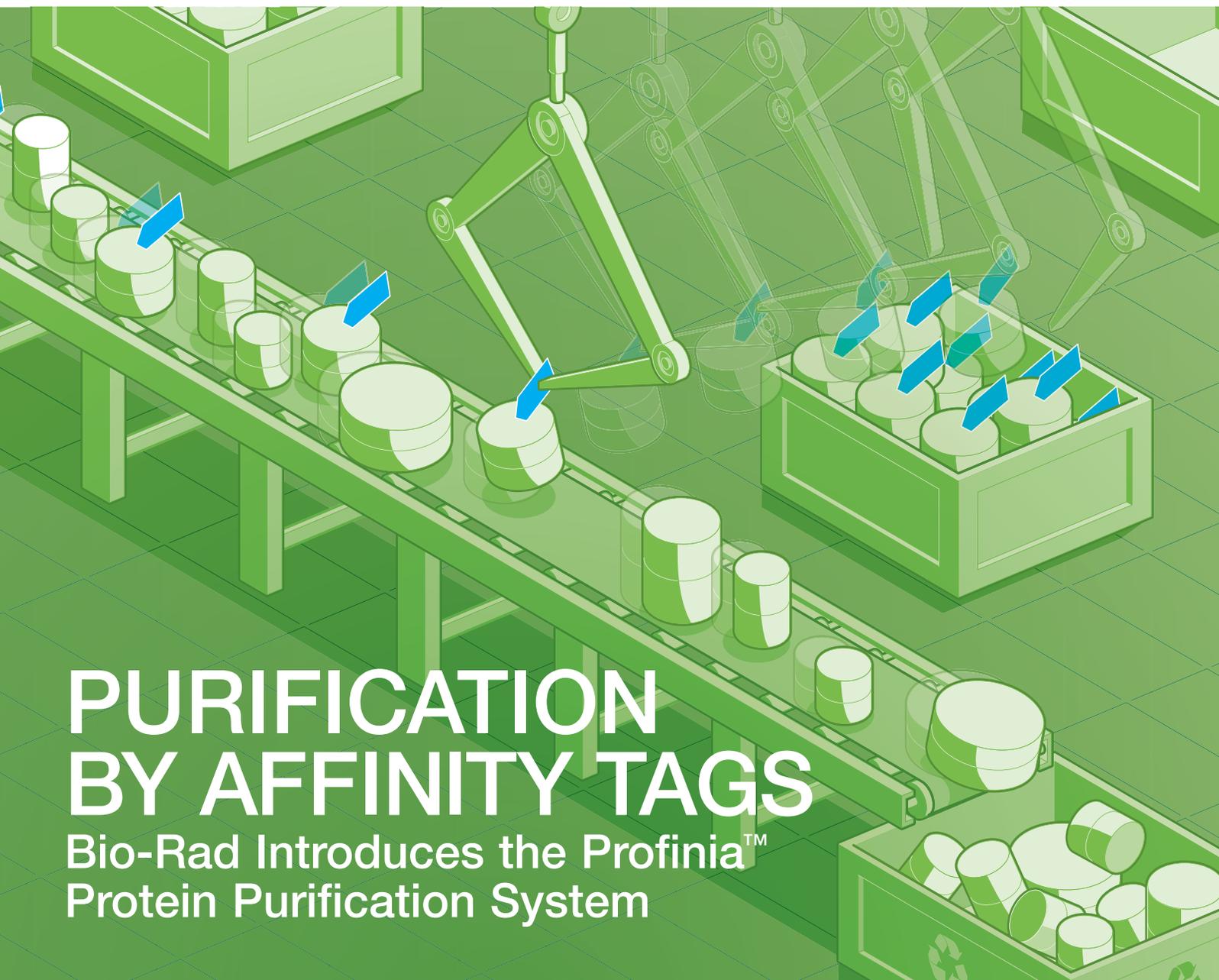


BioRadiations

A Resource for Life Science Research



PURIFICATION BY AFFINITY TAGS

Bio-Rad Introduces the Profinia™
Protein Purification System

In this issue:

Protein-Protein Binding Analysis
Sanitizing Anion Exchangers for Validation and Scale-Up
Clinical Qualification of Microarrays
Normalization Methods for Quantitative PCR

BIO-RAD



Protein in your hands faster.

Introducing the new Profinia™ protein purification system, an automated system that keeps your hands free for unraveling the really interesting questions.

Purify Your Samples, Simplify Your Life

A convenient and automated alternative to existing methods of purification, the Profinia protein purification system brings unprecedented speed and simplicity to the purification of affinity-tagged proteins.

- Preprogrammed methods for IMAC (native and denaturing), GST, and desalting applications
- Large, informative touch screen interface allows easy navigation through protocol steps
- Optimized kits and reagents match the methods and the instrument for greater reproducibility and reduced preparation time
- Automatically calculated run data includes protein yield and concentration

For more information on the Profinia purification system, visit us on the Web at www.bio-rad.com/ad/profinia/



Automated Purification: 30 Minutes
Profinia system affinity and desalting

Manual Purification: 0.5–4 Hours
Gravity-flow affinity (time for dialysis not shown)

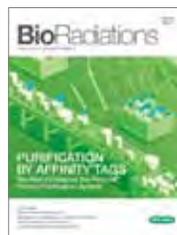
Purification of fusion proteins may require a license from third parties.

Australia 61-2-9914-2800
Austria 43-1-877-89-01
Belgium 32-9-385-55-11
Brazil 55-21-3237-9400
Canada 905-712-2771
China 86-21-6426-0808
Czech Republic 420-241-430-532
Denmark 45-44-52-10-00
Finland 358-9-804-22-00
France 33-1-47-95-69-65
Germany 49-89-318-84-0
Greece 30-210-777-4396
Hong Kong 852-2-789-3300
Hungary 36-1-455-8800
India 91-124-402-9300
Israel 972-3-963-6050
Italy 39-02-216-091
Japan 81-3-5811-6270
Korea 82-2-3473-4460
Latin America 00-1-305-894-5950
Mexico 55-52-00-05-20
The Netherlands 31-318-540666
New Zealand 64-9-415-2280
Norway 47-23-38-41-30
Poland 48-22-331-99-99
Portugal 351-21-472-7700
Russia 7-095-721-14-04
Singapore 65-6415-3188
South Africa 27-861-246-723
Spain 34-91-590-5200
Sweden 46-8-555-12700
Switzerland 41-61-717-9555
Taiwan 88-62-2578-7189
Thailand 662-651-8311
United Kingdom 44-20-8328-2000
USA Toll free 1-800-4BIORAD
(1-800-424-6723)

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Chris Crutchfield



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BioRadiations

issue 121, 2007

TO OUR READERS

Advances in genomics programs have dramatically increased the use of recombinant proteins containing a tagged sequence, and thus have created a demand for greater convenience in purification of tagged proteins for analysis and characterization. Bio-Rad now offers an automated affinity purification system with built-in speed and efficiency to overcome the limitations of traditional large-scale or kit-based recombinant protein purification techniques. Over many decades, Bio-Rad's chromatography products have answered the sample purification needs of thousands of scientists; in this issue of BioRadiations, the feature story describes the company's latest addition to the lineup. The Profinia™ protein purification system can deliver a purified protein in as few as 30 minutes of unattended operation using preprogrammed methods and prepackaged buffers and reagents. Its automated performance frees the researcher from standby duty, and the simplicity of the system means anyone in the laboratory can run the purifications.

COVER STORY

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Legal Notices — See page 32.

Profinia™ Protein Purification System

The Profinia system rapidly performs automated two-step affinity purification and desalting of histidine (His)- and GST-tagged proteins. Preprogrammed purification methods optimized for the most common affinity applications are accessed through a touch-screen user interface. Application-specific buffer and purification kits ensure rapid, reproducible protein purification. The Profinia system can purify affinity-tagged protein samples for immediate downstream use in as little as 30 minutes.

The Profinia system is the ideal upgrade from traditional gravity-flow column affinity purification systems. It provides a convenient, time-saving system that takes up minimal bench space; all reagent bottles, fraction and sample tubes, and tubing are held within the unit. In addition, the Profinia system requires minimal training and expertise to use, making it ideal for members of a large laboratory who frequently perform affinity-tagged purifications or for sharing among several laboratories.

Automation Enables Fast, Hands-Free Purification

Purification of affinity-tagged proteins with traditional gravity-flow columns is an intensive manual process that requires constant monitoring of the columns and frequent buffer additions and fraction tube changes. Traditional chromatography systems typically require extensive time and expertise. However, the Profinia system's simple operation eliminates the need to learn how to configure and program the instrumentation.

Simple, Automated, and Efficient

- Automatic detection, selection, and diversion of main tagged protein peak from affinity cartridge to size exclusion (desalting) cartridge
- Single-tube collection of flowthrough, wash, and elution fractions — eliminates the need to pool fraction tubes

Quick and Easy Setup for Purification

- Touch-screen interface displays component and reagent installation on the instrument
- Kit labels match those on the touch screen and instrument to ensure purification quickly and with confidence

Informative Touch-Screen Interface

Selection of methods and setup is simplified through the large and informative touch screen, which intuitively guides you through the process of selecting the method, number of samples, and size of cartridge for each purification run.

Profinia Consumables

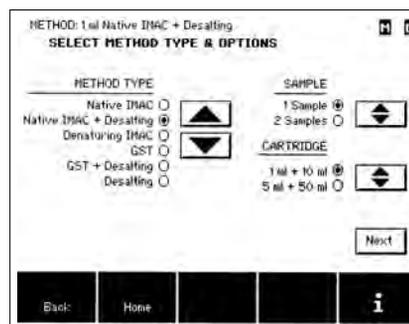
Each of the preprogrammed methods is paired with an optimized purification kit that contains all necessary buffers, reagents, and cartridges. The consumables work seamlessly with the Profinia system to simplify the operation and execution of all steps in the purification.

Profinia Reporting Software

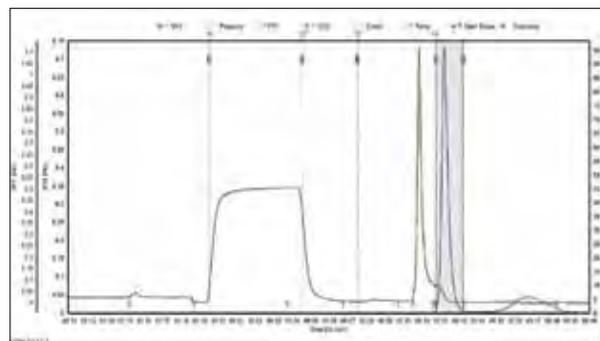
The Profinia system is a self-contained unit that does not require a computer to perform protein purification. However, Profinia software, an optional component that is run on a PC, displays purification data and allows you to create and view reports to document each purification run. Run data are transferred with a USB portable memory device to a PC running Profinia software, or by connecting the Profinia instrument directly to a PC via a USB cable for real-time data capture during the purification run.



Profinia protein purification system.



Touch-screen interface.



Profinia software displays a two-cartridge chromatogram. Shown are native IMAC and desalting, as well as yield, concentration, and run information from a Profinia protein purification run.

For more information on the Profinia system, go to www.bio-rad.com/affinitypurification/

Ordering Information

Catalog # Description

Profinia Systems

| | |
|----------|---|
| 620-1010 | Profinia Protein Purification System With Native IMAC Starter Kit, 100–240 V, includes Profinia instrument, accessory kit, Profinia native IMAC buffer kit, 1 x 1 ml IMAC and 1 x 10 ml desalting cartridge, <i>E. coli</i> lysate, Profinia software |
| 620-1011 | Profinia Protein Purification System With GST Starter Kit, 100–240 V, includes Profinia instrument, accessory kit, Profinia GST buffer kit, 1 x 1 ml GST and 1 x 10 ml desalting cartridge, <i>E. coli</i> lysate, glutathione reagent, Profinia software |

Profinia Purification Kits

| | |
|----------|---|
| 620-0225 | Profinia Native IMAC Purification Kit, 1 ml, includes Profinia native IMAC buffer kit, 2 x 1 ml IMAC and 2 x 10 ml desalting cartridges |
| 620-0235 | Profinia Native IMAC Purification Kit, 5 ml, includes 2 Profinia native IMAC buffer kits, 1 x 5 ml IMAC and 1 x 50 ml desalting cartridge |
| 620-0227 | Profinia Denaturing IMAC Purification Kit, 1 ml, includes Profinia denaturing IMAC buffer kit, 2 x 1 ml IMAC cartridges |
| 620-0237 | Profinia Denaturing IMAC Purification Kit, 5 ml, includes 2 Profinia denaturing IMAC buffer kits, 1 x 5 ml IMAC cartridge |
| 620-0226 | Profinia GST Purification Kit, 1 ml, includes Profinia GST buffer kit, 2 x 1 ml GST and 2 x 10 ml desalting cartridges |
| 620-0236 | Profinia GST Purification Kit, 5 ml, includes 2 Profinia GST buffer kits, 1 x 5 ml GST and 1 x 50 ml desalting cartridge |
| 620-0228 | Profinia Desalting Purification Kit, 10 ml, includes Profinia desalting buffer kit, 2 x 10 ml desalting cartridges |
| 620-0238 | Profinia Desalting Purification Kit, 50 ml, includes 2 Profinia desalting buffer kits, 1 x 50 ml desalting cartridge |

Bio-Scale™ Mini Cartridges

Bio-Scale Mini cartridges are disposable purification cartridges designed for use with low-pressure chromatography systems or with a Luer-Lok syringe. Their robust, leak-free design is based on a patent-pending double-wall construction that provides extra durability and reproducible, reliable, and easy runs. These cartridges are:

- Prepacked with Bio-Rad's chromatography media for ion exchange, affinity, and size exclusion purification
- Convenient to use for separation, method scouting, and testing media in new applications
- Compatible with aqueous buffers most commonly used in protein separations at pressures up to 45 psi*



Bio-Scale Mini cartridges are supplied in ready-to-use 1 ml and 5 ml volume sizes for most media types, and 10 ml and 50 ml sizes prepacked with Bio-Gel® P-6 media for size exclusion, buffer exchange, and desalting applications. The cartridges are compatible with a wide range of chromatography formats, including:

- BioLogic™ LP, BioLogic DuoFlow™, and other programmable chromatography systems suitable for general separations
- The Profinia™ system, an automated affinity-tagged purification platform that uses Bio-Scale Mini Profinity™ IMAC and GST affinity cartridges and Bio-Gel P-6 desalting cartridges to make purification more simple, convenient, and efficient (see above)

Ordering Information

| Description | 5 x 1 ml | 1 x 5 ml | 5 x 5 ml |
|--|----------|------------|------------|
| Prepacked Bio-Scale Mini Cartridges | | | |
| UNOsphere Q Support | 732-4100 | 732-4102 | 732-4104 |
| UNOsphere S Support | 732-4110 | 732-4112 | 732-4114 |
| Macro-Prep High Q Support | 732-4120 | 732-4122 | 732-4124 |
| Macro-Prep High S Support | 732-4130 | 732-4132 | 732-4134 |
| Macro-Prep DEAE Support | 732-4140 | 732-4142 | 732-4144 |
| Bio-Gel P-6 Support | — | 732-4502 | 732-4504 |
| Affi-Prep Protein A Support | 732-4600 | 732-4602 | — |
| Profinity IMAC Support | 732-4610 | 732-4612 | 732-4614 |
| Profinity GST Support | 732-4620 | 732-4622 | 732-4624 |
| DEAE Affi-Gel Blue Support | — | 732-4632 | 732-4634 |
| Affi-Gel Blue Support | — | 732-4642 | 732-4644 |
| | | Individual | Packs of 5 |
| Bio-Gel P-6 Support, 10 ml | | — | 732-5304 |
| Bio-Gel P-6 Support, 50 ml | | 732-5312 | 732-5314 |

* For more information, including specifications, refer to the 2007 Life Science Research catalog supplement or go to www.bio-rad.com/cartridges/

ProteinChip® SELDI System

Bio-Rad Laboratories has acquired CIPHERGEN Biosystems' ProteinChip surface-enhanced laser desorption/ionization (SELDI) technology products and services. These include instruments, arrays, software, and accessories and the services of Biomarker Research Center facilities worldwide.

SELDI technology delivers protein mass profiles quickly from complex biological samples. ProteinChip arrays use a variety of surface chemistries for protein binding; the captured proteins are then analyzed using the ProteinChip SELDI reader, a highly sensitive, powerful, and compact laser desorption/ionization time-of-flight mass spectrometer. The reader and arrays are supported by ProteinChip SELDI system software and numerous accessories to create exactly the right protein-binding conditions for your biomarker discovery experiments. Different versions of the ProteinChip SELDI system are available to match the size and activity level of any laboratory.

For more information, visit us on the Web at www.bio-rad.com/proteinchip/



Mini-PROTEAN® Tetra Cell

For over 20 years, the Mini-PROTEAN® systems have set the standard for excellence in protein electrophoresis. The Mini-PROTEAN Tetra cell has the same features you value in the Mini-PROTEAN 3 system, and allows you to increase your research throughput as well.

The Mini-PROTEAN Tetra cell, the next-generation mini cell for 1-D electrophoresis, provides flexibility for your research needs. It is versatile, easy to use, accommodates 1–4 handcast or Ready Gel® precast gels, and simplifies casting and running gels by eliminating tedious assembly procedures.

The Mini-PROTEAN Tetra cell is an ideal choice to:

- Run mini gels in less than an hour
- Run 2-D gels in less than a day
- Process discovery projects
- Screen new samples
- Evaluate sample preparation conditions

For more information on Bio-Rad's vertical electrophoresis cells, go to www.bio-rad.com/verticalelectro/



Ordering Information

| Catalog # | Description |
|---------------------------------|---|
| Mini-PROTEAN Tetra Cells | |
| 165-8000 | Mini-PROTEAN Tetra Cell, 10-well, 0.75 mm thickness; 4-gel system includes 5 combs, 5 sets of glass plates, 2 casting stands, 4 casting frames, sample loading guide, electrode assembly, companion running module, tank, lid with power cables, mini cell buffer dam |
| 165-8001 | Mini-PROTEAN Tetra Cell, 10-well, 1.0 mm thickness; 4-gel system includes 5 combs, 5 sets of glass plates, 2 casting stands, 4 casting frames, sample loading guide, electrode assembly, companion running module, tank, lid with power cables, mini cell buffer dam |
| 165-8002* | Mini-PROTEAN Tetra Cell, 10-well, 0.75 mm thickness; 2-gel system includes 5 combs, 5 sets of glass plates, casting stand, 2 casting frames, sample loading guide, electrode assembly, tank, lid with power cables, mini cell buffer dam |
| 165-8003* | Mini-PROTEAN Tetra Cell, 10-well, 1.0 mm thickness; 2-gel system includes 5 combs, 5 sets of glass plates, casting stand, 2 casting frames, sample loading guide, electrode assembly, tank, lid with power cables, mini cell buffer dam |
| 165-8004 | Mini-PROTEAN Tetra Cell for Ready Gel Precast Gels, 4-gel system includes electrode assembly, companion running module, tank, lid with power cables, mini cell buffer dam |

* The 2-gel systems do not include the companion running module.

Bio-Plex® Precision Pro™ Human Cytokine Assay Panel

The Bio-Plex Precision Pro human cytokine assay panel contains highly sensitive, highly reproducible magnetic bead-based assays. These assays allow accurate measurement of low levels of human cytokines with low intra- and inter-assay coefficient of variation (CV). The assay protocol is similar to Bio-Plex cytokine assays, with the option of using magnetic separation for wash steps instead of vacuum filtration. This option allows automation of many of the assay steps. This assay panel:

- Is offered in a convenient kit format that includes assay, reagent, and diluent components in a single box
- Includes standard diluents for both serum and plasma
- Contains controls for generating QC samples
- Uses the 25-bead map in Bio-Plex Manager™ 4.1 software (or later versions)

Specifications*

| | |
|-----------------------|---|
| Limit of detection | ≤1 pg/ml |
| Precision | |
| Intra-assay CV | <10% |
| Inter-assay CV | <15% |
| Accuracy (% recovery) | 80–120% |
| Cross-reactivity | ≤1% |
| Dynamic range | 1–2,500 pg/ml at high PMT |
| Matrices | Plasma, serum, culture supernatant, lysates, homogenates, other human biological fluids |

* Data vary by assay.

For more information, go to www.bio-rad.com/bio-plex/ or contact your local Bio-Rad sales representative.

Ordering Information

| Catalog # | Description |
|-------------|--|
| 171-A1001P* | Bio-Plex Precision Pro Human Cytokine 10-Plex Panel, 1 x 96-well, includes coupled beads, detection antibodies, standards, controls, assay buffer, wash buffer, detection antibody diluent, streptavidin-PE, filter plate, sealing tape, serum standard diluent, plasma standard diluent, and sample diluent for the detection of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-13, IFN-γ, TNF-α |

* This all-in-one assay does not require separate reagent and diluent kits.

Representative Working Assay Range*

| Analyte | Serum | Plasma |
|-------------|-----------|------------|
| IL-1 | 0.2–520 | 0.2–560 |
| IL-2 | 1.9–3,200 | 3.13–3,200 |
| IL-4 | 0.4–1,707 | 0.3–3,200 |
| IL-5 | 2.7–3,200 | 3.7–3,200 |
| IL-6 | 0.3–2,760 | 0.9–3,200 |
| IL-10 | 0.2–2,320 | 0.3–3,200 |
| IL-12 (p70) | 0.5–3,200 | 1.4–3,200 |
| IL-13 | 0.2–2,320 | 0.4–3,200 |
| IFN- | 0.9–3,200 | 1.2–3,200 |
| TNF- | 0.2–1,880 | 0.2–2,720 |

* Results (in pg/ml) based on an average of 5 assays meeting specifications for precision and accuracy. For both serum and plasma samples, intra-assay CV was ≤8% and inter-assay CV was ≤10%.

Bio-Plex Pro™ Human Isotyping Assay Panel

The Bio-Plex Pro human isotyping assay panel allows detection of seven immunoglobulin (Ig) isotypes (IgG₁, IgG₂, IgG₃, IgG₄, IgA, IgE, and IgM) in a single multiplex assay. This panel offers excellent sensitivity using either a magnetic washing system or a standard vacuum manifold. The Bio-Plex Pro human isotyping assay panel:

- Contains controls for generating QC samples
- Uses the 25-bead map in Bio-Plex Manager™ 4.1 software (or later versions)

For more information, go to www.bio-rad.com/bio-plex/ or contact your local Bio-Rad sales representative.

Ordering Information*

| Catalog # | Description |
|--|---|
| Bio-Plex Pro Human Isotyping Assay Panels | |
| 171-A3001M | Bio-Plex Pro Human Isotyping 7-Plex Panel, 1 x 96-well |
| 171-A3002M | Bio-Plex Pro Human Isotyping 7-Plex Panel, 10 x 96-well |

Bio-Plex Pro Reagent Kits

| | |
|------------|--|
| 171-304020 | Bio-Plex Pro Isotyping Reagent Kit, 1 x 96-well, includes Bio-Plex reagent kit and isotyping instructions |
| 171-304021 | Bio-Plex Pro Isotyping Reagent Kit, 10 x 96-well, includes Bio-Plex reagent kit and isotyping instructions |

Bio-Plex Pro Diluent

| | |
|------------|--|
| 171-305030 | Bio-Plex Pro Isotyping Diluent, 1 x 96-well |
| 171-305031 | Bio-Plex Pro Isotyping Diluent, 10 x 96-well |

* For isotyping, you will need a panel, a reagent kit, and a diluent. The panel includes coupled magnetic beads, detection antibodies, standards, and controls.

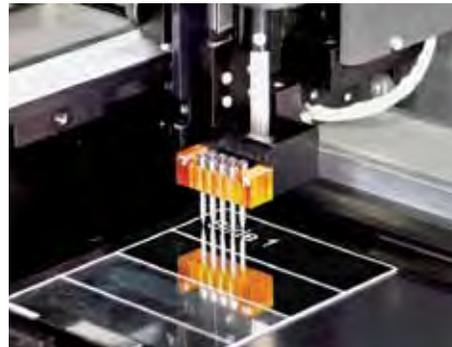
Specifications

| | |
|-----------------------|---|
| Precision | |
| Intra-assay CV | ≤15% |
| Inter-assay CV | ≤20% |
| Accuracy (% recovery) | 70–130% |
| Cross-reactivity | Negligible |
| Matrices | Plasma, serum, culture supernatant, lysates, homogenates, other human biological fluids |

BioOdyssey™ MCP™ Pins

MCP pins are available for use in the BioOdyssey™ Calligrapher™ miniarrayer. These high-performance microarray pins come in three types: MCP100 and MCP360 are for repetitive spotting of even high-viscosity samples such as protein solutions, and MCP310S is a solid pin for printing single samples. MCP pins offer pinpoint precision for your microarrays.

- Superior spot morphology and uniformity
- Exceptional deposition repeatability, ensuring reproducible spots
- Easy cleaning and maintenance to reduce risk of cross-contamination
- Optimized design to print the sample of your choice, including high-viscosity protein solutions and cell lysates
- Suitable for printing onto multiple substrates: nitrocellulose-coated glass slides, membranes, and wells of a 96-well plate
- Precise printing from a 1,536-well source plate
- Easy-to-use pin loading tool (optional)



For more information, request bulletin 5498.

Ordering Information

| Catalog # | Description |
|-----------|---|
| 169-2017 | BioOdyssey Pin, MCP100 |
| 169-2018 | BioOdyssey Pin, MCP360 |
| 169-2019 | BioOdyssey Pin, MCP310S |
| 169-2020* | BioOdyssey MCP Loading Tool |
| 169-2080* | BioOdyssey Calligrapher MCP Printhead Upgrade |

* The loading tool is not required but facilitates loading pins into the printhead. Depending on when it was purchased, your BioOdyssey Calligrapher miniarrayer may require a printhead upgrade for use with the MCP pins. For more information, contact your local Bio-Rad sales representative.

siLentMer™ Validated Dicer-Substrate siRNA Duplexes

siLentMer Dicer-substrate siRNA duplexes for the gene targets listed below are now available. These targets cover a variety of research interests and significance, including targets involved in cancer, cell cycle regulation, inflammation, cell signaling, and many diseases such as Parkinson's, Alzheimer's, rheumatoid arthritis, osteoarthritis, and cardiovascular, metabolic, and respiratory diseases. Additionally, siLentMer duplexes specific to several reference genes are also available to help optimize delivery conditions and establish appropriate controls, including Green Fluorescent Protein (GFP) and luciferase for use in cotransfection experiments involving plasmid-based reporter genes. For more information, go to www.bio-rad.com/RNAi/

Ordering Information

| Reference/Housekeeping Genes* | | | | Genes of Research Interest* | | | |
|-------------------------------|---------------------|---------------------|------------------|-----------------------------|---------------------|---------------------|------------------|
| Gene Target | Duplex 1, Catalog # | Duplex 2, Catalog # | NCBI Accession # | Gene Target | Duplex 1, Catalog # | Duplex 2, Catalog # | NCBI Accession # |
| Human GAPDH | 179-0100 | 179-0200 | NM_002046 | Human B2M | 179-0109 | 179-0209 | NM_004048 |
| Human HPRT | 179-0101 | 179-0201 | NM_000194 | Human APC | 179-0110 | 179-0210 | NM_000038 |
| Human Lamin A/C | 179-0102 | 179-0202 | NM_005572 | Human TP53 | 179-0111 | 179-0211 | NM_000546 |
| Human Cyclophilin | 179-0103 | 179-0203 | NM_021130 | Human MET | 179-0112 | 179-0212 | NM_000245 |
| Human β-Actin | 179-0104 | 179-0204 | NM_001101 | Human CDC2 (CDK1) | 179-0113 | 179-0213 | NM_001786 |
| Human β-Tubulin | 179-0105 | — | NM_178014 | Human CDK2 | 179-0114 | 179-0214 | NM_001798 |
| GFP | 179-0106 | — | M62653 | Human EGFR | 179-0115 | 179-0215 | NM_005228 |
| Luciferase | 179-0107 | — | X84846 | Human GSK3A | 179-0116 | 179-0216 | NM_019884 |
| | | | | Human CDK4 | 179-0117 | 179-0217 | NM_000075 |
| | | | | Human AKT1 | 179-0118 | 179-0218 | NM_001014431 |
| | | | | Human PLK1 | 179-0119 | 179-0219 | NM_005030 |

Catalog # Description

| | |
|----------|--|
| 179-0000 | **siLentMer siRNA Resuspension Buffer, 1.0 ml |
| 179-0001 | **siLentMer Fluorescently Labeled Nonsilencing siRNA, 1 nmol, control for delivery |
| 179-0002 | **siLentMer Nonsilencing siRNA, 1 nmol, negative control for silencing (unlabeled) |

* siLentMer Validated Dicer-Substrate siRNA Duplexes, 2 nmol, designed with proven criteria and functionally tested for >85% silencing.

** Kit components offered separately.

Mouse on Over to Comprehensive Support for All Your Amplification Needs

Whether you are a seasoned PCR expert looking for real-time quantitative PCR multiplexing tips or a beginner learning the fundamentals of conventional PCR, Bio-Rad's online amplification support can provide you with a host of guided tutorials, a PCR Doctor for troubleshooting problems, and detailed information on assay design and analysis to help you achieve successful research results.

Diagnosis From the PCR Doctor

With the PCR Doctor, you can troubleshoot problematic amplification experiments by comparing your results to real data. The PCR Doctor provides possible causes and treatments for specific symptoms present in gels and real-time data. Take advantage of the easy-to-use wizard format to quickly identify problems and find practical solutions.

- Conventional PCR Doctor provides solutions to improve specificity and yield with tips on reaction components and cycling conditions
- Real-Time PCR Doctor troubleshoots problems with reaction signal, efficiency, and unexpected C_T values

Watch, Listen, and Learn

Readily accessible training is available on your schedule with informative tutorials, including Fast PCR, Real-Time PCR Chemistries, and the Fundamentals of PCR. Now, at your own pace, learning is convenient and simple.

Rich Technical Content

Designing an optimized assay for your amplification experiment is not trivial. The Assay Design site is a rich technical resource developed to help you achieve successful experimental results. At this site, you will find information about critical elements of both real-time and conventional PCR, including primer design, assay validation, and relative quantification using a reference gene.



The screenshot shows the Bio-Rad Gene Expression Gateway website. The main navigation bar includes Home, Applications / Techniques, Products, Support, News | Events, and Contact Information. The 'Support' tab is active, leading to the 'Amplification Central' page. The page features three main sections: PCR Doctor, Tutorials, and Assay Design. Each section has a brief description and a list of sub-links.

| Section | Description | Sub-links |
|--------------|--|---|
| PCR Doctor | Cure your data ailments with this interactive troubleshooting tool for PCR and real-time PCR assays. | <ul style="list-style-type: none"> > Real-Time PCR Doctor > Conventional PCR Doctor > Bio-Rad Technical Support |
| Tutorials | Learn the key principles of conventional and real-time PCR. | <ul style="list-style-type: none"> > Real-Time PCR Fundamentals > Real-Time PCR Chemistries > Downloadable Presentations |
| Assay Design | Access design considerations for conventional and real-time PCR assays. | <ul style="list-style-type: none"> > Primer and PCR Product Design > Assay Validation and Optimization Overview > Quantitative PCR Analysis |

Mouse on over to www.bio-rad.com/genomics/pcrsupport/ and take advantage of this valuable new area of the gene expression gateway.

Normalization Methods for qPCR

Real-time PCR has been used for gene expression analysis for over a decade (Heid et al. 1996, Higuchi et al. 1992). The advent of better reagents and better techniques for assay design has increased the accuracy and efficiency of the nucleic acid quantification process, making quantitative PCR (qPCR) an even more powerful tool for gene expression studies.

Most gene expression assays are based on the comparison of two or more samples and require uniform sampling conditions for this comparison to be valid. Unfortunately, many factors can contribute to variability in the analysis of samples, making the results difficult to reproduce between experiments.

Variability is most often related to events upstream of the qPCR assay itself — namely, the quantity and quality of the extracted sample and the reverse-transcription efficiency (Fleige and Pfaffl 2006). Not only can the quantity and quality of RNA extracted from multiple samples vary, but even replicates can vary dramatically due to factors such as sample degradation, extraction efficiency, and contamination (Perez-Novo et al. 2005). The reverse-transcription efficiency can vary due to sample concentration, RNA integrity, the reagents used, and the presence of contaminants. Because the amount of starting material may vary greatly from sample to sample, accurate analysis requires that the samples be normalized.

This article describes two methods of sample normalization for accurate comparison of genes of interest: normalizing to input RNA and normalizing to a reference gene that has little variability as a function of treatment or due to the normal life cycle of the organism.

Normalization to Input RNA

Normalization to input RNA implies starting with the same amount and quality of material in each sample. This is typically done by measuring the absorbance at 260 nm (A_{260}) (Sambrook et al. 1989). This method cannot determine the integrity of the RNA molecules, however, because whole RNA and degraded RNA absorb light equally. Therefore, a secondary analysis, typically on a formaldehyde agarose gel, is required to determine whether there is degradation. Microfluidic electrophoresis on the Experion™ system is faster and requires much less RNA for this assessment than traditional agarose gel electrophoresis.

To illustrate the use of normalization in qPCR, we monitored the expression of four genes: annexin A3, CAPZB, cofilin, and destrin, in HeLa cells subjected to different treatments. Two sets of samples were transfected with small interfering RNAs (siRNAs, Act8 and Act9) that targeted different regions of the β -actin gene. A third set was transfected with siRNA targeting Green Fluorescent Protein (GFP) as a nonspecific control that is absent in HeLa cells. Two additional control samples were treated with lipid transfection reagent or buffer only. All analyses were performed using standard curves to determine individual amplification efficiencies.

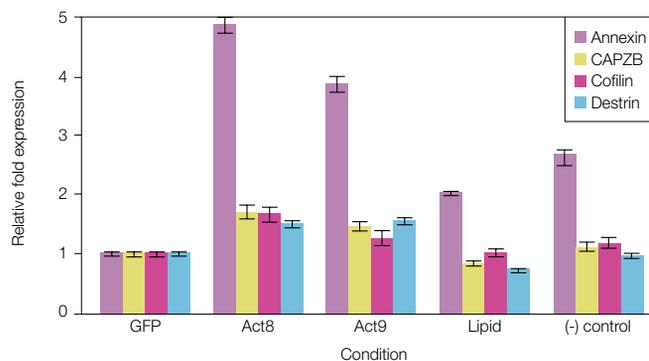


Fig. 1. Gene expression analysis of four genes across five conditions normalized to initial amount of starting RNA. GFP, Act8, Act9, treated with corresponding siRNA; Lipid, treated with lipid reagent; (-), control, treated with buffer only.

The relative expression of the four genes of interest across treatments was normalized to the amount of input RNA (Figure 1). This analysis showed that annexin A3 expression increased approximately 2- to 5-fold depending on the treatment. In addition, CAPZB, cofilin, and destrin displayed changes in expression varying from slightly below normal expression levels to a 50% increase in expression level.

Normalization to a Single Reference Gene

Although normalizing gene expression to the input amount of RNA ensures that equivalent amounts of RNA are compared, it cannot compensate for variations in the efficiency of reverse transcription, which is required to produce cDNA for PCR. Therefore, researchers often normalize expression levels of genes of interest to that of a reference gene. This can remove inaccuracies due to variations in reverse-transcription efficiency because the mRNA of the reference gene is reverse-transcribed along with that of the gene of interest.

Housekeeping genes such as β -actin, tubulin, GAPDH, and 18S ribosomal RNA have often been used as reference genes for normalization, with the assumption that the expression of these genes is constitutively high and that a given treatment will have little effect on the expression level.

To illustrate normalization to a reference gene, the data presented in Figure 1 were analyzed again, this time normalizing to the expression level of 18S RNA (Figure 2). The results of this analysis were qualitatively similar to those of the analysis using input RNA as the normalizer, but the calculated increases in expression were greater. For example, the expression level of annexin A3 in the Act8-treated sample showed a 4.9-fold increase when normalized to input RNA, but showed a 6.3-fold increase when normalized to 18S RNA expression. An obvious question is, which analysis is more accurate?

The question of which reference gene to use for normalization remains a key issue of debate. While it is agreed that the ideal reference gene is one that does not vary as a function of

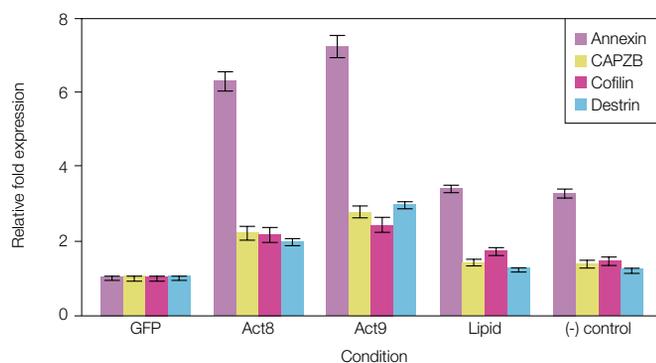


Fig. 2. Gene expression analysis of four genes across five conditions normalized to 18S ribosomal RNA.

treatment or condition, it is often difficult to identify even a single gene that meets this criterion (Glare et al. 2002, Kamphuis et al. 2005, Schmittgen and Zakrajsek 2000, Thellin et al. 1999). This difficulty is illustrated in Figure 3, which shows an analysis of three commonly used reference genes, 18S, GAPDH, and α -tubulin, from the same input. Although equivalent starting amounts of RNA were used, the expression levels of the three genes varied considerably depending on the treatment. These variations are unlikely to have resulted from errors in the starting amounts of RNA, because some of the genes are expressed at higher levels than the control, while others are expressed at lower levels. Therefore, these data indicate that genes that are generally considered to be housekeeping genes may in fact be expressed at variable levels across treatments or tissues.

Normalization to Multiple Reference Genes

A more accurate strategy for normalization has been proposed by Vandesompele and colleagues (2002). Instead of basing the normalization on a single reference gene that may or may not fluctuate, they propose carefully selecting a set of genes that display minimal variation across the treatment, determine the geometric mean of these genes, and normalize the gene(s) of interest to the geometric mean.

Figure 4 shows the expression levels of the four genes of interest across five treatments, as calculated using the multiple reference gene normalization strategy. This analysis reveals that treatment with either of the two siRNAs targeting β -actin (Act8 or Act9) increased annexin A3 expression levels to about 5.5-fold

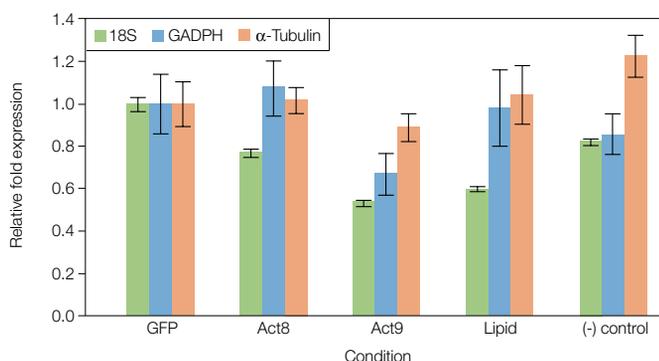


Fig. 3. Gene expression of three reference genes normalized to input amount of RNA.

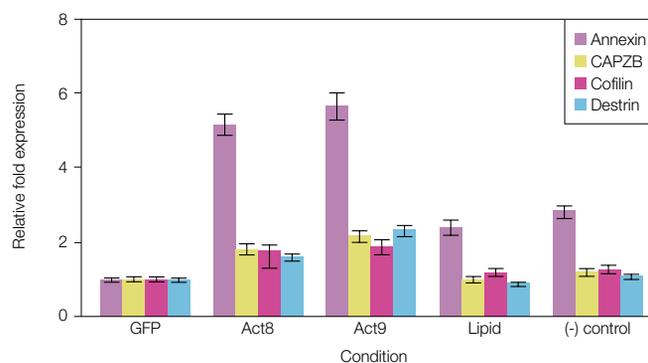


Fig. 4. Gene expression analysis of four genes across five conditions normalized to the geometric average of the three reference genes.

over basal level, and increased the expression of CAPZB, cofilin, and destrin to about 2-fold greater than basal levels. Annexin A3 also showed increased expression with lipid and buffer control treatments, but expression levels of the other three genes were similar in these controls to levels in the sample treated with nonspecific siRNA (GFP).

Summary

Proper normalization is essential for obtaining accurate gene expression studies. There are many strategies available for normalization, and with proper controls and replicates, all can be valid. The most comprehensive strategy uses a normalization factor calculated from the geometric mean of multiple reference genes.

To simplify data analysis, iQTM5 and MyiQTM real-time PCR detection systems come with analysis software that permits normalization to a standardized input amount, to a single reference gene, or to the geometric mean of multiple reference genes. Additionally, the software can take individual assay efficiencies into consideration, as well as combine multiple data sets to generate a complete gene study.

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Mechanisms of Protein-Protein Binding: Double-Mutant Cycle Analysis Using the ProteOn™ XPR36 System

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Introduction

Complex life processes require proteins to be able to transfer specific signals, build multiprotein complexes, control the function of enzymes, and regulate all other cellular activities. Many of these tasks are performed through specific protein-protein interactions. This is feasible due to the almost unlimited potential for the generation of protein binding sites, unique sites characterized by their shape and surface chemistry. A major research area today is the investigation of the structure, mechanisms, and dynamics of protein-protein interactions. One model system being utilized for basic research into the mechanisms of protein complex formation uses the interactions between TEM1 β -lactamase enzyme (TEM1) and its inhibitor, β -lactamase inhibitor protein (BLIP), shown in Figure 1 (Albeck and Schreiber 1999; Albeck et al. 2000).

Surface plasmon resonance (SPR) technology is a central, widely used tool for kinetic studies of interactions between unlabeled biomolecules in real time. However, there has been limited analytical, high-sensitivity SPR technology for monitoring multiple kinetic interactions in parallel. The ProteOn XPR36 protein interaction array system is a new system possessing all the qualities of high-level SPR biosensing technology combined with high-throughput and multiplexing capabilities. The principles and concepts of this array-format system are provided in Bronner et al. (2006). Briefly, XPR™ technology is based on the built-in orientation-controlled multichannel module of the ProteOn XPR36 system, which allows parallel measurement of multiple binding interactions between as many as six protein pairs. This kind of multiplexing is done efficiently on the ProteOn XPR36 system using the innovative technique of one-shot kinetics, which allows simultaneous monitoring of multiple protein pair interactions (Bronner et al. 2006).

The aim of this study was to measure the cooperativity between residues on TEM1 and BLIP, with the working assumption that while a single mutation provides information about the energy consequences of changing single residues, only analysis of multiple mutations can uncover the more complex, cooperative nature of noncovalent protein-protein interactions. Schreiber and colleagues are using the multiple-mutant cycle method to evaluate cooperativity of residue binding (Reichmann et al. 2005). With this method, the binding of two mutant proteins is measured individually and together; the interaction energy between the two proteins can then be determined by subtracting the free energy difference in the

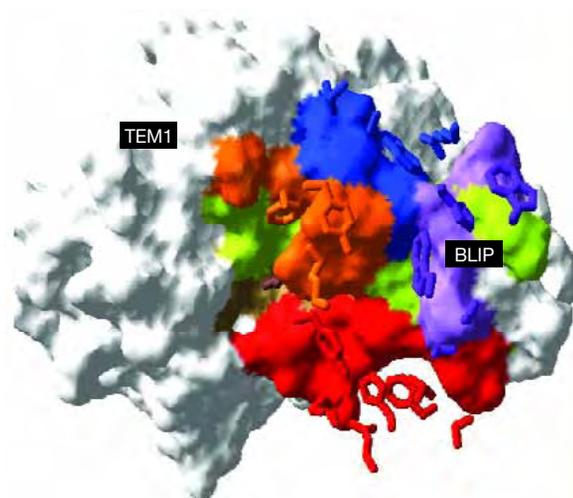


Fig. 1. Working model depicting the binding domain interactions between TEM1 and BLIP proteins.

binding of a double-mutant protein from that of the two single-mutant proteins. The data presented in this tech note are in the context of this larger study.

Here, five different TEM1 mutant protein ligands and a reference buffer sample were immobilized in the six ligand channels of a sensor chip. Six wild-type BLIP concentrations were injected in the analyte channels. This multiplex analysis provided kinetic and thermodynamic data on the binding of each of the six BLIP protein concentrations with each mutant TEM1 protein in a single experimental shot.

Methods

Instrumentation and Reagents

The experiment was performed using the ProteOn XPR36 protein interaction array system and a ProteOn GLC sensor chip. ProteOn phosphate buffered saline with 0.005% Tween 20, pH 7.4 (PBS/Tween) was used as running buffer throughout the experiment, which was performed at 25°C.

The TEM1 proteins used as ligands in the larger study included wild-type TEM1 (TEM1, 29 kD) and the following mutant proteins: E104A, Y105A, E104A/Y105A, R243A, K234A, S130A, SSR (TEM1 mutated to S130A, S235A, and R243A), KSSR (TEM1 mutated to S130A, S235A, R243A, and K234A), R243A/S130A, S235A, R243A/S235A, and K234A. The BLIP proteins used as analytes included wild-type BLIP (BLIP, 17.5 kD) and the following mutants: D49A, K74A, F142A, Y143A, K74/F142A, K74A/Y143A, F142A/Y143A, and K74A/F142A/Y143A.

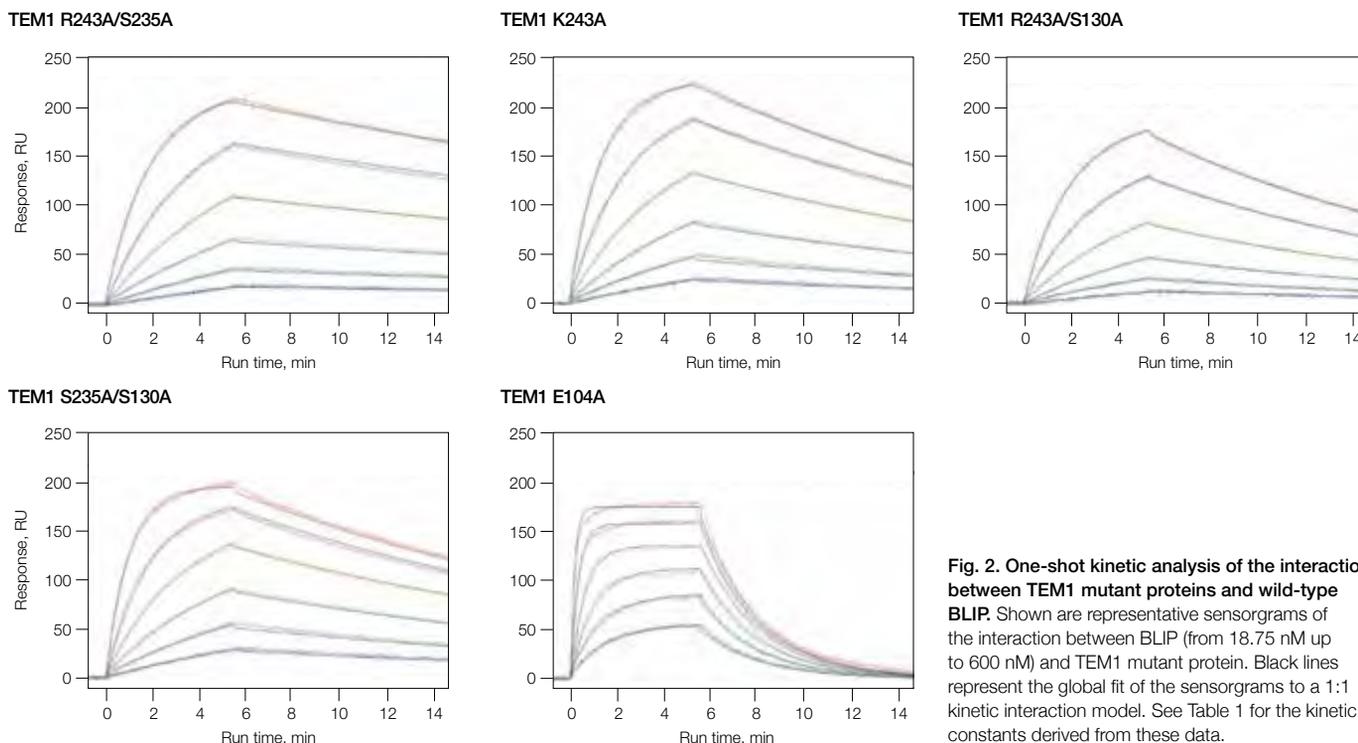


Fig. 2. One-shot kinetic analysis of the interaction between TEM1 mutant proteins and wild-type BLIP. Shown are representative sensorgrams of the interaction between BLIP (from 18.75 nM up to 600 nM) and TEM1 mutant protein. Black lines represent the global fit of the sensorgrams to a 1:1 kinetic interaction model. See Table 1 for the kinetic constants derived from these data.

The protein expression and purification procedures used are described in Reichmann et al. (2005). The following reagents were used for amine coupling: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC), *N*-hydroxysulfosuccinimide (sulfo-NHS), and ethanolamine HCl (ProteOn amine coupling kit).

Protein-Protein Interaction Experiment

For immobilization of the different TEM1 mutant proteins, the ligand channel surfaces were activated by amine coupling using 100 mM EDAC and 25 mM sulfo-NHS. The TEM1 mutant protein samples (180 μ l, 1 μ M prepared in sodium acetate buffer, pH 4.0) and a buffer sample (for use as a reference in the sixth channel) were injected in parallel in a single injection step at a flow rate of 30 μ l/min. Ethanolamine HCl (1 M, pH 8.5) was then injected to deactivate any remaining surface groups in the five ligand channels.

A 2-fold dilution series of six wild-type BLIP concentrations bracketing the K_D value (ranging from 0.1 to 10 times the K_D value) were prepared in PBS/Tween, pH 7.4. These six BLIP samples (250 μ l) were injected into the six analyte channels orthogonal to the ligand channels at a flow rate of 50 μ l/min, generating the 36-element interaction array (Bronner et al. 2006). The clustering studies and methods used are described in Reichmann et al. (2005).

Kinetic Analysis

Kinetic analysis was performed by fitting curves describing a simple 1:1 bimolecular reaction model to the resulting sensorgrams (Bronner et al. 2006). In addition to reference subtraction using a dedicated reference channel, in some

experiments, reference subtraction was performed using interspot references. Interspot references are computed by averaging the background signal obtained from the ligand-free spots on either side of each protein interaction spot.

Results and Discussion

Whereas the interface of protein-protein interactions is traditionally described by single-mutation analysis, in our larger study we analyzed a network of interactions in a multiplex method of multiple-mutant cycles. Such cycles reveal whether the contributions from a pair of residues are additive or whether the effects of mutations are coupled. Standard clustering techniques were used to separate the network of TEM1-BLIP interactions into five connecting binding units, or clusters. Of the five clusters (Reichmann et al. 2005), clusters C1 and C2 were investigated to determine (in terms of free energy of binding) the inter- and intracluster relationships of the TEM1 and BLIP residues. Various combinations of residues (single, double, and multiple) located in clusters C1 and C2 were analyzed using the ProteOn XPR36 system.

Figure 2 shows sensorgrams of the analysis of the TEM1-BLIP interactions. Each of the five panels shows six sensorgrams representing the interactions between six different wild-type BLIP concentrations and the TEM1 mutant protein indicated. Kinetic analysis of the interaction between the different ligand-analyte pairs is shown, and the kinetic properties of the different interactions are readily compared. The association rate constant, k_a , dissociation rate constant, k_d , and equilibrium dissociation constant, K_D , for these TEM1-BLIP interactions are presented in Table 1.

Table 1. Kinetic constants for the interactions between mutants of TEM1 and wild-type BLIP. The equilibrium dissociation constant, K_D , was calculated from k_d/k_a .

| TEM1 Mutant | k_a ($M^{-1}sec^{-1}$) | k_d (sec^{-1}) | K_D (M) |
|-------------|----------------------------|-----------------------|-----------------------|
| R243A/S235A | 1.61×10^4 | 4.48×10^{-4} | 2.78×10^{-8} |
| K234A | 2.11×10^4 | 8.86×10^{-4} | 4.20×10^{-8} |
| R243A/S130A | 1.29×10^4 | 1.22×10^{-3} | 9.46×10^{-8} |
| S235/S130A | 3.02×10^4 | 9.11×10^{-4} | 3.02×10^{-8} |
| E104A | 1.66×10^5 | 7.46×10^{-3} | 4.49×10^{-8} |

Similar experiments were performed for all combinations of TEM1 and BLIP wild-type and mutant proteins, and detailed results and conclusions of this larger study can be found in Reichmann et al. (2005). Briefly, the results indicated that interactions within a cluster are nonadditive, since the sum of $\Delta\Delta G$ values of the individual mutations is much larger than the value measured for the multiple-mutant protein.

This effect is clearly demonstrated when an entire cluster is mutated to alanine. Summing up the loss of free energy of binding of five single mutants of C2 (BLIP K74A, F142A, and Y143A; and TEM1 E104A and Y105A) yields a value of 31.1 kJ/mol. This number is composed of an additive loss of 25.3 kJ/mol for the three BLIP mutant proteins, and 5.8 kJ/mol for the two TEM1 mutant proteins. The $\Delta\Delta G$ value of the triple mutant BLIP was 16.3 kJ/mol, and that of the double mutant TEM1 was 4.3 kJ/mol. However, removing all five residues simultaneously (by mutation to alanine) resulted in a loss of only 10.1 kJ/mol of binding free energy. The same phenomenon was detected for the C1 cluster.

In contrast to the intracluster mutations showing nonadditive relationships, all the tested combinations of intercluster mutations were additive. These measurements were between five different TEM1 mutant proteins in cluster C1 (three single mutations, S130A, K234A, and R243A, and two multiple mutations, K234A, S130A, S235A, and R243A, and R243A, S130A, and S235A) and the seven BLIP proteins with mutations in C2 (three single mutations, K74A, F142A, and Y143A, and four multiple mutations, K74A and F142A, F142A and Y143A, K74A and Y143A, and K74A, F142A, and Y143A). Although the clusters are in close structural proximity, they are energetically independent. In other words, mutations in C1 do not affect residues in C2, and vice versa.

Extensive multiple mutant analysis of the two TEM1-BLIP clusters, C1 and C2, indicated that residue clusters are energetically independent of each other but have a high degree of cooperativity within each cluster. Additional detailed results and conclusions of this study can be reviewed in Reichmann et al. (2005). Results using interspot references were comparable to those using a dedicated reference channel, which confirms that the ProteOn XPR36 system can be used for analysis of a complete set of 36 protein-protein interactions.

Conclusions

In this study, the cooperativity between residues on TEM1 and BLIP was measured. The method most commonly used to analyze the contribution of residues toward the stability of a protein-protein complex involves evaluating the loss in free energy of binding upon mutation. However, this method is not without problems, because the loss in the measured free energy of binding caused by single mutations can equal, or even exceed, that of removing the entire cluster. Therefore, multiple mutants were analyzed efficiently and rapidly using the ProteOn XPR36 system. The results indicated that the sum of the loss in free energy of all of the single mutations within the C1 and C2 clusters far exceeds (up to 4-fold) the loss in free energy generated when all of the residues of the cluster are mutated simultaneously. These results demonstrate that the protein-protein interface is built in a modular fashion, where each cluster is an independent binding unit, and that two of these clusters (C1 and C2) show intracluster cooperativity as well as intercluster additivity between the protein residues.

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Signaling and Cytokine Profiling in Human Peripheral Blood Mononuclear Cells With the Bio-Plex® Suspension Array System

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Introduction

Cytokines, originally characterized as leukocyte effectors, are now recognized as pleiotropic messengers produced by and acting on virtually every known cell type. These soluble proteins mobilize specific cellular receptors, thereby regulating diverse cellular functions, including development, activation, and metabolism. While cytokines are critical for normal cellular function, aberrant cytokine production has been associated with disease and infectivity. Recent studies have reported that the effects of cytokines are mediated by different stress- and mitogen-induced protein (MAP) kinases. These kinases conduct signaling transduction through an array of phosphorylation events that eventually lead to activation or inactivation of a number of cellular responses, including apoptosis, cell growth, and proliferation. Therefore, cytokine monitoring has become an increasingly important component of drug development, particularly in oncology, immunotoxicity, autoimmunity, and infectivity. At the same time, assessing the phosphorylation status of kinases and their substrates, as well as that of transcription factors, is becoming critical for unraveling the mechanisms of gene expression and regulation.

In this study, we used Bio-Plex suspension arrays to profile cytokines and phosphoproteins following stimulation with mitogens and/or cytokines. These profiles demonstrate the usefulness of this array system for examining immunoregulation and signal transduction pathways. The approach may complement other methods for rapid screening and identification of disease-related biomarkers, biological contaminants, and environmental pathogens, as well as methods for pharmacokinetic/pharmacodynamic studies in drug development.

Methods

Human peripheral blood mononuclear cells (PBMCs, SeraCare Life Sciences, Inc.) were cultured in RPMI medium and treated with the pyrogen lipopolysaccharide (LPS), pokeweed mitogen (PWM), or interleukin 10 (IL-10) (Table 1). In some experiments, cells were stimulated with a combination of IL-10 and LPS. After treatment, the culture medium was collected and analyzed for secreted cytokines using Bio-Plex multiplex cytokine assays. The PBMC cells were collected separately and lysed using a Bio-Plex lysis kit. One portion of each lysate was analyzed for intracellular cytokines while another portion was analyzed for phosphorylated proteins using Bio-Plex multiplex phosphoprotein assays. In total, 50 cytokines and chemokines (both secreted and intracellular) and 22 phosphoproteins were tested. The workflow is summarized in Figure 1.

Table 1. Ligands used for stimulation.

| Ligand | Source | Characterization | Working Concentration |
|-------------|--|------------------|-----------------------|
| Human IL-10 | Recombinant | Interleukin | 0.35 nM |
| LPS | <i>Escherichia coli</i> 055:B5 | Pyrogen | 40 µg/ml |
| PWM | <i>Phytolacca americana</i> (pokeweed) | Lectin | 10 µg/ml |

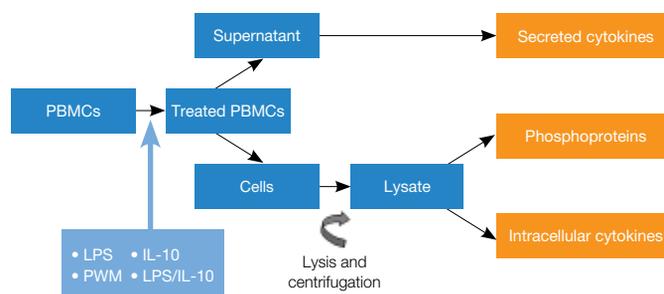


Fig. 1. Workflow used in this experiment.

The Bio-Plex system is a complete suspension array system that comprises a flow-based 96-well fluorescent microplate assay reader integrated with specialized software, automated validation and calibration protocols, and assay kits. Bio-Plex assays were run using a Bio-Plex system, following manufacturer instructions.

Results and Discussion

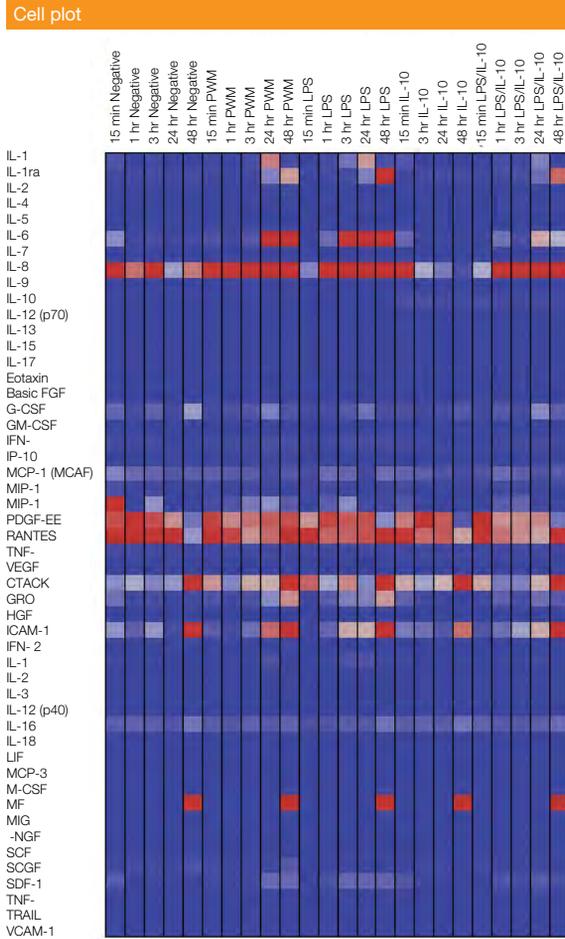
By profiling cytokine levels as well as phosphoprotein levels using a Bio-Plex suspension array system, we obtained a comprehensive picture of how PBMCs respond to three external stimuli — LPS, PWM, and IL-10 — and how signaling pathways are involved in these events. The three ligands induced distinct responses from PBMCs.

Pyrogen — LPS

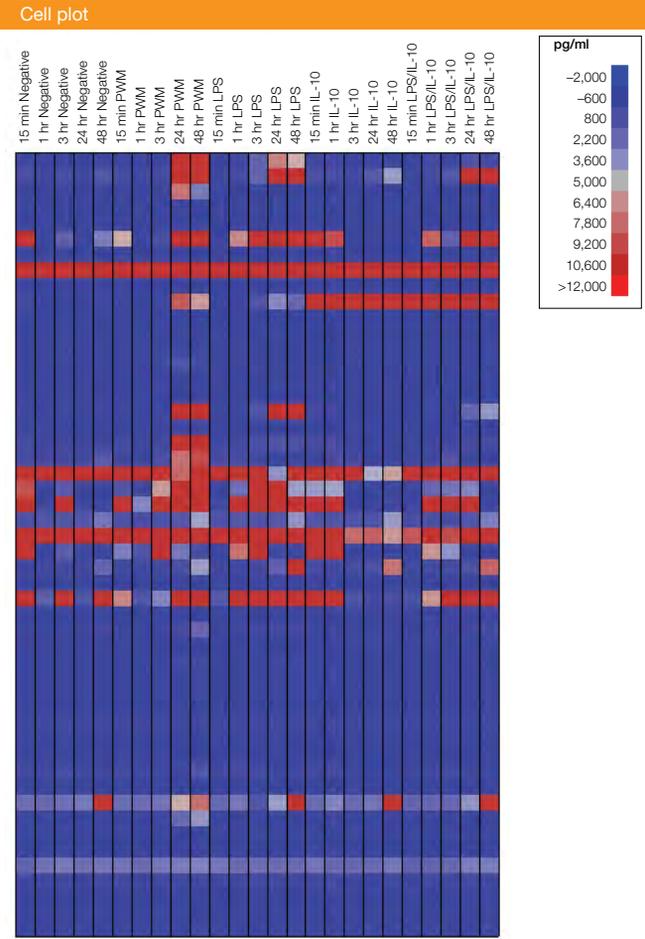
LPS, a component of the bacterial cell wall, induced an immune response as indicated by elevated cytokine levels both in the cytoplasm and in the culture medium (see IL-6 and IL-8 in Figure 2A). The elevation started after the cells were incubated with ligand for 1 hr and peaked at around 3 hr of incubation. Increased phosphorylation of the signaling molecules c-Jun, ERK1/2, IκB-α, MEK, and STAT3 also occurred (Figure 2B), with the peak phosphorylation of most occurring after 1 hr of incubation with LPS. Peak phosphorylation of STAT3 was observed at 3 hr of incubation (Figure 2B and Figure 3B). This indicates potential involvement of signaling pathways in the immune response.

A. Cytokine profiles

Intracellular cytokines



Secreted cytokines



B. Phosphoprotein profiles

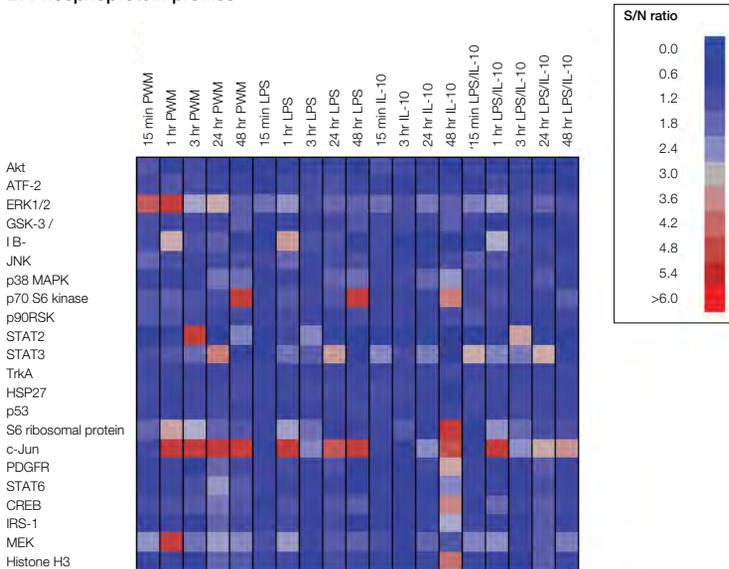
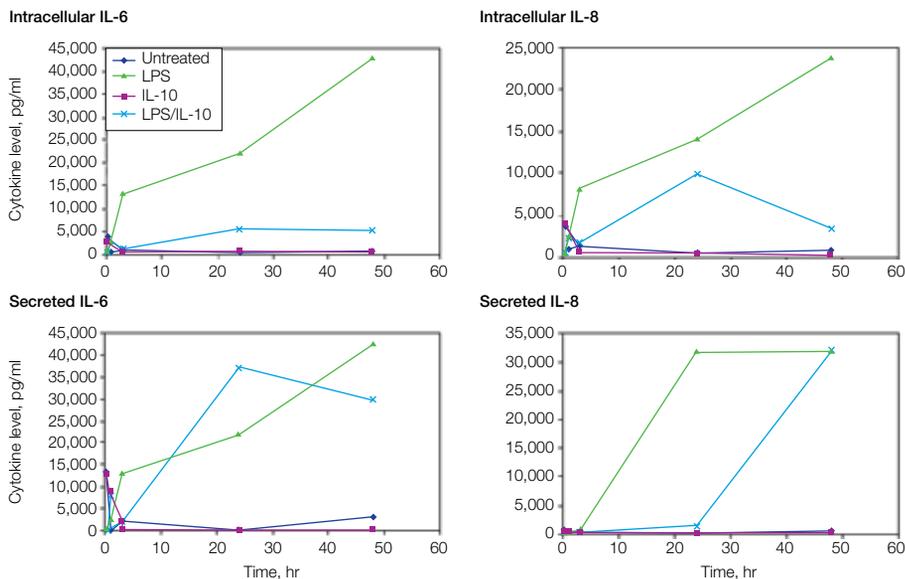


Fig. 2. Cytokine and signaling profiles in PBMCs. A, intracellular and secreted cytokine profiles in response to LPS, IL-10, PWM, and a combination of LPS and IL-10 (LPS/IL-10) at incubation times indicated. Negative, no ligand was added to the cells. **B,** same as A, but for phosphoprotein profiles.

A. Selected cytokines



B. Selected phosphoproteins

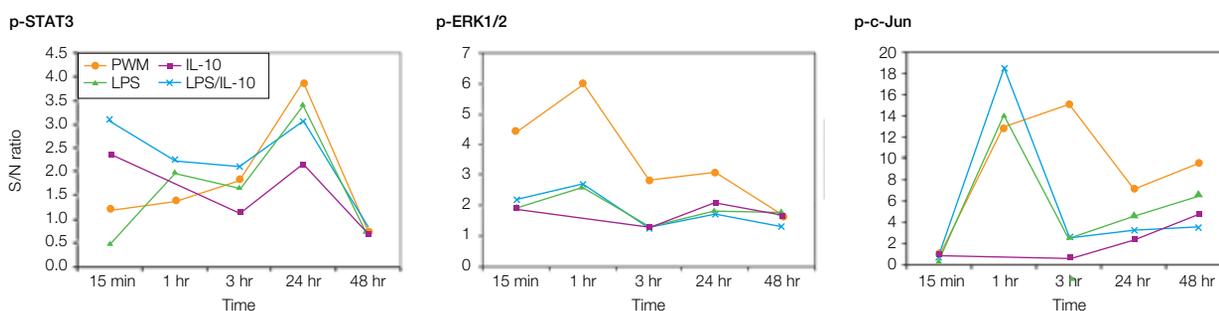


Fig. 3. Selected individual cytokine and phosphoprotein analyses. A, intracellular levels (upper plots) of IL-6 and IL-8 were compared to levels in culture media (lower plots). B, selected phosphoproteins: p-STAT3, p-ERK1/2, and p-c-Jun.

Mitogen — PWM

PWM stimulated an array of signaling pathways, and the most significant increase of phosphorylation was observed with c-Jun, MEK, and ERK1/2 (Figure 2B). Interestingly, an increase of ERK1/2 phosphorylation was observed as early as 15 min after addition of the mitogen, whereas the level of phosphorylated c-Jun peaked after 1 hr, indicating different signaling events. Levels of various cytokines and chemokines also increased in response to PWM, especially intracellular cytokines in cytoplasm (see for example IL-6, ICAM-1, and CTACK in Figure 2A).

IL-10

IL-10 treatment by itself had little effect on levels of the cytokines and phosphoproteins tested. Because IL-10 is thought to suppress expression of many LPS-induced genes, however, we examined the levels of these molecules in cells treated with a combination of IL-10 and LPS. In this case, the increase in intracellular IL-6 and IL-8 was reduced compared to LPS treatment by itself (Figure 2A, Figure 3A). In addition, the effect of IL-10 on LPS-induced increases in levels of secreted IL-6 and IL-8 appeared to be much more transient (Figure 3).

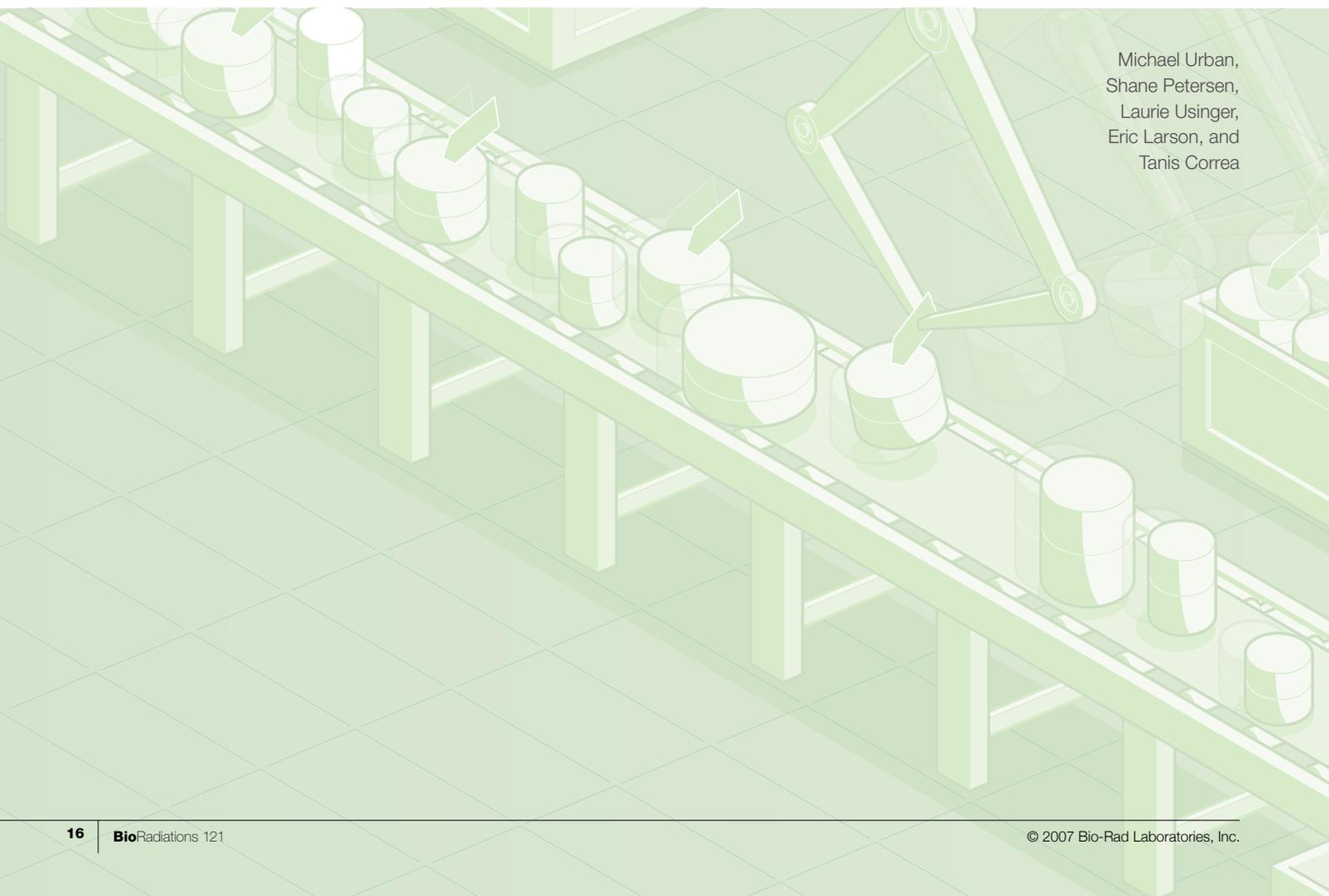
Conclusions

The Bio-Plex suspension array system is a useful tool for profiling intracellular and secreted cytokines as well as the phosphorylation of signaling molecules in comprehensive proteomic studies. The ability to test the same set of samples for different targets simultaneously permits well-controlled studies as well as significant savings in samples and labor. The ability to combine information from immune responses and signaling pathways is a powerful approach for basic research, biomarker development, and drug discovery processes such as pharmacokinetic and pharmacodynamic studies.

The Profinia™ Protein Purification System

An Automated Solution for Purifying Affinity-Tagged Proteins

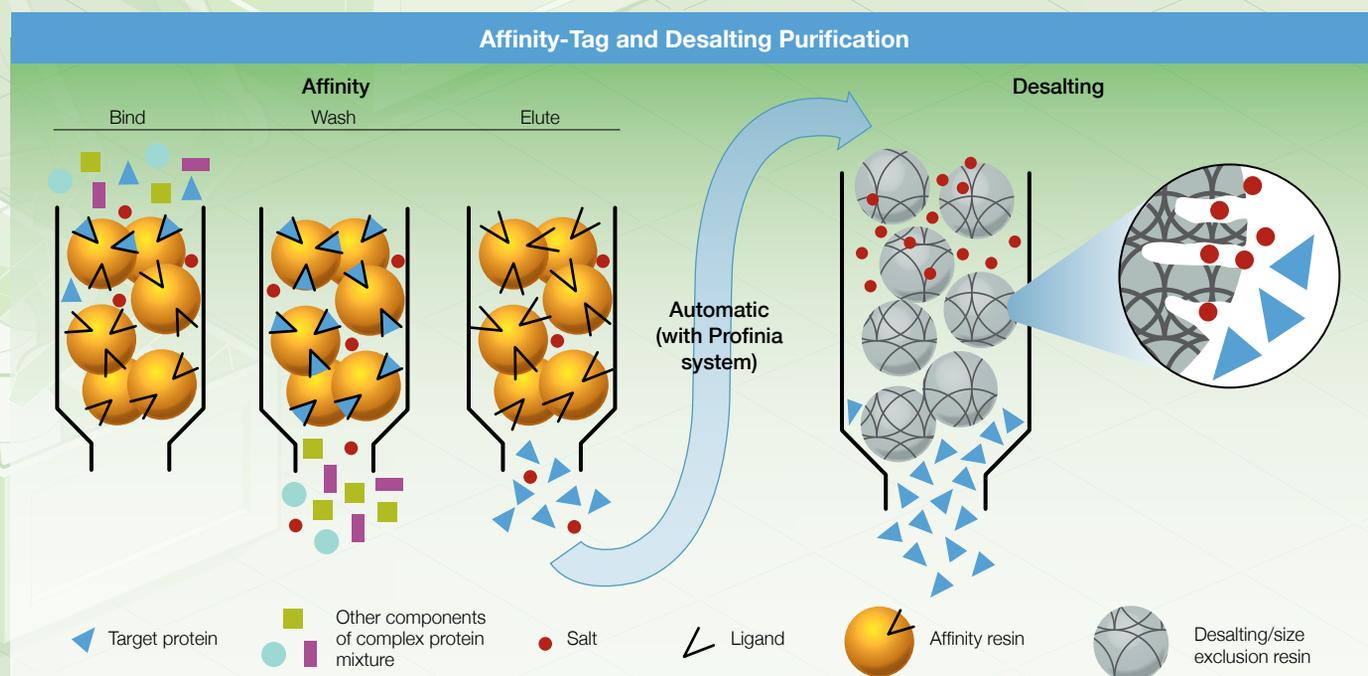
Michael Urban,
Shane Petersen,
Laurie Usinger,
Eric Larson, and
Tanis Correa



Ongoing genomics programs have driven both the development of protein expression methods and the generation of large numbers of recombinant proteins. A great deal of work is now directed toward characterizing the structure and biological function of these molecules. Such studies require that the proteins of interest be generated at desirable concentrations, be sufficiently pure, and in many cases, be biologically active. Protein purification can be a daunting exercise. One way to simplify the process is to attach a functional molecular affinity tag to the recombinant protein. Such tags bind to chromatographic affinity supports, so any tagged protein can easily be washed free of impurities, released from the support, and desalted.

Manual protein purification techniques are labor intensive and include a great number of time-consuming procedures. Most liquid chromatography systems are complex to learn and expensive. Either way, current approaches can be an inefficient use of research personnel. What is needed is a reliable means for automating and accelerating affinity-tagged protein purification without compromising protein quality or yield.

The Profinia protein purification system addresses this need. It offers a push-button alternative to existing lengthy methods of purification. The Profinia instrument is preprogrammed with methods that are optimized to work with prepackaged buffer and cartridge kits. The instrument and reagents together form a system that provides fast, dependable, and reproducible protein purification at a modest cost.



In two-step affinity-tagged protein purification, a protein is first purified by affinity chromatography, then desalted. In the Profinia system, the two steps are automated.

In the first step, a recombinant protein mixture is passed over a chromatography support containing a ligand that selectively binds proteins that contain an affinity-tag sequence (typically His or GST). Contaminants are washed away, and the bound protein is then eluted in pure form.

Affinity tags have different advantages. In IMAC, His binds with good selectivity to Ni^{2+} or other transition metals immobilized to the ligand; the tagged protein can be selectively eluted with imidazole. GST-tagged proteins bind to glutathione as the ligand, and are eluted with solutions of glutathione. Proteins with an enzymatically active GST fusion tag can only be purified under native conditions. In contrast, His-tagged proteins may be purified under native or denaturing conditions.

During the second step of desalting, affinity-purified samples can simultaneously undergo buffer exchange to remove salts in preparation for downstream applications. A number of desalting techniques, including size exclusion chromatography, dialysis, and ultrafiltration also allow buffer exchange. Desalting often includes not only the removal of salt, but also of other foreign substances, such as detergents, nucleotides, and lipids.

Affinity-Tag Purification Methods

The sequencing of genomic DNA over the past few decades has led researchers to investigate the structure and function of genetically encoded proteins. While recombinant DNA techniques allow the expression of proteins in quantities sufficient for investigation of protein structure and function, purification of these proteins remains a challenge. Many studies require proteins of interest to be homogeneous and contaminant free. Additionally, protein structural complexity can make purification a challenging task, since different proteins can behave very differently under the same conditions.

Advances in recombinant DNA techniques now enable the addition of affinity tags to a protein sequence* to facilitate purification. This advance has made possible the expression of huge numbers of proteins in a tagged format, which enables standard chromatography schemes to be used for purification (Gaberc-Porekar and Menart 2001, Hui and Usinger 2006,

Nilsson et al. 1997, Terpe 2003, Waugh 2005). Tag-based purification methods are protein independent, which allows the application of a common methodology to all proteins a researcher might be interested in purifying. Affinity tag-based methods offer the possibility of easy single-step purification with minimal impact on protein structure and biological activity, applicability to a wide range of proteins, and yield of nearly homogeneous protein (90–99% purity) starting from the crude recombinant protein mixture (Terpe 2003).

The applications of affinity tagging are wide ranging. Affinity purification of protein complexes followed by downstream identification is a powerful tool for generating maps of protein-protein interactions and cellular locations of complexes. The method also enables the study and identification of posttranslational modification sites. If necessary, the affinity tag can be enzymatically cleaved from the purified protein using a protease cleavage site. For certain downstream assays, removal

Why Use Affinity Chromatography?

Understanding cellular processes requires knowledge of the structure, function, posttranslational modifications, and interactions of proteins. Until recently, progress in understanding proteins has been slowed by the difficulty in purifying proteins. The advent of easy-to-use and high-throughput methods for protein purification should accelerate proteomics research. Useful applications of affinity-tag purification schemes include:

Developing and producing proteins for therapeutic applications. The human augments of liver regeneration (hALR), a hepatotropic protein that can stimulate hepatic cells to grow regardless of genus, has been expressed and purified from *Escherichia coli* (Sheng et al. 2006). The expression of hALR enables further study of its biological function, and also suggests that recombinant hALR could be developed for repair of hepatic damage.

Developing vaccines. Purification of a His-tagged spike glycoprotein, one of the major structural proteins of SARS-associated coronavirus (CoV), allowed production of a large amount of the protein, which maintained antigenicity and immunogenicity and induced strong IgG responses in mice (Zhao et al. 2005).

Similarly, serine proteinase inhibitor (serpin) from *Schistosoma japonicum* (Sj serpin) was His-tagged, expressed in *E. coli*, and purified by affinity chromatography (Yan et al. 2005). Mice immunized with the purified protein produced high levels of specific antibodies and developed moderate protection against infection by *S. japonicum*.

Generating sufficient protein for crystallization and structural analysis. Studies of *Mycoplasma pneumoniae* aim to find novel protein structures that may have similar functions across species (Chen et al. 2004; see also BioRadiations 117).

Identifying protein binding partners to elucidate functional pathways. Hardwidge et al. (2006) created GST-fusions with virulence proteins from enteropathogenic *E. coli* (EPEC), an enteric human pathogen responsible for much worldwide morbidity and mortality. These fusion proteins were expressed in *Saccharomyces cerevisiae*, and the yeast proteins that interact with the proteins were isolated by affinity purifying against the GST tag. These complexes were subjected to isotope-coded affinity tagging combined with electrospray ionization-tandem mass spectrometry, and the peptide sequences were searched against a database, which provided a list of proteins that bound specifically to each EPEC virulence protein.

Determining cellular localization, identifying of posttranslational modifications, and biochemically characterizing proteins. Tristetraprolin (TTP), an antiinflammatory protein that destabilizes mRNA, has not been

adequately characterized, due to the difficulties in protein purification. Purification of a fusion protein expressed in *E. coli* (Cao 2004) allowed generation of antibodies that could be used for cellular localization of the protein. Additional studies measured the binding affinity of TTP under different conditions, which suggested that phosphorylation and other posttranslational modifications reduce TTP's mRNA binding affinity by half.

Characterizing protein properties via site-directed mutagenesis.

Site-directed mutagenesis was used to target the putative NADP binding site of maize photosynthetic NADP-malic enzyme (Detarsio et al. 2003), which catalyzes the oxidative decarboxylation of L-malate to yield pyruvate, CO₂, and NAD(P)H. In maize and other C4 plants, this enzyme is involved in a CO₂ concentrating mechanism that increases photosynthetic yield. Following expression in *E. coli* and purification of the recombinant protein, the participation of mutated residues in substrate binding and the catalytic reaction was inferred by kinetics and by circular dichroism and fluorescence spectra.

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* The tag sequence is incorporated into an expression vector alongside the DNA sequence encoding the protein of interest. Induction of the vector results in expression of a fusion protein — the protein of interest fused to the affinity tag, which can then be purified from the cell lysate.

of the tag aids in determining the biological activity of the purified protein (for more on applications, see sidebar previous page).

A number of affinity-tag-based purification systems are available. The two most common approaches use immobilized metal affinity chromatography (IMAC) (Porath et al. 1975) for purification of polyhistidine (His)-tagged proteins, or glutathione S-transferase (GST) affinity tags (see sidebar on page 17). Following affinity purification, proteins are typically desalted in preparation for downstream applications.

Need for New Technology

While affinity-tag chromatography has provided a basis for effective protein purification, current techniques, especially traditional chromatographic techniques, can require significant time, specialized expertise, and large expense. Researchers are presented with a range of purification technologies, from low-cost manual methods to sophisticated systems based on high-end instrumentation. Each approach has advantages, but each is also characterized by an intrinsic set of limitations that makes it a less than satisfactory solution.

Current manual purification methods use spin and gravity-flow columns. These methods require hands-on time, can lack reproducibility, and do not facilitate collection of data during purification. They also require a second manual desalting procedure to deliver purified proteins. Low-pressure liquid chromatography systems provide more automation and can achieve faster throughput than the corresponding manual methods. Unfortunately, these systems can require a significant amount of chromatography expertise and user involvement, especially in programming and maintenance. While low-pressure systems can automate the desalting run, manual intervention is still necessary to program a separate desalting method. In addition, the affinity-purified eluate must be transferred onto the desalting column manually.

The considerable amount of time and attention demanded by these purification methods limit the effort that can be spent on the downstream activities that generate the proteomic data — the ultimate objective of the investigation.



Fig. 2. Time and labor savings with the Profinia system over manual methods of affinity-tagged protein purification. Typical ranges in time required for purification of target proteins are shown. The Profinia system's self-contained hardware and preprogrammed software methods accelerate His- and GST-tagged protein purification. The system combines affinity purification with automated desalting, compared to separate processes for affinity and desalting in the manual methods. ➡, hands-on step.



Fig. 1. Simple, automated, and reproducible affinity-tagged protein purification. The Profinia system works with prepackaged buffer and cartridge kits. The instrument features a small benchtop footprint (49 x 33 cm) and a space-saving, compartmental configuration that houses all reagents, sample and collection tubes, and cartridges on the instrument. A central compartment provides convenient storage for a touch-screen stylus and access to a USB drive for run data capture and export.

The Profinia Protein Purification System A Total Solution for Affinity-Tagged Protein Purification and Desalting

The Profinia protein purification system is an easy-to-use, automated liquid chromatography system for the purification and desalting of affinity-tagged proteins (Figure 1). The Profinia system replaces manual and more complex automated affinity purification methods with preprogrammed, reproducible, and time-saving protocols that enable completely unattended operation (Figure 2).

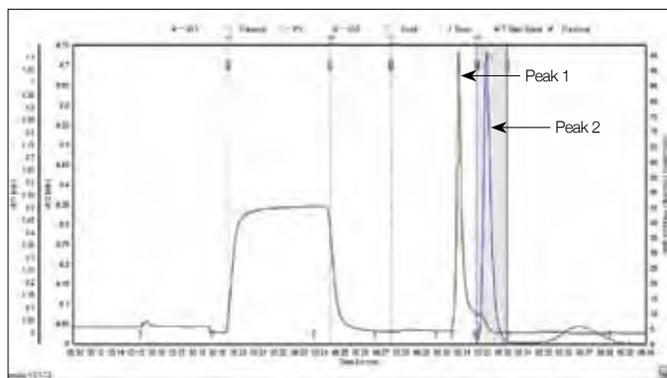


Fig. 3. Automated two-stage affinity and desalting protein purification. The chromatogram illustrates the Profinia instrument's ability to automatically detect, select, and transfer the main affinity fraction eluting from the affinity cartridge (peak 1) to the desalting cartridge. When it elutes as a purified and desalted protein (peak 2); it is collected in a single fraction tube.

A variety of purification kits are available for the Profinia instrument; they allow use of widely used purification methods, such as IMAC, GST, and desalting. Reagents are specially formulated to work with the instrument's preprogrammed (Bio-Rad) methods, and are designed to successfully and automatically carry out equilibration, binding, wash, and elution steps. Routine maintenance procedures, such as cartridge cleaning and storage, occur automatically at the end of each run. Three main reagent kit configurations are available: the Profinia purification, starter, and buffer kits. All kit components, including buffer bottles and cartridges, are designed for simple plug-in installation.

Automation That Lets You Focus on Furthering Discovery

On the Profinia system, up to two samples can be run in sequence, with the choice of using a single cartridge for both samples or individual cartridges for each. The two-step purification works by automatically detecting, selecting, and diverting the main affinity peak from the affinity cartridge to the desalting cartridge for buffer exchange (Figure 3). Flow-through, wash 1 and 2, and elution fractions are collected in designated individual fraction tubes, making it easy to locate the purified protein without the need to pool fractions. Once elution is complete, an estimate of protein yield and concentration is displayed on the instrument touch screen. This information can

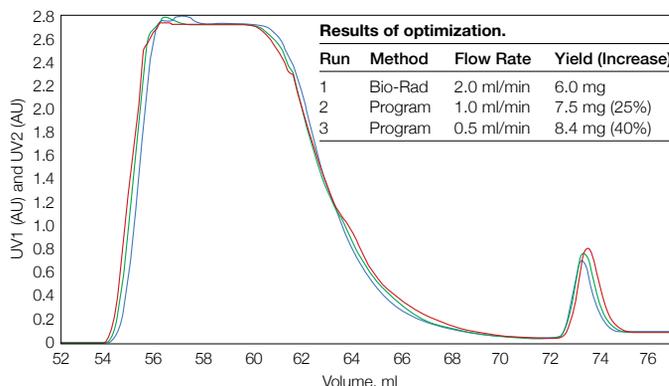


Fig. 4. Optimization of Profinia IMAC purification. Overlay of UV absorbance profiles of three IMAC purifications performed on the Profinia system. A 30 kD His-tagged protein was purified three times in sequence, with the flow rate for sample application adjusted to optimize yield. As the flow rate decreases, yield increases, as seen in the elution peak profile and the results table (inset). Run 1, —; run 2, —; run 3, —. The Profinia system allows adjustments in the methods, like flow rate, to accommodate desired optimization.

be transferred to a USB portable memory device for import into a PC with optional Profinia software installed. For real-time data acquisition, the instrument can be connected to a PC running Profinia software.

The optimized Bio-Rad methods for the most common affinity applications (programmed into the instrument) are accessed through a touch-screen interface that guides selection and setup. These automated templates can be edited in the system's program-method mode. The default values of system parameters, such as flow rates and wash times, can be adjusted to optimize the yield of a specific protein of interest. Once a program method has been customized (Figure 4), it can be stored and recalled as a saved method.

Gauging Profinia System Performance

Ideally, delivery of purified and desalted proteins for downstream applications should meet three criteria equally: quality (purity, homogeneity, activity), yield, and throughput. Since quality is absolutely essential, one or more of the other performance criteria are often compromised to achieve high-quality results.

As demonstrated empirically using the Profinia system, there is no longer any need for a tradeoff: a 51 kD dual His- and GST-tagged protein was affinity purified and desalted using the Profinia IMAC or GST starter kit or with a low-pressure

Table 1. Yield and purity data for a 51 kD protein. Protein was purified and desalted with the Profinia consumables (IMAC or GST starter kit) and instrument, a low-pressure chromatography system with the manufacturer's buffers, and manually using an affinity gravity-flow and desalting spin column kit with appropriate buffers. (For more information, refer to Berkelman and Urban 2006, Hui and Usinger 2006, Petersen and Usinger 2007).

| Purification Method | Purification Time | | Average Yield | Average Purity |
|--|-------------------|-------------------|---------------|----------------|
| | Affinity | Desalting | | |
| IMAC and Desalting | | | | |
| Profinia system — IMAC starter kit and Profinia instrument | | 34 min (combined) | 7.0 mg | 96.6% |
| Low-pressure system — IMAC cartridges and His buffer kits | 55 min | 20 min | 7.3 mg | 96.7% |
| GST and Desalting | | | | |
| Profinia system — GST starter kit and Profinia instrument | | 50 min (combined) | 4.76 mg | 96.2% |
| Manual — gravity-flow and spin column kits | | 90 min (combined) | 4.34 mg | 96.1% |

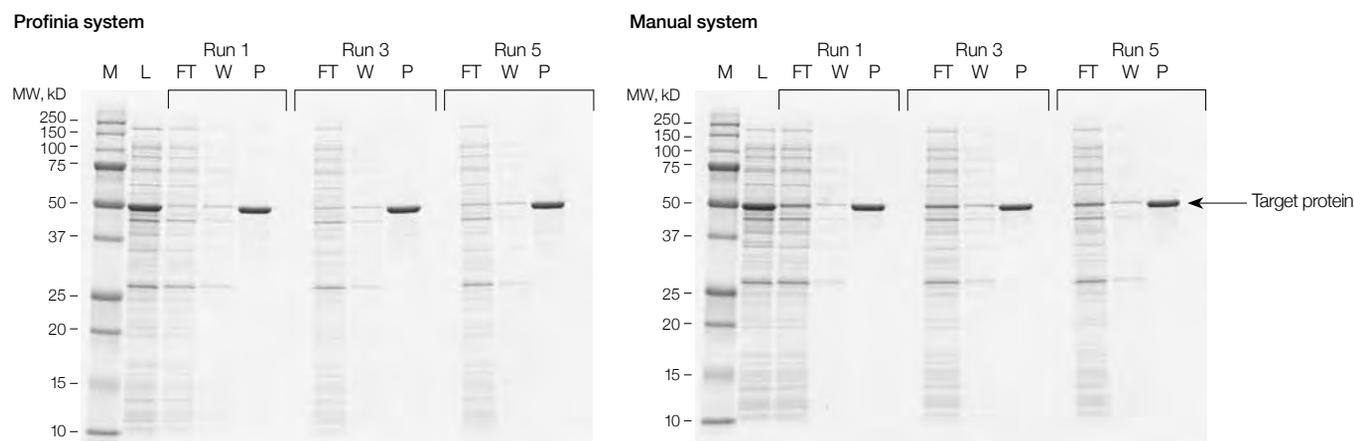


Fig. 5. SDS-PAGE analysis of purification fractions. M, markers (Precision Plus Protein™ dual color standards); L, lysate (unfractionated); FT, flowthrough; W, wash 1; P, eluted protein. Note comparable results for the two methods.

chromatography system and manual kits (gravity flow for affinity and centrifugation for desalting). Identical volumes of rehydrated *E. coli* lysate containing a 51 kD protein were used for all separations. Five consecutive purification runs involving IMAC or GST-based purification followed by desalting were performed using columns and buffers from the respective kit or system's manufacturer. The results obtained with the Profinia system demonstrate good reproducibility (similar results for different runs on the same purification cartridge) for GST-tagged proteins. The elution time for both the affinity and the desalted peak varied by no more than 10 sec (Berkelman and Urban 2006).

Yield and purity for both IMAC and GST purifications using the Profinia system were equivalent to results obtained using both manual and automated methods. An SDS-PAGE comparison (Figure 5) showed that the 51 kD GST-tagged protein was purified to apparent homogeneity using either the Profinia system or a manual method. Table 1 summarizes the results of both affinity methods used and demonstrates the utility of the Profinia system compared to low-cost manual solutions, as well as slightly higher-priced instrumentation that requires chromatographic expertise.

The dramatic difference in purification time is attributable to the fully automated Profinia system operations that require no user handling or intervention between the application of sample and collection of the purified and desalted protein (Figure 2). With manual methods, protein purification and desalting are two separate operations requiring manual transfer between the two columns. In addition, Profinia prepackaged reagents and other kit components are ready to use and do not require additional preparation time. Neither of these time-saving advantages applies to the low-pressure chromatography system or to manual methods. Furthermore, with the other methods, buffers and other kit reagents must be prepared by the researcher, so the hands-on requirements of these procedures add processing time.

Conclusions

Affinity tagging of recombinant proteins has made it possible to purify a large number of diverse proteins using a single purification technique. Until now, the technologies available for purifying affinity-tagged proteins have required a significant investment in hands-on time and expert technique to deliver purified recombinant proteins suitable for downstream applications. The Profinia system is the solution for fast, reproducible, and cost-effective purification of affinity-tagged proteins. As an automated system that is easy to learn and easy to use, the Profinia system enables the redirection of research time from purification activities to downstream applications and high-level tasks. This is accomplished by speeding the purification process and dramatically reducing the amount of hands-on time required, without sacrificing either the yield or the quality of the purified protein.

For more information, see page 2 or go to www.bio-rad.com/affinitypurification/

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Qualification of Microarrays for Clinical Research: Results From Testing of the BioOdyssey™ Calligrapher™ MiniArrayer

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Introduction

While both genomics and proteomics play a crucial role in unraveling the mysteries of molecular interactions, the information garnered from each area is vastly different. Gene arrays provide information describing changes in mRNA expression, while proteomics provides insights into deranged cellular signaling mechanisms. This proteomic cellular signaling is a complex interplay between kinases and phosphatases, resulting in specific posttranslational modifications of proteins such as phosphorylation, lipidation, glycosylation, or cleavage.

Advances in protein microarray technology have been made possible by gleaning information from genomics technologies such as cDNA arrays. Although different information is derived from genomics and proteomics, a common bond is shared: the microarray. In the simplest sense, a microarray is a series of samples immobilized on a substratum. The immobilized sample can be an antibody, cellular lysate, nucleic acid, serum, or aptamer.

Gene expression can be evaluated via cDNA or oligonucleotide microarrays. The arrays are constructed with immobilized nucleic acid strands as a capture molecule on a glass surface. A fluorescence labeled cDNA probe is applied to the array and the complementary strands are allowed to hybridize, thus permitting analysis of changes in mRNA expression levels.

Protein microarrays can be constructed in two formats: forward phase (also referred to as an antibody array) or reverse phase. A forward-phase array consists of different immobilized capture molecules, usually antibodies. The array is probed with serum or a cellular lysate sample as the bait molecule. A second labeled antibody probe provides the detection molecule. The disadvantages of a forward-phase array are (1) requirement for two distinct antibodies directed against the same epitope, and (2) inability to match the antibody affinities to the sample protein concentration.

In contrast, a reverse-phase array is constructed by immobilizing the bait molecule on a substratum and probing with a single antibody. The bait molecule may be a serum protein, a cellular lysate, or subcellular protein fraction. By immobilizing the bait molecule in a dilution series, it is possible to effectively match the protein concentration of the sample with the antibody probe affinity, facilitating measurement within the linear dynamic range of the array. The advantages of the reverse-phase array format are (1) ability to match the antibody affinity to the protein concentration, and (2) need for only one antibody directed against the epitope.

Widespread adoption of microarray technology to clinical research and clinical trials emphasizes the need to assess the technical and quality aspects of robotic devices used to construct the arrays. Instruments approved for use in human clinical diagnostic laboratories must undergo extensive quality assessments, including documentation of accuracy, precision, linearity, and sensitivity for a given assay. As in a clinical setting, it is necessary to understand both the capabilities and limitations of a robotic microarray printing device. Criteria to be considered when selecting a robotic printing device include (1) the type of material to be deposited, (2) the number of samples and number of microarrays to be printed (throughput), (3) the pin type/style, and (4) the pin-washing capabilities of the arraying device.

The ability to print a plethora of materials, including but not limited to cell lysates, microdissected material, whole tissue lysates, and serum, makes robotic printing technology exceptionally desirable. To determine the reliability, throughput, and reproducibility of the BioOdyssey Calligrapher for reverse-phase protein microarrays (RPPAs), we printed epidermal growth factor (EGF)-treated and untreated A549 human carcinoma cell lysates. The arrays were subsequently stained to evaluate multiplexed protein expression changes over time for selected phosphorylated protein endpoints. The results presented here represent a series of experiments typically used for evaluation of robotic printing devices.

Methods

Cell Line and Treatments

A549 cells (American Type Culture Collection, ATCC) were cultured in F-12K medium (ATCC) supplemented with 10% fetal bovine serum (ATCC). Approximately 2.75×10^5 cells were plated per well in 6-well microplates. The following day, growth medium was replaced with serum-free medium. After 24 hr, medium supplemented with EGF peptide (Cell Signaling Technology, Inc.) or medium alone was added to the treated and untreated cells, respectively, at different time points. At each time point, the cells were washed twice with Dulbecco's phosphate buffered saline and then lysed in a 2.5% solution of β -mercaptoethanol in T-PER (Pierce)/2x SDS Tris/glycine/SDS buffer (Invitrogen).

Arraying

The BackTracker™ program provided with the BioOdyssey Calligrapher miniarrayer, which assumes all slides will be read vertically, was used to determine sample placement on the arrays. All samples were plated into a 384-well microplate in a four-point, 2-fold dilution curve (neat, 1:2, 1:4, 1:8), and arrayed onto FAST nitrocellulose slides (Whatman, Inc.) using the miniarrayer equipped with Stealth SNS15 solid pins (TeleChem

International, Inc.). Samples were printed at three depositions per feature to ensure ample protein concentration. Assuming that the pins deposited 7.0 nL, the specified delivery volume, each spot in the dilution curve contained approximately 24, 12, 6, and 3 cells, respectively. All spots were replicated four times on the arrays (Figure 1). To limit potential carryover, the washes were carried out as shown in Table 1.

Table 1. Wash conditions for arraying. Humidity was set at 57% to help prevent evaporation of samples.

| Wash | Flow-Through Bath | Passive Bath | Vacuum |
|-----------------------|-------------------|-----------------------|---------|
| Prewash | 2.5 sec | 2.5 sec (10% ethanol) | 2.0 sec |
| Cycle wash (5 cycles) | 3.0 sec | 5.0 sec | 0.5 sec |
| Final wash | 5.0 sec | 5.0 sec | 2.0 sec |

Two batches of eight slides were printed from the same sample microplate in order to determine interprinting variability for total protein. Another set of samples was printed onto a batch of eight slides to establish inter- and intraslide variability for phosphorylated protein detection.

Staining

Arrays were blocked (I-Block, Applied Biosystems) and subsequently stained for Akt (Ser⁴⁷³) and p90RSK (Ser³⁸⁰) (Cell Signaling Technology, Inc.) in triplicate using a Dako autostainer with a catalyzed signal amplification system (CSA) (Dako) according to manufacturers' recommendations. Negative control slides were stained with secondary antibody alone (goat anti-rabbit IgG (H+L), Vector Laboratories). Chromogenic detection was achieved with diaminobenzadine (DAB) (Dako) and arrays were imaged on a flatbed scanner (UMAX PowerLook 1120) (Umax Technologies, Inc.). Arrays were also stained for total protein using SYPRO Ruby (Invitrogen Corporation) and visualized with a Molecular Imager[®] PharosFX™ Plus system (Bio-Rad Laboratories, Inc.).

Spot Analysis

Spot intensity was determined with MicroVigene image analysis software, version 2.5 (VigeneTech, Inc.). Local spot background was calculated for each spot. Additionally, negative control spot intensities (secondary antibody only) were calculated. Each antibody background-corrected spot intensity value was normalized to total protein. Coefficient of variation (CV) was determined for both raw background-corrected spot intensities and normalized data in order to compare inter- and intraslide variation.

Sample Carryover Experiment

Spot carryover was investigated by arraying streptavidin-conjugated Qdot 655 quantum dots (Invitrogen Corporation) at full strength and diluted 2-fold in 5% bovine serum albumin. A series of spots was printed in duplicate at two, three, and four hits per spot. An additional three empty wells were printed following the spotted samples to measure carryover. Default wash settings were used (Table 2). Arrays were imaged with a Kodak image station 4000MM digital imaging system at 385 nm excitation and 670 nm emission.

Table 2. Wash conditions for sample carryover experiment.

| Wash | Flow-Through Bath | Passive Bath | Vacuum |
|-----------------------|-------------------|-----------------------|---------|
| Prewash | — | 2.5 sec (10% ethanol) | 2.0 sec |
| Cycle wash (5 cycles) | 3.0 sec | 5.0 sec | 0.5 sec |
| Final wash | 3.0 sec | 5.0 sec | 2.0 sec |

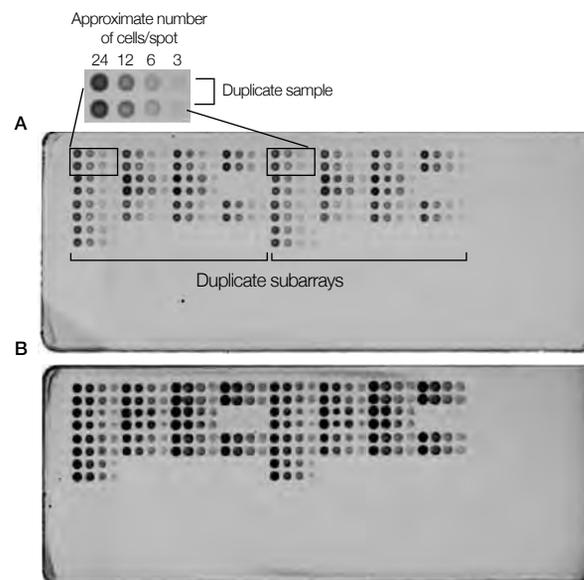


Fig. 1. Comparison of slide printing batches. Two batches of arrays were printed with identical samples from the same source microplate. Samples were printed in duplicate and the entire subarray was also replicated. Approximate number of cells per spot is shown. **A**, SYPRO Ruby-stained slide from first batch of eight printed slides; **B**, SYPRO Ruby-stained slide from second batch of eight printed slides. The more intense staining in slide B illustrates the effect of significant sample evaporation due to the elevated temperature of the platen after a 4 hr print run.

Results and Discussion

Evaluation of instrumentation performance is a necessary process for analytical instruments used in clinical settings. As clinical translational research bridges the gap between basic research and the clinic, researchers should be increasingly aware of the quality assessment processes required for clinical testing. This is particularly important if these technologies are being applied to “home-brew” clinical testing. Instrument quality assessments provide the initial qualification studies needed to develop diagnostic assays. As such, it is imperative to evaluate new instrumentation for precision, accuracy, and throughput as applicable for the particular instrument and testing volume (Lasky 2005). The results presented here represent a series of experiments typically used for evaluation of robotic printing devices.

RPPAs are an innovative technology that applies the principles of conventional antigen-antibody immunoassays to quantitatively identify changes in multiple protein expression patterns (Charboneau et al. 2002, Espina et al. 2003). RPPAs are constructed using whole cellular lysates prepared in a dilution series and subsequently deposited on nitrocellulose substratum by a robotic printing device. The array is constructed with multiple samples, including positive and negative controls,

as well as reference standards. Each array is probed with a single primary antibody directed against the antigen of interest and the signal is amplified via horseradish peroxidase-mediated deposition of biotinyl tyramide (Bobrow et al. 1989, King et al. 1997). DAB, which provides a relatively stable visual endpoint, is then employed for colorimetric detection. These features make RPPAs a promising tool in clinical translational research, such as individualized patient therapy (Petricoin III et al. 2005). Therefore, all parameters of the experiment from sample collection, manipulation, array printing, and staining require tremendous precision and accuracy to ensure reliable results.

RPPAs may be printed in a variety of formats using various printing devices. In this evaluation, arrays were printed with whole cell lysates, prepared from cell lines, in a denaturing buffer. The initial precision experiment included intra- and interslide reproducibility as well as evaluation of the effects of the printing chamber temperature and humidity conditions on the spot quality. This is crucial because small sample volumes (10–20 μ l), as used for the RPPA, are subject to evaporation and consequently increase in concentration in low-humidity and high-temperature conditions.

The Calligrapher was equipped with four out of eight possible flat-tip solid pins. We were able to print eight slides with 256 spots (16 samples, each in a four-point dilution curve spotted in quadruplicate) at three depositions per spot in 4 hr. We printed two sets of eight slides without a cooled platen at 57% humidity.

Visual inspection of the slides for spot consistency, size, and morphology proved that the results were satisfactory. Spot morphology was consistent with an average diameter of 658.3 μ m for spots with ample protein concentrations. For less concentrated samples, spots in the dilution curve were smaller, with an average diameter of 442.1 μ m.

Abundant evaporation of the samples in the microplate was observed between the first print run (eight slides) (Figure 1A) and the second set of eight slides (Figure 1B). This was also evident by quantitatively and qualitatively analyzing the slides. The intraslide CV ($n = 12$) was acceptable with an average of 2.7% (van Hijum et al. 2005). However, significant evaporation did lead to increased interslide variation with CVs as high as 32% ($n = 12$).

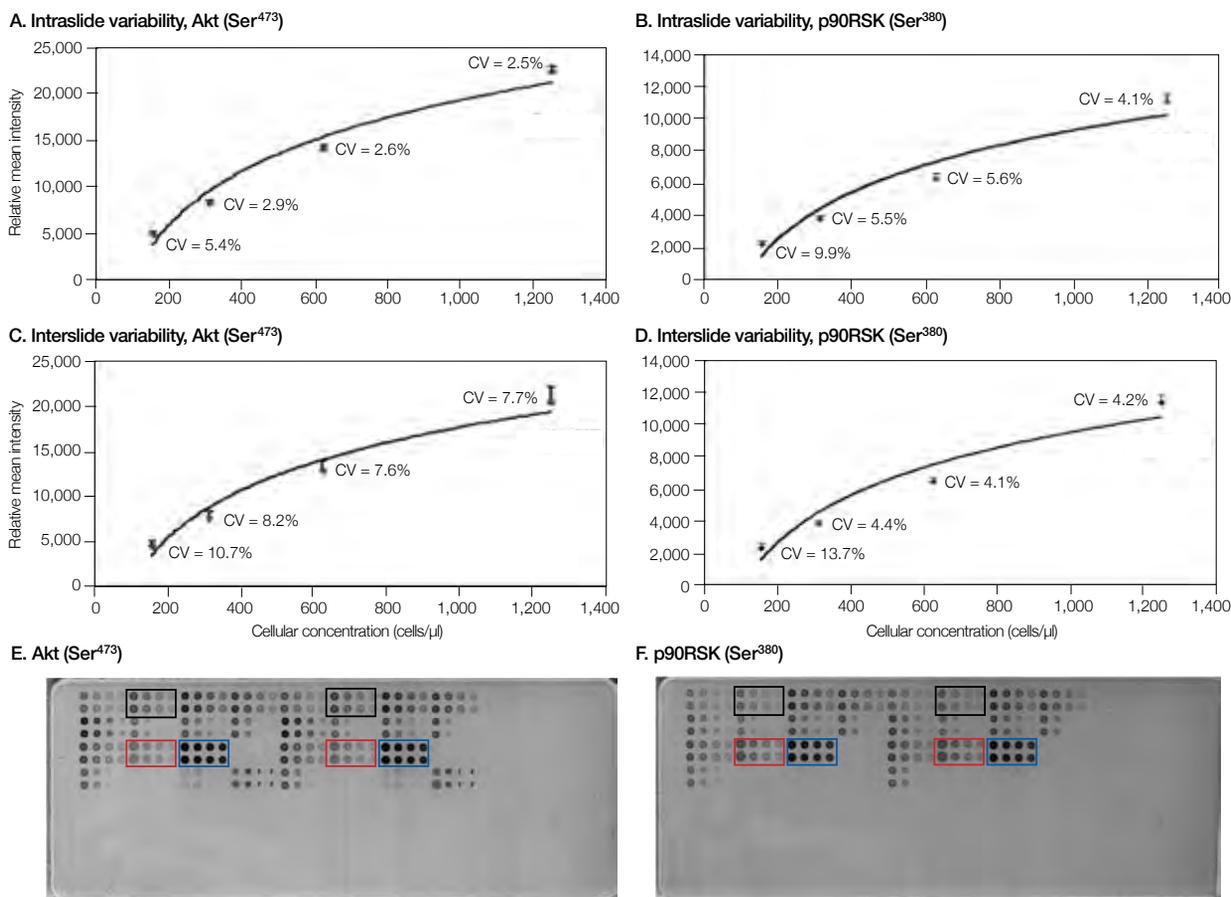


Fig. 2. Precision and linearity studies. The linear dynamic range for two different antibody probes was assessed for arrays printed with cellular concentrations of 156–1,250 cells/ μ l. **A** and **B**, Intraslide values obtained from one sample printed four times on one array. Arrays probed with **A**, Akt (Ser⁴⁷³) and **B**, p90RSK (Ser³⁸⁰). Ser⁴⁷³ showed good intraslide linearity ($r^2 = 0.9657$ and $r^2 = 0.94$, respectively); **C** and **D**, interslide values obtained from one sample printed four times on three separate arrays. Interslide r^2 values for **C**, Akt (Ser⁴⁷³) and **D**, p90RSK (Ser³⁸⁰) were $r^2 = 0.9656$ and $r^2 = 0.9388$, respectively. Intraslide CVs were $\leq 5.4\%$ for Akt (Ser⁴⁷³) and $\leq 9.9\%$ for p90RSK (Ser³⁸⁰). Interslide CVs were $\leq 10.7\%$ for Akt (Ser⁴⁷³) and $\leq 13.7\%$ for p90RSK (Ser³⁸⁰); **E**, Akt (Ser⁴⁷³)-stained array; **F**, p90RSK (Ser³⁸⁰)-stained array. The dilution curve inside the black boxes represents samples whose raw background-subtracted intensities were used for analysis above. The dilution curves of A431 and A431 + EGF control samples printed on the arrays are indicated in red and blue boxes respectively.

Although we did not evaluate this feature, the Calligrapher is available with a cooled platen. The cooled platen can be set at 22°C to maintain samples at room temperature, thus limiting the undesirable side effect of sample precipitation at lower platen temperatures. Without a cooled platen, the platform temperature increased from room temperature to 35°C after 4 hr of printing. In our experience, with humidity levels set to 57% and a cooled platen set at 22°C, undesirable evaporation of samples should be limited.

For the eight slides that were printed in the same run, inter- and intraslide variability were within acceptable limits. As shown in Figure 2, the first three dilution points (neat, 1:2, and 1:4) had excellent intraslide CVs ranging from 2.5 to 5.6% and interslide CVs ranging from 4.1 to 8.2%. The 1:8 dilution spot had higher variability due to lower protein concentration. These results illustrate the necessity of printing reverse-phase arrays in dilution curves in order to match the sample protein concentration with antibody probe affinity that is within the linear dynamic range of the assay.

Contamination of sample due to sample transfer from pin to pin or spot to spot is commonly termed carryover. Carryover using the Calligrapher equipped with TeleChem solid pins was evaluated by printing streptavidin-conjugated quantum dots, 655 nm, directly on the FAST slide. A fluorescent molecule, such as a quantum dot, is an ideal molecule for evaluating carryover because the quantum dot is not photobleached or quenched during long exposure times. Long exposure times permit larger dynamic range analysis and the ability to detect very low-abundance signal that may be present if contamination or carryover is occurring.

The most common source of carryover contamination is inadequate pin washing between sample depositions. The Calligrapher was programmed to the default setting as listed

above with a combination of water and 70% ethanol solutions to maximize the dissolution of sample on the pins during the wash cycle. In general, longer wash cycle times and multiple pin immersions provide enhanced pin cleaning and less contamination. The optimum pin washing steps are sample dependent, as highly viscous samples require longer wash times and/or a series of various wash solutions for effective pin cleaning.

The experiments performed by printing Qdot 655 showed no evident carryover when visualized with the Kodak image station 4000MM imager (data not shown). To emulate actual studies, two, three, and four hits per spot were all tested to ensure that carryover did not occur when greater concentrations of protein were printed on arrays. Using the default wash setting to determine carryover gave assurance that the minimal wash cycles were efficient. A more stringent wash cycle was then used when printing the lysates due to their increased viscosity.

Additionally, empty wells were printed on the array in quadruplicate for evaluation of carryover. Evaluations were performed colorimetrically with DAB and fluorescently with SYPRO Ruby. After staining with primary and secondary antibodies, the arrays were visualized with DAB. Total protein slides were visualized with SYPRO Ruby. Both revealed no apparent contamination resulting from carryover of the previous samples (Figure 3).

Conclusions

This evaluation confirms that the BioOdyssey Calligrapher is technically effective for printing reverse-phase microarrays in batches up to 16 slides. For spots with ample protein concentrations, the average spot diameter was 658.3 μm with variation of 7.3%. Inter- and intraslide CVs were also <10% for samples with adequate protein concentrations. Therefore, based on our precision and carryover experiments, the BioOdyssey Calligrapher meets the technical quality characteristics that are desirable for printing small sample sets of RPPAs.

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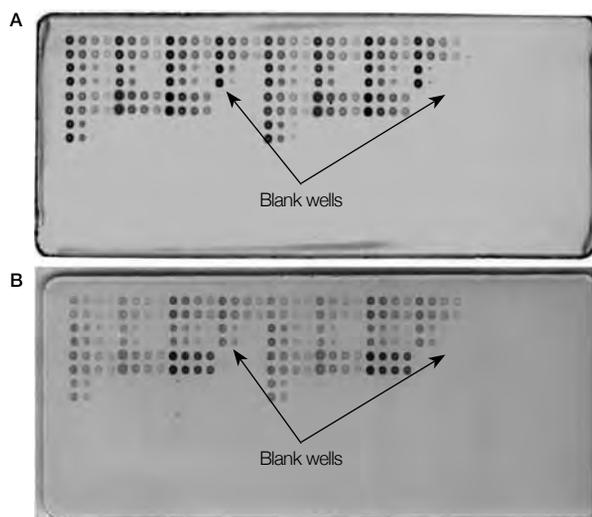


Fig. 3. Assessment of sample carryover. The blank wells represent areas on the array in which carryover was assessed by intentionally leaving microplate wells empty directly adjacent to sample-containing wells for assessment of sample carryover. Arrays stained with **A**, SYPRO Ruby, and **B**, p90RSK (Ser³⁹⁰), did not show any evidence of sample carryover with fluorescence and colorimetric detection (DAB), respectively, in the indicated areas.

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Comparison of Protein Quantitation Using the Experion™ Automated Electrophoresis System, Bradford Assays, and SDS-PAGE

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Introduction

Protein quantitation is a routine procedure used to determine the appropriate amount of sample to use in protein separations and analyses and to calculate the purity, yield, or percent recovery of purified proteins. A number of methods are available for protein quantitation, including ultraviolet (UV) spectroscopy at 280 nm, colorimetric dye-based assays, such as Bradford assays (Bradford 1976), and SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Each protein quantitation method has its benefits and drawbacks. UV spectroscopy works well for quantitating purified proteins with known extinction coefficients or for tracking relative amounts of protein through a purification or modification process; however, this method is limited by its relatively poor sensitivity and by the fact that nonprotein species in the sample buffer can also absorb at 280 nm. Colorimetric dye-based assays are sufficiently sensitive for most applications and offer good dynamic range, excellent reproducibility, and higher throughput through microplate protocols; however, these assays require the generation of a standard curve each time a protein is quantitated, are often incompatible with commonly used buffer constituents (for example, detergents or reducing agents), and can deliver different responses for different proteins, thereby compromising accuracy (Bio-Rad Laboratories 2003, Bradford 1976). The separation step of SDS-PAGE affords sizing and purity information and is virtually unaffected by buffer constituents; however, traditional SDS-PAGE is laborious, requires several hours to complete, and requires specialized imaging equipment and analysis software for quantitation.

The Experion automated electrophoresis system, based on microfluidic separation technology, provides a revolutionary method for protein quantitation (Nguyen and Strong 2005). It is rapid (requiring ~30 min), accurate, and reproducible. It requires minimum amounts of samples and reagents, and generates results that are displayed in real time and stored in a digital format. The Experion Pro260 analysis kit, used for protein analysis, includes a built-in standard (the 260 kD upper marker) in the sample buffer for use in relative concentration estimates (relative quantitation). Experion software also permits relative quantitation against a user-defined internal standard and absolute quantitation against a standard curve generated by a purified protein standard.

In this report, we examine the accuracy and reproducibility of quantitation achieved with the Experion system, Bradford-based assays, and SDS-PAGE coupled with a gel documentation and analysis system. This article is based on data originally published by Zhu and Strong (2006).

Methods

Protein Samples

Purified bovine serum albumin (BSA, 66 kD), bovine erythrocyte carbonic anhydrase (CA, 29 kD) (Sigma-Aldrich), and bovine plasma γ -globulin (IgG, 150 kD) were used. Each protein was dissolved and diluted in proteomics-grade water. Concentrations of the three proteins were independently determined using UV spectroscopy and extinction coefficients for 1 mg/ml solutions of 0.667 (at 279 nm) for BSA concentrations of 200 ng/ μ l and higher, and 1.73 and 1.38 (at 280 nm) for CA and IgG, respectively, at concentrations of 100 ng/ μ l and higher.

Experion Pro260 Analysis

The Experion Pro260 analysis kit was used for protein quantitation with the Experion system. Samples (4 μ l) were mixed with 2 μ l sample buffer either with β -mercaptoethanol (BSA and CA samples) or without β -mercaptoethanol (IgG samples), heated at 95°C for 5 min, diluted with proteomics-grade water, and loaded onto chips primed according to the protocols provided with the kit.

For absolute quantitation, five chips were run, each with 25, 100, 250, 500, 1,000, and 2,000 ng/ μ l samples as standards and with four sample concentrations (50, 200, 750, and 1,250 ng/ μ l). Protein concentrations (total protein or primary peak) were taken directly from the data reported by Experion software, and statistical analysis was performed using JMP version 5.1 software (SAS Institute, Inc.).

Bradford Assays

Two Bradford protein assays were evaluated using microplate protocols. The first used the Bio-Rad protein assay dye reagent concentrate, and the second used the Coomassie (Bradford) protein assay kit reagent (Pierce Biotechnology). To generate calibration curves, an ultrapure water blank and 11 standard concentrations of each protein (2.5, 10, 50, 100, 250, 500, 750, 1,000, 1,500, 1,750, and 2,000 ng/ μ l) were loaded in triplicate on separate plates. For analysis, each protein at four different concentrations was loaded in five replicate wells ($n = 5$) containing diluted dye reagent (the Bio-Rad reagent required 10 μ l protein and 200 μ l diluted dye reagent, and the Pierce assay required 5 μ l protein and 250 μ l dye reagent). The samples and reagent were then incubated for 5 min at room temperature, and the absorbance at 595 nm of each well was measured using a Benchmark™ Plus microplate reader.

SDS-PAGE

SDS-PAGE was performed using the Criterion™ cell and 7.5%, 10%, and 15% Criterion Tris-HCl precast gels to separate IgG, BSA, and CA, respectively. Protein sample (4 µl) was mixed with 4 µl 2x Laemmli sample buffer either with 5% β-mercaptoethanol (BSA and CA samples) or without β-mercaptoethanol (IgG samples), heated at 95°C for 5 min, and loaded onto the gel. Electrophoresis was performed at a constant 200 V for 55 min. Gels were stained for 1 hr with Bio-Safe™ Coomassie G-250 stain, destained in water overnight, scanned on a GS-800™ densitometer, and analyzed using Quantity One® software. Five gels were run for each protein (n = 5), and on each gel a calibration curve was generated by plotting either the combined signal densities of all bands (total protein) or the signal density of the primary band versus the concentration of six standards (25, 100, 250, 500, 1,000, and 2,000 ng/µl); concentrations (total protein or primary band) of four samples (50, 200, 750, and 1,250 ng/µl) were determined based on these curves.

Results and Discussion

Three purified proteins (BSA, CA, and IgG) were quantitated by the Experion system, Bradford assays, and SDS-PAGE, and the protein concentrations derived from these approaches were compared to those obtained by UV spectroscopy. As a measure of reproducibility, the coefficient of variation (%CV) was calculated as the standard deviation/mean x 100. Accuracy was measured by the percent difference between the measured concentration and the spectroscopically determined (UV-based) value. A summary of the results is presented in Figure 1.

Experion Automated Electrophoresis System

The Experion system automatically determines the relative concentration of a protein using a single-point calibration, wherein the peak area of the protein is compared to that of an internal marker protein present in each sample at a known concentration. Inclusion of an internal standard provides the added benefit of allowing automatic correction of any sample-to-sample differences in injection or separation (for example, those caused by differences in the concentration of salt or other buffer constituents), as all the proteins in the sample are affected by these differences to the same extent.

With this system, the user also has the option of using known concentrations of the purified protein to create a calibration curve on the chip. Such absolute quantitation takes into account inherent differences in the efficiency of protein-dye binding that can generate protein-to-protein variations in quantitation accuracy in any dye-based assay (Bio-Rad Laboratories 2003, Bradford 1976). The Experion Pro260 analysis kit, for example, simplifies this process by allowing the user to create a standard curve by selecting the wells to be used for generating the plot and entering their concentrations into the corresponding fields.

As indicated in Figure 1, the absolute quantitation method was used to measure the concentrations of the three test proteins for use in quantitation accuracy and reproducibility calculations. Each protein sample was analyzed on five chips. Each chip contained six known concentrations of protein,

which were used to generate calibration curves, as well as four sample concentrations, whose concentrations were reported by Experion software. The average r^2 values of the calibration curves were 1.0 for BSA, 0.965 for CA, and 0.995 for IgG, indicating excellent linear fit.

The concentrations of both the primary peak and the total protein per well were recorded, the latter for comparison with the other colorimetric methods and the former for comparison with SDS-PAGE. The capability of the microfluidics-based system to report both peak and total protein concentrations also facilitates determination of the percentage of total protein for any species detected. As expected, the accuracy of the peak and total protein concentrations was equivalent (Figure 1).

Bradford Assays

The Bio-Rad protein assay kit, which has a linear range of 50–500 ng/µl, was used to perform this assay. The r^2 values of the calibration curves generated by BSA, CA, and IgG in this range were 0.963, 0.981, and 0.999, respectively, and the values for the linear fit over the 0–2,000 ng/µl range were 0.919, 0.784, and 0.953. However, when a second-order polynomial fit was used in the same range, the r^2 values increased to 0.994, 0.957, and 1.00 (not shown).

Similar results were obtained with the Coomassie (Bradford) protein assay kit from Pierce Biotechnology (not shown). The published linear range of this assay is 100–1,500 ng/µl, but for comparison with the Bio-Rad protein assay, the 50–500 ng/µl range was used for the linear fit equations, and the 0–2,000 ng/µl range was used for the second-order polynomial fit. With both methods, the 50 ng/µl sample generated large variations, and the 750 ng/µl and 1,250 ng/µl samples demonstrated improved accuracy when analyzed with the second-order polynomial fit rather than with the linear fit of the data. Overall, the two assay kits produced similar results, with average %CV values within 1%, and accuracy values within 6% of each other (not shown).

SDS-PAGE

IgG, BSA, and CA samples were analyzed by SDS-PAGE using 7.5%, 10%, and 15% Tris-HCl precast gels, respectively. Each gel also contained separations of six standard concentrations (25–2,000 ng/µl), which were used to generate calibration curves. The intensities of all bands detected by Quantity One software were exported to Excel software for determination of concentration against the calibration curves. As with the microfluidics-based analyses, both the primary band intensity and the sum of all bands (total protein) per lane were used, since with BSA and IgG, more than one band was detected in lanes loaded with high protein concentrations.

The average r^2 values were 0.999 for BSA and IgG, and 0.973 for CA, indicating excellent linear fit. Higher protein concentrations were quantitated with higher accuracy; 50 ng/µl samples were quantitated with the lowest reproducibility and the poorest accuracy (Figure 1).

For traditional SDS-PAGE, samples were loaded on gels, separated by electrophoresis, stained, destained, scanned, and analyzed with Quantity One and Excel software in a process that

typically required 3–4 hr to complete. The Experion automated electrophoresis system generated comparable quantitation results for ten samples in only 30 min.

Comparison of Quantitation Methods

To examine the differences in the reproducibility of quantitation by the various methods, all data were evaluated using the %CV and plotted in a scatter plot (Figure 1A). Generally, the Bradford assays generated the most reproducible results, likely because no protein separation was involved. Samples with the lowest protein concentrations (50 ng/μl) displayed poorer quantitation reproducibility than those with higher concentrations; this was particularly prominent in the SDS-PAGE data (Figure 1A). The Experion Pro260 assay, in most cases, had a %CV of <20% for all three proteins, indicating good reproducibility.

The accuracy of quantitation for all methods was also compared, and the percent differences were plotted for three of the four concentrations examined (Figure 1B). (For all three proteins and for all methods, the 50 ng/μl samples showed the least accuracy; therefore, these data were excluded.) The Bradford assays generated comparable results, and most data fell within 20% of the UV-based concentrations. The Experion Pro260 assay performed similarly to SDS-PAGE and the dye-based assay kits. For BSA and IgG, the peak and total protein concentrations determined by the Experion Pro260 analysis were very similar, or the peak values showed slightly higher accuracy. For CA, the accuracy using the microfluidics-based quantitation method was not as good as the other methods when total protein concentrations were examined; this is likely due to differences in protein staining efficiency between CA and the upper marker (Nguyen and Strong 2005). However, a dramatic

improvement in quantitation accuracy was observed with absolute quantitation of peak protein concentrations. Overall, some of the most accurate measurements generated in this study were those made with the Experion system (Figure 1B).

Conclusions

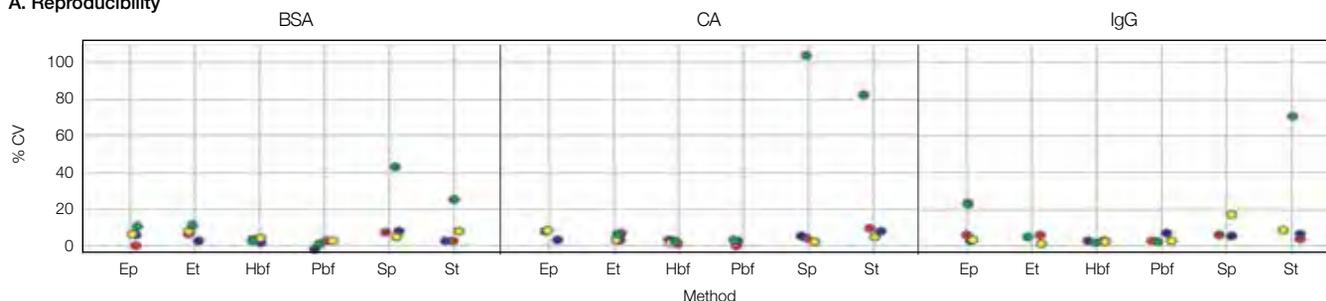
The Experion system and Pro260 analysis kit offer rapid and reliable protein quantitation. As shown here, this method provides comparable reproducibility and slightly higher accuracy than other traditional protein quantitation methods, such as SDS-PAGE and Bradford assays, yet this automated system covers a wider range of linearity, requires lower sample and reagent volumes, offers significantly reduced time to results and hands-on time, and decreases exposure to hazardous chemicals. In addition, the Experion Pro260 assay provides both the primary peak and total protein concentrations, whereas other dye-based colorimetric methods only determine total protein concentrations. Therefore, for mixed protein samples, the Experion Pro260 assay is an excellent choice for either relative or absolute quantitation of all protein components within a sample.

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A. Reproducibility



B. Accuracy

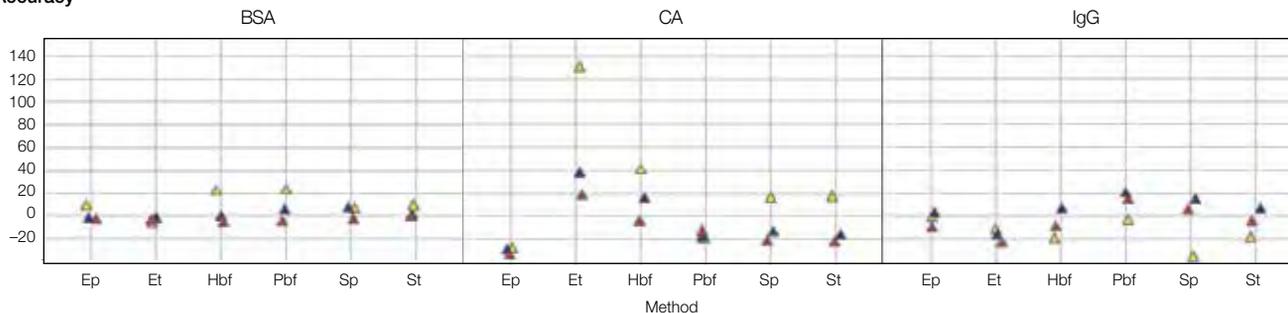


Fig. 1. Scatter plot comparison of quantitation reproducibility (A) and accuracy (B). Data from the Experion Pro260 analysis using absolute quantitation: Ep, peak concentration; Et, total protein concentration. Data from the Bio-Rad protein assay (Hbf), Pierce Coomassie (Bradford) assay (Pbf), and SDS-PAGE (Sp, primary band concentration and St, total protein concentration) are also shown. Data for both Bradford assays were obtained using the polynomial fit. Data from 50 ng/μl samples were excluded from B due to the large variations in accuracy observed with these samples with all methods. Protein concentrations: —, 50 ng/μl; —, 200 ng/μl; —, 750 ng/μl; —, 1,250 ng/μl.

Effective Cleaning and Sanitizing of UNOsphere™ Q Chromatography Resins

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Abstract

UNOsphere Q anion exchange chromatography columns soiled with DNA or endotoxin were decontaminated in situ using common, readily available chemicals. Mixing studies and analysis confirmed that eluted fractions contained >99% clearance of DNA with <0.5 M NaCl. Clearance of endotoxin was >10⁶-fold. Follow-up gradient separation of model proteins confirmed that the cleaning and sanitization methods reported here do not impair the selectivity of the chromatographic resin. These results are suggestive and promising for a variety of high-throughput chromatographic workflow applications.

Background

Chromatography columns may become contaminated by a variety of protein and nonprotein species during a purification campaign. Consequences of column contamination include an increase in column backpressure, selectivity, altered product yield, and medium discoloration. Common chromatographic contaminants include:

- Residual proteins
- Nucleic acids
- Lipids
- Endotoxins
- Viruses and bacteria
- Metal ions

Generally, methods for cleaning-in-place (CIP) and sanitization-in-place (SIP) of chromatographic resins are selected based on the interplay and relevance of three factors:

- Ease of operation
- Historical experience
- Performance requirements

In most cases, the column decontamination method chosen in the laboratory forms the basis not only for validation but also for subsequent scale-up. For this reason, an ideal scenario would be development and use of a generic decontamination method. At present, however, even taking into account the varying types of resins available, considerable disparity of CIP/SIP procedures is apparent in instructions available from manufacturers of chromatography resins.

Many traditional cleaning solutions are used for CIP and SIP. Target contaminants of these solutions are listed in Table 1.

Table 1. Traditional cleaning solutions for specific contaminants.

| Cleaning Solutions | Contaminants Removed |
|---|---------------------------|
| 1–3 M NaCl, 1–2 M NaOH | Residual proteins, DNA |
| Guanidine hydrochloride | Residual proteins, lipids |
| Urea, ethanol, isopropyl alcohol | Residual proteins, lipids |
| 1–2 M NaOH, tri(n-butyl)phosphate/Tween | Viruses, endotoxins |
| Citric acid, EDTA | Metal ions |

Unquestionably, the cleaning strategy that has attracted the most attention and produced the most dependable results is the combination of NaCl and NaOH. This combination has repeatedly proven effective in chromatography column decontamination. The key advantage of NaOH is its bactericidal action; NaOH inhibits the growth of and kills many bacteria and microorganisms. When NaOH is applied to base-resistant chromatography resins supplied by various resin manufacturers, it has proven to be highly effective in validation studies for the removal of residual proteins, viruses, and endotoxins (Conley et al. 2005, Dasarathy 1996).

In our investigation, we addressed the clearance of DNA since there is limited relevant information or supporting data on mass balance. DNA, being highly negatively charged, has strong affinity for the positively charged surfaces of anion exchange resins. But any DNA not removed by a cleaning procedure will gradually accumulate over time and diminish column binding capacity and selectivity. Accordingly, we chose to examine chromatography issues such as post-cleaning DNA recovery and selectivity. Simultaneous clearance of endotoxin and residual proteins will also be discussed.

Methods

DNA

Sheared salmon sperm DNA (catalog #9610-5-D, R&D Systems) was used for DNA recovery studies. As indicated in the manufacturer's package insert, the material contains DNA fragments ranging in size from 200 to 500 base pairs.

Quantitation of DNA

Absorbance at 260 nm (A_{260}) was used for monitoring DNA concentration with a conversion factor of 50 µg/ml DNA per absorbance unit. DNA concentration was also measured using a dye-based assay with PicoGreen (Invitrogen), which fluoresces on binding to double-stranded DNA. After addition

of the working solution of PicoGreen reagent to the sample, and incubation at room temperature for 2–5 min, fluorescence was measured using a Cary Eclipse spectrophotometer (Varian, Inc.) with excitation at 480 nm and emission at 520 nm. The detection limit of the PicoGreen assay is 250 pg/ml of double-stranded DNA (75 pg in a 300 µl sample volume). Linearity, with a regression coefficient of >0.99, was routinely obtained in a standard curve spanning 0–500 ng/ml.

Quantitation of Endotoxin

For single sample assays, we used the Endosafe-PTS reader (Charles River Laboratories), a point-of-use test system that uses existing FDA-licensed *Limulus* amoebocyte lysate (LAL) reagents in a test cartridge with a handheld spectrophotometer. Sensitivity of the assay is 0.05 EU/ml.

UNOsphere Q Chromatography Support

All chromatography experiments were conducted using UNOsphere Q support packed in Bio-Scale™ MT2 or MT10 columns (Bio-Rad Laboratories, Inc.). The MT2 column dimensions are 0.7 cm in diameter and 2.6–5.2 cm in height. The MT10 column dimensions are 1.2 cm in diameter and 8.8 cm in height.

Endotoxin Concentrate

The endotoxin concentrate consisted of equal amounts of liposaccharides from *Escherichia coli*, *Salmonella enterica* serotype *abortus equi*, and *Pseudomonas aeruginosa* 10. It was assayed with the Endosafe-PTS reader and determined to have 6.64×10^6 EU/ml.

Selectivity

A Bio-Rad Laboratories protein standard for anion exchange chromatography (catalog #125-0561), consisting of equine myoglobin, conalbumin, chicken ovalbumin, and soybean trypsin inhibitor, was separated using a gradient method (buffer A, 20 mM Tris, pH 8.5; buffer B, 20 mM Tris, 1.0 M NaCl, pH 8.5). The retention time of each protein was determined from the chromatogram.

Chromatography System

All chromatography experiments were automated and performed using a BioLogic DuoFlow Maximizer™ chromatography system and software (Bio-Rad Laboratories, Inc.). Flow rates of the columns were maintained at 300 cm/hr throughout all experiments.

Results and Discussion

DNA Recovery

The standard column hygiene sequence developed during this investigation is:

- Clean — 2.0 M NaCl, 3 column volumes
- Sanitize — 1.0 M NaOH, 3 column volumes
- Store — 0.02 M NaOH, 3 column volumes

A study was made of this decontamination sequence with fractions collected from stepwise elution of increasing NaCl concentrations up to 2.0 M. A 0.15 µg sample of salmon DNA was injected into a 1 ml UNOsphere Q column. The percentage yield (the ratio of A_{260} recovery relative to A_{260} injection) is shown in Table 2.

Table 2. Effect of wash sequence on DNA recovery.

| Process Step | Percentage Yield |
|---------------------------------|------------------|
| Flowthrough with wash | 0 |
| Eluted fractions at 0.1 M NaCl | 0 |
| Eluted fractions at 0.5 M NaCl | 0 |
| Eluted fractions at 1.0 M NaCl | 35.4 |
| Eluted fractions at 2.0 M NaCl | 10.8 |
| 2.0 M NaCl with 1.0 M NaOH wash | 37.8 |
| 1.0 M NaOH wash | 7.8 |
| 0.02 M NaOH wash | 0 |
| Cumulative | 91.8 |

The tested range of NaCl concentrations shown in Table 2 is a commonly used diagnostic elution zone for many proteins of research interest. These data show that insignificant clearance was obtained across the fractions from 0 to 0.5 M NaCl using anion exchange chromatography. Since there was no detectable absorbance at 260 nm in these fractions, their DNA fractions were determined with the PicoGreen assay. These results (Table 3) indicated significant DNA clearance, and agree with data published previously (Dasarathy 1996).

Table 3. Measured DNA clearance using the PicoGreen assay.

| Sample | Total DNA (ng) | Percentage Remaining |
|--------------------------------|----------------|----------------------|
| Feed | 147,500 | 100 |
| Fractions from 0 to 0.5 M NaCl | 417 | 0.28 |
| DNA remaining in column | 147,083 | 99.7 |

Endotoxin Clearance

Subsequent to soiling with a challenge of 3.3×10^5 units of endotoxin, the column was washed in sequence with 2.0 M NaCl and 1 M NaOH. After holding in 1.0 M NaOH for 3 hr, the column was washed with 0.02 M NaOH. The wash solution was neutralized with phosphate buffered saline prior to LAL assay. The results of the experiment (Table 4) demonstrated excellent clearance of endotoxin. These data are consistent with the most frequently employed CIP/SIP protocols that use NaOH as the sanitizing agent. A clearance factor of more than 6 orders of magnitude was obtained; however, this exceptional efficiency is restricted to artificially high endotoxin feed concentration. At feed concentrations significantly lower than the challenge used in this case, which is a more common endotoxin contamination level, total clearance of the endotoxin is anticipated with NaOH inactivation. The results are consistent with data previously published (Conley et al. 2005).

Table 4. Endotoxin removal from UNOsphere Q column.

| Total Challenge Reduction (EU) | Total in Eluate (EU) | Percentage Removal | log Value |
|--------------------------------|----------------------|--------------------|-----------|
| 3.3×10^5 | <0.16 | >99,999 | >6 |

Residual Contaminant Clearance

Following CIP/SIP and between runs, it is possible that small amounts of residual proteins, microorganisms, and endotoxins could be present. Their concentration will differ among various process applications and is a strong function of feed stream. It is necessary to employ practical methods to quantify such residual materials. Three tests, including A_{280} , microbial load, and LAL, can be used to verify that the resin is consistently meeting necessary and achievable acceptance criteria. The process developer simply compares initial and eluted buffer values to determine the magnitude of residual contaminants.

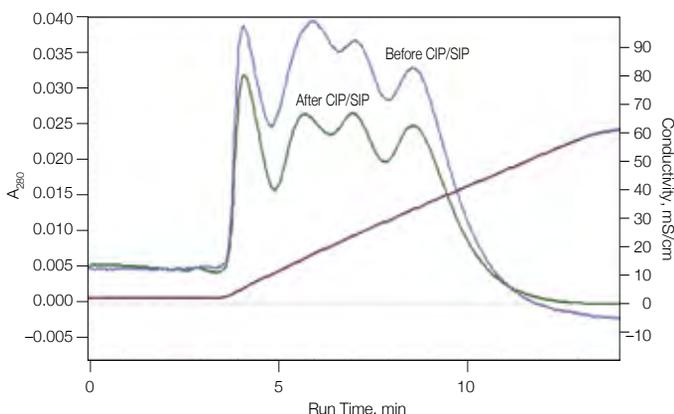


Fig. 1. Protein standard separation before and after CIP on a 1 ml UNOsphere Q column. —, conductivity.

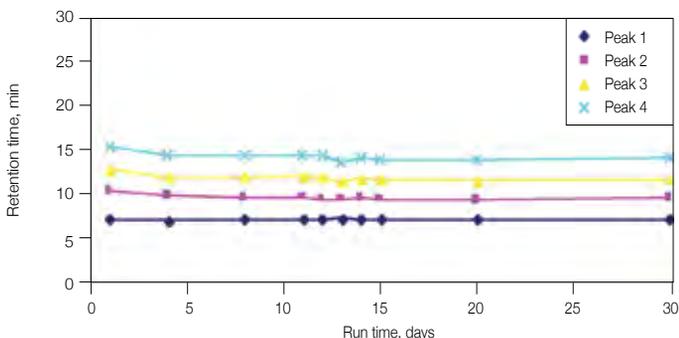


Fig. 2. Retention time of protein standard on a 10 ml UNOsphere Q column before and after CIP.

Selectivity of Column Before and After CIP

By using the recommended conditions (see DNA Recovery section), a study was made of the effect of CIP on selectivity. The data in Figure 1 show that the selectivity of the column was unaffected by the decontamination treatment. As would be expected, any change must be carefully monitored over the lifetime of a column. Such a study should be addressed prior to scale-up by the process developers.

To evaluate the sanitization/decontamination method further, the 1 ml column was scaled up to 10 ml, and the following cycles were repeated after each run: 2.0 M NaCl wash, 1.0 M NaOH for ≥ 3 hr, and storage at 0.02 M NaOH for ≥ 16 hr. We then measured the protein’s retention time, which is the time between injection and the appearance of the peak maximum. As shown in Figure 2, selectivity remained constant over ten cycles in a duration of 30 days. The data are consistent with the superior base stability reported previously (Franklin et al. 2002).

Conclusions

A cleaning cycle using 2.0 M NaCl and 1.0 M NaOH has been shown to give good chromatographic clearance of DNA and endotoxin. Excellent base stability of UNOsphere Q anion exchange support was evidenced by no change in its selectivity. This CIP/SIP protocol appears to be suitable for both validation and scale-up.

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 Dasarathy Y, A validatable cleaning-in-place protocol for total DNA clearance from an anion-exchange resin, Biopharm 9, 41–44 (1996)
 Franklin SG et al., UNOsphere Q support technical data, Bio-Rad bulletin 2729 (2002)

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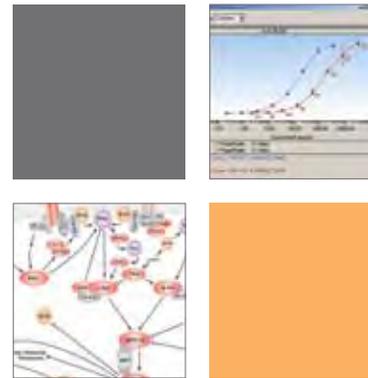
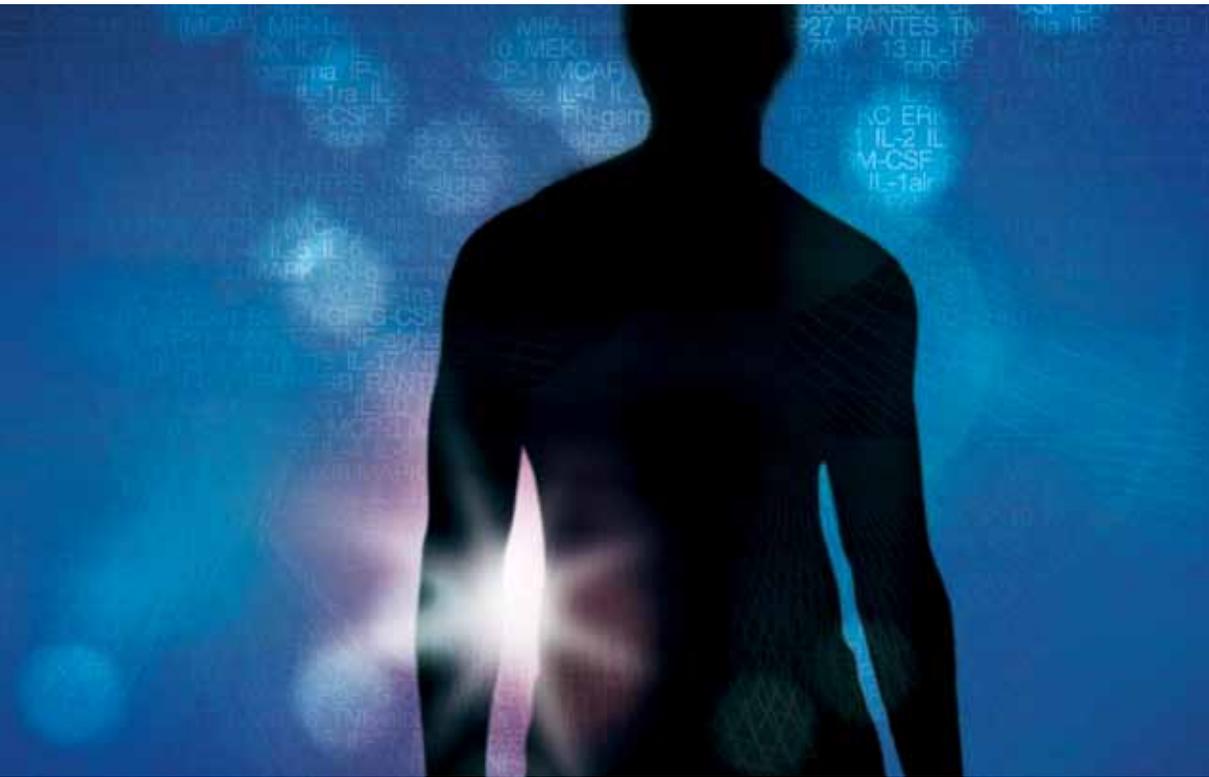
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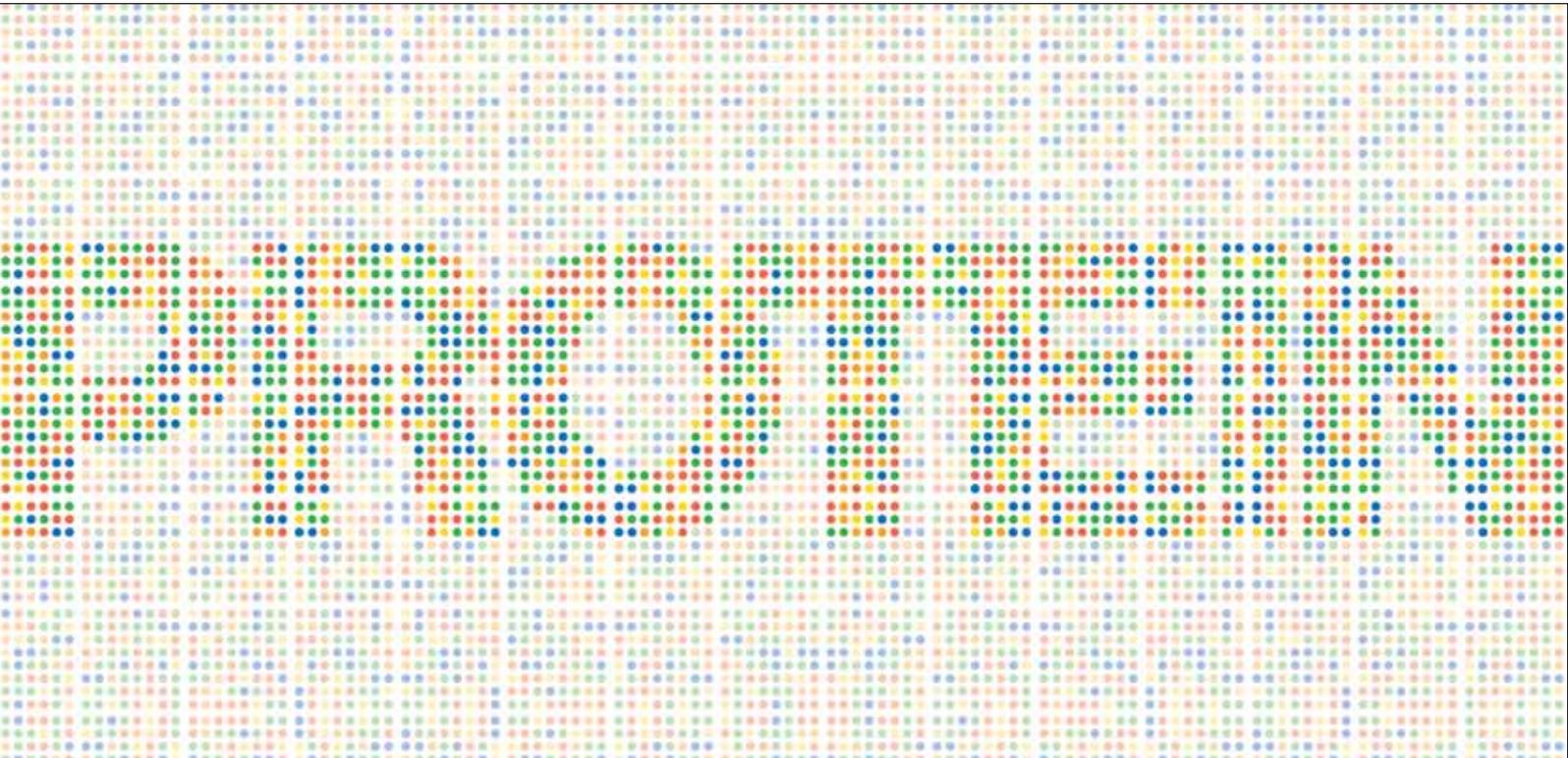
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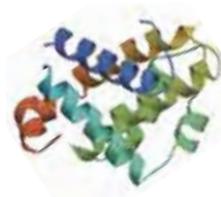


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