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A Resource for Life Science Research

In this issue:

INNOVATING ELECTROPHORESIS

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Bio-Rad's CFX384™ real-time PCR system is the best solution for your high-throughput qPCR needs. It can be adapted to your unique workflow to accelerate your discoveries with the unparalleled performance and reliability you know and trust from Bio-Rad.

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Greece 30-210-777-4396
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If you have comments or suggestions regarding *BioRadiations*, please email us at **bioradiations@bio-rad.com**.

TO OUR READERS

Electrophoresis was first described more than 200 years ago, but it wasn't until the second half of the 20th century that its application as a basic tool in protein discovery began to gain momentum. A widely used technique, electrophoresis is not without its drawbacks (including tedious and lengthy processes) — many of which have posed challenges for researchers for decades. For the past 50 years, Bio-Rad has worked to streamline the steps involved in the electrophoresis workflow while ensuring high reliability and reproducibility of results. The latest innovation are the Mini-PROTEAN® TGX™ precast gels, which offer an extended shelf life in the gold standard, Laemmli system format.

COVER STORY

DEPARTMENTS

TECHNICAL REPORTS

Mini-PROTEAN® TGX™ Precast Gels

For more information, visit **www.miniprotean.com**, or request the Mini-PROTEAN electrophoresis platform brochure, **bulletin 5535**.

Innovative Mini-PROTEAN TGX precast gels have a long shelf life and provide Laemmli-like separation patterns using a standard Tris/glycine/SDS running buffer system. Mini-PROTEAN TGX gels are compatible with Mini-PROTEAN® Tetra (accommodate 1–4 gels) and Dodeca™ cells (accommodate 1–12 gels). These gels can also be used in the discontinued Mini-PROTEAN 3 cell. For blotting applications, the gels are compatible with Bio-Rad's precut membrane sandwiches and Mini Trans-Blot® cell.

Mini-PROTEAN TGX gels are currently available in several acrylamide percentages (7.5%, 10%, 12%, 4–15%, 4–20%) and in a unique formulation, the Any kD™ precast gel. The Mini-PROTEAN TGX Any kD precast gel has a wide separation range of 10–250 kD, making it a suitable screening gel for 1-D separations. The Any kD gel delivers its best resolution in the 20–100 kD range, making it ideally suited for the second dimension of 2-D electrophoresis in rapid proteomic analysis. Additional acrylamide percentages will continue to be introduced; please visit **www.miniprotean.com** for their availability.

Benefits include:

- Formulated for Laemmli system (direct drop-in)
- Long shelf life
- Short run times
- High transfer efficiencies
- Inexpensive buffer system — low running costs
- Accurate molecular weight estimation
- Robust system for difficult samples
- Compatibility with all Mini-PROTEAN systems
- Bottom-open, ergonomic cassette design for easier gel handling and faster downstream setup
- Compatibility with native PAGE applications

Specifications

Gel dimensions (W x L x thickness)	8.6 x 6.7 x 0.1 cm
Cassette dimensions (W x L x thickness)	10.0 x 8.0 x 0.46 cm
Cassette material	Styrene copolymer
Comb material	Polycarbonate
Gel storage conditions	Store flat at 4°C; do not freeze
Shelf life at recommended temperature*	12 months
Recommended sample buffer	(Laemmli, dilute 1:1 with sample) 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue
Recommended running buffer	(Tris/glycine/SDS) 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3

* From date of manufacture.

Ordering Information

Description	10 Gels/Box 10-Well 30 µl	10 Gels/Box 15-Well 15 µl	10 Gels/Box IPG/Prep 7 cm IPG Strip/450 µl	2 Gels/Box 10-Well 30 µl
7.5% Resolving Gel	456-1023	456-1026	456-1021	456-1023S
10% Resolving Gel	456-1033	456-1036	456-1031	456-1033S
12% Resolving Gel	456-1043	456-1046	456-1041	456-1043S
4–15% Resolving Gel	456-1083	456-1086	456-1081	456-1083S
4–20% Resolving Gel	456-1093	456-1096	456-1091	456-1093S
Any kD Resolving Gel	456-9033	456-9036	456-9031	456-9033S

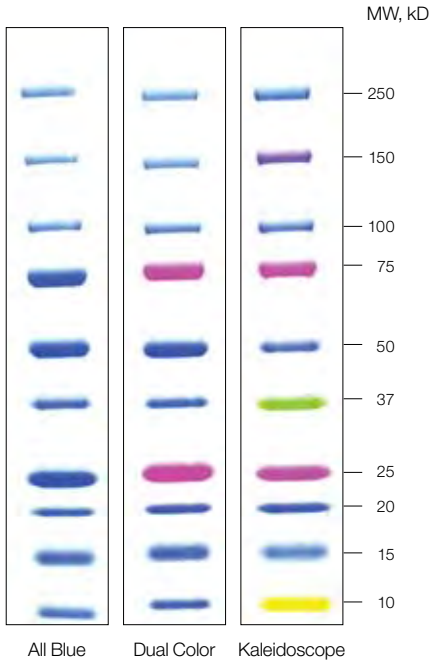
Precision Plus Protein™ Standards Value Packs

To make ordering more convenient and cost-effective for customers, Bio-Rad now offers value 5-packs of Precision Plus Protein™ All Blue, Dual Color, and Kaleidescope™ standards. These value packs of our most popular standards allow you to save on shipping costs, receive high-volume discounts, order less frequently, and avoid multiple trips to the supply center.

Features of Precision Plus Protein standards include:

- Clean, sharp bands for accurate molecular weight (MW) estimation
- MW confirmed by mass spectrometry
- Proprietary staining technology that provides batch-to-batch MW consistency and reproducible electrophoretic migration
- Natural fluorescence properties for multiplex fluorescent detection (request bulletins 5685 and 5723 for details)

Contact your local selling region for pricing details.



The Precision Plus Protein standards family offers accurate and consistent protein standards for electrophoresis and western blotting experiments.

For more information, visit **www.bio-rad.com/pppstandards/**.

Precision Plus Protein Standards Value Pack Specifications

Product	Volume	Number of Applications	Band Characteristics	Shelf Life
All Blue	2.5 ml	250	10 blue bands (10–250 kD) including 3 reference bands	1 year at –20°C
Dual Color	2.5 ml	250	10 bands (10–250 kD) with 8 blue bands and 2 pink reference bands	1 year at –20°C
Kaleidoscope	2.5 ml	250	10 multicolor bands (10–250 kD)	1 year at –20°C

Ordering Information

Catalog #	Description
161-0393	Precision Plus Protein All Blue Standards Value Pack, 2.5 ml, 250 applications
161-0394	Precision Plus Protein Dual Color Standards Value Pack, 2.5 ml, 250 applications
161-0395	Precision Plus Protein Kaleidoscope Standards Value Pack, 2.5 ml, 250 applications

Oriole™ Fluorescent Gel Stain

The novel Oriole fluorescent protein gel stain allows researchers to visualize their protein gels in less than 2 hours with high sensitivity. The stain has an excitation in the UV range and emission in the visible range, allowing visualization and imaging of the samples with essentially any UV-based imager. In a typical proteomics workflow, this stain dramatically speeds up time to results for the researcher without compromising sensitivity, dynamic range, or signal intensity. Oriole stain is supplied as a ready-to-use solution. The stain is also available in a 5 L kit that is sufficient for 100 mini-format precast gels.

For more information, visit **www.bio-rad.com/oriole/**.

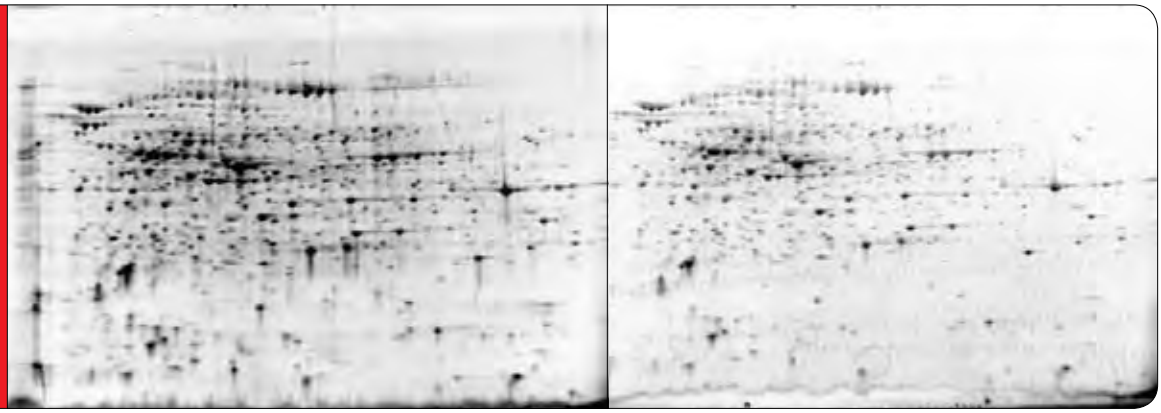
2-D SDS-PAGE gel stained with Oriole fluorescent gel stain. *E. coli* protein (40 µg) was run on an 11 cm pH 5–8 ReadyStrip™ IPG strip for the first dimension and an 8–16% Criterion™ Tris-HCl linear gradient gel for the second dimension.

Oriole fluorescent protein gel stain features:

- 90 min total staining time
- Nanogram sensitivity and 3 orders of magnitude dynamic range
- Compatibility with mass spectrometry
- Faster time to results (no fixing or destaining steps required)
- Low protein-to-protein staining variability

Molecular Imager® VersaDoc™ MP 4000 Imaging System

EXQuest™ Spot Cutter



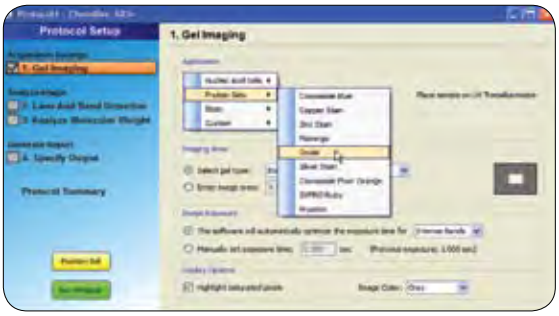
2-D SDS-PAGE gel stained with Oriole fluorescent gel stain. 2-D gels were run using 40 µg of *E. coli* total protein. The first dimension was run on 11 cm pH 5–8 ReadyStrip IPG strips, and the second dimension was run on Criterion Tris-HCl 8–16% linear gradient SDS-PAGE gels. The gels were imaged on the Molecular Imager VersaDoc MP 4000 imaging system (**left**) and EXQuest spot cutter (**right**). Gels were imaged for 10 sec on each instrument.

Ordering Information

Catalog #	Description
161-0495	Oriole Fluorescent Gel Stain, 1x solution, 200 ml
161-0496	Oriole Fluorescent Gel Stain, 1x solution, 1 L
161-0497	Oriole Fluorescent Gel Stain, kit for 5 L

Image Lab™ Software

Image Lab software is a next-generation product developed to simplify imaging and analysis of gels and blots. The software controls the Molecular Imager® Gel Doc™ XR+, ChemiDoc™ XRS+, and Criterion Stain Free™ imaging systems via a single protocol, automating the entire workflow from gel or blot image capture to results and reporting. With Image Lab software, no training is required; imaging is easy and takes only seconds to complete.



Easy protocol setup.

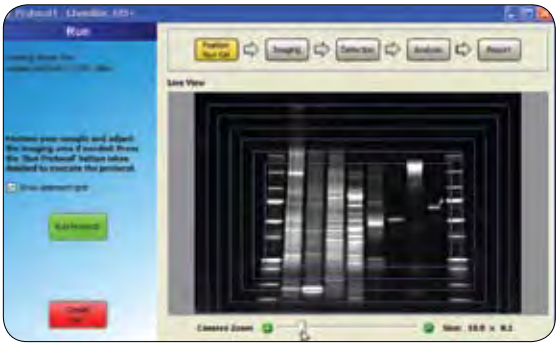
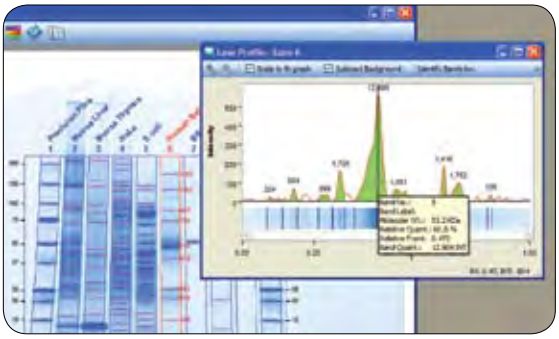
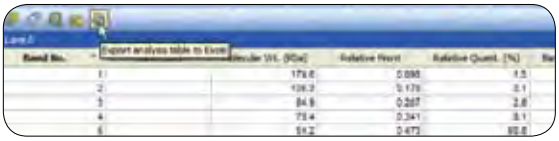


Image is maintained in focus at any zoom level.





Data analysis and reporting.

Image Lab software:

- Executes preprogrammed and user-created protocols to image and analyze gels and print reports with a single click of the mouse
- Uses proprietary algorithms to provide automated focus at any zoom level and automatically correct imaging artifacts
- Completes all image analysis steps automatically; can be user-modified for custom band detection, control of background levels, and choice of lanes
- Ensures that workflows are reproducible

Ordering Information

Catalog #	Description
170-8195	Molecular Imager Gel Doc XR+ System with Image Lab Software, for PC/Mac
170-8265	Molecular Imager ChemiDoc XRS+ System with Image Lab Software, for PC/Mac
170-8199	Molecular Imager Gel Doc XR+ Image Lab Upgrade Kit
170-8299	Molecular Imager ChemiDoc XRS+ Image Lab Upgrade Kit
170-9690	Image Lab software, version 2.0.1

For more information, visit **www.bio-rad.com/imaging/**, or request **bulletins 5838**, **5837**, and **5793**.

Instructional Video on Transfection of Primary Cells with High Efficiency

Electroporation is an extremely effective way to introduce plasmid DNA or siRNA into primary and difficult-to-transfect cells. Bio-Rad's Gene Pulser MXcell™ electroporation system and Gene Pulser® electroporation buffer were developed to easily transfect nucleic acids into mammalian and difficult-to-transfect cells, such as primary and stem cells.

Bio-Rad has produced an instructional video, "Using the Gene Pulser MXcell Electroporation System to Transfect Primary Cells with High Efficiency." The video highlights key factors that can lead to the success or failure of electroporation experiments, assists you in troubleshooting your experiment, and demonstrates how to:

- Easily get started with primary cell transfection projects
- Quickly identify the best electroporation conditions
- Conduct an experiment at the same time optimization is performed
- Switch from electroporation plates to electroporation cuvettes (or vice versa) while maintaining the same electroporation efficiency

The peer-reviewed video can be viewed online at **www.bio-rad.com/transfectiontips/** or **http://www.jove.com/index/details.stp?ID=1662**.

High-efficiency transfection of primary cells using the Gene Pulser MXcell electroporation system is demonstrated in Bio-Rad's new instructional video (image from JoVE website).

Choosing the Best Bio-Rad PCR Reagent Has Never Been Easier

Two online selection guides have been developed to enable fast and accurate selection of Bio-Rad PCR reagents in just a few quick mouse clicks. The instrument-based selection guide helps you identify the right reagent for real-time PCR systems from Bio-Rad, Applied Biosystems, Roche, Stratagene, Eppendorf, QIAGEN/Corbett, and Idaho Technology. The application-based selection guide allows users to select the appropriate reagent for standard PCR, quantitative real-time PCR (dyes and probes), high resolution melt analysis, high-fidelity, long, or fast PCR, multiplexing, cDNA synthesis for two-step and one-step RT-qPCR. The final reagent selection window in both guides is conveniently linked to the product detail page that displays technical and pricing information.

These tools allow error-free PCR reagent selection, freeing up more time for experimental design and data analysis. The red (instrument-based) and blue (application-based) reagent selector icons are easily located at **www.bio-rad.com/pcrreagents/** and related website pages. Visit us at the iTunes App Store for the soon-to-be released iPhone application version of these selectors.

To locate the PCR reagent selectors, visit **www.bio-rad.com/pcrreagents/**.

Research, Career Paths, Friendship, and Kaleidoscope™ Standard Tattoos

Dr Ann Aguanno (middle) with her students Jessica Stevens (left) and Devin Columbus (right).

Devin Columbus and Jessica Stevens recently graduated from Marymount Manhattan College, a small liberal arts school in New York City. While there, they worked in Dr Ann Aguanno's research lab studying the CDK5 enzyme and how its presence or absence affects the mammalian nervous system.

At the start of their college years, neither student had any idea that lab research would become such a significant part of their undergraduate experience. Stevens entered as a theater major, but switched to biology after an influential anatomy class. Columbus, on the other hand, enjoyed AP biology courses throughout high school, so majoring in biology was the natural next step. When Aguanno made a request for research students, it seemed merely like a good resume building opportunity for both Stevens and Columbus, but the experience became so much more than that.

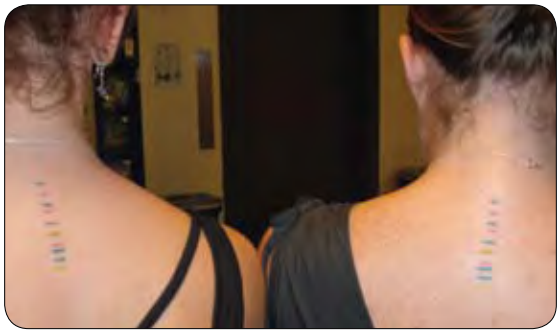
Aguanno is enthusiastic about undergraduate research training. She believes that many of her colleagues unfortunately don't invest the time necessary to train undergraduates. "Just having them make up solutions is a good way to drive someone out of science. It takes a good year to get to the point where they are going to be productive and go forward in research," she says.

> "I have this cool science thing that I will always have to commemorate these four years in love with science in my life."

To assist with the early training process, Aguanno has implemented a peer mentoring program in which existing students help those just starting out in the lab. As part of her time in the program, Columbus developed a protocol for training new undergrads. The hope is to not only apply this mentoring activity in the Aguanno lab, but to share it with other research labs in liberal arts colleges that don't benefit from a larger graduate research program.

Columbus and Stevens have participated in all aspects of biological research, from autoclaving glassware and making solutions to designing experiments and preparing posters. This training afforded them a greater understanding of the field and a richer overall experience. Their efforts culminated in attendance and participation at conferences, where Stevens says that she quickly recognized the difference between the experiences she had compared to students coming from other schools.

Both Columbus and Stevens agree that their lab work was a pivotal part of their undergraduate career. At the 2009 Experimental Biology Conference in New Orleans, the last conference of their undergraduate careers, they decided that they wanted to get a permanent symbol to commemorate their time in the lab — a tattoo.

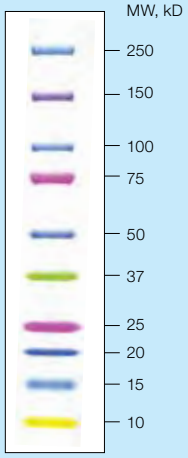


Precision Plus Protein Kaleidoscope standard tattoos (Devin Columbus, left, Jessica Stevens, right).

Stevens explains that when they were trying to decide on the perfect tattoo she instantly said, "Kaleidoscope." For her, the Precision Plus Protein™ Kaleidoscope™ standard is symbolic of her first four unsuccessful attempts at running a protein gel and it didn't work — and the excitement of seeing the protein marker separate for the first time, knowing that she had done it right. Running gels was one of the first techniques they learned together, so the Kaleidoscope protein standard was a fitting representation of the memory.

When people see each tattoo they ask what it means, and Columbus and Stevens are reminded of the work they have done, the knowledge they have gained, and the connections that they made as undergraduates. Stevens summed it up by saying, "I have this cool science thing that I will always have to commemorate these four years in love with science in my life."

To see a video of our interview with Devin Columbus, visit **www.myTetraCell.com/video/**.



The Kaleidoscope standard is a member of the Precision Plus Protein™ family of standards.
It features:

- 5 bright colors
- 10 sharp, recombinant bands from 10 to 250 kD
- Ability to monitor electrophoresis and western transfer
- Accurate MW estimation
- Lot-to-lot migration consistency

For more information on the Precision Plus Protein family of standards, please visit **www.bio-rad.com/pppstandards/**.

The Lucid Proteomics System™: Next-Generation SELDI-Based Biomarker Research Platform

The Lucid™ Advantage

- **Flexibility** — ability to perform top-down proteomic analysis on the same high-performance MALDI TOF/TOF mass spectrometer used for the bottom-up workflow, providing both wide molecular mass coverage (1.5–70 kD) and high-confidence MS/MS identification capabilities
- **Functionality** — derivatized array surfaces enable rapid, reproducible capture of subsets of peptides and proteins, providing on-chip separation and increasing the number of directly detectable proteins
- **Reliability** — Bruker's FLEX series instruments (ultraflex, autoflex, and ultrafleXtreme) provide unmatched sensitivity and resolution, and the Lucid system qualification kit provides a means for instrument optimization and standardization, promoting reproducibility of results over time
- **Utility** — detection of native proteins and peptides preserves posttranslational modifications and truncations, which may be important markers of disease
- **Compatibility** — ProteinChip® array chemistries are compatible with many diverse sample types, as well as salts, detergents, and other buffer components, eliminating the need for time-consuming cleanup steps and reducing sample volume requirements

Introduction

The demand for protein biomarkers to serve as biological indicators of phenotypically altered states continues to grow within clinical, drug development, and basic research programs. Digest-based bottom-up ("shotgun proteomics") approaches on high-resolution mass spectrometry instruments have led to the discovery of many candidate biomarkers, but differential expression of native proteins (via a top-down approach) permits the detection of many posttranslational modifications, including proteolytic events that may be relevant to or indicative of disease or drug response processes.

The Lucid Proteomics System, codeveloped by Bio-Rad Laboratories and Bruker Daltonics, couples Bio-Rad's chromatographic ProteinChip SELDI arrays with Bruker's FLEX series MALDI TOF/TOF mass spectrometers. The Lucid profiling access pack, ID access pack, and system qualification kit simplify and accelerate proteomic biomarker discovery and development programs by expanding the functionality of Bruker's FLEX platform, permitting both top-down and bottom-up methods on a single instrument.

Key Components

SELDI Chromatographic Arrays

The chromatographic nature of the SELDI surface allows direct analysis of complex biological samples across a broad mass range in a robust, reproducible manner. Cationic, anionic, hydrophobic, and metal affinity ProteinChip arrays are used to capture and enrich peptides and proteins with specific biochemical properties. The chromatographic surface enables easy removal of nonspecifically bound proteins. The surface also facilitates removal of interfering, signal-suppressive compounds (such as salts and detergents) commonly used during sample preparation, prefractionation, or depletion. These capabilities provide a distinct advantage over MALDI profiling, which commonly requires an additional cleanup step to avoid signal suppression.

Bruker MALDI TOF/TOF Spectrometers

Bruker's FLEX series of TOF and TOF/TOF mass spectrometers has long provided consistent performance and high-value data for MALDI applications. The newest member of this distinguished family is the ultrafleXtreme, featuring patented smartbeam-II laser technology which enables ultrahigh 1 kHz data acquisition speed in both MS and MS/MS modes (Figure 1). The combination of proprietary PAN ("panoramic") ion source technology, a unique FlashDetector, and new 4 GHz digitizer provides superior mass accuracy and resolution across a broad mass range. In addition to rapid profiling of intact peptides and proteins on SELDI arrays, this powerful instrument is amenable to a broad range of applications including MALDI imaging, TLC-MALDI, and top-down sequencing of purified proteins.

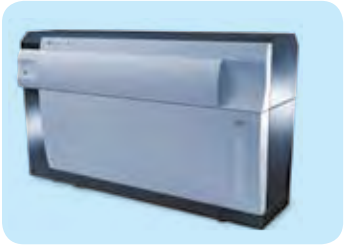


Fig. 1. Bruker Daltonics ultrafleXtreme MALDI TOF/TOF mass spectrometer.

Lucid Proteomics Software

An integral component of the system, Lucid proteomics software provides an intuitive interface for fast, easy input of sample properties and acquisition parameters. In addition, the software supplies a ready means for analysis of complex proteomic data generated by the Lucid system. The Virtual Notebook allows the user to input comprehensive information for sample tracking as well as the processing conditions associated with each spot on the ProteinChip arrays. It also provides a simple interface with Bruker's flexControl software, creating AutoXecute runs for data collection. Biomarker-driven data analysis tools include multiple modes of spectral visualization and step-by-step (mass) cluster generation. Differentially expressed proteins are sought through univariate analyses, including Mann-Whitney and Kruskal-Wallis tests, receiver operating characteristic curves, and trend plots. Relationships between samples may be further explored with multivariate analysis tools, including hierarchical clustering and principal component analysis. Built-in analyses of system qualification tests track and ensure instrument reliability over time.

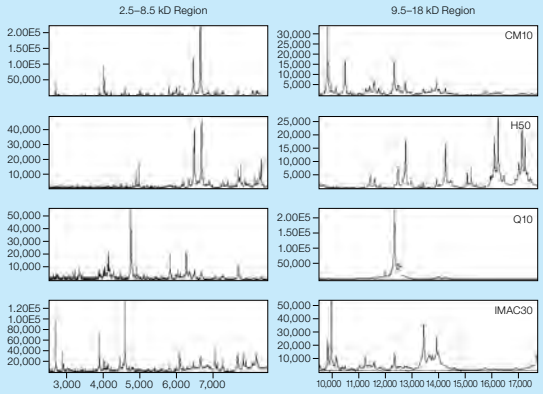


Fig. 2. Serum bound to ProteoMiner™ beads was eluted with 8 M urea, 2% CHAPS, 5% acetic acid and profiled on ProteinChip SELDI arrays (CM10, H50, Q10, and IMAC30).

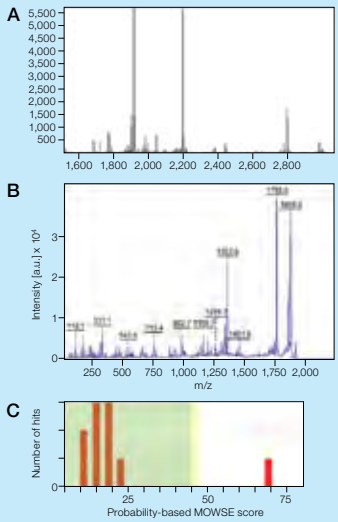


Fig. 3. Identification of a fragment of uromodulin from human urine after enrichment (binding to reverse phase beads, elution by 30% acetonitrile, and SELDI profiling). A, Reflectron MS analysis on a ProteinChip CM10 array; **B**, direct, on-chip MS/MS LIFT analysis of the 1,912 Da peptide; **C**, identification of the 1,912 Da peptide as a fragment of uromodulin.

For complete system and ordering information, go to **www.lucidproteomics.com**.

To order Bruker Daltonics instruments, go to **www.bdal.com/**.

High-Resolution Profiling for Biomarker Discovery

The Lucid Proteomics System permits high-resolution differential "fingerprinting" of native proteins from crude biological samples, including serum, urine, cerebrospinal fluid, and cell and tissue lysates (Figure 2). Maximizing the number of proteins detected increases the likelihood of discovering high-value biomarker candidates. The Lucid profiling access pack includes tools and consumables for high-throughput, top-down biomarker discovery applications. Both the ProteinChip system starter kit and the Lucid system qualification kit are provided with this access pack, increasing confidence and reliability in both array preparation and instrument performance. The profiling access pack also includes the powerful Lucid proteomics software package and a profiling consulting agreement, enabling users to benefit from Bio-Rad's expertise in study design and SELDI-derived data analysis to maximize study value.

For clinical samples such as serum and plasma, in which a small number of proteins constitute the majority of the protein concentration, fractionation prior to analysis increases the number of detectable proteins. The preparation of ProteinChip arrays in a 96-well bioprocessor enables rapid sample processing of the statistically significant numbers of samples or sample fractions often required for clinical proteomics studies. The Lucid array holder increases throughput by permitting loading of up to six ProteinChip arrays (for a total of 48 spots) into the instrument. Data may be collected across a broad mass range (1.5–70 kD); however, the Lucid profiling approach is particularly valuable for the reproducible detection and relative quantitation of low-mass proteins (<30 kD).

High-Confidence Target Identification

Identification of candidate biomarkers provides insight into disease biology and facilitates the development of analyte-specific assays; the Lucid Proteomics System provides a means for identification of both peptide and protein targets (Figure 3). The Lucid ID access pack includes tools and consumables, as well as an ID consulting agreement. This agreement entitles the user to customized guidance from Bio-Rad's team of experts for the purification and identification of up to five biomarker targets within a single project submission.

Biomarker candidates less than 4 kD discovered on the Lucid platform may be directly identified on Bruker's FLEX series instruments by MS/MS fragmentation from the chromatographic ProteinChip array of discovery. The candidate peak may be enriched by altering the array binding conditions (buffer stringency or sample load) or by the use of small-scale, bead-based chromatographic fractionation prior to capture on ProteinChip arrays. Candidates greater than 4 kD are typically subjected to multistep chromatographic separation (monitoring purification on appropriate ProteinChip arrays) and, once sufficiently enriched, are loaded on an SDS-PAGE gel for final purification before excision and digestion with a protease. Digests or native peptides are analyzed in MS and MS/MS modes on chromatographic ProteinChip arrays or Bruker MALDI targets for subsequent database searching through Bruker's BioTools software.

Conclusion

The Lucid Proteomics System enables the generation of high-value proteomic data and provides novel tools for biomarker-driven analysis. Bio-Rad's chromatographic SELDI arrays and Bruker's instrumentation are a powerful combination designed to meet current biomarker research needs.

Faster Results: Fluorescent Gel Staining in Less Than 2 Hours

Fluorescent stains are widely used in gel-based proteomics due to their high sensitivity and wide dynamic range. However, an intrinsic weakness of fluorescent gel stains is that they require multiple handling steps and long staining time. Researchers often have to wait up to 18 hours after gel electrophoresis to visualize their proteins, a major limiting step in an already complex and time-consuming proteomics workflow. Many researchers choose to use a visible gel stain such as Coomassie Blue or silver stain for this reason.

The simplicity of the Oriole stain protocol results in a staining process that takes only 90 minutes — saving researchers valuable time and energy.

The Oriole™ fluorescent gel stain was developed to combine the sensitivity and dynamic range of a fluorescent gel stain with the ease of use of Coomassie Blue stain. The unique composition of Oriole stain allows fast staining of proteins without the additional destaining, washing, and fixing steps typically required for fluorescent gel staining. The simplicity of the Oriole stain protocol results in a staining process that takes only 90 minutes — saving researchers valuable time and energy. The table compares the protocol and step-specific time requirements of Oriole fluorescent gel stain and SYPRO Ruby protein gel stain.

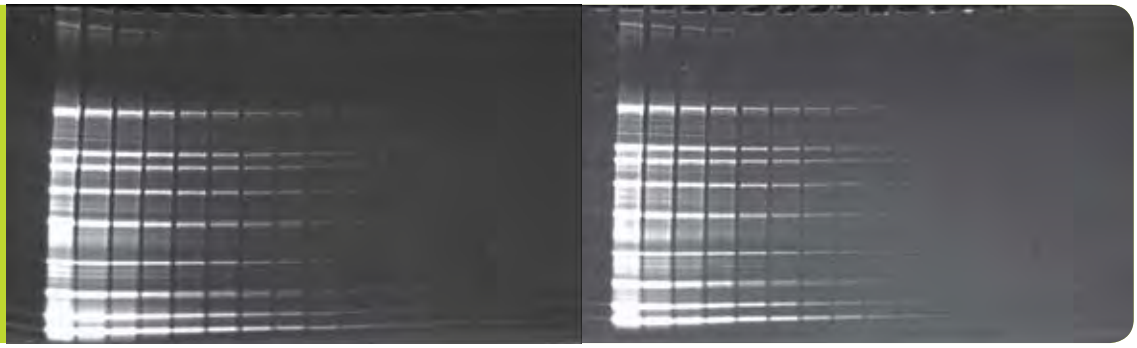
Comparison of basic fluorescence staining protocol and time requirement for Oriole and SYPRO Ruby protein gel stains, as recommended in their respective instruction manuals.

Processing Step	Oriole Stain	SYPRO Ruby Stain
Fixing step 1	None	30 min
Fixing step 2	None	30 min
Staining	90 min	Overnight
Washing	None	30 min
Total time	90 min	~18 hr

As with most protein gel stains, fixing and destaining help to remove background signals for imaging. Such steps are not found to significantly improve signal quality with Oriole stain. Since Oriole stain has an excitation maximum at 270 nm and emission maximum at 604 nm, it can be imaged with UV-based imagers (such as Bio-Rad's Molecular Imager® Gel Doc™ XR+, ChemiDoc XRS+, and Molecular Imager® VersaDoc™ MP series systems) or a simple UV transilluminator. The stained images yield similar sensitivity (<ng) and dynamic range (3 orders of magnitude). The figure below compares the images of gels stained with Oriole and SYPRO Ruby fluorescent gel stains.

Oriole fluorescent gel stain offers superior sensitivity and linearity with minimal background. The short protocol time allows the researcher to obtain same-day results without compromising quality and reproducibility. Data acquisition is simple and automated using Bio-Rad's Image Lab™ software. Whether you are running 1-D or 2-D gel electrophoresis experiments, Oriole fluorescent gel stain will shorten and simplify your protocols, allowing you to accomplish more in your role as a researcher.

Oriole Stain **SYPRO Rudy Stain**



Images of gels stained with Oriole fluorescent gel stain (left) and SYPRO Ruby fluorescent gel stain (right), photographed using the Bio-Rad Molecular Imager VersaDoc MP 4000 system. Similar sensitivity and band intensity are observed with both stains.

Highly Efficient Reverse siRNA Transfection of Human Epithelial Lung Carcinoma A549 Cells Using siLentFect™ Lipid Reagent

Claudia M Litterst and Luis Ugozzoli
Bio-Rad Laboratories, Inc., Hercules, CA 94547

Introduction

A549 cells are a cellular model system widely used to study the molecular biology of allergic and inflammatory responses in lung epithelial cells. These cells represent alveolar type 2 epithelial cells and release inflammatory mediators when stimulated with type 2 helper T cells (Th2) and proinflammatory cytokines (Sekiya et al. 2000, Shankaranarayanan and Nigam 2003, Taka et al. 2008).

PP2A is a serine/threonine-specific Ca^{2+}-independent protein phosphatase. PP2A, which is expressed ubiquitously in eukaryotic cells, has been shown to promote the IL-4 signaling pathway (Woetmann et al. 2003) and is potentially involved in the regulation of allergic responses in lung epithelial cells.

Short interfering RNAs (siRNAs) are powerful tools to suppress gene expression in mammalian cells. Elbashir et al. showed that transfection of short RNA (21-mers) could induce the silencing of specific genes at a cellular level, thus providing a simpler method for the loss-of-function study compared with traditional technologies (Elbashir et al. 2001). Subsequently, J Rossi and colleagues demonstrated that 27-nt duplexes are often more effective at silencing than the corresponding 21-mer siRNAs (Kim et al. 2005).

We show here a highly efficient reverse siRNA transfection protocol for A549 cells using siLentFect lipid reagent for *GAPDH* and PP2A knockdown. No prior seeding of cells is required, since siRNA-lipid complexes can be directly added to cells in suspension. The simplicity of the protocol makes it suitable for high-throughput transfection. Using 27-nt siRNA duplexes, we achieved more than 97% knockdown for *GAPDH* and 93% for PP2A. Furthermore, we demonstrated that siLentFect lipid reagent does not impair cell viability under these conditions. We conclude that this protocol can be used in functional genomic studies to investigate the biology of allergies and inflammation.

Methods

Transfection

A549 cells (American Type Culture Collection, ATCC #CCL-185) were grown in F-12K nutrient mixture, Kaighn's modification (Invitrogen Corporation) with 10% fetal bovine serum. Transfection complexes were prepared in Opti-MEM I reduced-serum medium (Invitrogen). Human *GAPDH* siRNA and nonsilencing siRNA were used (siLentMer starter kit for human GAPDH, Bio-Rad Laboratories, Inc.). PP2A siRNA was obtained from Integrated DNA Technologies, Inc. (IDT; sequence can be obtained upon request). For transfections, varying amounts of siLentFect lipid reagent (Bio-Rad) were mixed with 100 µl of Opti-MEM I medium, added to 20 pmol siRNA, and incubated for 20 min at room temperature. During the incubation time, cells were prepared for transfection. They were detached by trypsinization and resuspended in growth media at 100,000–140,000 cells/ml. After the incubation, the siRNA-lipid mix and 1 ml of cell suspension were combined in 12-well tissue culture dishes, resulting in a 20 nM final siRNA concentration, and cells were cultured overnight at 37°C.

Analysis of Transfection Efficiency

RNA was extracted using an Aurum™ total RNA mini kit (Bio-Rad). cDNA was prepared with an iScript™ cDNA synthesis kit (Bio-Rad). Real-time PCR reactions were performed using iQ™ supermix on a CFX96™ real-time PCR detection system, and data were analyzed using CFX Manager™ software (all from Bio-Rad). Primers for amplification of β-actin, *GAPDH*, and PP2A were purchased from IDT (sequences can be obtained upon request). LDH levels in the cell culture supernatant were determined using a CytoScan LDH cytotoxicity assay kit according to the manufacturer's recommendations (G-Biosciences).

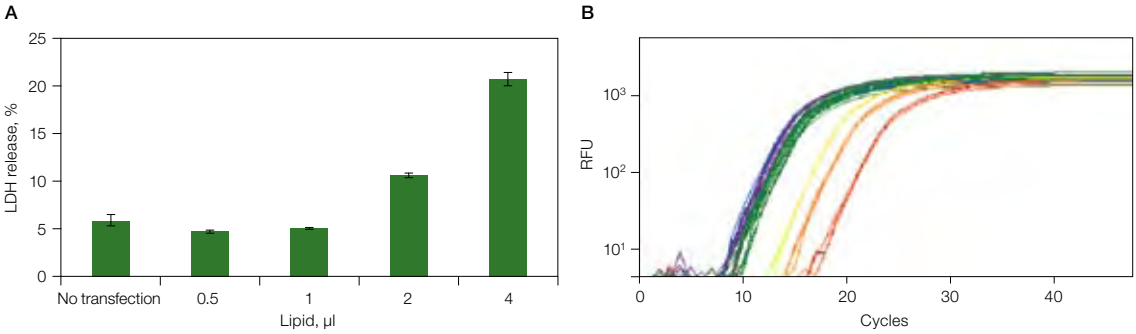
Results

Optimization of siRNA transfection conditions for A549 cells was carried out by varying the amount of lipid transfection reagent to achieve maximal siRNA uptake without affecting cell viability. As recommended for 12-well dishes in the siLentFect Lipid Reagent Instruction Manual, we tested the range of 0.5–4 µl of lipid. We transfected nonsilencing siRNA using a reverse transfection protocol that involves simultaneous transfection and plating of the cells. All transfections were carried out at a 20 nM final siRNA concentration.

Sixteen hours after transfection, we assessed cytotoxicity by measuring LDH release in cell culture supernatants. As shown in Figure 1A, 0.5 and 1 µl of lipid resulted in similar levels of LDH in the culture media as in the nontransfected control cells, indicating that these amounts of lipid did not impair viability of the cells. However, 2 and 4 µl of lipid resulted in increased LDH release. We also performed experiments 48 and 72 hr posttransfection with 1 µl of lipid, and did not observe any increase in cytotoxicity when this amount of lipid was used (data not shown).

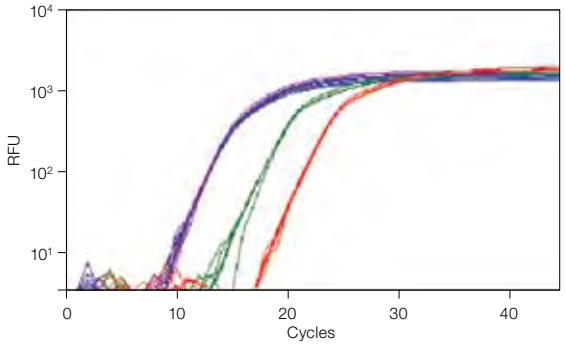
To quantify the knockdown of gene expression, we transfected a *GAPDH* siRNA and a nonsilencing control RNA using 0.5, 1, and 2 µl of lipid. Total RNA was prepared 16 hr after transfection, reverse transcribed, and *GAPDH* mRNA levels were determined by real-time PCR. Depending on the amount of lipid used, *GAPDH* gene expression was knocked down to 0.4–7.2% of the levels in control samples (92.8–99.6% knockdown, Figure 1B). One microliter of lipid (an amount that does not impair cell viability) resulted in 97% knockdown of *GAPDH* expression. In summary, siLentFect lipid reagent can be used for highly efficient reverse siRNA transfection of A459 cells without impairing the viability of the cells.

To confirm that the described siRNA transfection protocol can be used for the knockdown of scientifically relevant genes in A549 cells, we tested transfection of an siRNA targeting PP2A, a potential regulator of IL-4 signaling in A549 cells. Cells were transfected with a PP2A siRNA or a nonsilencing RNA control using 1 µl of lipid, which proved to be nontoxic for cells (see Figure 1A). As shown in Figure 2, we achieved 93% knockdown (7% remaining expression) for PP2A.



siRNA	Lipid, µl	C_T Target Gene	C_T Reference Gene	Δ C_T	ΔΔ C_T	Knockdown, %
GAPDH	0.5	16.08	11.38	4.69	3.79	92.76
GAPDH	1	17.48	11.32	6.16	5.22	97.31
GAPDH	2	20.15	11.50	8.65	7.84	99.56
Control	0.5	12.18	11.28	0.91		
Control	1	12.02	11.08	0.94		
Control	2	12.23	11.43	0.81		
No transfection		12.84	12.10	0.74		

Fig. 1. Optimization of siLentFect lipid reagent volume for siRNA transfection in A549 cells. A549 cells were transfected with nonsilencing siRNA or a *GAPDH* siRNA and a nonsilencing control siRNA using the indicated amount of siLentFect lipid reagent (20 nM final siRNA concentration). Cells were analyzed 16 hr after transfection. **A**, percentage LDH release; **B**, β-actin and *GAPDH* mRNA levels: β-actin (reference gene) expression in control samples (■), and in *GAPDH* siRNA–transfected samples (■); *GAPDH* levels in *GAPDH* siRNA–transfected samples using 0.5 µl (■), 1 µl (■), and 2 µl (■) siLentFect lipid reagent, and in control siRNA–transfected samples (0.5, 1, and 2 µl of lipid reagent) (■). RFU, relative fluorescence units.



siRNA	C_T Target	C_T Reference	ΔC_T	$\Delta\Delta C_T$	Knockdown, %
PP2A—a	20.71	11.94	8.77	3.80	92.81
PP2A—b	20.66	11.87	8.79	3.71	92.34
Control—a	16.77	11.80	4.97		
Control—b	16.87	11.79	5.08		

Fig. 2. Efficient knockdown of PP2A expression in A549 cells using siLentFect lipid reagent. A549 cells were transfected with a PP2A siRNA and a nonsilencing control siRNA (20 nM final siRNA concentration) in duplicate (samples a and b). PP2A and *GAPDH* (reference gene) mRNA levels were determined 16 hr after transfection. Chart shows expression of *GAPDH* in control samples (■) and in PP2A siRNA–transfected samples (■) and PP2A expression in control samples (■) and in PP2A siRNA–transfected samples (■). RFU, relative fluorescence units.

Conclusions

We designed a protocol for rapid, highly efficient siRNA transfection of A549 cells. This simple protocol can be easily adapted for high-throughput transfections. We achieved 97% knockdown for *GAPDH* and 93% for PP2A without impairing cell viability. Furthermore we showcased that our protocol is suitable for functional studies to identify and characterize new regulators of inflammatory responses in A549 cells.

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Fast Optimization of a Multiplex Influenza Identification Panel Using a Thermal Gradient

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[3]Tan Tock Seng Hospital, Singapore

Introduction

The year 2009 was marked by the emergence of a novel influenza A (H1N1) virus that infects humans. There is a need to identify the different strains of influenza virus for purposes of monitoring the H1N1 strain pandemic and for other epidemiological and scientific purposes. In preparation for an accurate and rapid identification of H1N1, a laboratory in Singapore established a multiplex influenza identification panel that detects H1N1 among influenza A subtype H1 (flu A), subtype H3 (H3N2) and influenza B (flu B) viruses. The panel uses a 5' nuclease (TaqMan) probe–based four-target multiplex real-time PCR assay.[1]

In this tech report we illustrate the use of the thermal gradient feature on the CFX96™ real-time PCR detection system to optimize reverse transcription (RT) and fast PCR conditions for a four-target multiplex PCR assay.

Methods

Viral RNA

The following virus strains were used: influenza A (H1N1), influenza A subtype H1 (flu A), influenza A subtype H3 (H3N2), and influenza B (flu B). Input concentration ranged between 10 and 1,000 copies.

Optimization of RT

Optimization of RT was performed with the four-target multiplex RT-qPCR MDX kit (Experimental Therapeutic Centre). The TaqMan probes were tagged with the following fluorophores: H1N1, HEX; flu A, FAM; H3N2, Cy5; and flu B, Texas Red.

RT-qPCR reactions were set up as indicated in Table 1 using the iScript™ one-step RT-PCR kit for probes (Bio-Rad Laboratories, Inc.).[2] Reactions were incubated in the CFX96 system (Bio-Rad) for 30 min at 45, 45.6, 47, 48.9, 51.3, 53.3, 54.4, and 55°C (temperature gradient), followed by 2.5 min at 95°C, then subjected to 42 three-step cycles of 95°C for 17 sec, 55°C for 31 sec, and 68°C for 32 sec. Fluorescence data were collected at the 55°C annealing step. Positive control RNAs equivalent to approximately 1,000 viral copies were used for optimization.

Multiplex RT-qPCR

RT-qPCR reactions were set up as indicated in Table 1. Reactions were incubated at 50°C for 30 min followed by 2.5 min at 95°C, then subjected to 42 three-step cycles of 95°C for 17 sec, 55°C for 31 sec, and 68°C for 32 sec. Fluorescence data were collected at the 55°C annealing step.

Table 1. Reaction setup (10 reactions).

Components	Volume, µl
2x RT-qPCR reaction buffer	125.0
H1N1 primer pair mix (7.5 µM each)	5.0
H1N1 probe–HEX (10 µM)	2.5
Flu A primer pair mix (7.5 µM each)	5.0
Flu A probe–FAM (10 µM)	2.5
H3N2 primer pair mix (7.5 µM each)	5.0
H3N2 probe–Cy5 (10 µM)	2.5
Flu B primer pair mix (7.5 µM each)	5.0
Flu B probe–Texas Red (10 µM)	2.5
RNA template (range from 0.01 pg–1 µg)	Up to 90
Reverse transcriptase and Taq polymerase mix	5.0
RNase-free H_20	Top up to 250

Optimization of Fast qPCR

RT-qPCR reactions were set up as indicated in Table 1, incubated for 10 min at 50°C followed by 2.5 min at 95°C, then subjected to 42 two-step cycles of 95°C for 17 sec and 31 sec at 55, 56, 58, 60.9, 64.5, 67.5, 69.2, and 70°C (temperature gradient). Fluorescence data were collected at the gradient range of the 55–70°C annealing-extension step. Positive control RNAs equivalent to about 1,000 viral copies were used for fast qPCR optimization.

Results and Discussion

RT Optimization Using the Thermal Gradient

The temperature gradient feature of the CFX96 real-time PCR system was used to determine the optimal RT temperature for the four-target multiplex RT-qPCR assay. The CFX96 system was programmed to perform RT at temperatures ranging from 45 to 55°C, followed by a three-step PCR protocol (denaturation-annealing-extension). Results from the RT gradient in Table 2 show that the optimal RT temperature range (in blue) was between 45.0 and 51.3°C for all primer-probe sets, with influenza A reactions working as high as 53.3°C. Selecting higher RT temperatures improves the specificity of the assay but compromises the assay sensitivity. In order to maintain both specificity and sensitivity, we have selected 50°C RT for the rest of the four-target multiplex assay validation.

[1]The test protocol described in this paper has not been cleared or approved by the FDA or any other regulatory agency for human diagnostic or other clinical use.

[2]This product may be used for scientific research purposes only. Please refer to the product insert for additional information.

Table 2. Optimization of RT reaction temperature for the four-target multiplex influenza RT-qPCR assay.

RT Reaction Temperature, °C	C_T* H1N1 (HEX)	Flu A (FAM)	H3N2 (Cy5)	Flu B (Texas Red)
55.0	32.68	33.70	32.41	31.71
54.4	32.34	33.70	32.46	30.67
53.3	32.09	32.50	31.81	30.57
51.3	31.67	32.48	31.70	29.26
48.9	31.45	32.66	31.04	28.89
47.0	31.18	32.02	31.09	29.45
45.6	31.16	32.24	31.15	29.47
45.0	30.93	32.97	30.98	29.43

* C_T values in blue reflect optimal RT temperatures yielding the lowest threshold cycle (C_T) ±0.5°C for each primer-probe set, while C_T values in red indicate suboptimal temperatures.

Table 3. Optimization of the four-target multiplex influenza RT-qPCR assay for fast PCR.

Annealing Temperature, °C	C_T* H1N1 (HEX)	Flu A (FAM)	H3N2 (Cy5)	Flu B (Texas Red)
70.0	—	—	—	—
69.2	—	41.98	—	—
67.5	—	31.11	—	—
64.5	—	29.47	27.67	—
60.9	30.81	29.79	27.55	31.68
58.0	31.26	30.01	27.78	30.54
56.0	31.83	30.15	28.18	30.54
55.0	31.53	30.39	28.44	30.06

* C_T values in blue reflect optimal annealing-extension temperatures yielding the lowest C_T ±0.5°C for each primer-probe set, while C_T values in red indicate suboptimal temperatures.

Multiplex RT-qPCR Assay Sensitivity

To determine the multiplex RT-qPCR assay sensitivity, we tested different concentrations of the four viral RNAs ranging from 1,000 to 10 copies. Results shown in Figure 1 clearly show that the primer-probe sets were able to detect all the viral RNA controls with no cross talk between channels.

Optimization of Fast qPCR Conditions

Optimization of fast qPCR conditions was achieved by combining the annealing and extension steps. Combining these steps is essential for 5' nuclease (TaqMan) probe–based assays because the probes need to anneal to the target while the product is being extended.

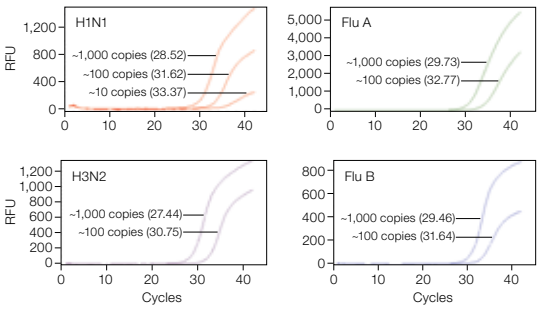


Fig. 1. Multiplexing of four-target influenza RT-qPCR. Amplification profiles of H1N1, flu A, flu B, and H3N2. Viral RNA isolates ranging from 1,000 to 10 copies were detected. RFU, relative fluorescence units.

Additional timesavings were achieved by reducing the RT time from 30 min to 10 min. C_T values in Table 3 and amplification plots in Figure 2 illustrate the optimal fast qPCR annealing-extension temperature range for H1N1 (58–60.9°C), flu A (60.9–64.5°C), flu B (55–58°C), and H3N2 (58–64.5°C). We selected 58°C for annealing-extension to perform the fast four-target multiplex RT-qPCR assay validation (total run time for this reaction was shortened from 128 to 73 min). The results generated using fast cycling conditions in Table 4 show that all viral RNA controls tested were detected with a 1–2 C_T delay compared to the standard 30 min RT using three-step qPCR cycling conditions.

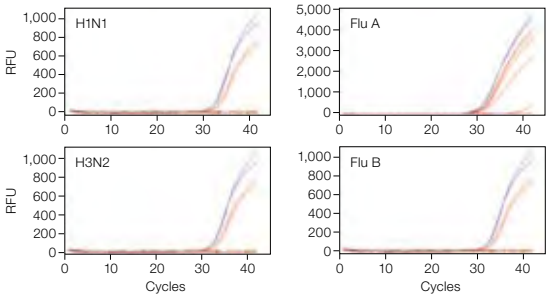


Fig. 2. Optimization of fast qPCR for the four-target multiplex influenza RT-qPCR. Amplification profiles of PCR reactions listed in Table 3. Blue and red traces indicate PCR reactions performed at optimal and suboptimal annealing temperatures, respectively. RFU, relative fluorescence units.

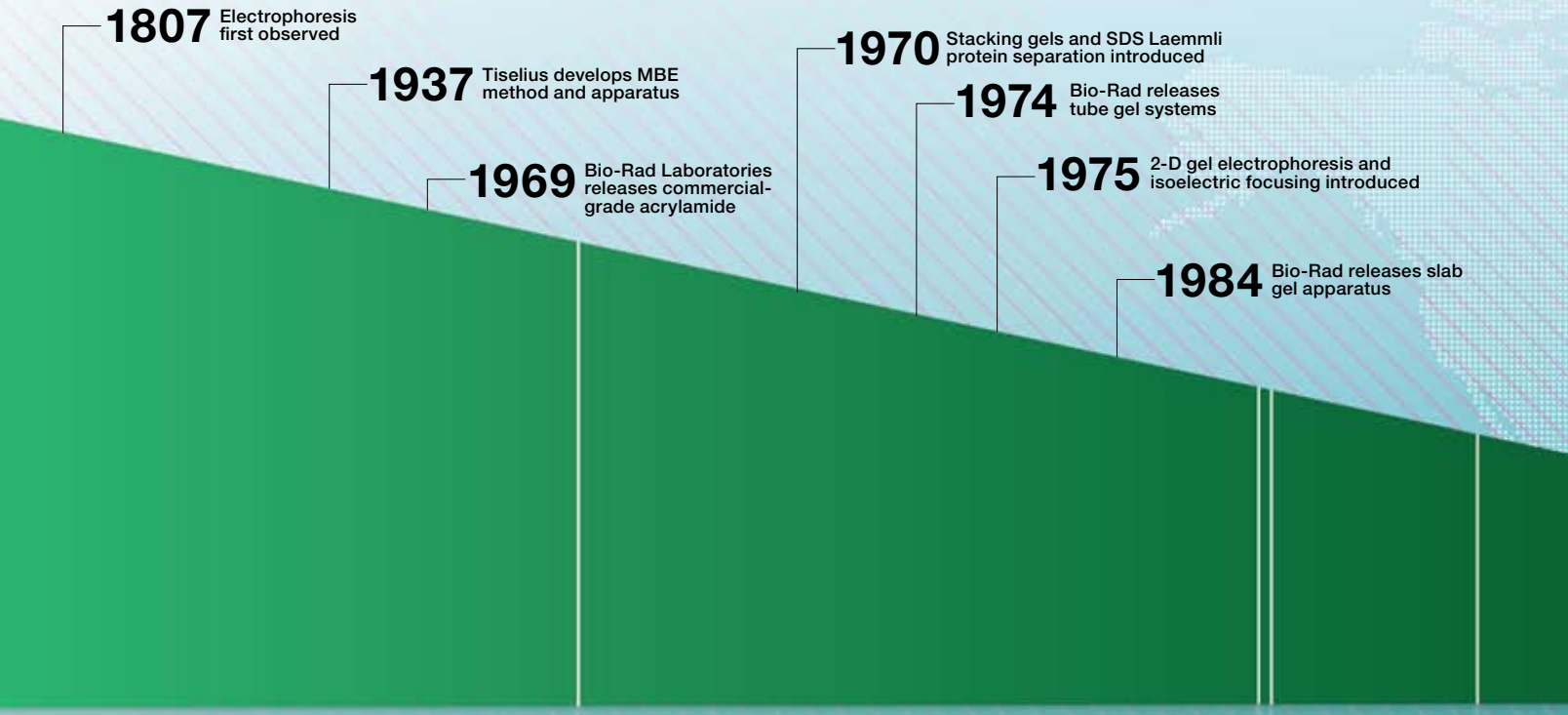
Table 4. Comparison of standard PCR and fast PCR efficiency.

Viral RNA	Copy Number	C_T Standard Run (128 min)	C_T Fast Run (73 min)	ΔC_T
H1N1	1,000	28.52	27.85	–0.67
	100	31.62	32.32	0.70
	10	33.37	34.42	1.05
Flu A	1,000	29.73	30.99	1.26
	100	32.77	34.32	1.55
H3N2	1,000	27.44	28.72	1.28
	100	30.75	32.38	1.63
Flu B	1,000	29.46	31.45	1.99
	100	31.64	33.54	1.90

Conclusions

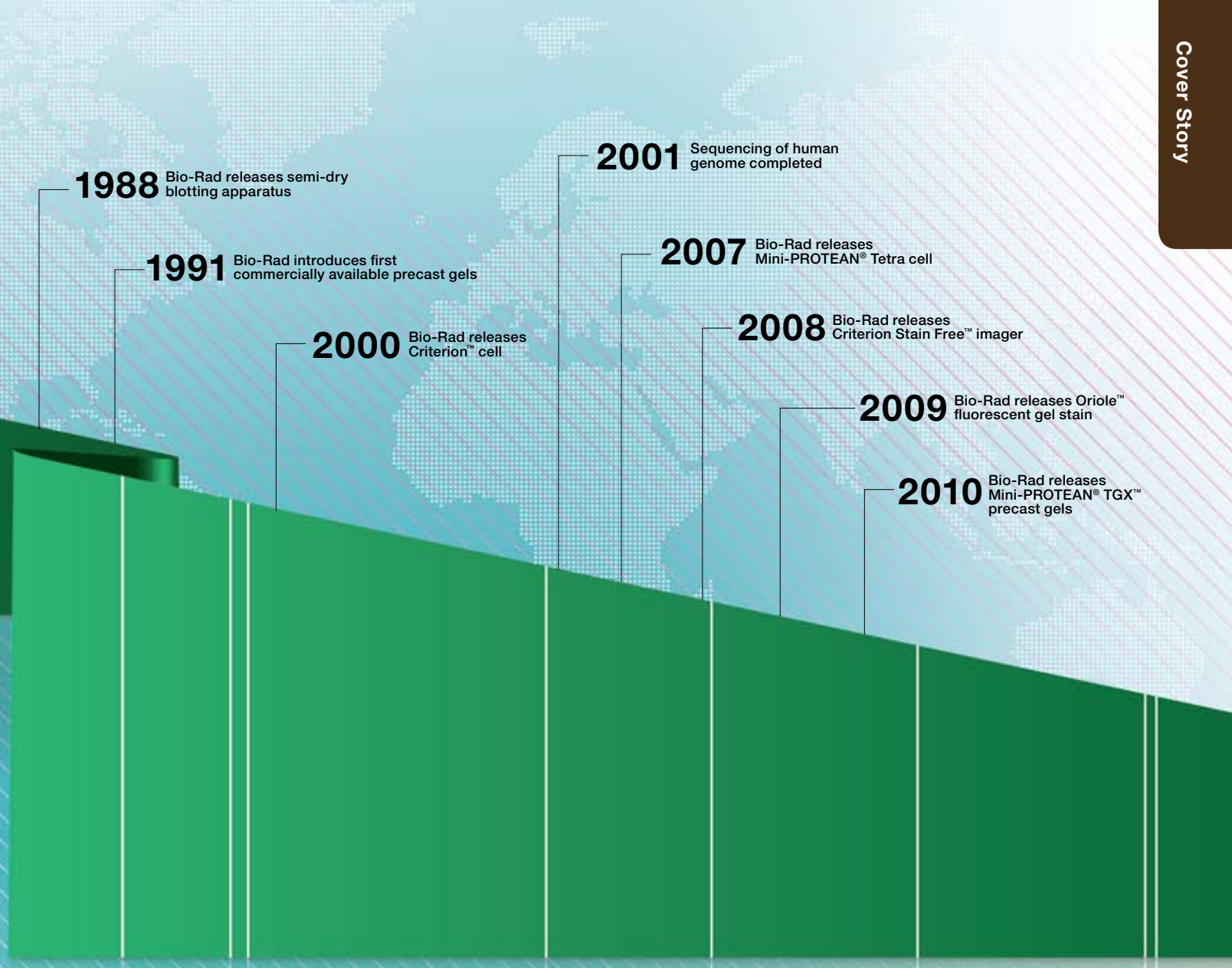
We have demonstrated that the gradient feature of the CFX96 system dramatically accelerates the optimization of the RT and PCR steps for multiplexing RT-qPCR assays. The thermal gradient also improved the ease of converting cycling conditions from a three-step to a two-step PCR assay, thus significantly reducing PCR run times while maintaining assay specificity and sensitivity.

For more information on optimizing multiplex RT-qPCR, refer to *BioRadiations* 113, pages 16–23, available in PDF format at **www.bio-rad.com**.



The discovery of electrophoresis can be traced back to 1807 with Dr Fedor Fedorovich Reuss's observation of the influence of an applied electric field on the migration of clay particles in water (Reuss 1809). But it wasn't until Dr Arne Tiselius developed "moving boundary electrophoresis" (MBE) in 1937 that the potential arose for its application as a routine tool in protein discovery. Since that time, the basic electrophoresis workflow — prepare samples, apply an electric field to observe mobility based on size or charge, then visualize and analyze results — has remained relatively unchanged. And though this fundamental technique has played an integral role in countless discoveries in protein research, there are intrinsic problems — margins of error can be high, optimization is required for each application step, and processes and time to results are often lengthy — that have posed challenges for researchers since its inception. Throughout the past 50 years, Bio-Rad scientists have sought to overcome these challenges with tools that help make research faster, more convenient, and more reliable — a goal that has most recently culminated with the launch of precast gels in a Laemmli system format.

Throughout the past 50 years, Bio-Rad scientists have sought to make research faster, more convenient, and more reliable . . .



Authors: Shawn Miller, Bill Gette, and Alpana Prasad.

INNOVATING ELECTROPHORESIS

Dr Stephen Mayfield

Professor of Molecular Biology at UCSD, Director of San Diego Center for Algae Biotechnology

Dr Stephen Mayfield researches the molecular genetics and biotechnology of algae (using algae to develop protein therapeutics, nutraceuticals, and biofuels). In typical experiments, protein expression or function is evaluated in response to the presence or absence of genes or the metabolic profiles of cells. "We run a lot of SDS-PAGE gels. We have for 25 years, and I think we'll continue to do so for another 25," says Mayfield.

Their greatest electrophoresis workflow challenge is in meeting today's throughput demands. "We used to say, 'One scientist, one year, one gene,'" he explains. "Nowadays, science is going too fast for that." In this quest for speed, Mayfield's lab typically runs gels at 40 mA. But high temperatures often correlate to a "smiling" effect in the gels. In his tests with TGX gels, however, this phenomenon was significantly minimized. In addition to increased speed, the key advantage of TGX gels observed in Mayfield's experiments was consistency over longer periods of time. "We want to think about new biology, not our gels," he explains.

A Work in Progress

The roots of today's biotechnology industry were planted in the 1960s, a period when traditional boundaries — whether they were in race relations, space exploration, or disease prevention and eradication — were continually challenged and redefined. As research expanded beyond vaccine development and production, determining ways to identify the proteins that play key roles in disease proliferation, food production, and energy efficiency was made possible, in part, through the use of fundamental tools such as electrophoresis.

Since the late 1960s, the SDS-PAGE workflow has become a widely used technique in almost every field of study. While electrophoresis is a workhorse technique, it is not without its challenges. These have included tedious procedures and long time to results. At its inception, tools to standardize electrophoretic processes were not readily available. Researchers were required to recrystallize commercially available acrylamide to obtain the purity required for good separations and had to craft their own homemade gel apparatuses. Variations in tube lengths and lack of uniformity in applied electric fields translated into results that were often unreliable and unreproducible. As the technique evolved from its early stages, a lack of checkpoints at the different steps of the workflow meant that any errors were not discovered until final results — requiring the entire experiment to be repeated from the beginning. Time brought with it many key developments in electrophoresis including capillary zone electrophoresis and immobilized pH gradients, which eliminated problems with gradient instability and poor sample loading capacity. However, many of the difficulties facing researchers performing electrophoresis remained.

Established in the early 1950s with products that were initially targeted for chromatographic applications, Bio-Rad soon saw the potential impact of electrophoresis on advances in protein research. Bio-Rad scientists responded to limitations of the electrophoretic workflow with the introduction of electrophoresis-grade chemicals in 1969, followed by carefully designed tube gel systems in 1974. Over the past decades, Bio-Rad researchers have continued to develop ways to overcome challenges without compromising quality of results (see the sidebar at the bottom of the page for Bio-Rad solutions to challenges in the electrophoretic workflow). Early innovations were followed by the introduction of a series of products improving the efficiency of electrophoresis — the slab gel apparatus in 1984; a ready-to-use, nonhazardous stain compatible with western blotting (unlike traditional Coomassie gel staining, which is irreversible); and the first semi-dry blotting apparatus in 1988, which reduced transfer time of multiple gels from several hours to as little as 15 minutes. But perhaps the most critical milestone occurred in 1991, with Bio-Rad's introduction of the first commercially available precast gels for vertical electrophoresis systems.

Evolving into the Present

Y2K brought with it an environment of seemingly constant change. Potential that was only suggested in decades past started to become realized with such monumental milestones as the sequencing of the human genome in 2001 (and of countless other species and organisms since). Once mere ideas originating in the realm of science fiction, achievements such as targeted drug therapies, cloned organs to replace the diseased, and the modification of agricultural production to meet the food and energy needs of billions of people are now concrete possibilities. Today, more than ever, the rapid pace of discovery and unprecedented competition for grant funds and journal space require that researchers have access to tools that accelerate fundamental workflows — such as that for protein electrophoresis — without compromising the quality and reliability of results.

Sample Preparation

Challenges	Bio-Rad Solutons
▪ Sample complexity	▪ **MicroRotofor™ system** — divides samples into multiple fractions according to pI
▪ Detecting low-abundance proteins in complex samples	▪ **ProteoMiner™ system** — enriches low-abundance proteins from large volumes of virtually any complex sample

Separation: Pouring and Running Gels

Challenges	Bio-Rad Solutions	
▪ Speed	▪ **Mini-PROTEAN line of cells** — easy-to-use, reliable apparatuses that speed up the electrophoresis workflow while ensuring consistency in results	▪ **Criterion cell** — midi format system that increases throughput in a leak-free design
▪ Reproducibility		
▪ Gel leakage		
▪ Throughput		

In addition to sophisticated technologies that allow researchers to analyze interactions as well as identify and characterize proteins, Bio-Rad continues to focus on improving the basics. As it was in the 1960s and the 1990s with major contributions to improving the gel running step, this is perhaps best exemplified in the recent introduction of Mini-PROTEAN TGX long shelf life precast gels.

Long Shelf Life — a Natural Selection

Key challenges for scientists using electrophoresis continue to be reproducibility of data and time to results. Researchers need to identify or evaluate proteins of interest in samples of increasing complexity or from a wide variety of sources. This requires buffers that might contain denaturants or high salt levels and which could have high levels of nonprotein components such as lipids, complex polysaccharides, or plant pigments. Such factors pose further challenges. In response to these and other challenges, Bio-Rad introduced the Mini-PROTEAN TGX precast gels in 2009. These long shelf life gels are based on a novel modification of the Laemmli system (Figure 1).

Challenges of Traditional Laemmli Precast Systems

The Laemmli system — utilizing a Tris-glycine-SDS buffer system to resolve proteins based on molecular mass — is regarded as the gold standard for SDS-PAGE techniques due to its ability to cleanly resolve and provide accurate estimation of the molecular weight (MW) of proteins in complex samples from a wide variety of sources. However, precast Laemmli-system gels typically have a shelf life of only a few months. Gel performance degrades steadily over time, reducing reproducibility. Commercially available precast gels that offer longer shelf life typically use alternative buffer systems (for example, MOPS or MES) that present different electrophoretic patterns, affecting both the resolution and order of elution of proteins in complex mixtures. These systems cannot be used for MW estimations (Figure 2). Specialized buffer systems usually cost more per run, so given the widespread use of SDS-PAGE, this can have a significant adverse impact on a laboratory's budget.

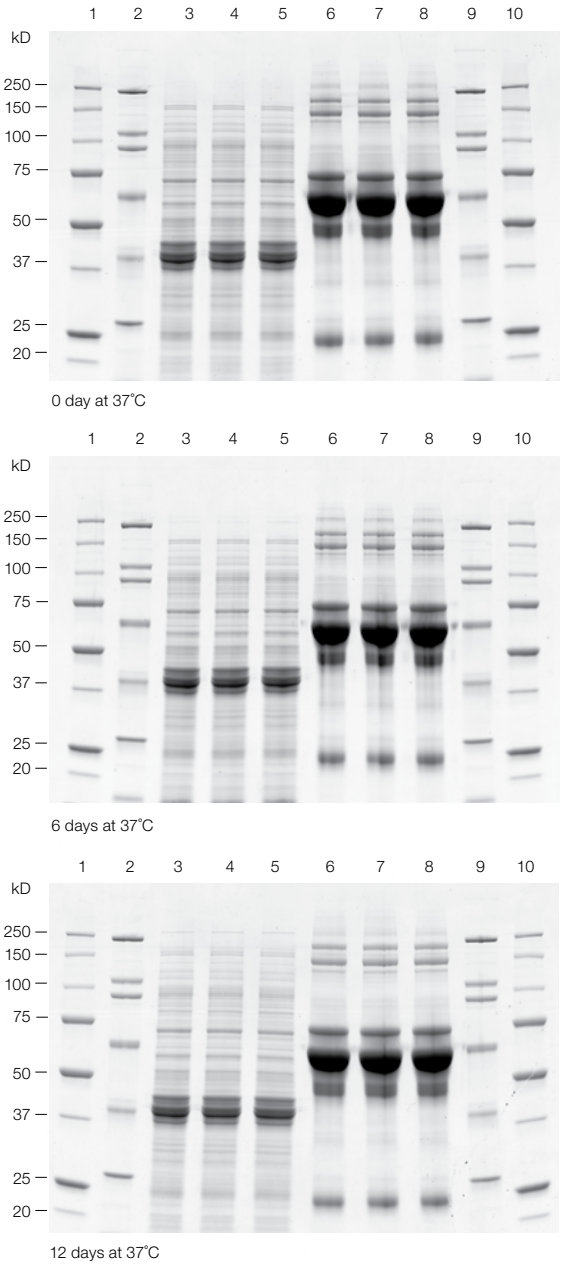


Fig. 1. Stability of Mini-PROTEAN TGX precast gels. The electrophoretic separation pattern is comparable on a 10% gel stored at 4˚C for 6 or even 12 months, relative to a gel run at day 0.

Dr Wolfgang Fischer

Staff Scientist, Clayton Foundation Laboratories for Peptide Biology
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Dr Wolfgang Fischer remembers a time before precast gels, when "we had to pour our own gels everyday, and it was the luck of the draw if they would polymerize properly, whether there were flaws, if we had degassed the solvents right." Today, the primary application of Fischer's laboratory is the characterization of proteins by mass spectrometry, and precast gels are an integral tool. Recently, Fischer conducted a comparative study in which the same complex sample was analyzed on TGX and competitor gels as well as by using the multi-dimensional liquid chromatography gel-free method. "The highest score in that comparison was obtained by the Bio-Rad gel," he states. "Not only was the score the highest, but I was also able to identify more proteins than by the other two approaches."

Challenges	Bio-Rad Solutions	
▪ Stability of gel matrix ▪ Choice of buffer systems ▪ Accuracy of MW estimations ▪ Time to results	▪ **Ready Gel® precast gels** — Tris-glycine; quality controls ensure reproducibility in results and precise gel gradients help improve the accuracy of MW estimations	▪ **Mini-PROTEAN TGX precast gels** — Tris-glycine; extended shelf life, gold standard Laemmli system, 15 min run times

Dr Ryan Jensen

Post-doctoral scholar, Stephen C. Kowalczykowski laboratory, University of California, Davis

Dr Ryan Jensen's research focus is on DNA repair and homologous recombination as they relate to breast cancer. He uses precast gels frequently when purifying the proteins involved in DNA repair. Currently, Jensen's work involves purifying a very fragile, 470 kD protein. "Since there's so much time invested in purifying the protein and doing the assay," he says, "the gels have to run perfectly every time. If a gel fails, that's a huge disaster." When using TGX gels, Jensen found that sample loading was easy and results were consistent. But the main appeal for him was the time savings. "In the environment that I'm in, with a very competitive project," he explains, "time is of the essence."

Gold Standard Laemmli — Long Shelf Life Format

TGX gels retain superior Laemmli-like separation characteristics using standard sample and Tris-glycine running buffers. The patented modification of the Laemmli system has significantly reduced hydrolysis of the gel matrix over time, thereby extending shelf life up to 12 months and delivering consistent, high-quality performance. A TGX gel stored at 4°C for 12 months provides resolution comparable to that of a new gel.

At 200 V, a typical run can be completed in 30 min (compared to 45–90 min); run completion time at 300 V is ~15 min and at 400 V is ~9 min. These already short run times can be further reduced by running TGX gels at highter voltages — without loss of separation quality and with no significant heat generation in the electrophoresis cell. The subsequent transfer step can be completed in 20 min (compared to 1 hr to overnight). TGX gels preserve workflow flexibility by enabling fast membrane transfers with excellent efficiencies and allowing the use of all stains with low backgrounds.

This new gel technology delivers uniform band shape and symmetry — lot-to-lot and within-lot reproducibility ensure consistency of results — and higher MW estimation accuracy when compared to traditional Laemmli precast gels or NuPAGE Bis-Tris gel systems (Figure 2). A linear regression analysis of estimated MW vs. known MW yields a coefficient of linearity (R^2) of greater than 0.98 for TGX gels, vs. an R^2 of only 0.91 for a representative Bis-Tris gel.

Dynamic range of quantitation and resolution are also superior with TGX gel technology (Figure 3). TGX gels exhibit greater lane and band symmetry than traditional Laemmli gels or alternative long shelf life gels, even when overloaded (Figure 4). TGX gels are currently available in 7.5%, 10%, 12%, 4–15%, 4–20%, and the unique Any kD™ gel formulation. Any kD gels offer resolution of proteins in the 10–250 kD range and can be used in screening experiments. These gels deliver superior resolution in the 10–100 kD range, the range most evaluated in 2-D samples, making it ideal for 2-D applications. Furthermore, all precast gels offered by Bio-Rad are manufactured without SDS and therefore can be used for native PAGE applications.

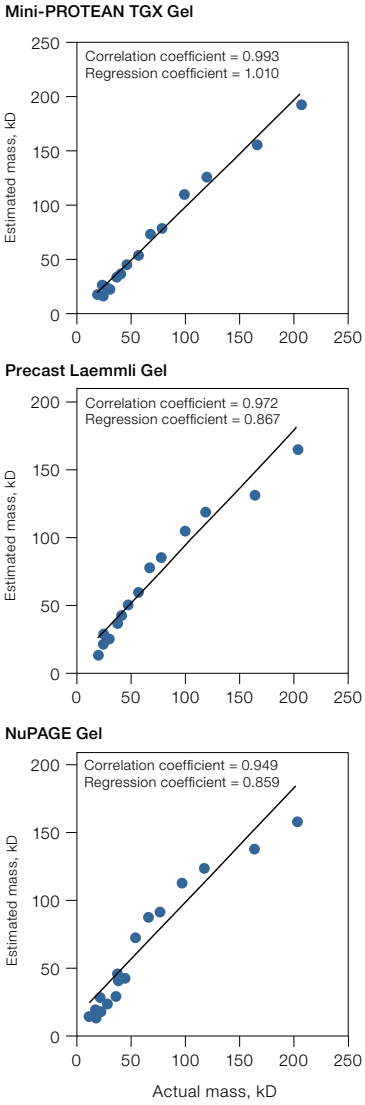

Fig. 2. Accuracy of MW estimation among different gel types. Different purified proteins (18) were run on the gel types indicated along with Precision Plus Protein™ Unstained standards. The MW of each purified protein was estimated by fitting the mobility of each protein to the line generated by plotting log MW vs. mobility (R_f) for the standards. The graphs present the mass of each protein estimated in this manner vs. its true mass (as estimated from the gene sequence or determined by mass spectrometry).

Challenges	Bio-Rad Solutons		
▪ Sensitivity ▪ Time to results ▪ Hazardous by-products ▪ Optimization	▪ **Bio-Safe™ colloidal Coomassie stain** — premixed, ready-to-use, nonhazardous formulation	▪ **Oriole fluorescent gel stain** — nanogram sensitivity; enables gel imaging within 2 hr post-run (vs. 18 hr with other stains), and eliminates the need for prior gel fixing or subsequent destaining	▪ **Criterion Stain Free imaging platform** — visualize proteins separated on gels in 5 min post-run; no staining required; preset protocols, 1-button operation

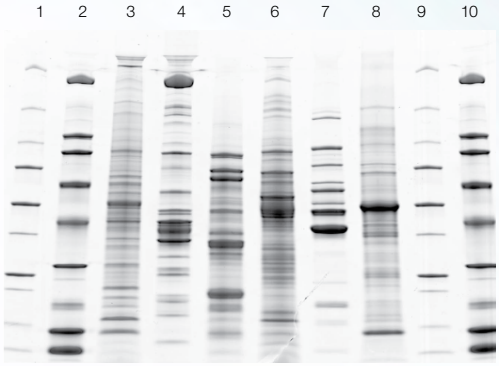

Fig. 3. Superior resolution of a variety of samples. Samples were loaded onto a 4–20% TGX gel in a volume of 5 µl. Lanes 1 and 9, Precision Plus Protein Unstained standards; lanes 2 and 10, broad range SDS-PAGE standards; lane 3, rat midbrain extract; lane 4, salmon muscle extract; lane 5, soybean extract; lane 6, rat liver microsomes; lane 7, bacteriophage T5; lane 8, soluble spinach protein.

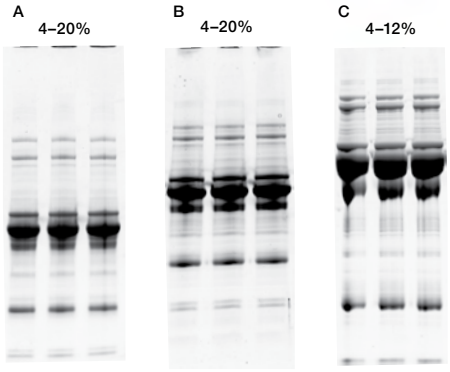

Fig. 4. Comparison of gel performance when overloaded with mouse serum. Mouse serum was diluted 20-fold into Laemmli sample buffer for Mini-PROTEAN TGX gels (**A**) and precast Laemmli gels (**B**) or into NuPAGE sample buffer for NuPAGE Bis-Tris gels (**C**) (Invitrogen Corporation). Samples (10 µl) were run according to manufacturers' instructions. Gels were stained with Bio-Safe Coomassie stain and scanned with a Molecular Imager® GS-800™ calibrated densitometer.

Looking Forward

The current electrophoresis workflow — from sample preparation to separation, visualization, and analysis — can still take as long as 5 days to complete. As the course of innovation and discovery continues its rapid pace through the new millennium, researchers will continue to seek faster processes — without compromise to accuracy, sensitivity, or reproducibility. Novel electrophoretic separation technologies, real-time monitoring of the electrophoresis run, alternative protein transfer and immunodetection methods, and faster imaging methods will all need to be explored to meet the need for higher throughput and sensitivity of detection in fewer steps.

Researchers will continue to look for modular systems that can be expanded without requiring complete replacement with each new innovation. Automation will be required to increase throughput and reduce time to results as well as hands-on time. In addition, life scientists today value "green" products that reduce the impact of research efforts on the environment. Bio-Rad's current and planned solutions pipeline will continue to include products and services that help overcome these remaining challenges posed by the electrophoresis workflow.

For more information, request **bulletins 5871, 5910, 5911, and 5933**.

Reference

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Downstream Analysis: Estimation of MW

Challenges	Bio-Rad Solutons
▪ Linearity ▪ Accuracy	▪ **Mini-PROTEAN TGX gels** — greater MW estimation accuracy ▪ **Precision Plus Protein standards** — provide sharp bands with highly reproducible relative migration rates

Downstream Analysis: Western Blotting

Challenges	Bio-Rad Solutons	
▪ Membrane transfer can take up to 16 hr to complete ▪ Time-consuming steps ▪ Sensitivity of detection	▪ **Trans-Blot™ SD semi-dry transfer cell** — reduces the time required to do transfers with multiple gels from several hours to as little as 15 min	▪ **Precut membranes and sandwiches** — improve the ease of use and speed of western blotting ▪ **Mini-PROTEAN TGX gels** — complete transfers in 30 min

Application of the Profinity eXact™ Fusion-Tag System to Eukaryotic Expression Systems

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Introduction

Protein overexpression and affinity purification are used in various biological research fields and play an important role in the postgenomic era. While expression of recombinant proteins in bacteria is often the preferred method, eukaryotic expression systems, especially those using insect and mammalian cells, are indispensable for producing proteins that are difficult to express in bacteria or that have specific required posttranslational modifications. Currently, different purification tags, like 6xHis and GST, are used with eukaryotic expression systems, but they have serious drawbacks. For example, insect and mammalian cells contain a higher percentage of His residues in their proteins than *E. coli*, which can lead to significant background binding to immobilized metal ions (Kimple and Sondek 2004). Furthermore, current affinity purification systems require a tag-removal step, which is necessary if the tag interferes with the function of the protein.

The Profinity eXact protein purification system offers a unique approach to affinity purification. It utilizes an immobilized, engineered protease that specifically recognizes and binds with subnanomolar affinity to an 8 kD Profinity eXact affinity tag fused to the N-terminus of the recombinant protein (Bryan 2000, Ruan et al. 2004). After column washing to remove impurities, a specific, controlled cleavage and removal of the tag from the target protein is rapidly performed directly on the column, resulting in the release of highly purified recombinant protein with a native or desired N-terminus. Since the tag remains firmly attached to the resin, the result of this simple process is a true, single-step affinity purification and tag removal procedure.

The Profinity eXact tag is derived from the Pro region of a protease isolated from *Bacillus subtilis*. Further, the Profinity eXact system was developed for bacterial expression, allowing single-step purification of tag-free proteins with high purity and high yields compared to other protein-based affinity resins. Potential concerns for utilization of this expression and purification system in eukaryotic cells include: (1) poor expression of the tag, (2) potential degradation of the tag by eukaryotic proteases due to its prokaryotic origin, (3) potential posttranslational modification or tertiary structure misfolding of the tag affecting binding and proper cleavage, and (4) excessive background contamination in the eluate because of high nonspecific binding of eukaryotic proteins to the immobilized protease.

In this report, we investigated these concerns using small-scale transfection of insect Sf9 cells and mammalian HeLa cells followed by expression and purification analysis. The results indicate that the Profinity eXact system can be used in eukaryotic cells for single-step purification of tag-free proteins with low background and without compromising the obvious function of the system. We tested expression and purification using GFP, MBP, and AKT protein kinases. The AKT kinases are key signal transducers in different biological processes including cell survival, cell growth, gene expression, and oncogenesis (Crowell et al. 2007). Our results show that all proteins tested can be expressed and purified well using the Profinity eXact system.

Methods

Cell Line, Vectors, Affinity Resin, and Assay

Vector pIEx6, Sf9 insect cells, BacVector insect cell medium, and Insect GeneJuice transfection reagent were from EMD Biosciences. Polyclonal primary antibody to green fluorescent protein (GFP) was from MBL International and monoclonal antibody to maltose binding protein (MBP) was from New England BioLabs. Secondary antibodies conjugated with HRP and Immun-Star™ WesternC™ chemiluminescence assay kit were from Bio-Rad Laboratories, Inc. pcDNA3.1 vector was from Life Technologies Corporation. The pPAL-MBP and pPAL-GFP vectors and the Profinity eXact resin were also from Bio-Rad.

Expression Vector Construction

The DNA fragment encoding the Profinity eXact tag was amplified by PCR using pPAL7 plasmid as a template and inserted into the pIEx6 plasmid digested with NcoI and NotI. The multiple cloning site downstream of the Profinity eXact tag was carried over to the pIEx6 vector for use in subcloning. The GFP gene was amplified by PCR using pPAL-GFP plasmid as a template and inserted into either pIEx6 vector or pIEx vector containing the Profinity eXact tag in order to obtain N-terminal-6xHis or Profinity eXact fusion–tagged GFP. The AKT1 cDNA clone was purchased from OriGene Technologies, Inc., amplified by PCR, and inserted in the tag-containing pIEx vector, downstream of the Profinity eXact tag. Both GFP and AKT1 protein sequences are immediately downstream of the Profinity eXact tag cleavage site FKAL.

The MBP gene was amplified by PCR using the pPAL-MBP plasmid as a template and inserted into the pcDNA3.1 vector to make the nontagged MBP construct pcDNA-MBP. The fragment from pPAL-MBP containing the Profinity eXact fusion tag and MBP was digested and inserted into the pcDNA3.1 vector to make the Profinity eXact fusion–tagged MBP gene, pcDNA–Profinity eXact tag–MBP.

Insect Sf9 Cells Expression System

Insect Sf9 cell cultures were maintained routinely in BacVector insect cell medium in a shaking flask at 28°C and 150 rpm. Endotoxin-free pIEx–Profinity eXact tag–GFP and pIEx-GFP plasmids (20 µg) were transfected into a 10 ml suspension of Sf9 insect cells with 100 µl of Insect GeneJuice transfection reagent according to the manufacturer's instructions. After 3 days' incubation, the cells were harvested, resuspended, and sonicated in Profinity eXact bind/wash buffer (0.1 M sodium phosphate, pH 7.2) or in lysis buffer (50 mM Tris-acetate, 100 mM NaOAc, 5% glycerol, 5 mM β-mercaptoethanol, pH 7.2). The supernatant was then applied to 20 µl of equilibrated Profinity eXact resin in a Mini Bio-Spin™ spin column (Bio-Rad) and incubated at room temperature for 10 min. The flow-through fraction was collected and the resin was washed with Profinity eXact bind/wash buffer or the above lysis buffer three times with ten column volumes (CV) each time. The column was then incubated in 40 µl of the above binding buffer or lysis buffer containing 10 mM sodium azide on a rotator. Following a 30 min room temperature cleavage incubation, the tag-free protein was eluted from the resin by a simple spin, and the elution process was repeated once more. Posttransfection expression was monitored on a 4–20% Criterion Stain Free™ gel followed by image acquisition and analysis using a Criterion Stain Free gel imaging system. The purification process was monitored by loading 10 µl of each fraction onto a 4–20% Criterion Tris-HCl gel followed by Bio-Safe™ Coomassie staining. Image acquisition and analysis were performed using the Molecular Imager® GS-800™ calibrated densitometer and Quantity One® 1-D analysis software.

HeLa Cell Expression System

Mammalian HeLa cell cultures were maintained routinely in medium containing 90% DMEM, 10% FBS, 1 mM NEAA, and 1 mM sodium pyruvate in culture dishes at 37°C with 5% CO_2. pcDNA-MBP or pcDNA–Profinity eXact tag–MBP plasmids (2 µg) were transfected using TransFectin™ lipid reagent (Bio-Rad) into HeLa cells cultured in 6-well plates following manufacturer's instructions. The transfected cells were harvested after a 21 hr incubation at 37°C. The cells were resuspended and lysed in Profinity eXact bind/wash buffer containing 0.5% NP-40. The supernatant was then applied to 20 µl of equilibrated Profinity eXact resin. The flow-through fraction was collected and the resin was washed in the Profinity eXact bind/wash buffer 2 to 3 times with 10 CV each time. The tag-free target protein was cleaved for 30–60 min and eluted from the resin using the Profinity eXact elution buffer with 0.1 M sodium fluoride as the cleavage trigger. The purification process was monitored by loading 10 µl of each fraction on a 4–20% Criterion Tris-HCl gel followed by Bio-Safe Coomassie stain. Image acquisition and analysis were performed using the GS-800 densitometer and Quantity One software.

Western Blot Analysis

Protein samples separated on SDS-PAGE gels were transferred onto PVDF membranes, and the standard protocol was followed for western blot analysis. The membranes were incubated for at least 1 hr in blocking buffer (TBS with 0.5% Tween 20 and 3% BSA) before incubation with either 1:10,000 or 1:500 diluted anti-MBP or anti-GFP antibodies, respectively. After three washes, the membranes were incubated with 1:10,000 diluted HRP conjugated goat–anti-mouse or goat–anti-rabbit secondary antibodies, respectively. The signal was developed using the Immun-Star WesternC kit and imaged using the Molecular Imager® ChemiDoc™ XRS imaging system (Bio-Rad).

Molecular Weight Determination of the Profinity eXact Tag by Mass Spectrometry

Following the standard resin cleaning procedure using 0.1 M H_3PO_4, the Profinity eXact tag was stripped from the resin used for the purification of Profinity eXact fusion–tagged MBP from HeLa cell and bacterial lysates, vacuum-dried, and resuspended in 2.5% TFA. The solutions were desalted using ZipTip pipet tips with C_{18} resin (Millipore) and eluted with 50% acetonitrile containing 0.1% TFA. A small aliquot of each eluate was loaded separately or combined onto a ProteinChip® NP20 array with SPA matrix, and the mass spectra obtained using the ProteinChip SELDI system (all from Bio-Rad).

Results and Discussion

Expression and Purification of Profinity eXact Fusion–Tagged Proteins in Insect Sf9 Cells

To examine the expression and purification of Profinity eXact fusion–tagged proteins in insect cells, genes encoding GFP and AKT1 were fused immediately downstream of the Profinity eXact tag sequence in the plasmid-based insect cell expression vector, pIEx6. The transcription of the chimeric genes is driven by the IE1 promoter, which together with the hr5 enhancer recruits the endogenous insect cell transcription machinery, thereby avoiding baculovirus

infection and associated cytotoxic effects. Small-scale transfection was performed and the expression of the Profinity eXact fusion–tagged GFP was analyzed by western blot using anti-GFP antibody. The expression of the protein was about 2-fold lower than the expression of the 6xHis fusion–tagged GFP in the cell lysate (Figure 1), which is not surprising because the Profinity eXact tag is much bigger than the 6xHis tag, and the Profinity eXact tag used here is not codon-optimized specifically for insect cells.

The cell lysate was then loaded onto Profinity eXact resin, and the tag-free GFP was obtained in high purity (Figure 2). Another protein, human AKT1 kinase, was also tested and the results confirmed expression of the protein by western blot analysis (data not shown). The purity of the eluted tag-free AKT1 protein was lower than that of GFP mainly due to lower overall expression. The major contaminant band is likely undigested Profinity eXact fusion–tagged AKT1, which can easily be removed by incubating with the elution buffer for a prolonged period. The results indicate that the Profinity eXact system can be used in insect cells for protein expression and single-step purification to obtain tag-free proteins.

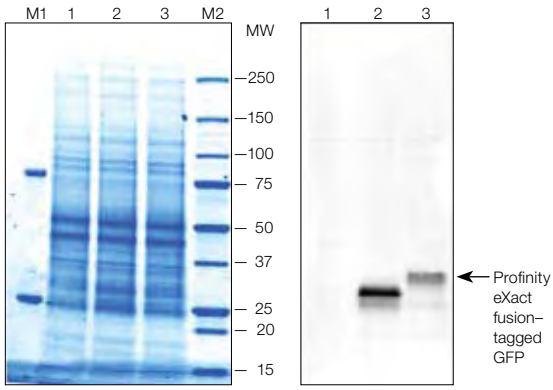

Fig. 1. Expression analysis of Profinity eXact fusion–tagged GFP by western blot. Small-scale transfection of insect Sf9 cells was conducted using the constructs containing genes that encode GFP tagged with His or the Profinity eXact tag. The total cell lysate was separated on a 4–20% Criterion Stain Free gel (**left panel**) and imaged before transferring onto a PVDF membrane. Western blot analysis using polyclonal anti-GFP antibody is shown (**right panel**). **M1**, Precision Plus Protein™ Dual Color standards; **1**, insect cell control; **2**, His-GFP; **3**, Profinity eXact tag–GFP; **M2**, Precision Plus Protein Unstained standards.

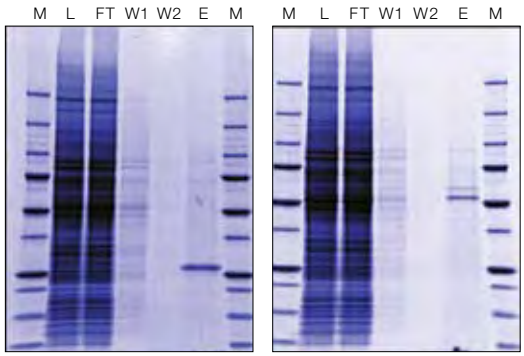

Fig. 2. Small-scale purification of tag-free GFP (left) and AKT1 (right) from the Sf9 cell transfectants. L, lysate; **FT**, flowthrough; **W1** and **W2**, washes; **E**, elution. **M**, Precision Plus Protein Unstained standards.

Expression and Purification of Profinity eXact Fusion–Tagged Proteins in HeLa Cells

Protein expression in mammalian cells is an alternative means of expressing and purifying target proteins, especially those that do not express well in bacteria, or that need posttranslational modifications for their activity. To analyze the applicability of the Profinity eXact system to mammalian cell expression, chimeric genes encoding Profinity eXact fusion–tagged or non-tagged MBP were constructed in the CMV promoter–based mammalian expression vector pcDNA3.1. The constructs were transfected and transiently expressed in rapidly growing HeLa cells. After a 21 hr incubation at 37°C, cell lysates were analyzed by western blot for the expression of the target proteins. The results indicate that Profinity eXact fusion–tagged MBP can be expressed well in HeLa cells, and that the expression level is comparable to the MBP without any tag, suggesting that the tag does not affect the expression or the stability of the proteins (Figure 3).

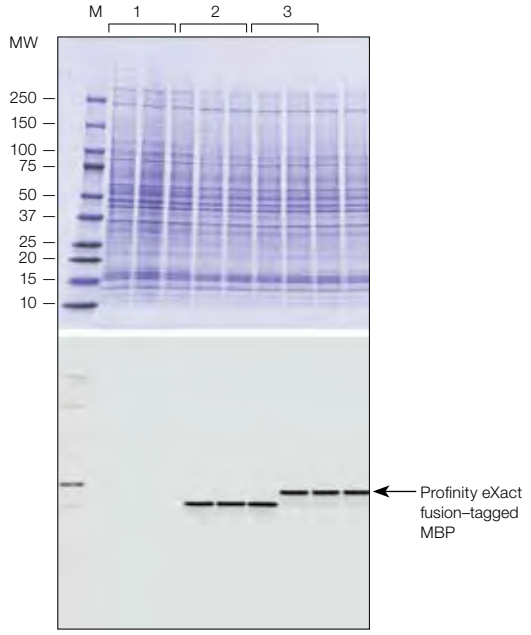

Fig. 3. Expression of Profinity eXact fusion–tagged MBP in HeLa cells. Protein extracts from each HeLa cell transfectant were loaded in triplicate. **M**, Precision Plus Protein Dual Color standards; **1**, HeLa cells without plasmid; **2**, HeLa cells transfected with MBP gene construct; **3**, HeLa cells transfected with the chimeric gene construct of the Profinity eXact fusion tag and MBP. Monoclonal anti-MBP antibody was used as the primary antibody.

Furthermore, small-scale purification of the tag-free MBP was conducted using the cell lysate from the HeLa cell transfectant (Figure 4). The tag-free MBP was obtained in the elution fraction with high homogeneity, indicating the Profinity eXact system can be used to express Profinity eXact fusion–tagged proteins in mammalian cells and purify tag-free proteins.

Proteins or peptides expressed in mammalian cells often undergo posttranslational modifications, which may affect their function. To determine whether the Profinity eXact tag, which is of prokaryotic origin, is modified in HeLa cells, the Profinity eXact tags stripped from the column after purification from either bacterial or HeLa cell lysates were analyzed for their molecular mass using a SELDI mass spectrometer. The results (Figure 5) showed that the Profinity eXact tag expressed in mammalian cells has the same molecular weight as the tag expressed in *E. coli*. This indicates that the Profinity eXact tag is not modified after translation, thus its function as a purification tag and its cleavability are most likely not impacted in mammalian cells. The successful purification of the tag-free MBP (Figure 4) confirms that the Profinity eXact system is fully functional in HeLa cells.

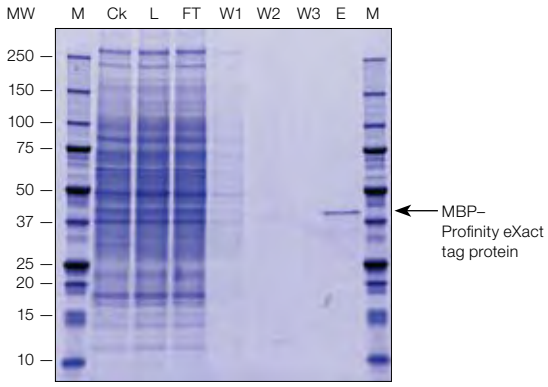

Fig. 4. Small-scale purification of tag-free MBP from HeLa cells. The purification of the MBP was analyzed by SDS-PAGE by separating all the purification fractions on 4–20% Criterion gel. **Ck**, HeLa cells without plasmid transfection; **L**, cell lysate; **FT**, flowthrough; **W1**–**W3**, washes; **E**, elution. **M**, Precision Plus Protein Dual Color standards.

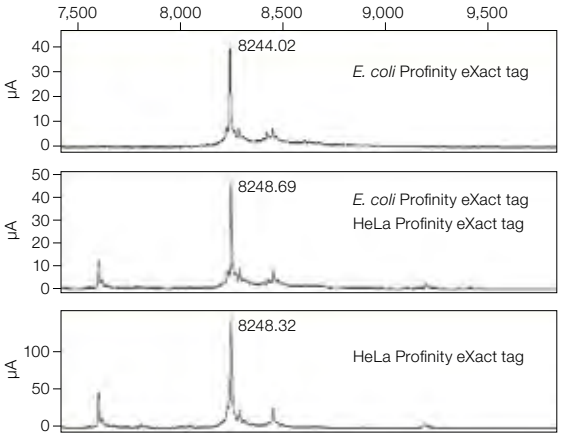

Fig. 5. Mass analysis of the Profinity eXact tags expressed in bacteria and HeLa cells. The Profinity eXact tags stripped from the column after purification from bacterial or HeLa cell lysates were spotted on uncoated ProteinChip arrays and their molecular mass was determined by mass spectrometry using the ProteinChip SELDI system.

Conclusions

This work demonstrates that the Profinity eXact purification system is adaptable for use in mammalian and insect cells. Both expression of the Profinity eXact fusion–tagged proteins and single-step purification of the tag-free proteins with high purity can be achieved: (1) with equal or slightly poor expression of the tag in HeLa and insect cells, (2) without obvious degradation of the tag by eukaryotic proteases due to its prokaryotic origin, (3) without potential posttranslational modification or tertiary structure misfolding of the tag in HeLa cells, and (4) with very low background contamination in the eluate from nonspecific binding of eukaryotic proteins to the immobilized protease. Enhanced expression in either the insect or mammalian cell systems may be observed by utilizing a host-appropriate, codon-optimized version of the Profinity eXact tag and target proteins.

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Multiplexing Across Cytokine Panels: Bio-Plex Pro™ Human and Mouse Group I and Group II

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Introduction

Cytokines, chemokines, and growth factors play an important role in a wide range of physiological processes including immune response, inflammation, and hematopoiesis. They have great utility as biomarkers for a spectrum of disease states. Bio-Rad's Bio-Plex Pro cytokine assays are designed to rapidly quantitate these proteins in diverse matrices such as serum, plasma, and tissue culture supernatant.

The cytokine assay menu includes 48 human and 32 mouse assays that are available in distinct panels: human group I and II and mouse group I and II. These assays are based on magnetic beads, which allow automation of wash steps (using the Bio-Plex Pro wash station), and simplify the assay process by eliminating the need for manual washes on a vacuum manifold. The assays are configured into all-in-one kits that contain the required buffers and diluents. Detailed information about the magnetic bead–based cytokine panels has been described previously (see bulletins 5800 and 5803).

The effectiveness of immunoassays depends on the analytical sensitivity and specificity of the antigen-antibody reaction for the analyte. In this article, the cross-reactivity of antibodies within each species (human or mouse) is described. We show that the majority of human group I and II cytokines may be combined in a 48-plex assay, and all analytes within mouse groups I and II may be combined into a 32-plex assay.

Methods

To evaluate the ability to combine group I and II assays, human group I (27-plex) and group II (21-plex) analytes were tested individually and in combination as a 48-plex. Similarly, mouse group I (23-plex) and group II (9-plex) analytes were tested individually and in combination as a 32-plex.

Evaluation of Assay Specificity

The assay specificity when mixing the analytes from the two assay groups was determined by examining the ratio of background median fluorescence intensity (MFI) of group I and II assays when tested alone and in combination. Background MFI is the signal obtained in the absence of specific binding and is attributable to assay noise such as instrument and reagent noise. It was determined in the presence of multiplexed beads coupled to capture antibodies and detection antibodies in standard diluent lacking the standard proteins. Specificity within each assay group was also established in single detection antibody cross-reactivity experiments for individual analytes.

Evaluation of Assay Sensitivity

The impact on the assay sensitivity upon mixing of analytes from the two assay groups was determined by comparing the differences in the limit of detection (LOD) between individual and combined panels. LOD is defined as the concentration of analyte (measured in the standard diluent) from the standard curve for which the corresponding MFI is two standard deviations above the background.

Evaluation of Assay Accuracy

The accuracy of the assay is determined by the standard recovery of each analyte. It is calculated as the percentage of the observed value of a spiked standard of known concentration relative to its expected value; the acceptable recovery is 70–130% of the expected value.

The ability to multiplex is defined when:

- Assays display background MFI ratio <10
- LOD values do not change considerably between groups I and II when tested alone and in combination
- Analyte recovery is between 70 and 130%

Results

For human cytokine analytes, the background MFI values did not change considerably in the 48-plex assay, except for seven group II analytes: CTACK, IL-1α, IL-3, IL-12 (p40), M-CSF, SDF-1α, and TNF-β. These analytes also exhibited high LOD values in the 48-plex assay. To identify which detection antibodies of human cytokine group I contributed to the high background MFI values, single detection antibody cross-reactivity tests were conducted. The group I detection antibodies that cross-reacted nonspecifically to group II capture antibodies are noted in Table 1.

Mouse cytokine assays (groups I and II either individually or in combination) did not show considerable increases in either background MFI or LOD values.

Table 1. Identification of human cytokine group I detection antibodies that cross-react with group II capture antibodies.

Human Cytokine 27-Plex Panel, Group I Detection Antibodies	Human Cytokine 21-Plex Panel, Group II Capture Antibodies																				
	CTACK	GRO-α	HGF	IFN-α2	IL-1α	IL-2Rα	IL-3	IL-12 (p40)	IL-16	IL-18	LIF	MCP-3	M-CSF	MIF	MIG	β-NGF	SCF	SCGF-β	SDF-1α	TNF-β	TRAIL
IL-1β																					
IL-1ra																					
IL-2					•		•	•				•								•	
IL-4																					
IL-5																			•		
IL-6																					
IL-7	•																				
IL-8																			•		
IL-9	•																				
IL-10																					
IL-12 (p70)							•	•				•									
IL-13					•																
IL-15																					
IL-17																					
Basic FGF																					
Eotaxin																					
G-CSF	•																				
GM-CSF																					
IFN-γ																					
IP-10																					
MCP-1 (MCAF)																					
MIP-1α																					
MIP-1β																					
PDGF-BB																					
RANTES																					
TNF-α																					
VEGF	•																				

• Denotes cross-reactivity between group I detection antibodies and group II capture antibodies.

The recovery of all the analytes was 70–130%, within the working assay range, except for CTACK, IL-3, and IL-12 (p40) from the human group II cytokine panel, which resulted in a narrower working range. For mouse group I and II assays, the standard recovery for the 23-plex and the 9-plex panels was comparable to that of the 32-plex format (70–130%), with the exception of these four analytes: IL-1α, GM-CSF, IL-18, and MIP-2. This effect was observed only at the high end of the standard curve and did not impact the sensitivity; therefore, they may be multiplexed with the other analytes.

While cross-reactivity in the majority of the assays was less than 2%, higher cross-reactivity was detected among some analytes, especially those with high sequence similarities. All analytes, with the exception of MIP-1α and MIP-1β, and IL-12 (p40) and IL-12 (p70), demonstrated <2% cross-reactivity for both human and mouse.

Conclusions

The cross-reactivity of group I and group II cytokine panels within each species (human and mouse) was determined.

Based on background MFI and LOD values, all but the following seven assays from group II may be combined with group I assays: CTACK, IL-1α, IL-3, IL-12 (p40), M-CSF, SDF-1α, and TNF-β. The single detection antibody cross-reactivity test identified the specific detection antibodies that caused the high background, with IL-2, IL-12 (p40), and IL-12 (p70) detection antibodies being the major contributors.

Based on the background MFI and LOD values, all mouse groups I and II assays may be combined with minimal impact on assay performance.

Guide to Ligand Immobilization for Protein Interaction Studies

Introduction

Surface plasmon resonance (SPR) has revolutionized the study of biomolecular interactions by providing a platform that does not require that the ligand or analyte be labeled. SPR measures the interaction of ligands immobilized on the surface of a sensor chip with analytes in solution, in real time, providing kinetic, equilibrium, and concentration data. Performing interaction analysis on an active and stable ligand surface is the key to generating robust data.

However, determining the optimal conditions for ligand immobilization can be a time-consuming activity. The ProteOn™ protein interaction array system has a unique 6 x 6 array in which six ligand channels intersect with six analyte channels, creating 36 interaction spots. In this manner, six different ligand immobilization conditions (surface activation, pH, ligand concentration, etc.) can be tested in parallel, allowing rapid optimization of immobilization conditions.

Multiple analytes can also be measured and analyzed in parallel to speed assay screening. The multiplexing capabilities of the ProteOn system are advantageous during both optimization and assay screening, generating data rapidly for analysis.

Major Steps in the Ligand Immobilization Process

Five major steps are involved in ligand immobilization:

- Conditioning
- Activation
- Immobilization
- Deactivation
- Stabilization

The conditioning step involves cleaning the chip surface to obtain the best quality data and rapid stabilization of the baseline prior to the start of an experiment. Activation prepares the sensor chip surface for amine coupling. In the third step, ligand is bound to the surface of the chip. Following ligand immobilization, any residual activated carboxyl groups are deactivated by ethanolamine HCl. Addition of this reagent also removes any remaining electrostatically bound proteins. Finally, a stabilization solution such as 50 mm NaOH can be used to wash nonspecifically bound molecules from the chip surface. Additional details on the activation and ligand immobilization steps are described here.

Activation Step

Reactive groups are formed on the sensor chip surface by addition of an equal-volume mixture of the activation chemicals EDAC and sulfo-NHS from the ProteOn amine coupling kit. The ligand is attracted to these reactive groups and covalently binds through amine coupling; any primary amine can bind. The EDAC/sulfo-NHS mixture is not stable and has a half-life of 30–60 min; it must be prepared fresh for each experiment. Dilutions of the EDAC/sulfo-NHS mixture in water achieve a lower degree of surface activation. This is useful for testing kinetic conditions under different ligand densities.

Ligand Immobilization Step

The most important factor affecting the level of immobilization is electrostatic attraction of the ligand to the surface: without it, immobilization is not possible. Additional factors are the levels of surface activation and ligand concentration, ligand size, and injection parameters such as injection volume, flow rate, and contact time.

Electrostatic Attraction

For the ligand to be immobilized on the sensor chip, it must be attracted to the surface of the chip. After activation, the sensor chip surface will have an overall negative charge; therefore, the ligand must have an overall positive charge. Because each ProteOn sensor chip has six ligand channels, it is easy to test multiple immobilization conditions (for example, immobilization buffers of different pH) to determine which gives the highest level of immobilization (Figure 1). Care must be taken to ensure that the immobilization conditions used allow the ligand to retain its activity. If buffers with very high or very low pH values are

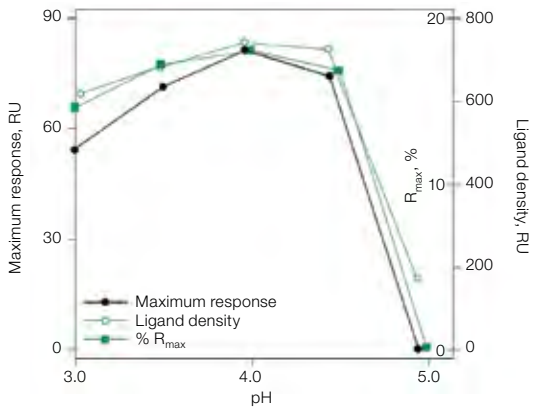


Fig. 1. Effect of immobilization buffer pH on ligand density, ligand activity (% R_{max}), and subsequent analyte response (R_{max}). The scales have been adjusted to align the response of each parameter to the same plot.

used, the ligand may be denatured or unfolded and so lose its activity. A detergent can be added to the immobilization buffer to enhance binding.

Level of Surface Activation

Different levels of activation of the surface can be used to create surfaces with different ligand densities.

Ligand Conditions

The concentration of the ligand will also affect how much of it can be immobilized. Typically, concentrations of 5–100 µg/ml should be sufficient to attain a good level of immobilization.

Ligand Injection Parameters

Flow rate and contact time can have significant impact on ligand immobilization. The default injection flow rate for the ProteOn system is 30 µl/min for 5 min. Reducing flow rate will increase immobilization, as will increasing the injection volume, the contact time, or both.

Guidelines for Immobilization Levels

Importance of R_{max}

The ratio of the mass of the ligand to the mass of the analyte can be used to determine how much ligand should be immobilized to produce a specific analyte response.

The level of ligand immobilization depends on the type of interaction you want to study, but a good guideline is "less is more." If the surface density is too high, you may observe mass transport effects whereby the rate of diffusion of the analyte from bulk solution to the sensor chip surface is slower than the rate of binding of the analyte to the ligand. The result is a shortage of analyte on the surface. If you are working with a bivalent analyte, you may also see altered kinetics from cross-reactivity. An easy way to determine optimum surface density is to calculate the theoretical R_{max} of the interaction you are studying (Figure 2). The theoretical R_{max} is the maximum analyte response assuming that the ligand is 100% pure and 100% active and that all binding sites are available.

$$R_{max} = \frac{MW_A}{MW_L} \times R_L \times n$$

R_{max}, maximum theoretical response of the analyte for a given ligand level.
L, ligand.
A, analyte.
R_L, amount of ligand immobilized.
MW, molecular weight.
n, stoichiometry of the reaction.

Fig. 2. Determining R_{max}. The standard analyte response that gives the best data is 100–200 RU (response units).

It is assumed that amine coupling is random between the ligand and the sensor chip and that not all ligand binding sites will be available, because the ligand is not present in a homogenous orientation on the sensor chip surface.

Choosing a Sensor Chip for Amine Coupling

Which ProteOn sensor chip you use depends on the level of ligand immobilization that is required and on your specific application. Each chip — GLC, GLM, and GLH — has a different ligand binding density. The GLC sensor chip offers the lowest binding capacity and the GLH chip the highest. By calculating the R_{max} for your interaction, you can determine which chip to use. For protein-protein interactions, the GLC and GLM chips are sufficient (Figure 3).

For related information, request **bulletin 5367.**

For a full-length version of the guide to ligand immobilization, request **bulletin 5821.**

$$200\ RU = \frac{5\ kD}{10\ kD} \times R_L \times 1$$

$$200\ RU = \frac{1}{2} \times R_L$$

$$400\ RU = R_L$$

RU, response units.
R_L, amount of ligand immobilized.

Fig. 3. Using R_{max} in protein-protein interactions. At least 400 RU of protein A ligand (10 kD) must be immobilized for a 200 RU response of protein B analyte (5 kD) to be seen, assuming maximum ligand activity, purity, and correct orientation. However, it is usual to immobilize twice as much (800 RU) of protein A, to counter the fact that not all protein A ligand may be active or in the correct orientation.

For protein–small molecule experiments in which you would expect to immobilize larger amounts of ligand, the GLM and GLH chips are the best choice (Figure 4). A complete guide to the different ProteOn sensor chips is provided in the ProteOn system manual.

$$200\ RU = \frac{1\ kD}{75\ kD} \times R_L \times 1$$

$$200\ RU = \frac{1}{75} \times R_L$$

$$15{,}000\ RU = R_L$$

RU, response units.
R_L, amount of ligand immobilized.

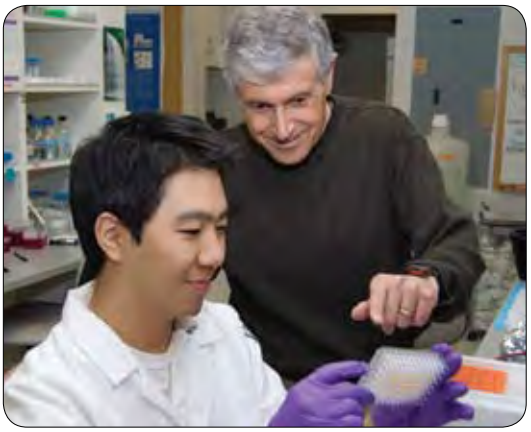
Fig. 4. R_{max} for protein–small molecule interactions. At least 15,000 RU of protein Y ligand (75 kD) must be immobilized for a 200 RU response of compound X analyte to be seen, assuming maximum ligand activity, purity, and correct orientation. More ligand may be immobilized to counter activity and orientation effects, see Figure 3.

Conclusion

The suggestions made in this article are to guide the researcher in assay development and optimization using the ProteOn XPR36 protein interaction array system. Combining these ideas with thorough preparation and planning will result in exceptional interaction data.

Targeting Suppression of B-Cell Lymphoma Proliferation

For more information, request **bulletin 5942**.



Dr John J Rossi supervising research in a laboratory at the City of Hope Comprehensive Cancer Center.

Dr John J Rossi's titles and accolades are many and varied — and well earned. In his current affiliation with the City of Hope Comprehensive Cancer Center, Rossi serves as chair and professor of molecular and cellular biology, dean of the graduate school of biological sciences, and associate director for laboratory research. He is co-leader of the cancer biology program and the first holder of the Lidow Family Research Chair. These professional accomplishments are complemented by numerous awards, including a 2002 Merit Award in the Division of AIDS, National Institute of Allergy and Infectious Diseases. The common thread that weaves all of these activities and achievements together continues to be an unabashed enthusiasm for and curiosity toward scientific discovery — specifically in the molecular genetics of disease.

Rossi received his doctoral degree in microbial genetics in the late 1970s. At the time, cloning was only just becoming a tool that researchers could use, and with Rossi's exposure to this now basic technique, his fascination with genetics turned to the molecular aspects of the discipline. Rossi was drawn to postdoctoral studies in Dr Arthur Landy's lab at Brown University because of Landy's groundbreaking work in sequencing genetic information for the bacteriophage lambda. Landy's work focused on trying to understand some of the sequences of the attachment site of the bacteriophage in its host chromosome. He also completed the first restriction map of any lambda phage. Rossi was particularly attracted by the technology he would have access to in this forward-thinking environment.

In the early 1980s, Dr Keiichi Itakura with the City of Hope, whom Rossi refers to as "one of the fathers of DNA synthesis," was looking for a person to work with synthetic DNA and apply it to some problems in biology and medicine. "Today, it's a routine tool; you don't even think twice about ordering a piece of DNA," says Rossi. "But at the time, it was a hot area that was just evolving, so I came here [to the City of Hope] initially to learn how to make DNA, and then how to use it."

Rossi's early projects with the City of Hope focused on developing antisense technology. "Because we had synthetic DNA capabilities, we wanted to see if we could use it to block gene function," he explains. "We probably had the first example of using an antisense oligonucleotide to block replication of a virus." But because the research was conducted in bacteria and not considered publication worthy, his group never submitted the findings. This project did, however, lay the foundation for similar studies related to ribozymes. "Ribozymes have the capability of cleaving the target — this became fascinating because I wanted to understand more about the mechanisms and how they would function inside a living cell," Rossi explains. His research focus continued primarily on ribozymes — until RNA interference (RNAi) was discovered.

Rossi considers the discovery of RNAi as pivotal to antiviral discovery. "RNAi has become another important, powerful genetic tool we can use to further our antiviral work," he says. Rossi's lab became the first to publish research demonstrating the expression of small interfering RNAs (siRNAs) to inhibit HIV replication in human cells. Various aspects of RNAi and siRNAs continue to play a key role in his research on viral diseases and lymphomas.

One of the stumbling blocks inhibiting progress in his latest research efforts, however, has occurred in the transfection step of the RNAi workflow. "When studying siRNAs, you have to get the synthetic molecules into the host cells, and for many cultured cells — especially if they're adherent to the place in which they're grown — it's common to use lipid-based transfection agents. But for primary cells such as B cells, T cells — they're impossible to transfect," Rossi explains. This is where electroporation comes into play.

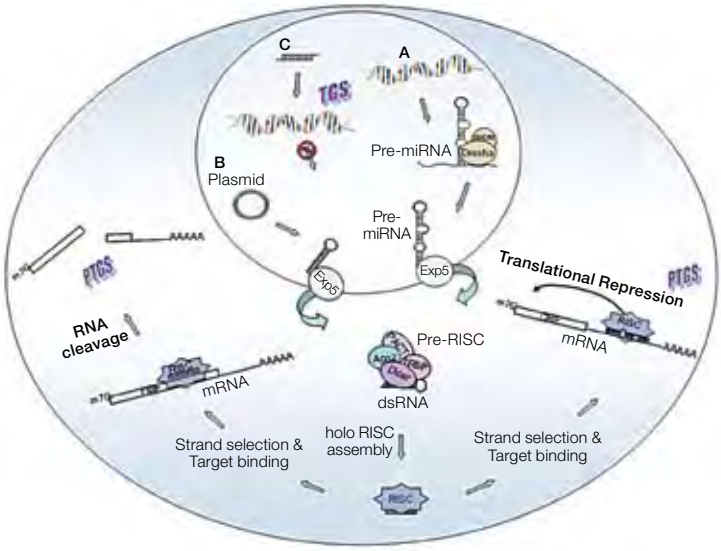


Fig. 1. Mechanism of cellular gene silencing. **A**, Primary microRNAs (pri-miRNAs) are processed by Drosha and its partner DGCR8 into precursor miRNAs (pre-miRNAs) and transported to the cytoplasm by exportin 5 (Exp5). In the cytoplasm, they are processed to yield the guide sequence containing all the components required for gene silencing. The guide sequence binds to the target sequences in the 3' UTRs. If the miRNA guide sequence is fully complementary to its target (**left pathway**), site-specific cleavage and degradation of the mRNA is triggered. If the base pairing is incomplete (**right pathway**) but includes pairing of the seed region with the target, translational inhibition occurs, often accompanied by messenger degradation. **B**, Artificially transcribed shRNAs are transported to the cytoplasm similar to miRNAs. The dsRNA in the cytoplasm is recognized and processed by Dicer into ~21–25 nucleotide siRNA fragments that are loaded into the RISC. The siRNAs target complementary sequences of cellular mRNAs and trigger their degradation through AGO2-mediated cleavage. **C**, When siRNAs are present in the nucleus and are complementary to promoter regions, they can trigger transcriptional gene silencing (TGS).

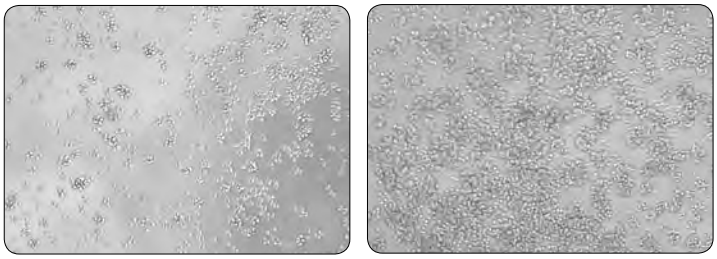


Fig. 2. Bright field image of human B cells. Jeko (**left**) and Z138 (**right**) cells were transiently transfected with siRNAs using the Gene Pulser MXcell electroporation system. Cells were viewed 24 hr posttransfection under an inverted microscope using bright field imaging. Other attempts (amaxa electroporator and lipid transfection) to transfect into these cell lines were unsuccessful.

Currently, Rossi's group is seeking the genetic mechanisms critical to the suppression of B-cell lymphoma proliferation. Lymphomas are cancers derived from lymphocytes, a type of white blood cell that protects the organism from foreign bodies such as bacteria and viruses. B-cell lymphoma is caused primarily by chromosomal translocations and constitutes approximately 85% of all non-Hodgkin's lymphomas. In a recent experiment, his group introduced bifunctional siRNAs (each strand targets different genes) that target suspected anti-growth pathways into various B-cell lines to effectively knock out the ability of these cells to proliferate (Figure 1).

The cells used in Rossi's experiments were all in suspension, so each line was different, and determining electroporation conditions became a major obstacle to pursuing this avenue of experimentation. "We tried the amaxa electroporator, ventromers — you name it and it seems like we tried it," says Rossi. But nothing enabled them to electroporate siRNAs into B cells and retain cell viability — until his lab was offered the opportunity to work with Bio-Rad's Gene Pulser MXcell™ electroporation system (Figure 2).

The Gene Pulser MXcell system is a flexible platform allowing both manual and preset programming. Because the Gene Pulser MXcell system allowed Rossi's group to perform multiwell analyses and test multiple conditions simultaneously, they were finally able to determine conditions in which "the cells weren't dying, we were getting transfection, and we could monitor target knockdown," says Rossi. "The Gene Pulser MXcell system proved very, very useful — I think it's a great little invention."

Ultimately, the group was able to demonstrate that the bifunctional siRNAs of interest can independently knock out their targets in B cells (data pending publication).

As it was in the beginning of his career with the fascination of cloning as a tool to uncover genetic mysteries, Rossi today remains intrigued by the application of cutting-edge tools and technologies to help further research.

BioRadiations 1975: Using Hydroxylapatite to Improve DNA Isolation and Separation

In this column, we look back at previously published issues of *BioRadiations* to see how tools and applications have changed since Bio-Rad first began publishing the journal in 1965.

Then!

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BIO-RADIATIONS

No. 18 March 1975

Hydroxylapatite—Super Separator!

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