of the genitourinary tract. A sample collected early in the morning will contain more cells than a sample collected during the day. Cellular contaminants must be removed prior to preparing the sample for ProteinChip SELDI analysis.

Because of the presence of high concentrations of small molecule interferants, such as nitrates, creatinine, and urea, most common assays for total protein content don't work well in urine. The protein content is normally measured using a precipitation assay, which is routinely carried out in clinical laboratories.

Preparation for Array Binding Protocol

Immediately after collection, urine samples should be centrifuged at 16,000 x g for 5 minutes at 4°C to sediment cellular material. The supernatants should be frozen and stored at -80°C until further analysis. The supernatants should subsequently be subjected to only one freeze-thaw cycle, and should only be thawed on ice.

Preparation for array binding:

1. To 160 µl urine, add 60 µl denaturing buffer (9 M urea/2% CHAPS/50 mM Tris, pH 9.0).
2. Vortex for 30 minutes at 4°C.
3. Aliquot and freeze at -80°C.
4. Centrifuge samples at 16,000 x g for 5 minutes at 4°C prior to use.

Chapter 3: Sample Preparation From Solid Tissue andTissue Lysates

Introduction

Preparation of solid tissue involves disruption of the tissue structure and separation of the cells from the connective tissue, followed by lysis of the dissociated cells. Tissue disruption is performed mechanically, or sonication can be used to both disrupt the tissue and lyse the cells. If proteins in a specific organelle (e.g. the nucleus) are to be profiled, see the "Subcellular Fractionation Protocol" on page 17.

Lysis Using the FastPrep Homogenizer

This protocol describes the use of a tissue homogenizer for one-step tissue disruption and cell lysis of mammalian tissue. This is Bio-Rad's recommended method because it has been successfully used to generate excellent data using ProteinChip® SELDI technology.

Materials

- Dewar flask 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)
- FastPrep homogenizer (also called a ribolyzer) (Thermo Scientific)
- Lysis buffer — 9.5 M urea + 2% (w/v) CHAPS+ 1% (w/v) dithiothreitol (DTT)
- Aluminum foil

Protocol

1. Prepare the lysis buffer — Add 30 g urea to 30 ml deionized water. Stir until dissolved. To the urea solution, add 1.0 g CHAPS, 0.5 g DTT, and fill volume to 50 ml. Aliquot and freeze at -80°C.

NOTE

Do not heat the urea-containing solution above 37°C.

2. Remove the tissue(s) from a -80°C freezer and place on dry ice.
3. Approximately 1 ml lysis buffer is required per 100 mg of tissue. Place 0.75–1.0 ml lysis buffer in a separate FastPrep homogenizer tube for each sample to be prepared. Note the volume of lysis buffer in each tube.
4. Line a ceramic pestle and mortar with aluminium foil and carefully pour in a small amount of liquid nitrogen.

5. Weigh out approximately 100 mg of tissue and note the weight of the tissue.
6. Place the tissue in the mortar with the liquid nitrogen and crush the tissue with the pestle.
7. When all the liquid nitrogen has evaporated, but before the tissue defrosts, place the powdered tissue in a FastPrep homogenizer tube of lysis buffer. Leave the sample at room temperature while you prepare the rest of the samples.
8. When all the tissues have been prepared, place the tubes in the FastPrep homogenizer. Check that the tube lids are tightly sealed to avoid spillage.
9. Run the FastPrep homogenizer for two 10-second bursts at speed 6. Place the tubes on ice between homogenizations to cool the samples.
10. Spin the FastPrep homogenizer tubes in a microcentrifuge for 5 minutes at 13,000 rpm to reduce air bubbles.
11. Pipet the lysate into appropriate tubes and spin at 42,000 x g for 1 hour at 15°C.
12. Remove the supernatant from the tubes, aliquot it into 50 µl samples. Store the samples at -80°C.
13. Check the protein concentration of the samples using a Bradford quantitation assay.

Lysis by Sonication

Cells are ruptured by sonication using a probe-style sonicator (bath sonication does not yield sufficient 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 need to be determined empirically for each type of probe.

Materials

- Buffer A — 0.1X phosphate-buffered saline (PBS)
- Buffer B — 50 mM NaCl, 0.1% dodecyl maltoside, 25 mM HEPES

NOTE

Either of these buffers can be used successfully for lysis by sonication. The amount of detergent in Buffer B can be increased. Optimization must be done empirically.

Protocol

1. Dilute cells in buffer A or buffer B to a final concentration of ~10^7 cells/ml.
2. Sonicate the cell suspension in short bursts, keeping the sample cold between sonications. For example, sonicate 5 seconds on a low setting, then place sample on ice for 1 minute. Repeat until most or all cells are lysed.
3. The amount of cell lysis may be estimated by spinning the sample on high speed in a microcentrifuge. The pellet will decrease in size as lysis proceeds.
4. Keeping the sample cold between sonication bursts will minimize the proteolytic degradation that results from heating the sample.

Subcellular Fractionation

Purification of Nuclei From Mammalian Tissues

Typical tissue preparation procedures are relatively harsh and cause lysis of all subcellular compartments. In order to isolate nuclei, it is necessary to disrupt the tissue and lyse the cells without lysing the nuclear membrane. The nuclei are purified away from cellular debris and subsequently lysed at a high salt concentration (0.45 M NaCl) to extract all proteins other than the highly abundant histones off the chromatin, which is subsequently removed by centrifugation. Doing this lysis and extraction step at a higher salt concentration yields an extract with a very high concentration of histone proteins, which can prevent the detection of other, less abundant proteins.

Subcellular Fractionation Protocol

The following protocol details purification of nuclear protein from cultured cells and nonmuscle cells of most mammalian tissues.

NOTE

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 PTFE pestle (or in a microcentrifuge tube with a blue Eppendorf plastic pestle) in a solution of 0.15 M NaCl, 10 mM HEPES, or Tris-HCl, pH 7.5. Cultured cells should be collected by centrifugation at 3,000 rpm in a microfuge and washed with the same solution.
2. Filter through 2 layers of cheesecloth.
3. Centrifuge at 3,000 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 X-100 in 5:1 v/v (solution/cells).

5. Leave on ice for 15 minutes, resuspending gently with a thin glass or plastic rod at 5 minute intervals. Try to disperse any aggregates.
6. Spin at 3,000 rpm in a microfuge for 5 minutes at 4°C. Collect the supernatant, which is the cytoplasmic 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. Agitate 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, doing so will extract large amounts of histone.
10. Spin for 5 minutes at maximum speed in a microfuge.
11. Collect the supernatant and store in aliquots at -70°C.

Cytoplasmic and Membrane Fraction Preparation

The following procedure is used to separate membrane from cytosolic proteins in cultured mammalian cells. The cytosolic proteins are extracted without any detergent. At least 4 x 10^7 cells in the active log phase of growth are required.

Materials

- 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, fresh 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% Nonidet P-40 (NP-40), fresh protease inhibitors at 1X concentration

Protocol

1. Harvest the cells in the log 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 a 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 1 ml cell suspension.

4. Dounce homogenize with 20 strokes on ice. Verify cellular disruption using a light microscope.
5. Centrifuge at 3,300 x g for 10 minutes at 4°C.

CAUTION

If the cells are centrifuged at too high a speed, some of the mitochondria and microsomes may be lost.

6. Transfer the supernatant to a fresh Eppendorf tube.
7. Overlay the supernatant on a 35% (w/v) sucrose solution.
8. Centrifuge the pellet at 18,000 x g for 60 minutes at 4°C.
9. Remove the interface. Resuspend interface in 1 ml buffer B, then centrifuge at 100,000 x g for 60 minutes at 4°C.
10. Resuspend the pellet thoroughly in 1 ml buffer C containing 1X protease inhibitors, then vortex.
11. Incubate for 30 minutes at 4°C. Centrifuge at 10,000 rpm for 20 minutes at 4°C.
12. 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.
13. Aliquot into freezing vials and store at -70°C until use.

Recovering Proteins After RNA Preparation Using TRIzol

TRIzol (Invitrogen Corporation) is a monophasic guanidine isothiocyanate and phenol solution developed for single-step purification of RNA. The strongly denaturing TRIzol solution is used to disrupt and lyse the cells and acts to keep the RNA intact during the process by inactivating RNases. After lysis is complete, a two-phase extraction with chloroform is performed — the RNA remains in the aqueous phase, while the DNA and proteins partition into the organic (chloroform) layer. After first precipitating the DNA using ethanol, proteins are recovered out of the organic layer by isopropanol precipitation.

Obviously, after such harsh treatments, virtually all proteins in the preparation will be completely denatured, some irreversibly so. Most proteins will likely be inactive in protein interactions. However, some proteins in the resuspended isopropanol pellet will bind to ProteinChip arrays.

Protocol

1. Follow the manufacturer's instructions for using TRIzol for RNA preparation. Keep track of the amount of TRIzol originally added, since subsequent volumes of reagents will be based on this.
2. After centrifugation to separate the phases, the mixture partitions into three layers: a red organic phase (containing protein and DNA), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA). The aqueous phase is removed and used to prepare RNA.
3. To precipitate the DNA from the organic and interphase, add 0.3 ml 100% ethanol per 1 ml TRIzol reagent initially used. Mix by inversion and incubate at room temperature for 2–3 minutes.
4. Centrifuge at 2,000 x g for 5 minutes at 4°C.
5. Remove the supernatant and keep at 4°C for protein isolation.
6. Add 1.5 ml isopropanol per 1 ml of TRIzol initially used. Incubate for at least 10 minutes at room temperature.
7. Centrifuge at 12,000 x g for 1 minute at 4°C.
8. Discard the supernatant.
9. Wash the pellet 3 times:
    a. Incubate the pellet in 2 ml 0.3 M guanidine hydrochloride and 95% ethanol per 1 ml of TRIzol initially used, for 20 minutes at room temperature.
    b. Centrifuge the samples at 7,500 x g for 5 minutes at 4°C to repellet the protein.
    c. Repeat twice more for a total of 3 washes.
10. After completing the 3 washes, discard the supernatant and add 2 ml 100% ethanol, and vortex the protein pellet. Incubate for 30 minutes at room temperature. Centrifuge at 7,500 x g for 5 minutes at 4°C and then discard the supernatant.
11. Dry the protein pellet under vacuum for 5–10 minutes.
12. 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 ProteinChip H4 or H50 array, resuspend in 10% acetonitrile.

Laser Capture Microdissection

Laser capture microdissection (LCM) is a technique for isolating the cells in a small subsection of a tissue specimen. The protein content of LCM capsules can be profiled to look for differential expression in adjacent cells.

Particularly when using an LCM lysis protocol for the first time, Bio-Rad recommends using as many cells as possible (5,000–10,000 cells).

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 using a 1-D gel or ProteinChip SELDI 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
- Staining by hematoxylin and eosin can diminish the yield of proteins
- Use fresh stain every time. Some people use protease inhibitors in the staining solution
- If possible, microdissect for only a short time (e.g., 10–15 minutes) to avoid heating the sample and subsequent proteolysis

Two lysis methods are given below. You may want to test both methods to determine which works best for you. Because the volumes used are so small, the buffer used in the initial step may dry up in low humidity. Keeping the capsules in a humidity chamber during this step will cause a slight increase in volume due to moisture absorption

Urea Lysis Method

1. Prepare urea lysis buffer — 8 M urea, 1% CHAPS in PBS.
2. Add 2 µl urea buffer to a capsule containing 2,000–5,000 cells and pipet up and down 3–5 times.
3. Leave the lysis buffer on the capsule for ~5 minutes.
4. Drop the capsule and the lysis buffer into a 0.5 ml Eppendorf tube containing 6 µl PBS. The final buffer concentration is 2 M urea, 0.25% CHAPS.

Guanidinium Lysis Method

1. Prepare guanidinium lysis buffer — Mix equal volumes of 6 M guanidine thiocyanate in 50 mM HEPES, pH 7.5, and 1% deoxycholate or Triton X-100.
2. Add 2 µl guanidinium buffer to a capsule containing 2,000–5,000 cells and pipet up and down 3–5 times.
3. Leave the lysis buffer on the capsule for ~5 minutes.
4. Drop the capsule and the lysis buffer into a 0.5 ml Eppendorf tube containing 8 µl 50 mM HEPES. The final concentration is 0.6 M guanidine thiocyanate, 5 mM HEPES, 0.1% deoxycholate.

Bacterial Cell Lysates

The following procedure is based on bacteria grown on solid agar media under the conditions required for the bacteria under study. The number of plates required will depend on how well the bacteria have grown. In many cases, a single plate incubated overnight provides sufficient material for analysis. For slow-growing bacteria, the material from a number of plates incubated for 24–48 hours can be pooled together.

Method 1: Detergent/Urea Lysis Protocol

Materials

- Bacterial lysis buffer — 0.01 M Tris-HCl, pH 7.4, 1 mM EDTA, 8 M urea, 0.05 M DTT, 10% (v/v) glycerol, 5% (v/v) NP-40, 1 mM phenylmethylsulphenylfluoride (PMSF)

Protocol

1. Scrape the bacteria from the surface of the agar using either a sterile swab or plastic disposable plating loop. Although the swab allows direct resuspension into the bacterial lysis buffer, there may be some sample loss. In contrast, the loop method can lead to clumping of the bacteria, and it is generally preferable to resuspend the bacteria in a wash buffer (see below) rather than into the lysis buffer immediately.
    - *Haemophilus influenzae*, *Neisseria meningitidis* — Lyse an overnight plate culture
    - *Escherichia coli* — For fast-growing isolates, lyse a half- (or one-third) plate after overnight incubation
    - *Streptococcus pneumoniae* — Lyse 3 plates of bacteria (24–48 hour growth)

2. Dislodge the bacteria from the swab or loop directly into 0.5 ml lysis buffer (held in 1.5 ml microfuge tube) and resuspend the bacteria
3. Leave on ice for 5 minutes
4. Remove insoluble cell debris by centrifugation on a microfuge at 11,000 x g for 5 minutes
5. Freeze and store the supernatant containing the soluble bacterial proteins at -70°C until required

Method 2: Sonication Protocol

Materials

- TEN buffer — 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl

Protocol

1. Harvest bacteria from agar plate into 5 ml of TEN buffer.
2. Centrifuge at 5,000 x g for 10 minutes and discard the supernatant.
3. Freeze the pellet by placing the tube in a dry ice and ethanol bath, then thaw at 37°C.
4. Repeat step 3 twice for a total of 3 rounds of freezing and thawing.
5. Resuspend bacterial pellet in a small volume (0.1–0.5 ml) of ice-cold microtubule stabilizing (MTBS) buffer (16 mM Na_2HPO_4, 4 mM NaH_2P0_4, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF).
6. Sonicate the suspension with a 15-second burst followed by 30-second incubation on ice (four rounds of sonication and incubation).
7. Pellet the bacterial debris at 14,000 x g.
8. Transfer the supernatant to a clean tube. Aliquot and store at -70°C until ready to be assayed.

Chapter 4: Differential Expression Profiling

Differential Expression Analysis Using ProteinChip® Arrays

The goal in differential expression profiling is to capture as many different proteins as possible on the surface of various ProteinChip arrays and read the arrays under conditions which will allow the maximum number of protein species to be detected by the ProteinChip SELDI reader. In order to avoid binding interference or ion suppression from any highly abundant proteins in the sample, the standard differential expression protocol begins with an anion exchange fractionation step that uses a pH step gradient. The support is washed with each of the six elution buffers twice to ensure complete reequilibration of the support, and the two elutions are pooled. This gives greater reproducibility in the fractionation as well as better partitioning of proteins into their respective fractions.

An aliquot of each of the six fractions collected in the prefractionation step is bound to a variety of chip surfaces under moderate-stringency binding conditions. Since the sample has already been prefractionated by anion exchange, complementary ProteinChip array chemistries are typically tried first: cation exchange ProteinChip CM10 array reversed-phase ProteinChip H50 array, and metal affinity ProteinChip IMAC array. Since the goal in differential expression profiling is to capture as many proteins as possible, rather than any specific protein motif, the ProteinChip IMAC30 array is charged with Cu^{+2}, rather than Ni^{+2} or Ga^{+3}, which selectively bind specific forms of protein histidine (His)-tagged or phosphorylated, respectively).

Anion Exchange Prefractionation

Introduction

The protocol given below is that used in the ProteinChip serum fractionation kit. This protocol has been optimized for the fractionation of human serum or plasma, although with suitable adjustments it can be used with any complex biological sample. If fractionating a large number of serum samples, we strongly advise that you simply use the kit and follow the detailed instructions that come with it. The kit includes a 96-well Proteinchip Q filtration plate containing premeasured, dried Q ceramic HyperD F sorbent. If fractionating only a few samples, you can use ProteinChip Q spin columns.

Regardless of which format is used for the assay, the outline of the protocol is the same:

1. Serum is mixed with 9 M urea and detergent to break up any protein-protein associations.
2. Simultaneously, the Q ceramic HyperD F sorbent is prewashed and equilibrated to pH 9.0. Please refer to the ProteinChip Serum Fractionation Kit product bulletin# 10008245 for important details on sorbent rehydration.

3. The urea concentration in the sample is diluted, and the sample is mixed with the Q ceramic HyperD F sorbent.
4. Vacuum filtration or centrifugation is used to collect the flowthrough and the first pH wash (pH 9.0), which are combined.
5. The sorbent is washed with successively lower-pH buffers, twice with each buffer.

Materials

Buffers:

- ProteinChip U9 buffer— 9 M urea, 2% CHAPS, 50 mM Tris-HCl, pH 9.0
- U1 buffer— 1 M urea, 0.22% CHAPS, 50 mM Tris-HCl, pH 9.0
- Wash buffer 1 (WB1) — 50 mM Tris-HCl with 0.1% OGP, pH 9.0
- Wash buffer 2 (WB2) — 50 mM HEPES with 0.1% OGP, pH 7.0
- Wash buffer 3 (WB3) — 100 mM Na acetate with 0.1% OGP, pH 5.0
- Wash buffer 4 (WB4) — 100 mM Na acetate with 0.1% OGP, pH 4.0
- Wash buffer 5 (WB5) — 50 mM Na citrate with 0.1% OGP, pH 3.0
- Wash buffer 6 (WB6) — 33.3% isopropanol, 16.7% acetonitrile, 0.1% trifluoracetic acid (TFA)

Other materials required:

- ProteinChip serum fractionation kit or ProteinChip Q spin columns
- Seven 96-well V-bottom microtiter plates
- One microtiter plate-format vacuum filtration manifold (Innovative Microplate)
- One MicroMix 5 Microtiter plate shaker (Diagnostic Products Corp.)

Protocol Using ProteinChip Q Filtration Plate

1. Prepare Q ceramic HyperD F sorbent by washing 3 times with 5 bed volumes 50 mM Tris-HCl, pH 9.0. Afterwards, store at 4°C as a 50% slurry in 50 mM Tris-HCl, pH 9.0.
2. Disaggregate the serum proteins.
    a. Thaw frozen serum on ice.
    b. Centrifuge at 20,000 x g for 10 minutes at 4°C in a tabletop centrifuge.
    c. Aliquot 20 µl of each sample into a well in one of the 96-well V-bottom plates, one sample per well.
    d. Add 30 µl ProteinChip U9 buffer to each sample, then shake for 30 minutes at 4°C on the MicroMix 5.

3. Equilibrate the sorbent by adding 180 µl Q ceramic HyperD F support to each well in the filtration plate. Remove the buffer from the sorbent by filtering on the vacuum manifold for 1 minute. Wash twice with 200 µl 50 mM Tris-HCL, pH 9.0, vacuum filtering each time.
4. Bind the serum with the sorbent. Pipet the entire sample (50 µl) from each well of the V-bottom plate into the corresponding well in the filtration plate. Add 50 µl of U1 buffer to each well of the sample plate. Mix 5 times. Transfer the rinse solutions to the corresponding wells in the filtration plate. Shake 30 minutes at 4°C on the MicroMix 5.
5. Collect fractions:
    a. Label six of the 96-well V-bottom plates F1–F6.
    b. Assemble the vacuum manifold with a 96-well V-bottom plate under the filtration plate. Collect flowthrough in plate F1 by applying vacuum for 2 minutes. Release the vacuum and make sure it has fully dissipated by carefully lifting one corner of the filtration plate. Add 100 µl more WB1 to each well of the filtration plate. Shake 10 minutes at room temperature on the MicroMix 5. Replace the filtration plate on the manifold, and collect this wash in the same collection plate (F1). Fraction 1 contains both the flowthrough and the pH 9.0 eluent.
    c. Add 100 µl of WB2 to each well of the filtration plate. Shake for 10 minutes at room temperature on the MicroMix 5. Assemble the vacuum manifold with a fresh 96-well V-bottom plate under the filtration plate (plate F2). Collect the eluate in plate F2 by applying vacuum for 2 minutes. Release the vacuum and make sure it has fully dissipated by carefully lifting one corner of the filtration plate. Add 100 µl more WB2 to each well of the filtration plate. Shake 10 minutes at room temperature on the MicroMix 5. Replace the filtration plate on the manifold, and collect this wash in the same filtration plate (F2). Fraction 2 contains the pH 7.0 eluent.
    d. Repeat step 5c with each of the succeeding wash buffers to collect the entire pH gradient.

Protocol Using Spin Columns

ProteinChip Q Spin Columns are prefilled with the correct amount of anion exchange sorbent to carry out the protocol above. The product insert for the spin columns contains detailed instructions for use, which should be followed. Prefractionation is carried out using the protocol above with the following modifications:

1. Follow the instructions in the product insert for equilibrating the spin columns with buffer.

2. Instead of shaking on a MicroMix 5, the spin columns should be agitated on a vortex with a microcentrifuge tube adaptor.
3. Instead of vacuum filtration, the uncapped columns are placed in a collection tube and centrifuged at 80 x g for 30 seconds in a tabletop centrifuge.

Array Binding

Introduction

The optimal starting protocol for array binding in differential expression profiling is the same regardless of sample type, except for the amount of sample dilution (see below). Experience has shown that in the initial "fishing" for biomarkers, this protocol maximizes the chance of finding differences. The standard differential expression array-binding protocol has five basic steps:

1. Array pretreatments (for IMAC and reversed-phase arrays)
2. Equilibration (prewashing) of arrays
3. Sample dilution and binding
4. Post-binding stringency washing
5. ProteinChip energy absorbing molecule (EAM) application

The same buffer is used for array equilibration, sample binding, and post-binding washing. The detailed protocol given below was optimized for human serum or plasma, but it can be used for almost any sample type, provided that the dilution of the sample into binding buffer is adjusted appropriately. It is intended for use with arrays in a ProteinChip bioprocessor, and can either be done manually or robotically using a Biomek 3000 laboratory automation workstation with a ProteinChip SELDI system integration package.

Sample Dilution

In the array-binding step of differential expression profiling, the sample is diluted in the binding and washing buffer. Correct sample dilution is achieved by balancing several factors. Obviously, the sample cannot be so dilute that proteins won't bind to the surface. However, if the sample is too concentrated, the more abundant proteins in it will saturate the surface, preventing less abundant proteins from being detected. It is also important that the addition of sample not alter the pH of the moderate-stringency binding and wash buffer, or excessively dilute other buffer components. This is especially important if prefractionation has been carried out, since the fractions will have very different pHs. If too much of the fraction is added to the binding buffer, the pH of the final diluted sample will be different for each fraction and the results will not be reproducible. For serum, we have found that a 1:10 dilution (10 µl sample +

90 µl binding buffer) of the anion exchange fractions to binding buffer gives the optimal results. For different sample types, try several different dilution factors, bracketing 1:10. If the pH of the sample is far from neutral, you should check that the sample addition won't cause a pH shift. This can easily be done by diluting a large volume of the sample buffer into the binding/wash buffer at the same dilution factor, and measuring the pH of the resulting solution with a standard pH meter. For example, to check a 1:5 dilution, add 2 ml of sample buffer to 8 ml of binding and wash buffer, mix, and measure the pH.

Protocol

Table 4.1.ProteinChip Arrays and corresponding buffers for array binding.

ProteinChip Array	Moderate-Stringency Wash Buffer	High-Stringency Wash Buffer
IMAC30	100 mM $NaPO_4$, pH 7.0; 0.5 M NaCl	
H50	10% acetonitrile, 0.1% TFA, pH 1.9	
CM10	100 mM Na acetate, pH 4.0	50 mM HEPES, pH 7.0
Q10	50 mM Tris-HCl, pH 8.0	

1. Pretreat arrays:

   ProteinChip IMAC30 Arrays

   a. Charge with Cu^{+2} — Place arrays in a ProteinChip bioprocessor. Load 50 µl 100 mM $CuSO_4$ onto each spot on the ProteinChip IMAC30 array. Shake 5 minutes at room temperature. Remove $CuSO_4$ after vortexing. Rinse with deionized water.

   b. Neutralize surface — Load 50 µl 100 mM sodium acetate, pH 4.0 onto each spot on the ProteinChip IMAC30 array. Shake 5 minutes at room temperature. Remove sodium acetate after shaking. Rinse with deionized water.

   ProteinChip H50 Arrays

   a. Place arrays in a ProteinChip bioprocessor. Add 50 µl 50% acetonitrile in deionized water to each bioprocessor well. Shake arrays for 5 minutes. Remove wash solution after shaking.

2. Equilibrate arrays — Add 150 µl appropriate array binding buffer to each well. Shake 5 minutes at room temperature. Remove buffer. Add 150 µl appropriate buffer into each well. Shake 5 minutes at room temperature. Remove buffer before adding samples.
3. Sample dilution and binding — Add 90 µl of appropriate buffer to each well. Add 10 µl of fractionated sample. Shake 30–60 minutes at room temperature. Remove sample and buffer.
4. Post-binding stringency washes — Add 150 µl of corresponding buffer into each well. Shake 5 minutes at room temperature. Remove buffer after shaking. Repeat wash step two more times for a total of 3 washes. Rinse twice with deionized water.
5. EAM application:

   ProteinChip sinapinic acid (SPA) EAMs

   a. Add 400 µl of 50% acetonitrile, 0.5% TFA to SPA EAM vial. Shake 5 minutes at room temperature.

   b. Apply 1.0 µl to each spot. Allow spots to air-dry. Apply 1.0 µl to each spot again. Allow spots to air-dry.

   ProteinChip alpha-cyano-4-hydroxycinnamic acid (CHCA) EAMs

   a. Add 200 µl of 50% acetonitrile, 0.25% TFA to CHCA EAM tube. Shake 5 minutes at room temperature. Centrifuge at 10,000 rpm for 1 minute at room temperature. Remove supernatant and dilute with an equal volume of 50% acetonitrile, 0.25% TFA.

   b. Apply 1.0 µl to each spot. Allow spots to air-dry. Apply 1.0 µl to each spot again. Allow spots to air-dry.

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