In this issue:

ProteOn™ System Tools Aid Regulatory Compliance in Protein Studies
Enhancing Sensitivity in SDS-Protein Electrophoresis
Using the SmartSpec™ Plus Spectrophotometer With Low-Volume Samples
Evaluating Magnetic Microsphere Assays

Bio-Rad’s powerful siLentMer™ siRNA duplexes keep genes silenced longer using lower concentrations. And they’re validated.

siLentMer siRNA duplexes are a new class of dsRNA molecules with increased length and altered end structure, capable of initiating an enhanced, more specific silencing compared to traditional siRNAs.

- Lower usage amounts — effective at concentrations as low as 5 nM, reducing the risk of off-target effects
- High purity — HPLC purification ensures specific gene silencing
- Validated qPCR primers — included to help assess knockdown with qPCR assays using SYBR® Green

For information on products and promotions that support your RNAi research, visit us on the Web at www.bio-rad.com/ad/RNAi/.

SYBR is a trademark of Invitrogen Corporation.
Practice of the polymerase chain reaction (PCR) may require a license.
Nearly 50 years ago, scientists developed the radioimmunoassay to measure insulin levels in biological samples. Enzyme-linked immunosorbent assays (ELISAs), soon became the standard for detecting analytes of interest for several subsequent decades. Today, necessity continues to drive progress in the development of techniques that aid discovery. The current information-intensive research environment requires tools that allow higher content analysis in an easy-to-use format. That’s why Bio-Rad has invested research and development over the past two decades into multiplexing technology. The Bio-Plex® multiplex suspension array system allows up to 100 markers to be measured simultaneously at a cost per analyte that is similar to that of low-multiplexing technologies. And with the system’s most recent evolution — the incorporation of magnetic bead-based technologies — more data points per sample can now be achieved with both accuracy and ease.

**COVER STORY**

16 Second-Generation Multiplex Immunoassays: xMAP Technology and Magnetic Appeal Put Multiplexing in the Fast Lane
Emily Dale, Bill Gette, Efthalia Gerasimopoulos, Jessica Dines, and Todd Yeck, Bio-Rad Laboratories, Inc., Hercules, CA USA
New Supermixes for Fast qPCR

Bio-Rad introduces two new supermixes for qPCR — iTaq fast SYBR® Green supermix with ROX and iTaq fast supermix with ROX. These supermixes have been optimized for fast real-time qPCR. They deliver maximum PCR efficiency, sensitivity, specificity, and a robust fluorescent signal under fast or standard thermal cycling conditions with any real-time detection chemistry. With the introduction of these new supermixes, Bio-Rad now offers a comprehensive line of high-performance qPCR reagents suitable for use on a variety of real-time PCR instruments.

**iTaq™ Fast SYBR® Green Supermix With ROX**

iTaq fast SYBR® Green supermix with ROX is formulated for optimal results in fast real-time qPCR assays. It is validated for use on the ABI 7500 and Stratagene Mx series real-time PCR systems. The formula yields sensitive, specific amplification over several orders of magnitude with cDNA as well as genomic and plasmid DNA templates. This fast SYBR® Green supermix is blended with iTaq DNA polymerase, optimized buffer, nucleotides, SYBR® Green I dye, and ROX passive reference dye.

- Excellent amplification efficiency for increased sensitivity and specificity using fast cycling protocols for SYBR® Green qPCR
- Rapid activation and polymerization kinetics for fast qPCR results in less than 40 min
- Reproducible, highly uniform results across a wide range of template concentrations

**Ordering Information**

<table>
<thead>
<tr>
<th>Catalog #</th>
<th>Description</th>
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<tr>
<td>172-5100</td>
<td>iTaq Fast SYBR® Green Supermix With ROX, 200 x 20 µl reactions, 2x mix contains dNTPs, iTaq DNA polymerase, 6 mM Mg²⁺, SYBR® Green I, ROX passive reference dye, stabilizers</td>
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<tr>
<td>172-5101</td>
<td>iTaq Fast SYBR® Green Supermix With ROX, 500 x 20 µl reactions</td>
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<tr>
<td>172-5102</td>
<td>iTaq Fast SYBR® Green Supermix With ROX, 1,000 x 20 µl reactions</td>
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<td>172-5103</td>
<td>iTaq Fast SYBR® Green Supermix With ROX, 20 ml bottle, 2,000 x 20 µl reactions</td>
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**iTaq™ Fast Supermix With ROX**

iTaq fast supermix with ROX is formulated for optimal results in fast real-time qPCR of up to two different gene targets. It is validated for use on the ABI 7500 and Stratagene Mx series real-time PCR systems. The formula yields sensitive, specific amplification over several orders of magnitude with cDNA as well as genomic and plasmid DNA templates. This fast supermix is blended with iTaq DNA polymerase, optimized buffer, nucleotides, and ROX passive reference dye.

- Robust, simultaneous detection of up to 2 different gene targets under fast qPCR conditions
- Rapid activation and polymerization kinetics for fast qPCR results in less than 40 minutes
- Compatibility with any real-time detection chemistry

**Ordering Information**

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<tr>
<td>172-5105</td>
<td>iTaq Fast Supermix With ROX, 200 x 20 µl reactions, 2x mix contains dNTPs, iTaq DNA polymerase, 6 mM Mg²⁺, ROX passive reference dye, stabilizers</td>
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<td>172-5106</td>
<td>iTaq Fast Supermix With ROX, 500 x 20 µl reactions</td>
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<td>172-5107</td>
<td>iTaq Fast Supermix With ROX, 1,000 x 20 µl reactions</td>
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<td>172-5108</td>
<td>iTaq Fast Supermix With ROX, 20 ml bottle, 2,000 x 20 µl reactions</td>
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Streamlined RNA Sample Preparation for Gene Expression Analysis

iScript™ RT-qPCR sample preparation reagent delivers efficient cell lysis, RNA stabilization, and removal of genomic DNA for sensitive quantitative gene expression analysis without RNA purification. This novel reagent accelerates and streamlines RT-qPCR analysis of cultured cells by eliminating the need to purify RNA. Reverse transcription PCR and real-time PCR can be performed directly from cell lysates. This system is ideal for rapid, high-throughput gene expression analysis, such as validation of siRNA-mediated gene knockdown.

- Rapid protocol efficiently removes genomic DNA and stabilizes RNA in 5 to 10 minutes
- Sensitive detection of high-, medium-, and low-copy gene targets directly from cell lysates
- Enables multiplex real-time detection of up to 4 targets from as few as 10 cells

**Ordering Information**

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<thead>
<tr>
<th>Catalog #</th>
<th>Description</th>
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<tr>
<td>170-8888</td>
<td>iScript RT-qPCR Sample Preparation Reagent, 100 reactions (10 ml), contains RNase inhibitors and RNA stabilizers</td>
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<tr>
<td>170-8899</td>
<td>iScript RT-qPCR Sample Preparation Reagent, 500 reactions (5 x 10 ml), contains RNase inhibitors and RNA stabilizers</td>
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siLentMer™ siRNA Gene Targets — Grouped by Research Interest

siLentMer™ siRNA gene targets cover a variety of research interests, including apoptosis, inflammation, cell signaling, and many diseases and disorders. To simplify finding targets in your area of research, we have categorized our collection of validated siRNA duplexes into preliminary gene target panels. The variety of panels will continue to expand. Currently, our validated gene targets are grouped into eight panels according to areas of research interest:

- Apoptosis
- Cell Cycle/Checkpoint
- Inflammation
- Angiogenesis
- Chromatin Regulation
- Carcinogenesis
- Metabolism
- Toxicology/ADME

**Current list of targets in apoptosis panel**

- ABL1
- AKT1
- BAD
- BAX
- CASP1
- CASP2
- CASP4
- CASP6
- CASP7
- IGF1R
- MAPK8
- MDM2
- MMP2
- NFKB1
- RAF1
- TNFRSF1A
- TP53

**Potent Silencing**

siLentMer siRNA duplexes are fully validated to achieve at least 85% knockdown for up to 6–9 days.

Bio-Rad’s collection of validated siRNA duplexes is continuously growing. The current list of available gene targets and panels can be found at [www.bio-rad.com/RNAi/](http://www.bio-rad.com/RNAi/).
Hard-Shell® Full-Height 96-Well Semi-Skirted PCR Plates

This versatile Hard-Shell PCR plate fits most available thermal cyclers. Features of these semi-skirted plates include:

- Full-height wells that fit most cyclers, real-time PCR systems, and sequencers
- Half-height skirt for stiffness and labeling surface
- Superior stability due to 2-component design, minimizing well-to-well variability and allowing precise positioning for automation
- Black lettering for easy well identification
- Color-coding option for improved laboratory processes while providing the convenience of clear wells
- White-well option for stronger fluorescent signal
- Low-cost, user-readable bar codes for convenient database tracking

Specifications

<table>
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<tr>
<th>Compatible instruments</th>
<th>DNA Engine® family, PTC-100®, C1000™, S1000™, and iCycler® thermal cyclers, MyiQ™, iCycler IQ®, IQ™5, and Chromo4™ real-time systems, Applied Biosystems sequencers, and certain other cyclers</th>
</tr>
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<tr>
<td>Recommended sealing options</td>
<td>Strips of 8 caps, domed, Strips of 8 caps, flat, optical clarity, Strips of 12 caps, domed, Microseal® ‘A’ film, Microseal ‘B’ adhesive seals, optical clarity, Chill-out™ liquid wax</td>
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<tr>
<td>Catalog#</td>
<td>Description</td>
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<tr>
<td>HSS-9601</td>
<td>Hard-Shell Full-Height 96-Well Semi-Skirted PCR Plates, clear shell, clear well, 25</td>
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<tr>
<td>HSS-9641</td>
<td>Hard-Shell Full-Height 96-Well Semi-Skirted PCR Plates, green shell, clear well, 25</td>
</tr>
<tr>
<td>HSS-9665</td>
<td>Hard-Shell Full-Height 96-Well Semi-Skirted PCR Plates, black shell, white well, 25</td>
</tr>
<tr>
<td>HSS-9901</td>
<td>Hard-Shell Full-Height 96-Well Semi-Skirted PCR Plates With Bar Codes, clear shell, clear well, 25</td>
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Mini-PROTEAN® System Social Networking Site — myTetraCell.com

Bio-Rad introduces myTetraCell.com, a social networking site developed to connect present and past Mini-PROTEAN electrophoresis cell users for exchange of ideas and information. Register to explore the many features of this site and earn points that can be redeemed for promotional items.

Features include:

**Forum** — Share your thoughts and explore the ideas of others. myTetraCell.com provides a forum where users can post comments and questions, and submit links to relevant publications and posters. Bio-Rad will lend support and expertise, and answer questions related to Bio-Rad products as well as general electrophoresis protocols. The Forum is a way to communicate with the rest of the electrophoresis community from your computer.

**Brainbuster** — Test your general trivia knowledge — categories include science, business, current events, and entertainment — and earn points for correct responses. You may be an expert in science, but do you know who Alexander Calder is?

**Photo Gallery** — Share pictures with friends and colleagues. Show off your proudest moments and your creativity. Redeem points for decals to decorate your Mini-PROTEAN cell. The Photo Gallery is a place where you can express yourself to the rest of the community.

**Product Tour** — Are you interested in a Mini-PROTEAN Tetra cell? Take a quick product tour and learn about all the new features in this next-generation electrophoresis cell. The Product Tour is updated periodically with fresh interactive content and videos to help you meet your electrophoresis needs.

**Tetrastore** — The Tetrastore offers a variety of promotional items that you can purchase with points earned throughout the site. Choose from items like T-shirts and sweatshirts, mugs, and business card holders. The Tetrastore has an array of items that are both practical and appealing.

**Promotions** — See Bio-Rad promotions of the month before they are announced to the general public. Try our newest products by signing up to participate in sample programs.

Designed to foster information-sharing and networking among scientists all over the world, myTetraCell.com is your portal to the rest of your scientific community. See for yourself today at [www.myTetraCell.com](http://www.myTetraCell.com).
**Experion™ Starter Kits**

Get your Experion automated electrophoresis system up and running quickly with two new starter kits — one for protein and one for RNA applications. Each kit:

- Includes control sample, reagents, and materials needed for a successful Experion system run
- Provides a detailed step-by-step guide with tips for successful, reproducible results
- Is useful for training new users, troubleshooting, or validating assay performance

**Ordering Information**

<table>
<thead>
<tr>
<th>Catalog #</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>700-7110</td>
<td>Experion Pro260 Starter Kit, includes 3 Experion chips, 1 cleaning chip, Experion reagents, spin filters, IgG protein standard, DTT, cleaning swabs, electrode cleaner, tips and tubes to run 3 chips, ultrapure water, training DVD, instructions</td>
</tr>
<tr>
<td>700-7111</td>
<td>Experion RNA StdSens Starter Kit, includes 3 Experion chips, 2 cleaning chips, Experion reagents, spin filters, total RNA standard, cleaning swabs, electrode cleaner, tips and tubes to run 3 chips, DEPC-treated water, training DVD, instructions</td>
</tr>
</tbody>
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**Purification of Monoclonal and Polyclonal Antibodies Using Preprogrammed Protein A and G Methods on the Profinia™ System**

The Profinia protein purification system now features preprogrammed Protein A and G methods for the affinity purification of monoclonal and polyclonal antibodies from a wide variety of sources, including serum, ascites fluid, and hybridoma cell culture supernatant. The Protein A and G methods feature the ability to affinity purify an antibody followed by an integrated desalting step that automatically exchanges the elution buffer and stabilizes the antibody immediately after elution.

- Automated monoclonal and polyclonal antibody purification
- Immediate integrated desalting to neutralize the antibody after elution
- Large sample volume loading capability to accommodate even dilute monoclonal samples
- Compatibility with 1 or 5 ml protein A or G cartridges from a variety of commercial sources
- Large touch-screen user interface to guide preprogrammed purification system set-up for minimal training and high reproducibility

For a complete list of ordering configurations, refer to the 2008/09 Life Science Research product catalog, or go to [www.bio-rad.com/affinitypurification/](http://www.bio-rad.com/affinitypurification/).

**Ordering Information**

<table>
<thead>
<tr>
<th>Catalog #</th>
<th>Description</th>
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<tr>
<td>620-1010</td>
<td>Profinia Protein Purification System With Native IMAC Starter Kit, 100–240 V, includes cleaning tray, inline filter pack, 2 x 50 ml sample lids, 2 x 15 ml sample lids, bottle starter pack, waste/diluent bottle set, Profinia native IMAC buffer kit, 1 x 1 ml IMAC and 1 x 10 ml desalting cartridge, E. coli lysate, and Profinia software</td>
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<tr>
<td>620-1011</td>
<td>Profinia Protein Purification System With GST Starter Kit, 100–240 V, includes cleaning tray, inline filter pack, 2 x 50 ml sample lids, 2 x 15 ml sample lids, bottle starter pack, waste/diluent bottle set, Profinia GST buffer kit, 1 x 1 ml GST and 1 x 10 ml desalting cartridge, E. coli lysate, glutathione reagent, and Profinia software</td>
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<tr>
<td>732-4600</td>
<td>Bio-Scale Mini Affi-Prep Protein A Cartridges, 5 x 1 ml</td>
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<tr>
<td>732-4602</td>
<td>Bio-Scale Mini Affi-Prep Protein A Cartridge, 1 x 5 ml</td>
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<tr>
<td>732-5304</td>
<td>Bio-Scale Mini Bio-Gel P-6 Desalting Cartridges, 5 x 10 ml</td>
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<tr>
<td>732-5312</td>
<td>Bio-Scale Mini Bio-Gel P-6 Desalting Cartridges, 1 x 50 ml</td>
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</table>
ProteOn™ XPR36 Protein Interaction Array System: Regulatory Tools

ProteOn Manager™ 2.1 Software, Security Edition
ProteOn Manager software is now available with controls to help achieve U.S. FDA 21 CFR Part 11 compliance. Included in the many features of this software release are:

- Audit trails
- Electronic signatures
- Data validation
- User log-ins and permissions
- Closed-system security

ProteOn XPR36 Installation Qualification/Operation Qualification (IQ/OQ) Software
ProteOn XPR36 IQ/OQ software has been designed to test critical system functions to ensure reliability and consistency of system performance. Key features include:

- Wizard-driven software
- Printable electronic reports for document control
- Electronic log of IQ/OQ and test results
- Ready-to-use reagents and sensor chip for testing system performance
- Unattended operation

For more information, go to www.bio-rad.com/proteininteraction/.

Ordering Information
Catalog # Description
176-0210 ProteOn Manager Software, Security Edition
176-0220 ProteOn Manager Software Upgrade, upgrades to version 2.1
176-4200 ProteOn XPR36 IQ/OQ Software
176-4220 ProteOn Operation Qualification Kit

xMark™ Microplate Absorbance Spectrophotometer
xMark microplate absorbance spectrophotometer, a new addition to Bio-Rad absorbance microplate systems, combines precision and versatility of absorbance detection with high throughput capabilities of microplate assays.

- Broad wavelength range of 200–1000 nm allows versatile detection of absorbance peaks, from DNA in the UV to water in the infrared spectral range
- Spectral scanning feature precisely determines the absorbance properties of the contents in each well
- Compatible with all formats, from 6- to 1,536-well plates, eliminating restrictions on assay configuration, sample type, volume, or throughput
- Able to create absorbance intensity maps for a single well or an entire plate, providing higher content data and additional control over microplate assay development

xMark microplate spectrophotometer expands the limits of microplate reading with remarkable flexibility, robustness and precision, making this absorbance detection system an ideal platform for:

- Efficient development of highly reproducible microplate assays, such as ELISA, cell viability/proliferation, and various biochemical activity assays
- A wide range of applications, including biomarker discovery and quality and purity assessment

Ordering Information
Catalog # Description
168-1150 xMark Microplate Absorbance Spectrophotometer, PC or Mac, includes incubator, Microplate Manager software, USB2 and power cables
Preprogrammed Protein A and G Purification of Antibodies on the Profinia™ Protein Purification System

Antibody purification is a common task in modern biological research and biotechnology. Reasons to purify antibodies include the need to concentrate the antibody for downstream assays (such as ELISAs), to improve signal to noise for western blots and other in vitro assays, and to further study the characteristics of the antibody.

The most recent addition to the preprogrammed methods available on the Profinia protein purification system is protein A and G chromatography with integrated desalting. The preprogrammed Protein A and G methods allow purification of antibodies with high purity and yield. With an affinity plus desalting method, purified antibodies are immediately exchanged from the acidic elution buffer and placed into a neutral pH buffer suitable for antibody storage. This can greatly reduce antibody oxidation and instability after purification.

The Profinia system with Protein A and G methods can be used to purify monoclonal antibodies, polyclonal antibodies, and, theoretically, any protein fused to an Fc region. In addition to antibody isolation from human serum, the Protein A and G methods on the Profinia system have been tested for isolation of antibody from rabbit, mouse, rat, and goat sera, and monoclonal antibody from hybridoma cell culture (see bulletin 5726). Figure 1 shows a typical profile of a polyclonal antibody purified from mouse serum on the Profinia system with desalting using the Bio-Rad Affi-Prep® protein A cartridge.

The Profinia system can accommodate other manufacturers’ media and cartridges such as GE Healthcare’s protein A and protein G cartridges. Figure 2 demonstrates the purification profile of IgG from different sera using GE Healthcare’s HiTrap protein G HP cartridge.

Preprogrammed Protein A and G methods on the Profinia system add a powerful set of tools for purification of polyclonal and monoclonal antibodies. The automated system provides fast, easy-to-use, and reproducible purifications. Importantly, the integrated desalting step neutralizes the antibody immediately after it is eluted, and delivers stable and purified antibody that is ready for downstream experiments.

Fig. 1. Protein A plus desalting purification from mouse serum using the Profinia system. A, chromatograms showing different fractions; B, SDS-PAGE of protein A column fractions. Lane M, molecular mass standards; L, load; F, flowthrough; W, wash; E, elution; C, Experion™ system analysis of the purified protein A column fraction for mouse. The electropherogram is an overlay from three independent purifications. L, light chain; H, heavy chain.

Fig. 2. Analysis of purified antibodies from different sera using the Experton™ system. Antibody samples were purified on the Profinia system with a GE Healthcare protein G cartridge. Left panel, alignment of electropherograms of purified antibodies from different sera. L, light chain; H, heavy chain. Right panel, simulated gel view of the purified antibody samples. Lane L, ladder; 1 and 2, human serum; 3 and 4, rabbit serum; 5 and 6, mouse serum; 7 and 8, goat serum; 9 and 10, rat serum. Right panel lane numbers correlate with left panel sample numbers.
The regulatory guidelines set forth by the FDA are of the utmost importance to the safety of our food and drug manufacturing processes. Adherence to these regulations requires procedural (notification, training, standard operating procedures), administrative, and technical (software-related) controls. These controls support the good practices rulings observed within the pharmaceutical industry. Collectively known as GxP, these are: Good Laboratory Practice (GLP), Good Automated Manufacturing Practice (GAMP), Good Manufacturing Practice (GMP), and Good Clinical Practice (GCP).


ProteOn Manager Software, Security Edition

Over the years, paper records delivered to the U.S. FDA by the truckload have given way to electronic data sources. This led to the introduction of the 21 CFR Part 11 regulations in 1997, which detail how to manage electronic records for internal and external audits and submissions to the FDA.

The FDA defines a “closed system” as a private network managed by individual organizations. The operation and maintenance of the system is controlled by personnel working within the user organization, and is usually governed by strict standard operating procedures. Therefore, all data, result, and protocol files generated by ProteOn Manager software, along with the audit trail and instrument log database, are considered electronic records generated in a closed system environment. The following controls are built into ProteOn Manager software to assist with 21 CFR Part 11 regulatory compliance:

- **Electronic signatures** — reviewers and approvers can digitally sign records. The name, date, time, reviewer/approver status, and reason (user comments) are associated with each signature and logged by the software. Once a file has been approved, it is reflected in the software, and changes can only be saved as a new file name or revision. The software will automatically update the file name to reflect a new revision. All electronic signatures require a username and password

- **Audit trail** — all auditable changes are recorded, including the date and time, originator of the record, and other related information. The audit trail cannot be changed or deleted by the user

- **Data validation** — accuracy of electronic copies is confirmed using a secure checksum to detect invalid or altered records

- **Identification codes and passwords** — the system administrator must set up unique user identification codes for each individual. User identification codes cannot be reused or reassigned to others. The Windows operating system ensures that all active user identification codes are unique and that all identification code and password combinations are unique

- **Device check** — the software records the identity (serial number) of the ProteOn instrument that it is controlling

- **System access and authority** — access rights are based on those assigned within the Windows domain/workstation user database. ProteOn Manager software, Security Edition uses the Windows operating system security feature to authenticate users and retrieve access levels via group membership. User permissions will determine access to the software functions

- **Generation of copies** — accurate and complete copies of the data can be generated within ProteOn Manager software (using the Save As feature) and accessed later for inspection and review. The application also enables export of electronic records to ASCII (tab delimited) or XML (secure) file formats

ProteOn XPR36 Installation Qualification/Operation Qualification (IQ/OQ) Software

Under good practices rulings, all devices must meet installation and performance standards. After installation, all systems must be validated on a regular basis to ensure performance to manufacturer specifications. ProteOn XPR36 IQ/OQ software assists compliance with these rulings. The software comes with the ProteOn XPR36 IQ/OQ software wizard, OQ control reagents and sensor chip, and a user manual.

The IQ/OQ software provides an IQ protocol to verify the delivery of all system components, including electronic verification of system firmware and software. The IQ protocol verifies system operation within a series of defined tests and validation parameters. Electronic logs and printable PDF reports of the instrument’s IQ and OQ are provided to meet internal documentation requirements. This kit allows unattended qualification protocols to ensure proper system installation and performance.

These new regulatory tools complement one of the key advantages of using the ProteOn XPR36 protein interaction array system in the drug discovery and development workflow: increased data collection efficiency by reducing the time required for these procedures from days to hours.
Experion™ Automated Electrophoresis System: Enhancing Sensitivity in SDS-Protein Electrophoresis

Tim Wehr1, William Strong2, Shin Adachi2, and Aran Paulus1,
1 Bio-Rad Laboratories, Inc., Hercules, CA 94547 USA
2 Bio-Rad Laboratories KK, Tokyo, Japan

Introduction
The development, manufacture, and quality control of protein therapeutics require state-of-the-art analytical techniques to elucidate protein structure and to assure product purity, homogeneity, and stability (Ma and Nashabeh 2001). SDS-PAGE has been the benchmark methodology for monitoring impurities and assessing consistency in the manufacture of biologics, with silver-staining techniques required to detect minor impurities. However, SDS-PAGE is time consuming and requires the use of toxic reagents. An automated and quantitative alternative to SDS-PAGE has long been desired. The advent of capillary electrophoresis (CE) provided a solution (Wehr et al. 1998). The entangled polymer sieving systems used in CE exhibit separation power similar to that of SDS-PAGE, and automated injection, on-tube detection, and peak integration permit unattended analysis of multiple samples. However, the UV absorbance detectors employed in most commercial CE systems have insufficient sensitivity for detection of low-level impurities. By prelabeling proteins with a fluorescent tag and using laser-induced fluorescence (LIF) detection, CE can achieve sensitivity comparable to silver stains, and this method is currently in use in the biopharmaceutical industry (Hunt and Nashabeh 1999).

A drawback of both CE-UV and CE-LIF methods for separation of SDS-proteins is the requirement for extensive capillary preparation before each injection, which increases analysis time and reduces throughput. Automated microscale electrophoresis systems provide a rapid and convenient alternative to conventional CE instruments for the separation of SDS-proteins. The Experion automated electrophoresis system, for example, is a chip-based separation system that can process ten samples in 30 minutes. The protein mass range covered by the system is between 10 and 260 kD, and the resolution is similar to that of a 4–20% gradient gel. The system employs a dynamic staining-destaining chemistry with fluorescence detection that provides sensitivity comparable to colloidal Coomassie staining. To enhance the sensitivity of the Experion system for detection of low-level impurities in preparations of biotechnology products, a method for prelabeling proteins with a fluorescent dye was investigated. The effect of dye:protein molar ratio was also studied. The method also incorporates modifications in the Experion system sample preparation procedure to enhance injection efficiency, and a modification in the Experion gel-staining procedure to reduce background fluorescence of the Experion Pro260 stain. The method uses the Experion system with no hardware or software changes, and employs the Experion Pro260 chip and components of the Experion Pro260 analysis kit.

Methods
Protein Labeling
Prior to labeling, samples were buffer exchanged into labeling buffer (0.1 M sodium bicarbonate) using Micro Bio-Spin™ 6 columns equilibrated with 0.1 M sodium bicarbonate. Proteins were labeled with Alexa Fluor 647 carboxylic acid, succinimidyl ester dye (Invitrogen Corporation), which has an excitation wavelength that closely matches the Experion laser wavelength. A stock solution of dye was prepared in dimethyl sulfoxide (DMSO) at a concentration of 1.4 mg/ml, and a dye working solution was prepared by diluting the stock solution to 350 µg/ml in DMSO. BSA or porcine IgG (Sigma-Aldrich) were used as protein standards, and the final protein content of antibody samples was estimated by interpolating absorbance at 280 nm against a standard curve prepared with BSA. These standards were prepared in labeling buffer. Proteins were mixed with aliquots of dye stock or working solution to provide a dye:protein molar ratio of 1:1, 5:1, 10:1, or 100:1. Dye-protein solutions were incubated overnight at 4°C.

Sample Preparation
Prelabeled protein samples were mixed with an equal volume of Laemmli sample buffer with 5% β-mercaptoethanol and heated for 5–7 min at 95°C. Samples were then buffer exchanged into 10 mM Tris-HCl (pH 7.4) using Micro Bio-Spin 6 columns. Sample buffer was prepared by diluting Experion sample buffer containing 0.03% β-mercaptoethanol 10-fold with distilled water. For analysis, 4.5 µl of diluted sample buffer was mixed with 9 µl of prelabeled sample and heated at 95°C for 5 min. Unlabeled protein samples and the Experion Pro260 ladder were prepared according to the Experion Pro260 analysis kit instructions. Briefly, 4 µl of ladder or sample was mixed with 2 µl of Experion sample buffer containing 0.03% β-mercaptoethanol, heated at 95°C for 5 min, and then diluted with 84 µl of deionized water.

Preparation of Diluted Gel Stain
For experiments using diluted gel stain, the reagent was prepared by adding 2 µl of Experion stain and 18 µl of 6.75% SDS (w/v in DMSO) to one tube of Experion Pro260 gel. The diluted gel-stain mixture was vortexed and filtered according to the Experion Pro260 analysis kit protocol.
Experion System Electrophoresis and Analysis

Priming of the Experion Pro260 chip with gel stain and loading of the chip with gel stain, samples, and ladder were performed according to the Experion system instructions. Electrophoresis and analysis were performed using the Experion Pro260 analysis kit protocol.

Results and Discussion

Comparison of Experion System Analysis of Prelabeled and Unlabeled Proteins

To determine the sensitivity enhancement obtained by prelabeling proteins with Alexa Fluor 647, unlabeled and prelabeled samples of BSA and porcine IgG were prepared over an approximately 1,000-fold concentration range using 2-fold serial dilutions. Both proteins were prelabeled using a dye:protein molar ratio of 1:1. Sensitivity was measured as the slope of the corrected peak area vs. concentration plot. For BSA, sensitivity enhancement was 100-fold (Figure 1). For IgG, sensitivity was calculated using the heavy chain peak, and was found to be enhanced 27-fold relative to unlabeled IgG heavy chain (Figure 2). An overlay of the electropherograms of the prelabeled and unlabeled IgG samples is shown in Figure 3. The higher sensitivity gain for BSA versus IgG heavy chain may reflect differences in dye incorporation due to the higher lysine content in BSA.

Effect of Dye:Protein Molar Ratio on Sensitivity and Peak Width

To determine the effect of dye:protein molar ratio on sensitivity and peak width, BSA was prelabeled with varying amounts of Alexa Fluor 647 and analyzed on the Experion system. Results demonstrate that a more than 500-fold increase in sensitivity can be achieved at a dye:protein molar ratio of 1:100 (Table 1). However, significant peak broadening is observed using ratios greater than 1:1. In applications where minor contaminants are poorly resolved from major components, the loss in resolution could offset the gain in sensitivity.

Table 1. Effect of dye:protein molar ratio on peak width for prelabeled BSA.

<table>
<thead>
<tr>
<th>Dye:Protein Molar Ratio</th>
<th>Protein Concentration (µg/ml)</th>
<th>Sensitivity Enhancement Relative to Unlabeled BSA</th>
<th>Peak Width at Half Height (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10</td>
<td>7.4</td>
<td>99x</td>
<td>1.30</td>
</tr>
<tr>
<td>1:100</td>
<td>6.8</td>
<td>539x</td>
<td>2.09</td>
</tr>
</tbody>
</table>

Contribution of Sample Preparation Procedure to Sensitivity Enhancement

The amount of protein sample introduced into the separation channel by electrokinetic injection depends on the concentration of the sample, as well as on the stacking effects due to differences in ionic strength between the sample and the gel. When using the Experion system, the conventional sample preparation method requires dilution of 4 µl of the protein sample and 2 µl of Experion buffer in 84 µl of water prior to loading into the gel, which results in a 22.5-fold dilution of the protein sample and a 45-fold dilution of the buffer’s ionic strength. With the high-sensitivity sample preparation method, the protein sample is first diluted 2-fold (prior to the buffer exchange step using a Micro Bio-Spin 6 column) and then 9 µl of the diluted protein sample is mixed with 4.5 µl of Experion buffer that has been diluted 10-fold with water. This results in an overall 3-fold dilution of the protein sample and a 30-fold dilution of the buffer’s ionic strength prior to loading.

Consequently, with the high-sensitivity method, the protein sample is 7.5-fold more concentrated than with the conventional method. Because the ionic strength of the sample is 1.5-fold higher with the high-sensitivity method, its loading efficiency is reduced proportionally. Taking into account these two factors, the high-sensitivity method should allow loading of five times more protein into the Experion system than the conventional method.
To investigate this, IgG was prepared using the two procedures without addition of the Alexa Fluor dye. The results indicate that the labeling sample preparation procedure contributes a 4.7-fold increase in sensitivity (data not shown).

Effect of Gel Stain Dilution on Sensitivity
The fluorescent dye in the Experion gel-stain mixture serves two purposes. At the beginning of an Experion system run, the signal from the dye is used to autofocus the Experion instrument optics. During analysis, the dye is part of a dynamic protein detection system. The dye (stain) partitions into SDS micelles, which complex with proteins in the sample, and dye fluorescence intensity is enhanced in the hydrophobic interior of the micelle. Reduction of background fluorescence from the stain is accomplished by introducing stain-free medium by electromigration into the region between the separation segment and the detection segment of the channel. This forms an SDS-free region adjacent to the migrating sample bands. Diffusion of free SDS into this region reduces the detergent concentration below the critical micelle concentration (CMC), releasing dye from unbound micelles. This reduces background fluorescence (the destaining process). However, some residual fluorescence from the free dye remains.

With the Experion system analysis of prelabeled proteins, the stain is no longer needed for sample detection, but is required for the focusing step, detection of the Experion ladder, and detection of the upper alignment marker. The drawback is that its presence in the gel contributes to background fluorescence. To reduce this background, a reformulated gel stain was prepared in which the stain concentration was reduced 10-fold, yet still allowed for instrument autofocusing. The noise reduction of the diluted gel stain caused an additional 3-fold increase in sensitivity (data not shown).

The contributions of prelabeling, the sample preparation procedure, and Experion stain dilution are summarized in Table 2.

Table 2. Summary of sensitivity enhancements using fluorescent prelabeled IgG.

<table>
<thead>
<tr>
<th>Method</th>
<th>Sensitivity Enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experion sample preparation without prelabeling</td>
<td>1x</td>
</tr>
<tr>
<td>Modified sample preparation without prelabeling</td>
<td>4.7x</td>
</tr>
<tr>
<td>Modified sample preparation with prelabeling</td>
<td>27x</td>
</tr>
<tr>
<td>Modified sample preparation with prelabeling and Experion stain dilution</td>
<td>80x</td>
</tr>
</tbody>
</table>

Application to Monitoring Monoclonal Antibody Purification
SDS-PAGE, CE-UV, and CE-LIF are all currently used as analytical tools in the development and manufacture of monoclonal antibodies. These techniques are used in product and process development, and in determination of product purity and lot-to-lot consistency (Hunt and Nashabeh 1999, Ma and Nashabeh 2001). Of particular importance is the use of silver staining in SDS-PAGE and CE-LIF for the detection of low-level impurities that may be product-related (mass variants, proteolytic fragments) or nonproduct-related (host cell impurities, environmental contaminants). Therefore, it was of interest to investigate the use of the Experion system with prelabeling for characterization of monoclonal antibodies. The electropherogram shown in Figure 4 compares the level of monoclonal antibody in a crude cell culture filtrate and following purification. The relative intensities of light and heavy chain peaks demonstrate a 3.5-fold enrichment of antibody after purification, and the use of the prelabeling technique allowed visualization of contaminants such as those migrating at 40 and 44 seconds.

Conclusions
Prelabeling proteins with the fluorescent tag Alexa Fluor 647 prior to analysis with the Experion system can provide an 80- to 500-fold enhancement in sensitivity compared to the conventional Experion procedure. Sensitivity enhancement arises from the increased fluorescence of the tagged proteins combined with a modified sample preparation procedure. Total sensitivity gain depends on the dye:protein molar ratio, and is a tradeoff between increased signal and peak broadening. The prelabeling method requires no changes to the Experion instrument hardware or software, and uses the reagents from the Experion Pro260 analysis kit with the addition of a simple dilution of the sample preparation buffer and stain. This procedure extends the detection capability of the Experion system to trace components in biotechnology products such as contaminants in monoclonal antibody preparations.

References

Acknowledgement
We gratefully acknowledge Takayuki Yoshimori from Cyugai Pharmaceutical Co. Ltd., in Tokyo, Japan, for contributions toward the final protocol.

For an expanded version of this article, request bulletin 5719.
Measurement of Low-Volume DNA Samples Using the Hellma TrayCell Fiber-Optic Device With the SmartSpec™ Plus Spectrophotometer

Camille Diges1, Gene Friedmann2, and Rhonda Henshall-Powell1,
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2 Hellma USA, Inc., Plainview, NY 11803 USA

Introduction
UV spectrophotometry is a widely used method for determining the concentration and purity of nucleic acid samples in the laboratory. Traditionally, large sample volumes (on the order of 50–100 µl) have been required to produce accurate measurements. This has been a drawback to the method since precious experimental RNA and DNA samples are often obtained only in low volumes. The recent introduction of spectrophotometers that have the ability to measure sample concentrations using low volumes has alleviated this problem, but at the cost of purchasing an expensive instrument.

The Hellma TrayCell fiber-optic device (Hellma USA, Inc.) can be used to analyze small volumes of biological samples, such as proteins and nucleic acids, on a standard spectrophotometer. The Hellma TrayCell device uses integrated beam deflection and fiber-optic cables to measure the sample directly on the surface of its optical window (Figure 1) (www.traycell.com). The Hellma TrayCell device comes with two caps, each providing a well-defined optical path (either 1 or 0.2 mm). The caps ensure reproducible measurements of samples because evaporation is minimized. They also enable the measurement of low-surface-tension samples that may otherwise be difficult to measure using small volumes. The 1 mm cap is optimized for low-concentration biological samples (<850 ng/µl) and requires 3–5 µl of sample. The 0.2 mm cap is designed for higher-concentration samples (up to 4,250 ng/µl), and uses as little as 0.7 µl of sample to obtain a measurement. The dynamic range of the Hellma TrayCell device for double-stranded DNA (dsDNA) is reported to be 25–4,250 ng/µl; however, the actual dynamic range may vary depending on the spectrophotometer being used.

The Bio-Rad SmartSpec™ Plus spectrophotometer is an accurate and dependable UV/visible scanning benchtop spectrophotometer with an accessible user interface that instantly calculates sample concentrations and nucleic acid purity. In its standard configuration, the SmartSpec Plus spectrophotometer requires that samples be diluted to 50–100 ng/µl and a minimum sample volume of 50 µl is used with trUView™ cuvettes, resulting in accurate and precise photometry within its linear range. Coupling the SmartSpec Plus spectrophotometer with the Hellma TrayCell fiber-optic device combines the precision and accuracy of the instrument with the convenience of using low sample volumes for measuring DNA and RNA concentrations.

![Fig. 1. Schematic representation of the Hellma TrayCell device. A, loading the device; B, device in the SmartSpec Plus spectrophotometer.](image-url)
In this study, we determined the dynamic range of dsDNA concentration measurements using the Hellma TrayCell device with the SmartSpec Plus spectrophotometer. The results were compared to the same samples analyzed using either the SmartSpec Plus spectrophotometer with trUView cuvettes and 50 µl sample, or a competitor spectrophotometer that requires only 1 µl of sample and has a reported dynamic range of 2–3,700 ng/µl for dsDNA. We found that the Hellma TrayCell device increased the linear dynamic range of the SmartSpec Plus spectrophotometer to 25–3,000 ng/µl. The competitor spectrophotometer is limited to use with low-volume (1–2 µl) samples. Adding the Hellma TrayCell device to the SmartSpec Plus spectrophotometer adds low-volume capacity to the system, expanding the accurate measurement range to 1–50 µl of sample.

**Methods**

To determine the dynamic range of the Hellma TrayCell device, we made a dilution series of sheared human genomic DNA (Sigma-Aldrich) with calculated concentrations from 4,000 ng/µl down to 3.125 ng/µl. Absorbance at 260 and 280 nm was measured ten times for each dilution using either the SmartSpec Plus spectrophotometer or the competitor spectrophotometer. All readings using the Hellma TrayCell device were performed with 3 µl of sample, and both the 1 and 0.2 mm caps were used for each point in the dilution series. For comparison, 1 µl of each dsDNA dilution was measured on the competitor spectrophotometer.

As a positive control, 50 µl of each dsDNA dilution was measured with trUView cuvettes (standard cuvettes with a 1 cm pathlength) in the SmartSpec Plus spectrophotometer. For the sample set, dsDNA samples with calculated concentrations >100 ng/µl were further diluted to ensure that samples were within the linear range of the SmartSpec Plus spectrophotometer. The measured concentration was then multiplied by the appropriate dilution factor to obtain the actual concentration of the sample. This procedure also served to confirm the accuracy of the calculated concentrations of the dsDNA dilution series.

Data were analyzed by comparing the calculated DNA concentration to the measured concentration for each dilution and instrument/fiber-optic device combination tested. The average concentration and percent coefficients of variation (%CV) for each set of measurements were calculated and used to determine the precision and dynamic range of each tested instrument/fiber-optic device combination.

**Results**

Both the Hellma TrayCell device and the competitor spectrophotometer were able to accurately measure dsDNA concentrations over a wide range without requiring dilution of the original sample (Table 1). We found that the Hellma TrayCell device extended the dynamic range of the SmartSpec Plus spectrophotometer from 50–100 ng/µl to 25–3,000 ng/µl of

<table>
<thead>
<tr>
<th>Concentration of dsDNA (ng/µl) Based on Serial Dilutions</th>
<th>SmartSpec Plus Spectrophotometer With Hellma TrayCell</th>
<th>Competitor Spectrophotometer</th>
<th>SmartSpec Plus Spectrophotometer With trUView Cuvettes</th>
</tr>
</thead>
<tbody>
<tr>
<td>dsDNA, ng/µl</td>
<td>%CV</td>
<td>dsDNA, ng/µl</td>
<td>%CV</td>
</tr>
<tr>
<td>----------------------------------------------------------</td>
<td>-----------------------------------------------------</td>
<td>------------------------------</td>
<td>--------------------------------------------------------</td>
</tr>
<tr>
<td>4,000</td>
<td>3,327.95</td>
<td>2,894.36</td>
<td>0.32</td>
</tr>
<tr>
<td>3,000</td>
<td>2,666.55</td>
<td>2,230.76</td>
<td>0.79</td>
</tr>
<tr>
<td>2,000</td>
<td>1,848.36</td>
<td>1,515.91</td>
<td>0.78</td>
</tr>
<tr>
<td>1,000</td>
<td>903.30</td>
<td>771.22</td>
<td>0.41</td>
</tr>
<tr>
<td>500</td>
<td>449.51</td>
<td>411.89</td>
<td>1.61</td>
</tr>
<tr>
<td>100</td>
<td>88.91</td>
<td>95.91</td>
<td>0.83</td>
</tr>
<tr>
<td>50</td>
<td>42.62</td>
<td>49.31</td>
<td>1.37</td>
</tr>
<tr>
<td>25</td>
<td>22.25</td>
<td>24.71</td>
<td>1.22</td>
</tr>
<tr>
<td>12.5</td>
<td>7.94</td>
<td>11.99</td>
<td>3.70</td>
</tr>
<tr>
<td>6.25</td>
<td>—</td>
<td>5.84</td>
<td>4.71</td>
</tr>
<tr>
<td>3.125</td>
<td>—</td>
<td>2.96</td>
<td>14.17</td>
</tr>
</tbody>
</table>

* The 1 mm cap of the Helma TrayCell device was used to measure dsDNA concentrations between 3.125–500 ng/µl, while the 0.2 mm cap was used to measure the 1,000–4,000 ng/µl samples.

** n = 10.
dsDNA. The range of the Hellma TrayCell device was comparable to that of the competitor spectrophotometer, which was 3.125–1,000 ng/µl in our study.

Data collected using the SmartSpec Plus spectrophotometer with trUView cuvettes served as a positive control in these experiments. Samples measured using this traditional method had to be diluted to <100 ng/µl to be within the linear range of the photometer. Therefore, if the calculated concentrations were accurate, then plotting the measured versus the calculated concentration of dsDNA would give a slope of 1.0. The actual slope was 1.05 (Figure 2), indicating that the calculated dsDNA concentrations were accurate and could be used as the reference concentrations for the data analysis. The Hellma TrayCell device with the SmartSpec Plus spectrophotometer outperformed the competitor spectrophotometer for accuracy and had a slope of 0.86. The competitor spectrophotometer had the poorest correlation between measured and calculated DNA concentrations with a slope of 0.73.

The Hellma TrayCell device accurately measured up to 3,000 ng/µl of dsDNA, while the competitor spectrophotometer was inaccurate above 1,000 ng/µl of dsDNA (Table 1). The Hellma TrayCell device was accurate when measuring as little as 25 ng/µl dsDNA; however, the %CV at this lower concentration was 6.49%. The competitor spectrophotometer was the most accurate for low concentrations of dsDNA, giving accurate readings at 3.125 ng/µl with a %CV <5% for concentrations as low as 6.25 ng/µl. It should be noted that even though the SmartSpec Plus spectrophotometer with standard cuvettes had only a small dynamic range compared to the other conditions tested, it had the highest precision with a %CV <1.2% in all cases.

Conclusions
The Hellma TrayCell fiber-optic device is designed for measuring low-volume samples and extending the dynamic range of traditional spectrophotometers. Since the Hellma TrayCell device is compatible with traditional cuvette-style spectrophotometers, the scientist retains the flexibility to perform cell density, colormetric, and simple kinetic assays that require a cuvette, while gaining the additional ability to perform low-volume concentration measurements. We tested the ability of the SmartSpec Plus spectrophotometer to measure a wide range of dsDNA concentrations when combined with the Hellma TrayCell device. We found that only 3 µl of sample are needed to accurately measure dsDNA concentrations ranging from 25–3,000 ng/µl. The Hellma TrayCell device is extremely user-friendly — it is compatible with standard spectrophotometers, does not have to be removed from the instrument between readings, and requires only a few seconds to clean between samples. This fiber-optic device is an excellent choice for measuring low-volume nucleic acids in conjunction with the SmartSpec Plus spectrophotometer, without the need for a costly new instrument.
SECOND-GENERATION MULTIPLEX IMMUNOASSAYS

xMAP Technology and Magnetic Appeal Put Multiplexing in the Fast Lane

Authors: Emily Dale, Bill Gette, Efthalia Gerasimopoulos, Jessica Dines, and Todd Yeck
For almost 50 years, immunoassays have allowed sensitive and highly specific detection of analytes of interest in biological samples for use both in life science research and clinical diagnostics. Immunoassays provide information to researchers on the roles proteins and other biomolecules play in a myriad of biological processes, thereby providing insight to clinicians on the identification and assessment of disease progression.

**Early Immunoassays**

The first immunoassay was developed by Yalow and Berson (1960), who received the Nobel Prize for their efforts to measure insulin levels. These initial assays used radiolabels for detection. The radioimmunoassay (RIA) would remain the standard for detection of bioanalytes for more than ten years because of its extraordinary sensitivity, in spite of the health risks and disposal issues posed by the use of radioisotopes.

The search for a suitable alternative to the RIA led to the development of the enzyme-linked immunosorbent assay (ELISA) in the early 1970s (Engvall and Perlmann 1971, Van Weeman and Schuurs 1971). The ELISA uses an enzymatic reaction as the basis of detection, rather than a radioactive signal. While early versions did not rival the sensitivity of the RIA, the development of highly specific monoclonal antibodies and chemiluminescence detection resulted in ELISA assays with sensitivity that exceeds that of radiolabels.

Today, key advantages of ELISA are its ease of use, flexibility, and low cost. The impact of immunoassays on life science research and clinical diagnostics has been enormous, with almost 10,000 studies published per year that include the terms “enzyme immunoassay” and “enzyme-linked immunoassay” (Lequin 2005).

**Fit-for-Purpose Assays**

The growth of proteomics and genomic analysis is driving the need to discover and monitor large numbers of biomarkers indicative of human disease states. The output of the Human Genome project, for example, provides the ability to simultaneously monitor the roles of multiple genes during investigations of complex biological systems.

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**The Bio-Plex® Suspension Array System**

Suspension bead arrays provide the largest multiplexing capability for immunoassays. With xMAP bead technology, ~50 different target proteins (the theoretical limit is 100) can be simultaneously detected and quantitated in one sample. Following incubation, the sample in each well of a 96-well plate is read by the flow-based xMAP fluorescent reader in the Bio-Plex system. This platform offers not only the highest capability, but also the greatest flexibility in multiplexing according to the user’s needs. It is easy to use, inexpensive to run per analyte tested, and highly sensitive.

The Bio-Plex system is the most widely cited suspension array platform, with research applications in Alzheimer’s and Parkinson’s diseases, diabetes, obesity, cancer, asthma, cystic fibrosis, autoimmune diseases, viral infections, and vaccine development.

A wide array of assays are available, including those for the study of:

- Inflammation
- Signal transduction
- Diabetes
- Angiogenesis
- Acute phase response
- Isotyping

With the addition of the Bio-Plex Pro™ wash station, the Bio-Plex suspension array system represents an integrated solution for scientists performing high-throughput multiplex immunoassays.
Recent reviews have described the power of biomarkers in the drug discovery and clinical diagnostic development processes, while also emphasizing the need to ensure that the assay is fit-for-purpose. In other words, the assay must be proven reliable for its intended use (Allinson and Brooks 2004, Lee et al. 2005, Lee et al. 2006). Thoughtful consideration must be paid to the desired goals of the experiment. When the measurement of multiple biomarkers is needed, the choice of appropriate technology typically requires striking a balance between precision, sensitivity, sample throughput, multiplexing ability, and cost. On the low-cost, low-multiplexing end of the spectrum, quantitative PCR, ELISA, and western blotting allow up to five markers to be measured simultaneously and quantitatively. On the high-cost, high-multiplexing end of the spectrum, “-omics” technologies such as microarrays, SELDI, LC/MS, and 2-D gel electrophoresis allow measurement of several hundred potential markers, but the output is essentially qualitative. The Bio-Plex platform sits in the middle of this spectrum: up to 100 markers can be measured simultaneously, while the quantitative assay performance and cost per analyte are equal to or are better than those of the low-multiplexing technologies (Figure 1).

**Bio-Plex Assays: More Data Then — Increased Accessibility and Consistency Now**

When first introduced, Bio-Plex assays accelerated research for many laboratories by generating more data points per sample in a 96-well format than other technologies could deliver. The recent introduction of the Bio-Plex Pro wash station takes this throughput to a new level of ease and accuracy by eliminating the need for additional training and producing consistent results every time an experiment is run. With this new generation of technology, if you can perform an ELISA experiment, you can run a Bio-Plex assay. Rather than perform manual wash steps after each incubation (left), the researcher simply places the 96-well plate in the wash station (right) and starts the preprogrammed wash protocol.

**Fig. 1. Multiplex analysis and fit-for-purpose assays.**

<table>
<thead>
<tr>
<th>Type of Assay</th>
<th>Sample Required</th>
<th>Data Generated</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>10–100,000s of sample</td>
<td>Single parameter</td>
</tr>
<tr>
<td>Multiplex Assays</td>
<td>10–10,000s of samples</td>
<td>5–100 parameters</td>
</tr>
<tr>
<td>SELDI, LC/MS, Microarrays</td>
<td>1–10s of samples</td>
<td>1,000s of parameters</td>
</tr>
</tbody>
</table>

Completion time varies per user.
When biomarker assays are performed, it is the responsibility of the researcher to confirm that the performance of each assay is valid for its intended use in each study. Questions to assess include: Is the assay sufficiently sensitive, accurate, and precise? Does the working assay range (the lower and upper limits of quantitation) cover the desired concentration range? Are the sample dilutions required during sample preparation appropriate for the expected analyte concentrations in the samples?

Commercial developers of multiplex immunoassays therefore have two major challenges: that the assays are fit-for-purpose for the vast majority of researchers, and that the assays are fit-for-purpose in multiplex formats with different matrices (that is, with acceptably low levels of nonspecific cross-reactivity and matrix effects). Bio-Plex assays are developed using a rigorous validation process to meet these requirements.

**Evolution of Multiplex Immunoassays: Automation and Magnetic Beads**

While suspension bead arrays offer high multiplexing capability, full automation of this platform, as is attainable with traditional ELISA platforms, has been limited by the need to wash and retain the beads in each well of the microplate. This requires filter bottom plates and a vacuum washing system. However, Bio-Rad has overcome this limitation through the use of magnetic beads.

As early as the mid-1990s, Bio-Rad began looking for an alternative to washing beads by filtration for application to flow cytometry-based diagnostic methods. There had been a few publications demonstrating that polystyrene beads along with a flow cytometer could be adapted to perform the simultaneous analysis on multiple proteins in a solution. This method requires a separation step in which the sample (for example, human serum) is washed from the polystyrene beads by filtration. However, vacuum filtration can introduce variability into experimental data as a result of debris in the sample, filter clogging, bead overdrying, and cross-well reactivity due to wicking. Variability is also influenced by the experience level of the user.

In 1996, Bio-Rad researchers theorized that paramagnetic beads — polystyrene beads with an underlayer of magnetite — could replace conventional polystyrene beads for use in these assays. They determined that this would enable replacement of the filtration step by magnetic separation, a key step in automating the performance of protein measurements. When the beads are placed in a magnetic field, they are immobilized, which allows the liquid (and debris) to be removed by aspiration, leaving the analyte, which is attached to the beads, to be measured. A patent for performing immunoassays on magnetic beads was granted to Bio-Rad in 2001.

After adjusting the chemistry of these magnetic beads and the polymers used in the process, Bio-Rad researchers successfully developed a highly effective method for flow cytometric-based immunoassays, which can be automated. However, there remained the problem of measurement using the flow cytometer, an expensive, unreliable, and complex instrument. What was needed was an easy-to-use, reliable, and automated method of
testing. In 1997, Bio-Rad became aware of xMAP technology, which included a flow cytometer dedicated to the performance of multiplex bead-based immunoassays. Subsequently, Bio-Rad licensed xMAP technology for use in both life science research and clinical diagnostic applications (see the sidebar timeline and sidebar interview with Michael Barcellos). The first Bio-Plex system launched by Bio-Rad was intended for research applications, and did not incorporate magnetic beads or automation. However, many higher throughput immunoassay laboratories involved in drug development and clinical research require automation. Additionally, many automated systems utilize magnetic properties to automate sample preparation. Automating sample testing using Bio-Plex magnetic beads on a robotic sample preparation system minimizes hands-on technician time, improves precision, and streamlines workflow. Five multiplex assay panels based on magnetic beads (see timeline sidebar) are now available from Bio-Rad, and all future assays will be magnetic enabled. Bio-Plex Pro magnetic assays were developed using 25-bead map, and validated using 100-bead map xMAP technology (Figure 2), so these assays can be used by other xMAP software packages.

To facilitate automation with research assays on the Bio-Plex 200 system, Bio-Rad now offers an automated wash station (Bio-Plex Pro wash station) that can be used with both polystyrene and magnetic beads. Magnetic assays combined with the magnetic wash station help provide consistent results, regardless of user familiarity with the system and its workflow (Table 1). The addition of the magnetic wash station to the system also enhances its ease of use, making the multiplex assay workflow as simple as an ELISA. The combination of

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Table 1. Validation of the Bio-Plex Pro wash station using the magnetic carrier and Bio-Plex Pro human cytokine assay. *

<table>
<thead>
<tr>
<th>Sample</th>
<th>IL-1 (pg/ml)</th>
<th>IL-2 (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
<th>IL-5 (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
<th>IL-12 (p70) (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed concentration</td>
<td>6.3</td>
<td>Out of range</td>
<td>17.98</td>
<td>Out of range</td>
<td>459.06</td>
<td>Out of range</td>
<td>16.06</td>
<td>Out of range</td>
<td>30.17</td>
</tr>
<tr>
<td>Observed concentration %CV</td>
<td>16.04</td>
<td>NA</td>
<td>10.64</td>
<td>NA</td>
<td>10.3</td>
<td>NA</td>
<td>15.15</td>
<td>NA</td>
<td>19.25</td>
</tr>
</tbody>
</table>

---

* Parallel experiments were performed by expert users to validate that the magnetic separation method achieves equal or better performance than the manual vacuum method. Results of all samples are calculated from triplicates. Standards were a serial dilution from high (1) to low (8) concentration.
Bio-Plex 2200 Division, Clinical Diagnostics Group, Michael Barcellos, Division Marketing Manager, Clinical Diagnostic Market

Multiplex Assays in the Perspective on Automation of high-throughput and automation.

Can you share a case story where having this device has made a difference? Yes, we all share the same vision. The whole concept of developing more homogeneous format. Using magnetic beads in an automated format enabled our development teams to achieve the market-leading performance we wanted in our autoimmune and serology panels.

When did the Bio-Plex 2200 system launch, and can you comment on placements? The Bio-Plex 2200 system was launched in late 2005, and in 2006 we had our first customer installation in North America. We now have placements in the U.S., Canada, Europe, Eastern Europe, and South Africa.

How did the Bio-Rad Clinical Diagnostics Group discover the need for magnetic beads when developing xMAP assays for IVD? It was an issue of performance. We wanted to achieve certain levels of performance (sensitivity and specificity), and we couldn’t get there with a homogeneous format. Using magnetic beads in an automated format enabled our development teams to achieve the market-leading performance we wanted in our autoimmune and serology panels.

How long from sample to result? It is 45 minutes from first sample to first result, and every 36 seconds thereafter you get another result. The theoretical throughput with a 22-plex assay and 3 internal controls is 100 samples per hour, which equates to 2,200 tests/hr. That is how the Bio-Plex 2200 system got its name.

What assays does Bio-Rad currently offer to the IVD market? Our current menu of autoimmune and serology assays, the BioPlex 2200 system brings the benefits of multiplexing to an area of the lab that is dominated by semi-automated methods, thus bringing the additional benefit of automation, which further improves workflow.

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How long from sample to result? It is 45 minutes from first sample to first result, and every 36 seconds thereafter you get another result. The theoretical throughput with a 22-plex assay and 3 internal controls is 100 samples per hour, which equates to 2,200 tests/hr. That is how the Bio-Plex 2200 system got its name.

What assays does Bio-Rad currently offer to the IVD market? The ANA screen (13-plex), EBV IgG (3-plex), EBV IgM (2-plex), syphilis IgG (3-plex), and the most recent release is our vasculitis kit (3-plex). All assays are FDA cleared. ToRQ IgG is currently being reviewed by the FDA.

Can you mention what customers can look forward to (what’s in the pipeline)? In the area of serology, we have panels in development for syphilis IgM, ToRQ IgM, MMRV-Immunity, HSV, and Lyme disease. In the area of autoimmune diseases, we are developing a gastrointestinal panel and a rheumatoid arthritis panel. We are also in development for diabetes, cardiac risk and damage, a urine toxicology test for drugs of abuse, and other longer-term projects for biomarker panels in complex diseases.

Do you see any synergies between drug discovery research applications and the clinical diagnostics labs (for example, cytokines in clinical research)? Yes, we all share the same vision. The whole concept of developing more targeted therapies to small targeted populations and using biomarker panels to identify patients who can benefit most from drugs makes complete sense. Many pharma companies are interested in working with IVD companies to develop companion diagnostics to identify patients who can benefit most from drugs. This has evolved into a market reality, but it will happen. A number of companies have contacted us about putting their biomarker panels on the BioPlex 2200 system.

Can you share a case story where having this device has made a difference? From a workflow standpoint, our first customer used to spend an entire day to generate the lab’s daily volume of ANA results using a different (manual) multiplex product. With the BioPlex 2200 system, all the results are completed and reported out in 2 hours. This time savings underscores the real value of high-throughput and automation.

References
Development and Validation of a Novel Multiplex Immunoglobulin Isotyping Assay on Magnetic Microspheres

Candice Reyes, Joe Fedynyshyn, and Sophie Allauzen, Bio-Rad Laboratories, Inc., Hercules, CA 94547 USA

Introduction
Monitoring immunoglobulin (Ig) concentrations is a valuable approach in the evaluation of immune responses. Blood Ig concentrations vary depending on factors such as inheritance, disease state, drug treatment, and Ig class and subclass. Ig isotypes can be differentiated into classes and subclasses based on the domains of heavy and light chains in the constant region of the antibody molecule. Each Ig isotype plays a specific role in the immune system by binding to receptors on various cell types and mediating events such as complement activation and pathogen clearance (Hamilton 2001).

We developed a multiplex sandwich immunoassay based on microspheres (beads), that simultaneously quantitates the concentrations of seven Ig isotypes: IgG1, IgG2, IgG3, IgG4, IgA, IgE, and IgM (Figure 1). This Bio-Plex Pro™ human isotyping 7-plex panel determines concentrations of these seven isotypes from a single sample of serum, plasma, or tissue culture supernatant, with a recommended dilution of 1:10,000 in isotyping diluent. The multiplex format yields Ig profiles of the isotypes with significant time and cost savings over technologies such as ELISA, radial immunodiffusion, and nephelometry. A complete profile of Ig isotype concentrations from as little as 10 µl of sample is obtained in about 3 hr. In this report, we describe results of validation experiments, calculating the sensitivity and upper and lower limits of quantitation for the assay.

We validated the isotyping panel using samples from healthy humans and patients with rheumatoid arthritis (RA). We tested the assay against World Health Organization (WHO) reference standards: National Institute for Biological Standards and Control (NIBSC) 67/086 for IgG, IgA, and IgM and NIBSC 75/702 for IgE, and compared isotype concentrations across experiments and methods.

Methods
We studied the performance characteristics of the Bio-Plex Pro human isotyping panel and compared isotype concentrations across experiments and methods. Standards containing known concentrations of purified human Ig’s were prepared by adding 0.5 ml isotyping diluent to lyophilized standard (both provided in the Bio-Plex Pro kit) and incubating on ice for 30 min. Starting concentrations were: IgG1, 12,000 ng/ml; IgG2, 6,000 ng/ml; IgG3, 5,000 ng/ml; IgG4, 1,000 ng/ml; IgA, 2,000 ng/ml; IgE, 100 ng/ml; and IgM, 1,500 ng/ml. Isotyping diluent was used to make 1:4 serial dilutions for a total of eight standard concentrations. Per plate, 250 µl of antibody-conjugated beads, 312.5 µl of detection antibody, and 62.5 µl of streptavidin-PE were used.

Controls were prepared by adding 1 ml isotyping diluent to lyophilized control and incubating on ice for 30 min. The controls were spiked at three different concentrations (high, medium, and low) and a 5 µl serum sample from a healthy subject (diluted 1:10,000 in isotyping diluent) were run using 250 µl antibody-conjugated beads, 312.5 µl detection antibody, and 62.5 µl streptavidin-PE.

Igs were spiked into single donor serum and plasma to determine recovery in the sample matrix. Once these spiked samples were diluted 1:10,000, the concentration of each spike was: IgG1, 700 ng/ml; IgG2, 350 ng/ml; IgG3, 300 ng/ml; IgG4, 60 ng/ml; IgA, 100 ng/ml; IgE, 5; and IgM, 90 ng/ml. In addition, WHO reference standards (pooled sera), were run using 250 µl antibody-conjugated beads, 312.5 µl detection antibody, and 62.5 µl streptavidin-PE per plate.

We tested the assay against NIBSC 75/702 for IgE and NIBSC 67/806 for IgG, IgA, and IgM. Target activities were based on the reconstitution volumes of the WHO reference standards.
samples, international units (IU) of activity in each vial as described by the product sheet, and the concentration results obtained from the Bio-Plex Pro human isotyping panel: IgG, 0.103 ng/IU; IgA, 0.019 ng/IU; IgE, 3 ng/IU; and IgM, 0.006 ng/IU.

Experiments were performed according to the Bio-Plex Pro isotyping reagent kit instructions (Figure 1). Runs were done in triplicate on a 96-well plate, and five independent verification assays were performed. Although the standard vacuum assay separation method was used, these magnetic bead-based assays also allow magnetic separation and automation of wash steps. This innovation eliminates the need for a vacuum manifold, greatly simplifying assay processing. Assays were read on the Bio-Plex suspension array system.

**Results**

**Performance Characteristics of the Bio-Plex Pro Human Isotyping Panel**

Sensitivity (limit of detection, LOD) for each target was calculated as the concentration of each standard corresponding to the background median fluorescence intensity (MFI) values plus two standard deviations. Lower limit of quantitation (LOQ) and upper limit of quantitation (ULOQ) were defined for each target by determining the standard concentration range that met the assay’s technical specifications for both precision (≤15%) and ≤20% for intra- and inter-assay precision, respectively, as measured by percent coefficient of variation, %CV and accuracy (70–130% of expected concentration). These results are shown in Table 1.

**Table 1. Performance of the Bio-Plex Pro human isotyping 7-plex panel.***

<table>
<thead>
<tr>
<th>Target</th>
<th>LOD, ng/ml</th>
<th>LLOQ, ng/ml</th>
<th>ULOQ, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1</td>
<td>0.52</td>
<td>2.93</td>
<td>7,950</td>
</tr>
<tr>
<td>IgG2</td>
<td>1.65</td>
<td>19.92</td>
<td>6,000</td>
</tr>
<tr>
<td>IgG3</td>
<td>0.29</td>
<td>5.25</td>
<td>5,000</td>
</tr>
<tr>
<td>IgG4</td>
<td>0.05</td>
<td>0.06</td>
<td>813</td>
</tr>
<tr>
<td>IgM</td>
<td>0.05</td>
<td>0.42</td>
<td>1,500</td>
</tr>
<tr>
<td>IgA</td>
<td>0.12</td>
<td>0.12</td>
<td>500</td>
</tr>
<tr>
<td>IgE</td>
<td>0.003</td>
<td>0.01</td>
<td>100</td>
</tr>
</tbody>
</table>

* Concentrations are for 10,000-fold diluted samples.

Intra-assay %CV was calculated from the MFI variance of three replicate wells on a 96-well plate for each of eight standard concentrations. The mean intra-assay %CV for the five verification assays is shown in Table 2. The technical specifications (≤15%) were achieved for all points.

Inter-assay %CV was determined from the variance of measured concentration values of three Ig-spiked controls (high, medium, and low) and a healthy serum sample, run on five verification plates. Data are shown in Table 2. The technical specifications (≤20%) were met as required for the medium- and low-spike concentrations, and for all but two targets for the high-spike concentration.

Accuracy of the assay was determined from the mean percent recovery of Ig-spiked controls and Igs spiked into both single-donor serum and single-donor plasma, in triplicate, over the course of five verification runs. These results are shown in Table 3 and Table 4. The WHO reference standards results are shown in Table 4.

**Validation of Healthy and Diseased Sera and Plasma**

We compared Ig profiles from subjects that were healthy or had RA. We analyzed 20 lots of sera and plasma from individuals.

---

Table 2. Intra- and inter-assay precision and recovery.*

<table>
<thead>
<tr>
<th>Standard Curve Point</th>
<th>IgG1</th>
<th>IgG2</th>
<th>IgG3</th>
<th>IgG4</th>
<th>IgM</th>
<th>IgA</th>
<th>IgE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.6</td>
<td>8.3</td>
<td>6.1</td>
<td>7.9</td>
<td>6.1</td>
<td>3.5</td>
<td>5.4</td>
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<tr>
<td>2</td>
<td>2.1</td>
<td>2.8</td>
<td>2.3</td>
<td>2.2</td>
<td>3.2</td>
<td>1.7</td>
<td>2.2</td>
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<td>3</td>
<td>2.7</td>
<td>4.7</td>
<td>3.3</td>
<td>2.9</td>
<td>7.3</td>
<td>2.5</td>
<td>4.1</td>
</tr>
<tr>
<td>4</td>
<td>3.2</td>
<td>5.4</td>
<td>4.8</td>
<td>3.6</td>
<td>6.7</td>
<td>2.8</td>
<td>2.9</td>
</tr>
<tr>
<td>5</td>
<td>4.4</td>
<td>5.2</td>
<td>4.4</td>
<td>3.4</td>
<td>8.6</td>
<td>3.3</td>
<td>3.7</td>
</tr>
<tr>
<td>6</td>
<td>5.4</td>
<td>6.8</td>
<td>6.4</td>
<td>5.1</td>
<td>8.5</td>
<td>7.5</td>
<td>6.2</td>
</tr>
<tr>
<td>7</td>
<td>3.9</td>
<td>6.3</td>
<td>5.8</td>
<td>4.9</td>
<td>7.9</td>
<td>4.4</td>
<td>6.3</td>
</tr>
<tr>
<td>8</td>
<td>4.0</td>
<td>3.8</td>
<td>7.4</td>
<td>6.3</td>
<td>4.9</td>
<td>5.1</td>
<td>7.1</td>
</tr>
</tbody>
</table>

**Table 3. Accuracy of Ig quantitation in serum and plasma samples.***

<table>
<thead>
<tr>
<th>Target</th>
<th>Spiked-In Concentration x 10^-4, ng/ml</th>
<th>Serum % Recovery</th>
<th>Plasma % Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1</td>
<td>700</td>
<td>121</td>
<td>97</td>
</tr>
<tr>
<td>IgG2</td>
<td>350</td>
<td>120</td>
<td>109</td>
</tr>
<tr>
<td>IgG3</td>
<td>300</td>
<td>108</td>
<td>108</td>
</tr>
<tr>
<td>IgG4</td>
<td>60</td>
<td>87</td>
<td>72</td>
</tr>
<tr>
<td>IgM</td>
<td>90</td>
<td>110</td>
<td>104</td>
</tr>
<tr>
<td>IgA</td>
<td>100</td>
<td>143</td>
<td>100</td>
</tr>
<tr>
<td>IgE</td>
<td>5</td>
<td>99</td>
<td>100</td>
</tr>
</tbody>
</table>

* Accuracy for spiked Igs was determined by using the formula, [(observed concentration of sample + Ig spike) – (observed concentration of sample)]/ (observed concentration of Ig spike) x 100. Mean values from five verification runs are shown.

**Table 4. Quantitation of IgG, IgA, IgM, and IgE in WHO reference standards.***

<table>
<thead>
<tr>
<th>Target</th>
<th>WHO Standard Concentration, mg/ml</th>
<th>NIBSC 67/086 (IgG, IgA, IgM)</th>
<th>NIBSC 75/702 (IgE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1</td>
<td>0.103</td>
<td>10.89</td>
<td>8.62</td>
</tr>
<tr>
<td>IgG2</td>
<td>0.103</td>
<td>7.84</td>
<td>5.04</td>
</tr>
<tr>
<td>IgG3</td>
<td>0.103</td>
<td>1.61</td>
<td>5.60</td>
</tr>
<tr>
<td>IgG4</td>
<td>0.103</td>
<td>0.65</td>
<td>0.68</td>
</tr>
<tr>
<td>IgM</td>
<td>0.019</td>
<td>3.82</td>
<td>4.29</td>
</tr>
<tr>
<td>IgA</td>
<td>3.00</td>
<td>0.00067</td>
<td>0.03061</td>
</tr>
<tr>
<td>IgE</td>
<td>0.006</td>
<td>1.20</td>
<td>0.65</td>
</tr>
</tbody>
</table>

* Based on the reconstitution volumes of the WHO reference samples, international units (IU) of activity in each vial as described by the product sheet, and the concentration results obtained from the Bio-Plex Pro human isotyping panel. IgE activity was 3.210 ng/IU, IgG was 0.103 ng/IU, IgA was 0.019 ng/IU, and IgM was 0.006 ng/IU. WHO reference standards NIBSC 67/086 and 75/702 were used.
that were classified as healthy (16 males and 4 females, ages 21–48) and 20 lots from individuals diagnosed with RA (20 males, ages 24–70), totaling 40 different patient samples. Increased values were observed for all IgG subclasses, IgM, and IgA in RA samples relative to healthy samples (Figure 2). These results correlate with trends cited in the literature (Adhya et al. 1998). It is known that the distribution of antibodies specific for rheumatoid factors is predominately IgG1 and IgG4 (Chapuy-Regaud et al. 2005), and this may explain the observed higher concentrations for these IgG subclasses when compared to healthy controls. Interestingly, IgG2 and IgA levels were also higher in RA patients versus controls. It is not known whether any of the RA patients were undergoing treatment that could have influenced their Ig profiles.

**Conclusions**

The Bio-Plex Pro human isotyping 7-plex panel was used to determine the isotype profiles of seven Ig classes and subclasses in a single, small volume of sample. The assay panel proved specific (0–3% cross-reactivity), precise (≤15% intra-assay and ≤20% inter-assay CV within working assay range), and accurate (70–130% recovery) for samples at a 1:10,000 dilution. It is also linear for most targets at sample dilutions of 1:5,000 and 1:2,500 (data not shown). The suggested 1:10,000 dilution resulted in a robust assay. Diluting the sample matrix to this degree resulted in negligible matrix effects, making the assay suitable for diverse applications. Assay ranges allowed the measurement of normal and diseased samples. This enables evaluation of isotype profiles of different populations and the monitoring of effects of drug treatments.

The panel can be used with traditional vacuum filtration washing or can be adapted to a nonvacuum, magnetized, automated system for higher throughput. The flexibility and ease of use of the Bio-Plex Pro human isotyping 7-plex panel makes it ideal for a variety of different lab settings.

**References**


Hamilton RG (2001). The human IgG subclasses, Calbiochem-Novabiochem Corporation, La Jolla, CA.
Development and Validation of a High-Precision, High-Sensitivity Human Cytokine Assay on Magnetic Microspheres

Woei Tan, Joyce Eldering, Li Ma, Wei Geng, Joe Fedynyshyn, and Sophie Allauzen, Bio-Rad Laboratories, Inc., Hercules, CA 94547 USA

Introduction
Cytokines are present in a wide variety of body fluids. Their role in physiological states and in infection is usually inferred from measurements of serum or plasma cytokine levels. The level of circulating cytokines is frequently defined by assays such as ELISA that measure cytokines at concentrations greater than 1.0 pg/ml (de Jager and Rijkers 2006).

In this report, the development and validation of a robust human multiplex panel, the Bio-Plex® Precision Pro™ human cytokine 10-plex panel, is described. This panel is designed to provide researchers with consistent results both within replicates and across experiments.

This assay is distinguished by the incorporation of 8 µm diameter magnetic microspheres (beads) and use of standard diluents tailored specifically for the analysis of cytokines in human serum and plasma samples. The magnetic properties of these beads allow the separation and washing steps to be carried out using either the conventional vacuum filtration technique or the more easily automated magnetic separation technique.

Methods
Assay performance was evaluated in five independent verification experiments, which incorporated both standard curves and human serum and plasma samples spiked with recombinant cytokines. The study was conducted on the Bio-Plex® suspension array system, which integrates a series of color-coded beads covalently coupled to target-specific monoclonal antibodies for each of the ten cytokine targets (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-13, IFN-γ, and TNF-α). The beads serve as a solid-phase matrix for the capture of serum or plasma cytokines via a sandwich-based detection format.

Assay procedures are outlined in Figure 1. In the first step, beads were combined with a serum or plasma sample (diluted 1:4 with sample diluent), followed by 1 hr incubation with agitation at ambient temperature. The beads were then vacuum filtered and washed to remove unbound materials. After the wash cycle, biotinylated detection antibodies were added and the beads were incubated for 30 min at ambient temperature to allow the formation of antibody sandwich complexes. Following the removal of excess detection antibodies, the complexes were added to a solution of streptavidin-phycoerythrin (PE) conjugate and incubated for 10 min at ambient temperature. After excess streptavidin-PE conjugate was removed, the final mixture was passed through the Bio-Plex system array reader, and fluorescence of the bound streptavidin-PE conjugate was measured.

Specificity studies were performed using a single antigen (to determine the specificity of each capture antibody) or a single detection antibody (to evaluate specificity of the detection antibody). The sensitivity (limit of detection) for each target was defined as the concentration associated with the background median fluorescence intensity (MFI) plus two standard deviations. The lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) were defined for each target by determining the concentration range that met the assay’s technical specifications for both precision and accuracy. Intra-assay precision, measured by percent coefficient of variation (%CV), was determined from the MFI variance of replicate wells on a 96-well plate for eight standard points prepared in human serum and plasma diluent.

Assay Steps

1. Prewet filter plate
2. Add antibody-conjugated beads
3. Wash
4. Incubate with samples and/or standards, 60 min
5. Wash
6. Incubate with streptavidin-PE, 10 min
7. Wash
8. Resuspend beads
9. Read on Bio-Plex suspension array system with Bio-Plex Manager™ 4.1 software, 25-bead map

Standard Preparation

1. Lyophilize standards
2. Add standard diluent for testing serum samples, or add the culture medium used for cell culture sample
3. Incubate on ice, 30 min
4. Make serial dilutions

Serum Sample Preparation

1. Dilute samples with sample diluent (1:4 ratio)
2. Incubate with detection antibody, 30 min
3. Wash
4. Incubate with streptavidin-PE, 10 min
5. Wash
6. Resuspend beads

Fig. 1. Procedures for the Bio-Plex Precision Pro human cytokine 10-plex assay panel.
human plasma standard diluents. Inter-assay precision, measured by %CV, was determined from the percent variance of spiked antigen concentrations (in the form of spiked controls) measured in human serum and plasma standard diluents for each of five assays. Cross-reactivity was calculated by measuring the percent of capture antibody cross-reactivity with potential cross-reactants in a multiplex environment.

Standards and spiked controls were all assayed in duplicate. Accuracy was measured by calculating spike recovery. The percent recovery values were obtained by dividing the observed concentration by expected concentration and multiplying the value by 100.

Results
The standard curve profiles of each target are shown in Figure 2. In the study of assay specificity, each antigen was evaluated with multiplexed capture and detection antibodies, which permits determination of the overall assay specificity of the capture antibody. The study recorded ≤1% cross-reactivity of capture antibodies for all ten targets (Table 1), with variable assay range and sensitivity (Table 2). Table 2 illustrates the concentration range in which the assay is both precise (intra-assay %CV: ≤10%; inter-assay %CV: ≤15%) and accurate (80–120% standard and spike recovery). These parameters were used to derive the upper and lower limits of quantitation for each target cytokine.

Table 1. Cross-reactivity among cytokine targets in a human cytokine 10-plex panel.*

<table>
<thead>
<tr>
<th>Single Antigen</th>
<th>IL-1β</th>
<th>IL-2</th>
<th>IL-4</th>
<th>IL-5</th>
<th>IL-6</th>
<th>IL-10</th>
<th>IL-12 (p70)</th>
<th>IL-13</th>
<th>IFN-γ</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Cross-Reactivity of Multiplexed Capture and Detection Antibodies</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>100</td>
<td>0.02</td>
<td>0.00</td>
<td>0.27</td>
<td>0.05</td>
<td>0.00</td>
<td>0.00</td>
<td>0.03</td>
<td>0.00</td>
<td>0.03</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.00</td>
<td>100</td>
<td>0.00</td>
<td>0.29</td>
<td>0.05</td>
<td>0.02</td>
<td>0.02</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
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<tr>
<td>IL-4</td>
<td>0.00</td>
<td>0.09</td>
<td>100</td>
<td>0.28</td>
<td>0.13</td>
<td>0.03</td>
<td>0.07</td>
<td>0.01</td>
<td>0.00</td>
<td>0.02</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.01</td>
<td>0.03</td>
<td>0.00</td>
<td>100</td>
<td>0.04</td>
<td>0.00</td>
<td>0.04</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.00</td>
<td>0.04</td>
<td>0.00</td>
<td>0.24</td>
<td>100</td>
<td>0.03</td>
<td>0.06</td>
<td>0.01</td>
<td>0.00</td>
<td>0.01</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.03</td>
<td>0.04</td>
<td>0.00</td>
<td>0.16</td>
<td>100</td>
<td>0.09</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>IL-12 (p70)</td>
<td>0.00</td>
<td>0.02</td>
<td>0.00</td>
<td>0.15</td>
<td>0.06</td>
<td>0.00</td>
<td>100</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
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<tr>
<td>IL-13</td>
<td>0.00</td>
<td>0.03</td>
<td>0.00</td>
<td>0.25</td>
<td>0.13</td>
<td>0.01</td>
<td>0.07</td>
<td>100</td>
<td>0.00</td>
<td>0.01</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.01</td>
<td>0.07</td>
<td>0.02</td>
<td>0.22</td>
<td>0.08</td>
<td>0.01</td>
<td>0.10</td>
<td>0.01</td>
<td>100</td>
<td>0.01</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.14</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>100</td>
</tr>
</tbody>
</table>

* Percentage of cross-reactivity was calculated using the MFI of multiplexed capture bead and detection antibodies in the presence of a single spiked antigen. An antigen concentration of 800 pg/ml was used for all ten targets.

Table 2. Assay sensitivity (lower limit of detection, LOD) and assay ranges of ten target cytokines in human serum and plasma standard diluents.*

<table>
<thead>
<tr>
<th>Target</th>
<th>Assay LOD (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.41 ± 0.24</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.09 ± 0.05</td>
</tr>
<tr>
<td>IL-5</td>
<td>1.37 ± 0.95</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.34 ± 0.17</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>IL-12 (p70)</td>
<td>0.24 ± 0.23</td>
</tr>
<tr>
<td>IL-13</td>
<td>0.17 ± 0.07</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.27 ± 0.30</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.14 ± 0.06</td>
</tr>
</tbody>
</table>

* Assay sensitivity was determined from human serum and plasma standard curves. Values represent mean ± SD. LLOQ, lower limit of quantitation; ULOQ, upper limit of quantitation.

** Results based on an average of five assays meeting specifications for precision and accuracy.
The intra- and inter-assay %CV are shown in Table 3. For serum standard diluent, with the exception of IL-1\(\beta\) and IL-4 cytokines, all targets recorded inter-assay %CV of less than 15%. For plasma standard diluent, both IL-2 and IL-5 cytokines recorded %CV of greater than 15% (boldface values).

To address sample matrix effect, parallelism and linearity studies were conducted to demonstrate the correlation between the assay standard curve and the measured analyte in either serum or plasma. For example, Figure 3 illustrates that the standard curve is parallel to the curve drawn for a range of concentrations of IL-1\(\beta\) cytokines measured in the serum matrix. Linearity was demonstrated in three individual serum samples with \(R^2\) greater than 0.99 for all ten cytokines.

Conclusions
Bio-Rad has developed a sensitive and robust human cytokine 10-plex assay on 8.0 µm diameter beads for the Bio-Plex suspension array system. The magnetic beads are specially designed for automation and increased throughput. They are compatible with the current 96-well filter plate format or magnetic separation for versatility in life science and clinical research. The Bio-Plex Precision Pro human cytokine 10-plex magnetic bead panel is highly precise, accurate, sensitive, specific, and robust (Table 4). Newly developed human serum and plasma standard diluents demonstrate similar binding characteristics (parallelism and linearity) to clinical samples, making this assay ideal for clinical research applications.

### Table 3. Intra- and inter-assay %CV.

<table>
<thead>
<tr>
<th>Target, pg/ml</th>
<th>IL-1(\beta)</th>
<th>IL-2</th>
<th>IL-4</th>
<th>IL-5</th>
<th>IL-6</th>
<th>IL-10 (pg70)</th>
<th>IL-13</th>
<th>IFN-(\gamma)</th>
<th>TNF-(\alpha)</th>
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<tbody>
<tr>
<td>Serum standard curve</td>
<td>3,200</td>
<td>3.0</td>
<td>1.7</td>
<td>2.0</td>
<td>2.7</td>
<td>2.1</td>
<td>2.5</td>
<td>1.5</td>
<td>1.8</td>
</tr>
<tr>
<td>800</td>
<td>1.5</td>
<td>1.4</td>
<td>2.5</td>
<td>1.0</td>
<td>3.0</td>
<td>1.8</td>
<td>2.4</td>
<td>3.1</td>
<td>7.3</td>
</tr>
<tr>
<td>200</td>
<td>1.8</td>
<td>3.5</td>
<td>2.0</td>
<td>1.8</td>
<td>2.4</td>
<td>2.8</td>
<td>2.5</td>
<td>3.6</td>
<td>2.1</td>
</tr>
<tr>
<td>50</td>
<td>3.8</td>
<td>3.1</td>
<td>6.5</td>
<td>3.9</td>
<td>3.8</td>
<td>4.0</td>
<td>3.9</td>
<td>4.2</td>
<td>2.1</td>
</tr>
<tr>
<td>12.5</td>
<td>7.8</td>
<td>7.6</td>
<td>6.7</td>
<td>5.3</td>
<td>7.4</td>
<td>4.7</td>
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<td>4.4</td>
<td>5.2</td>
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<td>3.4</td>
<td>1.7</td>
<td>5.4</td>
<td>5.4</td>
</tr>
<tr>
<td>0.78</td>
<td>3.5</td>
<td>8.8</td>
<td>4.7</td>
<td>5.5</td>
<td>4.1</td>
<td>3.9</td>
<td>0.9</td>
<td>4.8</td>
<td>8.6</td>
</tr>
<tr>
<td>0.02</td>
<td>2.8</td>
<td>9.1</td>
<td>6.6</td>
<td>4.1</td>
<td>2.0</td>
<td>4.6</td>
<td>2.4</td>
<td>4.4</td>
<td>6.7</td>
</tr>
<tr>
<td>Plasma standard curve</td>
<td>3,200</td>
<td>1.9</td>
<td>2.8</td>
<td>1.0</td>
<td>2.3</td>
<td>1.8</td>
<td>1.0</td>
<td>2.2</td>
<td>1.6</td>
</tr>
<tr>
<td>800</td>
<td>1.8</td>
<td>3.8</td>
<td>2.4</td>
<td>2.2</td>
<td>2.4</td>
<td>2.3</td>
<td>1.9</td>
<td>2.5</td>
<td>1.3</td>
</tr>
<tr>
<td>200</td>
<td>1.6</td>
<td>2.3</td>
<td>5.4</td>
<td>3.0</td>
<td>2.4</td>
<td>2.4</td>
<td>3.6</td>
<td>3.1</td>
<td>4.8</td>
</tr>
<tr>
<td>50</td>
<td>1.9</td>
<td>3.4</td>
<td>3.5</td>
<td>4.5</td>
<td>3.3</td>
<td>1.0</td>
<td>4.6</td>
<td>2.0</td>
<td>3.1</td>
</tr>
<tr>
<td>12.5</td>
<td>4.6</td>
<td>5.6</td>
<td>8.8</td>
<td>3.0</td>
<td>5.3</td>
<td>4.5</td>
<td>4.0</td>
<td>5.3</td>
<td>5.6</td>
</tr>
<tr>
<td>3.13</td>
<td>3.1</td>
<td>3.0</td>
<td>5.4</td>
<td>5.0</td>
<td>7.2</td>
<td>4.0</td>
<td>5.2</td>
<td>4.7</td>
<td>2.8</td>
</tr>
<tr>
<td>0.78</td>
<td>5.1</td>
<td>3.9</td>
<td>6.4</td>
<td>4.3</td>
<td>5.2</td>
<td>6.9</td>
<td>3.5</td>
<td>3.9</td>
<td>7.3</td>
</tr>
<tr>
<td>0.02</td>
<td>5.2</td>
<td>4.8</td>
<td>6.0</td>
<td>3.0</td>
<td>6.8</td>
<td>5.4</td>
<td>2.4</td>
<td>4.8</td>
<td>7.2</td>
</tr>
</tbody>
</table>

| Plasma pooled sample | 1,000 | 25.8 | 9.5 | 15.4 | 8.0 | 10.9 | 10.7 | 4.2 | 10.9 | 6.4 | 6.6 |
| 333 | 10.2 | 7.1 | 8.7 | 7.7 | 7.6 | 2.4 | 5.8 | 9.3 | 8.0 | 4.6 |
| 111 | 6.8 | 1.7 | 7.8 | 4.1 | 4.1 | 5.3 | 5.4 | 2.9 | 3.7 | 10.1 |
| 37 | 9.3 | 6.0 | 9.9 | 6.1 | 6.4 | 8.4 | 8.9 | 7.4 | 9.7 | 8.3 |
| 9.3 | 5.2 | 9.7 | 2.1 | 4.9 | 5.1 | 4.8 | 2.0 | 5.1 | 8.0 | 3.0 |
| 2.3 | 6.7 | 14.6 | 8.6 | 6.3 | 9.1 | 8.7 | 6.9 | 7.3 | 4.2 | 10.4 |

| Serum pooled sample | 1,000 | 12.3 | 3.6 | 7.8 | 10.7 | 7.3 | 7.3 | 3.9 | 5.0 | 6.3 | 14.8 |
| 333 | 5.3 | 6.8 | 8.2 | 5.5 | 5.5 | 6.5 | 5.2 | 3.3 | 3.4 | 6.4 |
| 111 | 4.9 | 5.6 | 10.1 | 1.6 | 2.7 | 2.8 | 4.4 | 1.7 | 2.3 | 1.9 |
| 37 | 4.9 | 2.6 | 5.3 | 2.8 | 1.8 | 4.1 | 1.7 | 0.8 | 3.9 | 3.0 |
| 9.3 | 6.1 | 5.7 | 9.6 | 10.0 | 9.6 | 8.1 | 5.4 | 6.4 | 4.7 | 9.1 |
| 2.3 | 9.2 | 17.0 | 7.2 | 33.6 | 10.0 | 3.7 | 8.9 | 10.5 | 12.9 | 7.9 |

* Mean of five assays, each sample in duplicate.
** Boldface values indicate results beyond the target inter-assay %CV of 15%.

---

**Table 4. Summary of empirically confirmed technical specifications of the Bio-Plex Precision Pro human cytokine 10-plex assay panel.**

<table>
<thead>
<tr>
<th>Specification</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precision (%CV)</td>
<td></td>
</tr>
<tr>
<td>Intra-assay</td>
<td>&lt;10%</td>
</tr>
<tr>
<td>Inter-assay</td>
<td>&lt;15%</td>
</tr>
<tr>
<td>Accuracy (% recovery)</td>
<td>80–120</td>
</tr>
<tr>
<td>Cross-reactivity</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>&lt;1 pg/ml</td>
</tr>
</tbody>
</table>

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References

Electroporation Conditions for Neuroblastoma Cell Lines Using the Gene Pulser MXcell™ System

Elizabeth T Jordan, Joseph Terefe, Luis Ugozzoli, and Teresa Rubio, Bio-Rad Laboratories, Inc., Hercules, CA 94547 USA

Introduction
The ability to transfer exogenous nucleic acids into mammalian cells has allowed scientists to study gene expression and molecular pathways. Gene transfer can be mediated by one of several commonly used techniques, including lipid transfection, viral infection, and electroporation. Not all transfection techniques work equally well in all cell types.

Neuroblastoma is a childhood cancer that accounts for approximately 7% of all cancers in children under 15 years of age (Gurney et al. 1995). Because some neuroblastomas (Stage 4S) spontaneously regress in infants (Haas 1998), the identification of gene expression differences between nonlethal and lethal forms of the disease (Voth et al. 2007) may lead to a greater understanding of tumor biology, apoptosis, and therapies for the disease.

Neuroblastoma cell lines are used as model systems for the study of neural cell development because stimulation by commonly used differentiation agents such as phorbol esters, N-(4-hydroxyphenyl) retinamide, and cytosine arabinoside results in different responses by different cell lines (Thiele 1988). In addition, one study on the metastatic properties of neuroblastoma revealed two subpopulations within a cultured neuroblastoma cell line: one with high and the other with low-invasive properties (Xie et al. 2007).

This report describes how we optimized conditions for two neuroblastoma cell lines for which electroporation conditions had not yet been defined.

Methods
Neuroblastoma cell lines IMR-32 (American Type Culture Collection, ATCC, #CCL-127) and SK-N-SH (ATCC #HTB-11) were grown in RPMI-1640 medium (containing 25 mM HEPES and L-glutamine, Invitrogen Corporation) supplemented with 12.5% fetal bovine serum, 1% penicillin-streptomycin, and 0.85% sodium pyruvate.

Cells were trypsinized, washed with PBS, and resuspended in Gene Pulser® electroporation buffer at a final cell density of either 1 x 10⁶ cells/ml (IMR-32 and SK-N-SH cells) or 3 x 10⁶ cells/ml (SK-N-SH cells). A luciferase expression plasmid, pCMVi-Luc (Bio-Rad), was added to IMR-32 cells and SK-N-SH cells (1 x 10⁶ and 3 x 10⁶/ml) at a final concentration of 20 μg/ml. Alternatively, siLentMer™ Dicer-substrate siRNA duplexes (negative control and GAPDH) were added to 3 x 10⁶/ml SK-N-SH cells at a final concentration of 50 and 100 nM, respectively. Once plasmid DNA or siRNA was added to cells, the suspension was mixed gently, and 150 μl of the mixture was aliquoted into the appropriate well sets of a 96-well electroporation plate. Cells were electroporated using the Gene Pulser MXcell electroporation system.

Following electroporation, 100 μl of the cell suspension was removed from each well and added to 0.5 ml of medium in each well of a 24-well culture plate. The medium volume was increased to 1 ml for cells electroporated at a density of 3 x 10⁶/ml. Since we did not have prior experience electroporating IMR-32 and SK-N-SH cells, we initially used preset protocol 1 (Opt mini 96-well/Sqr, Exp) on the Gene Pulser MXcell system. This protocol is designed to test both square and exponential waveforms, with three different voltages applied for square and three different capacitance settings applied for exponential waveforms (Figure 1).

Cells were washed with PBS 24 hr following electroporation and used for either the luciferase activity assay or real-time RT-PCR analysis. For gene expression analysis, total RNA was extracted from cells using the Aurum™ total RNA mini kit and used as a template to prepare cDNA using the iScript™ cDNA synthesis kit. To assess gene silencing, real-time PCR reactions were performed with qPCR SYBR® Green supermix and the iQ™5 real-time PCR detection system.

Results and Discussion
When IMR-32 and SK-N-SH cells were electroporated with a luciferase expression plasmid, the highest luciferase activity was obtained with a square-wave protocol (Figures 2 and 3), suggesting that square waves and not exponential waves may be optimal for these cells. During our initial testing, we observed that the maximal luciferase activity for square-wave electroporations was obtained using the lowest voltage (200 V); without additional testing, we cannot be sure that optimal conditions were achieved.

Since we wanted to test a voltage lower than 200 V, we selected 150 V for the next electroporation experiment with IMR-32 and SK-N-SH cells (data not shown). Much lower luciferase activity was observed than at 200 V (data not shown).

Fig 1. Gene Pulser MXcell preset protocol 1 (Opt mini 96-well/Sqr, Exp). Conditions for each well set (a set of four rows in one plate column, as shown) are indicated in the appropriate column.
Neuroblastoma cells tend to be small, which may affect transfection efficiency, so we also tested cell density in this study. Increasing the cell density 3-fold (from 1 x 10^6 to 3 x 10^6/ml) resulted in a commensurate amount of activity: RLU obtained were at least 3-fold higher in the higher-cell-density electroporations as expected.

According to our study, the optimal conditions for electroporating a luciferase expression plasmid into 3 x 10^6/ml SK-N-SH cells were a square-wave pulse at 200 V, 2,000 μF, with a pulse duration of 20 msec. At both cell densities the square-wave protocol resulted in greater cell viability than the exponential decay protocol (data not shown).

Under conditions determined in the plasmid experiments, 3 x 10^6/ml SK-N-SH cells were electroporated with either the negative control or GAPDH-specific siLentMer siRNAs, and gene silencing was assessed. We observed over 90% GAPDH gene silencing after 50 or 100 nM GAPDH siLentMer electroporations compared to corresponding negative control siLentMer siRNAs (Figure 4).

Conclusions
In this study, we identified optimal waveform and voltage conditions for electroporating IMR-32 and SK-N-SH neuroblastoma cell lines with plasmid DNA or siRNA. The data indicate that the best conditions for electroporating these cells are a 20 msec square-wave pulse of 200 V and 2,000 μF. These conditions may be a good starting point when working with other neuroblastoma cell lines, which may or may not require subsequent optimization of the final parameters.

We also observed that using a higher cell density yielded results that were at least as good as those obtained with cells at lower densities. This demonstrates that cell concentration is another important variable to consider when performing electroporations.

The amount of material delivered is also crucial. We tested plasmid DNA at a final concentration of 20 μg/ml, while siRNA was tested at final concentrations of 50 and 100 nM. Further optimization of cell density and plasmid DNA and siRNA concentrations, accomplished quickly and easily with the Gene Pulser MXcell system, may provide even better results.

References
General Guidelines for Successful Protein Biomarker Research

In clinical proteomics research, biomarkers are generally discovered through differential expression analysis using any of a variety of proteomics technologies, including 2-D gel electrophoresis, liquid chromatography-mass spectrometry, and surface-enhanced laser desorption/ionization (SELDI) mass spectrometry (MS). The goal is to detect changes in protein expression that stem from biological differences related to a disease or treatment of interest. However, because of the high sensitivity of current proteomics techniques, some changes in protein expression may be nonspecific. Changes may simply reflect patient heterogeneity due to multiple study sites, inherent biological complexity and diversity of sample types, and even small differences in the way samples are collected, processed, and analyzed. As a consequence, results may be site-, study-, population-, or sample-specific, and therefore not of clinical utility (Baggerly et al. 2004, Fung and Enderwick 2002, Hu et al. 2005, Mischak et al. 2007, White et al. 2005).

This article provides general recommendations for improving the reproducibility and utility of biomarker research results. Although specific reference is made to the ProteinChip® SELDI system, these guidelines apply to any proteomics platform. For a more complete discussion of effective study and experimental design, visit our webinar at www.bio-rad.com/lsrwebinars/, or refer to bulletin 5642.

Minimize Bias
The key to maximizing reproducibility is identifying and minimizing all potential sources of preanalytical and analytical bias (Mischak et al. 2007, Poon 2007). These factors can have profound effects on the outcome of a discovery study and, more importantly, on the ability to apply discovered biomarker candidates to broader populations in subsequent validation studies.

Preanalytical Bias
Factors that impact preanalytical bias include any systematic differences in patient populations or sample characteristics, as well as in the procedures used for sample handling (Table 1). Minimizing preanalytical bias ensures that the differences observed between experimental and control samples reflect innate biological differences. To minimize preanalytical bias:

- Define the clinical or biological question and select appropriate samples, including those for all the experimental and control groups
- For retrospective studies of specimens from a sample bank, evaluate patient and sample histories and establish rigorous criteria for sample inclusion and exclusion
- For prospective studies, develop and apply standard operating procedures (SOPs) for all aspects of sample collection, handling, and storage
- Measure and document all potential sources of uncontrollable variation (Table 1) and consider them in the final data analysis

### Table 1. Factors that impact preanalytical bias.

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, sex, ethnicity</td>
</tr>
<tr>
<td>Disease subtype and/or severity</td>
</tr>
<tr>
<td>Type of control (healthy or disease)</td>
</tr>
<tr>
<td>Location of sample collection (single or multiple study sites)</td>
</tr>
<tr>
<td>Smoking status, diet, other risk factors</td>
</tr>
<tr>
<td>Drug treatments</td>
</tr>
<tr>
<td>Study inclusion and exclusion criteria</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type (blood, serum, plasma, urine, cerebrospinal fluid, cell lysate, etc.)</td>
</tr>
<tr>
<td>Number of individuals</td>
</tr>
<tr>
<td>Source (banked or prospectively collected)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample-Handling Procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Archived vs. new samples</td>
</tr>
<tr>
<td>Collection protocols (number of sites, procedure, timing, initial processing, type of anticoagulant, etc.)</td>
</tr>
<tr>
<td>Storage procedures (time, temperature, freeze-thaw cycles, aliquoting, etc.)</td>
</tr>
</tbody>
</table>

Analytical Bias
Factors that impact analytical bias arise from differences in how the samples are processed and analyzed (Table 2). Minimizing analytical bias maximizes the discovery of true biological differences from a properly selected sample set by minimizing differences in sample processing and analysis. Analytical bias can be controlled largely through rigorous training, instrument qualification, and the use of SOPs. To further minimize analytical bias:

- Select the most appropriate proteomics technique for each phase of the study. Consider the benefits and limitations of the technique and how they might impact the clinical question being asked
- Use sufficient numbers of replicates
- Randomize the order and placement of samples (for example, into a 96-well microplate format) during sample and array preparation. This ensures that any inadvertent or unavoidable sources of bias (such as variability in liquid handling, instrument fluctuations, or differences in array or reagent quality) affect all samples equally and do not appear as biological differences
- Process all samples together — including controls — and use reagents from the same lot if possible. Use automated liquid-handling and processing systems to help minimize variability
- Maintain detailed records of all sample-processing and data-analysis steps for each sample so that any differences are taken into account during data analysis
Bioinformaticists, epidemiologists, clinical and analytical chemists, and biologists or biochemists. A collaborative effort is most critical during study design and data analysis (Clarke et al. 2005, Mischak et al. 2007).

### Develop Data Analysis Strategies Before Acquiring Data

MS-based profiling techniques generate many peak intensity features per sample, significantly more than the total number of samples in a study. This results in high-dimensional data that carry a higher risk of false discovery and overfitting of multivariate models. Therefore, the involvement of a biostatistician is recommended for both study design and data analysis. A biostatistician can devise strategies for data analysis, develop solid statistical assumptions, and apply conservative feature selection and statistical cross-validation within a sample set.

#### Validate Results

To ensure predictive values, the validity of candidate biomarkers should be tested in a larger, more heterogeneous population than used for discovery. This entails analyzing a large number of samples under a reduced set of experimental conditions (for example, those yielding the biomarker candidates). Inclusion of a more diverse study population facilitates the selection of the most robust biomarkers. The design of the validation phase can vary depending on the study setting: it may repeat and confirm the findings from the discovery phase on a larger sample set, or it may explore different clinical variables that may affect the validity of the biomarkers in a large population and, ultimately, the clinical utility of the biomarkers themselves.

Validation ideally follows the discovery of candidate biomarkers and precedes any additional efforts at purification, identification, or clinical application. The throughput and data analysis capabilities of the ProteinChip SELDI system, however, allow rapid and efficient validation of candidate biomarkers from large numbers of samples, which can increase the statistical significance of a biomarker before identification. However, comprehensive validation studies often demand prospective sample collection and require extensive numbers of samples and long periods of time. It is, therefore, also not uncommon to proceed directly to purification and identification immediately following discovery.

#### References


Mischak H et al. (2007). Clinical proteomics: a need to define the field and to begin to set adequate standards, Proteomics Clin Appl 1, 148–156.


---

**Table 2. Factors that impact analytical bias.**

<table>
<thead>
<tr>
<th>Sample-Processing Procedures</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Liquid-handling methods (automated or manual, technique, equipment, etc.)</td>
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<tr>
<td>Processing steps (denaturation, buffer components, delipidation, etc.)</td>
<td></td>
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<tr>
<td>Fractionation and depletion methods</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Experimental Protocols</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Array types</td>
<td></td>
</tr>
<tr>
<td>Sample load and placement on arrays</td>
<td></td>
</tr>
<tr>
<td>Sample binding and washing procedures</td>
<td></td>
</tr>
<tr>
<td>Matrix addition (type and method)</td>
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</tr>
<tr>
<td>Instrument settings</td>
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</tr>
<tr>
<td>Number of instruments, locations</td>
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<table>
<thead>
<tr>
<th>Data Analysis Methods</th>
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<tbody>
<tr>
<td>Spectrum processing (baseline subtraction, noise reduction, normalization, etc.)</td>
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</tr>
<tr>
<td>Peak labeling</td>
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</tr>
<tr>
<td>Feature selection, statistical analyses</td>
<td></td>
</tr>
<tr>
<td>Classification approaches</td>
<td></td>
</tr>
</tbody>
</table>

When using the ProteinChip SELDI system, also do the following:

- Optimize the protein load and binding and wash buffers used for array preparation
- Maintain optimum instrument performance through operational qualification and regular detector calibration
- Account for lab-to-lab, instrument-to-instrument, and assay-to-assay variability, as well as instrument drift over time, by including reference and quality control samples for intensity normalization and comparison of relative peak quantitation
- Optimize data collection parameters for each experimental condition (fraction, array chemistry, matrix, and mass range)
- Directly compare peak intensity data collected for only one experimental condition using a single set of instrument parameters
- For multivariate analyses, combine biomarker candidates from different experimental conditions to improve diagnostic performance
- Analyze all data using consistent parameters for spectrum processing, feature selection, and statistical analyses

**Implement SOPs**

Effective use of any proteomics platform in biomarker research requires training of all personnel and adherence to strict guidelines and protocols. SOPs are critical for implementing the highest operating standards in order to achieve quality and reproducibility (Baggerly et al. 2004, Hu et al. 2005, Mischak et al. 2007). SOPs also facilitate the validation of biomarkers by other groups using different sample sets, thereby increasing the possibilities for robust biomarker discoveries.

**Collaborate With Specialists**

For clinical proteomics research, study design entails defining a clinical question, selecting appropriate patients and samples for analysis, and carefully planning a series of experiments that generate reproducible results. Since these considerations require expertise in a number of fields, it is often advantageous to design and conduct studies with input from a group of specialists. These specialists might include, for example, a clinician, proteomics researcher or mass spectrometry technician, and biostatistician. In addition, during appropriate phases of the project, it may be
Chromatography
5726 Purification of antibodies using preprogrammed methods on the Profinia™ protein purification system product information sheet
5701 Profinia buffers for protein A and protein G chromatography flier

Automated Electrophoresis
5719 Experion™ automated electrophoresis system: enhancing sensitivity in SDS-protein electrophoresis

Electrophoresis
5705 Sensitivity and protein-to-protein consistency of Flamingo™ fluorescent gel stain compared to other fluorescent stains

Surface-Enhanced Laser Desorption Ionization (SELDI) Technology
5695 Identification of a panel of cerebrospinal fluid biomarkers for Alzheimer’s disease by SELDI-TOF-MS

Multiplex Suspension Array Technology
5703 Bio-Plex Pro™ wash stations flier

Gene Transfer
5704 Electroporation conditions for Chinese hamster ovary cells using the Gene Pulser MXcell™ electroporation system
5686 Optimization of electroporation conditions for jurkat cells using the Gene Pulser MXcell electroporation system

Amplification/PCR
5721 The PCR song words and music
5713 Real-time qPCR as a tool for evaluating RNAi-mediated gene silencing brochure
5633 Thermal gradient feature brochure
5588 CFX96™ real-time PCR detection system specifications

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