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BioRadiations

A Resource for Life Science Research

HIGH-RESOLUTION MELT ANALYSIS

A Cost-Effective, Highly Sensitive Genetic Analysis Tool

In this issue:

BIO-RAD

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With Bio-Rad's Criterion Stain Free™ gel imaging system you get what you've been asking for: fast, clean, reliable protein gel results. You can now detect and quantitate proteins in 2.5 minutes without staining and destaining.

The Criterion Stain Free system is unlike any other protein visualization platform, offering you:

- Equal or better sensitivity than Coomassie staining
- Time savings of up to two hours compared to Coomassie staining
- Automatic band sizing and purity analysis
- Environmentally friendly technology using fewer chemicals than Coomassie staining
- Compatibility with Western blotting, mass spectrometry, and standard staining methods
- The Criterion Stain Free imager, Image Lab™ software and a formulation of Criterion™ Precast Gels in one complete package

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Denmark 45-44-52-10-00
Finland 358-9-804-22-00
France 33-1-47-95-69-65
Germany 49-89-31884-0
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Poland 48-22-331-99-99
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BioRadiations

issue 127, 2009

TO OUR READERS

As the field of genomics continues to evolve beyond identification of the genetic composition of biological organisms to defining how genotype impacts physiological function, options for tools used to aid these discoveries also continue to expand. Traditional methods used post-PCR include HPLC, SSCP, and RFLP. Though precise and highly sensitive, these techniques require labor-intensive screening protocols and use specially labeled probes — both of which are expensive to perform. High-resolution melt (HRM) analysis is emerging as an alternative to screening and probe-based genotyping assays that significantly reduces the cost and time involved with post-PCR genetic analysis. This simple, yet highly sensitive and accurate tool is quickly becoming a mainstream part of the genetic analysis workflow. The feature article defines HRM analysis and describes how this tool is impacting research in breast cancer, SNP genotyping, and its potential for application in the highly regulated forensic laboratory environment.

COVER STORY

DEPARTMENTS

TECHNICAL REPORTS

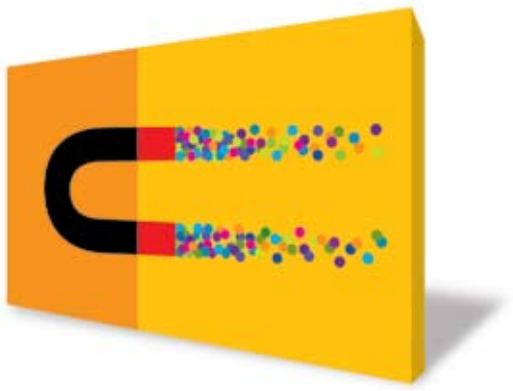
Legal Notices — See the inside back cover.

multiplexcellent.

Trusted immunoassays, improved workflow, greater throughput.

For the past eight years, you have trusted Bio-Rad's multiplex Bio-Plex® assays. And we've trusted you to tell us what we can do better. Your feedback has driven every improvement to our assays and workflow without sacrificing the reliability and reproducibility you require.

With Bio-Plex Pro™ assays, measuring important disease markers and modulators of immune response is now faster and easier than ever.



- Proven antibodies coupled to magnetic beads allow for automation and improved workflow
- New Bio-Plex Pro wash stations offer easier processing options
- Global service and support offer you ease of mind from delivery through validation

Please visit us at **www.bio-rad.com/bioplex/** to learn more about Bio-Plex Pro assays.

Research. Together.

Criterion Stain Free™ Gel Imaging System

The Criterion Stain Free gel imaging system allows direct visualization, analysis, and documentation of protein samples in SDS-PAGE gels with the single touch of a button, without staining, destaining, or gel drying procedures. The complete system is composed of the Criterion Stain Free gels, Criterion Stain Free imager, and Image Lab™ software. Criterion Stain Free gels have a proprietary compound that facilitates protein visualization and can be run under denaturing and nondenaturing conditions. This system is ideal for quick sample assessment prior to purification procedures, and as a precursor to blotting and profiling workflows in which Coomassie stain is ordinarily used. Benefits include:

- Fast results — gel images and complete analysis in as little as 2.5 min after electrophoresis
- Reproducibility — automation and standardization of methods eliminate the background variability
- Environmentally friendly methodology — acetic acid and ethanol are eliminated, reducing organic waste
- Ease of use — one-touch gel image and analysis processing
- Sensitivity — results are equal to or better than those from Coomassie stain

For more information, including system ordering information, visit www.bio-rad.com/stainfree/.

Bio-Plex Pro™ Cytokine, Chemokine, and Growth Factor Assays

The new Bio-Plex Pro cytokine, chemokine, and growth factor assays are built around MagPlex magnetic beads and are designed to run on the Bio-Plex® system. The antibodies and buffers in these assays are the same as those used in the Bio-Plex corresponding assays; however, magnetic beads afford compatibility with the Bio-Plex Pro wash stations, providing convenient, consistent, and hands-free assay processing.

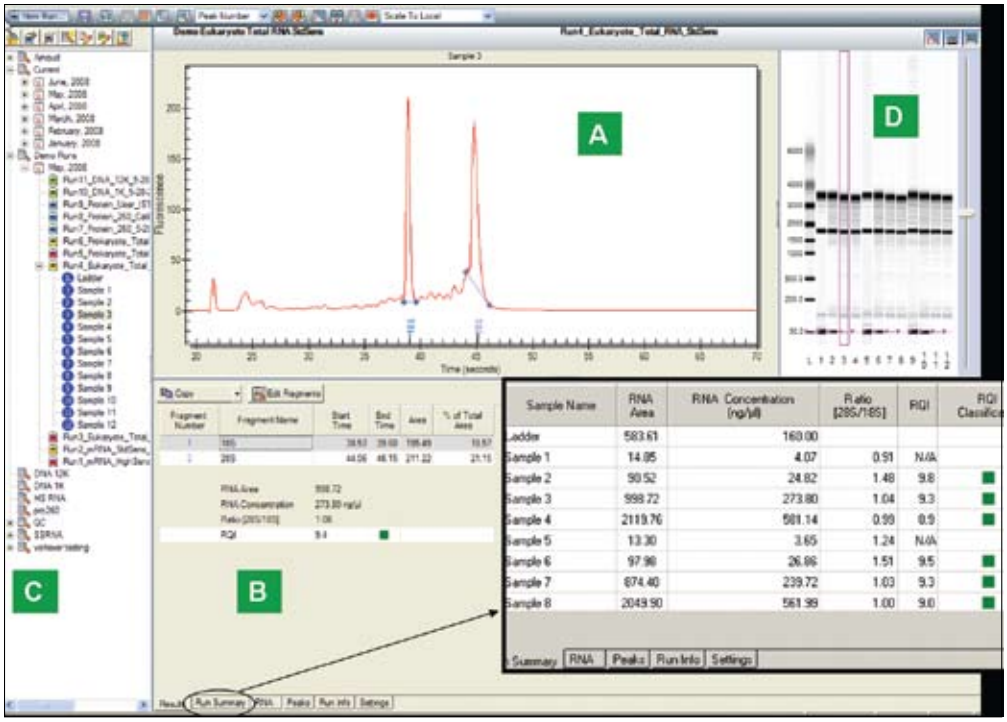
Bio-Plex Pro cytokine, chemokine, and growth factor assays can be ordered as premixed panels, Bio-Plex® x-Plex™ assays (any combination of available assays), Bio-Plex Express assays (for faster delivery, any combination of available assays up to a 10-plex maximum), and individual components (singleplex sets). All assay panels are available in the new all-in-one kit packaging format.

For a complete list of assays as well as product ordering information, go to www.bio-rad.com/bio-plex/.

Experion™ Automated Electrophoresis System: New Software, Version 3.0

The Experion automated electrophoresis system uses microfluidic technology to deliver fast, reproducible results for protein, RNA, and DNA separations. Experion software, an integral component of the system, provides added efficiency through an easy-to-use interface for performing runs and analyzing data. Experion software, version 3.0 builds on the automatic calculations and basic analysis tools of earlier versions. New key features:

- RNA quality indicator (RQI) function automatically generates a numerical value (from 1 to 10) that correlates with eukaryotic total RNA integrity; the RQI number complements the electropherogram and reported ribosomal peak area ratio
- Manual integration enables users to easily assign peaks and refine peak areas, further simplifying and increasing flexibility of data analysis
- Annotation and export tools simplify data presentation for reports and publications or for in-depth data analysis
- Logical, flexible tree-view browser makes run files easy to find
- Run summary tabs for easy viewing or exporting of data from all wells



Experion software provides a user-friendly interface for protein, RNA, or DNA analysis. For total RNA analysis, the software automatically provides an electropherogram for visualizing the RNA profile (**A**), results table showing an RQI value with convenient color-coded rankings (also shown within the run summary table, inset) (**B**), tree-view browser for organizing runs (**C**), and virtual gel image (**D**).

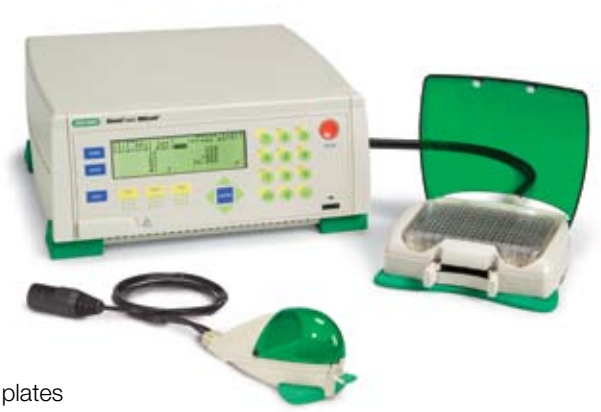
For more information, request bulletins 3171B, 5761B, and 3174A, or go to **www.bio-rad.com/experion/**.

Ordering Information

Catalog #	Description
700-7050	Experion Software, version 3.0, system operation and data analysis tools, includes software CD-ROM
700-7051	Experion Validation Kit, includes 3 test chips, qualification procedures, dongle
700-7052	Experion Software, Security Edition, standard and U.S. FDA 21 CFR Part 11 compliance data analysis tools, includes 3 test chips, qualification procedures, dongle

Gene Pulser MXcell™ ShockPod™ Cuvette Chamber

The Gene Pulser MXcell plate-based electroporation system has been expanded to include a new delivery tool, the Gene Pulser MXcell ShockPod cuvette chamber. The Gene Pulser MXcell system equipped with the cuvette chamber provides the added flexibility of electroporating samples in single cuvettes. The system can now be used to electroporate as few as 10^5 cells using a 96-well plate, and as many as 10^6–10^7 cells in a 12-well plate or cuvette. Key advantages:



- Highly reproducible results regardless of delivery format provide reliable data
- Cross-compatible protocols between cuvettes and plates minimizes optimization time
- Simple cable connection allows easy transition between delivery formats

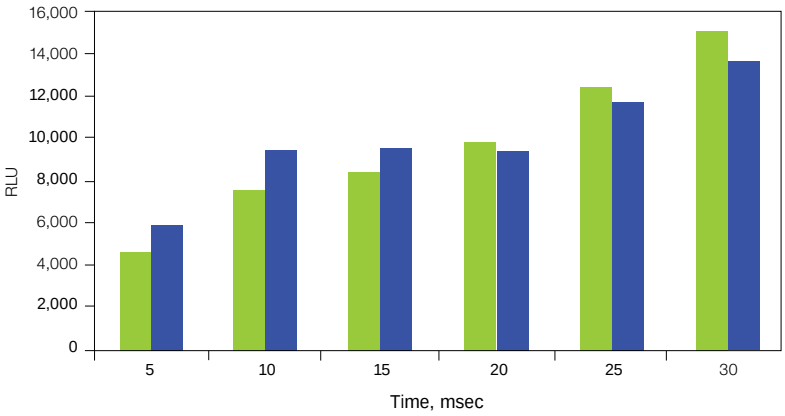


Plate vs. cuvette conversion equation test with variable duration. Electroporation performance was nearly identical in cuvettes and 96-well plates. The luciferase activity of HeLa cells electroporated in a 96-well plate was compared to HeLa cells electroporated in a cuvette with six different parameters. With all six parameters there was a 20% difference in luciferase activity between plate and cuvette formats. Cell concentration, 1 x 10^6 cells/ml; plasmid concentration, 10 µg/ml luciferase plasmid; sample volume, 150 µl/96-well plate and 600 µl/cuvette; 250 V, 2,000 µF, 1,000 Ω, 1 pulse. Plate (–), cuvette (–).

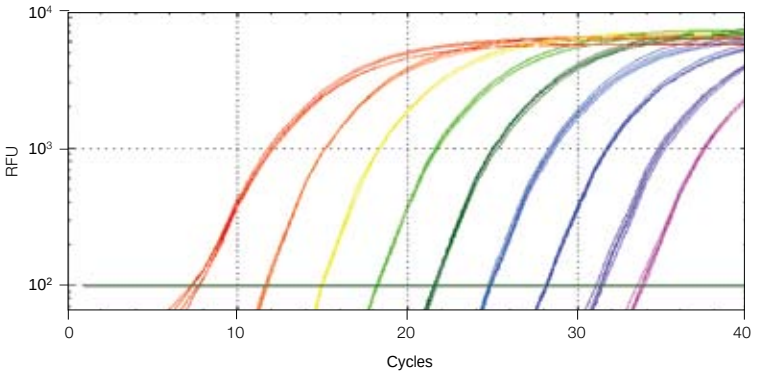
Ordering Information

Catalog#	Description
Gene Pulser MXcell Electroporation System	
165-2670	Gene Pulser MXcell Electroporation System, 100–240 V, 50/60 Hz, exponential-decay and square-wave delivery, includes power module, plate chamber, 1 x 96-well electroporation plate, instructions
165-2674	Gene Pulser MXcell Electroporation System With Gene Pulser MXcell ShockPod Cuvette Chamber, 100–240 V, 50/60 Hz, exponential-decay and square-wave delivery, includes power module, plate chamber, 1 x 96-well electroporation plate, Gene Pulser MXcell ShockPod cuvette chamber, instructions
165-2673	Gene Pulser MXcell ShockPod Cuvette Chamber, includes integral leads for connection to the Gene Pulser MXcell electroporation system
Cuvette Packs	
165-2081	Gene Pulser/MicroPulser Cuvettes, 0.4 cm gap, 5
165-2088	Gene Pulser/MicroPulser Cuvettes, 0.4 cm gap, 50
165-2091	Gene Pulser/MicroPulser Cuvettes, 0.4 cm gap, 500

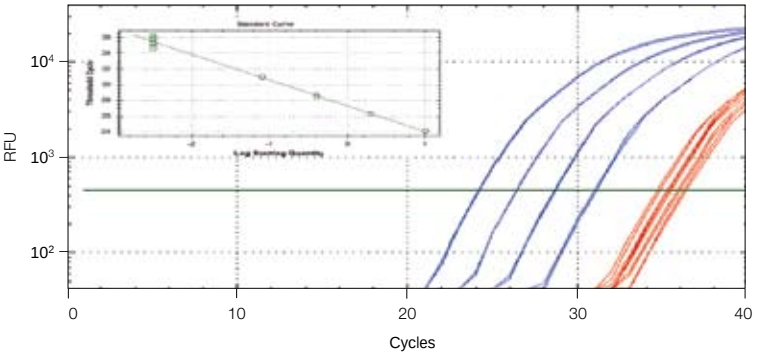
SsoFast™ EvaGreen® Supermix

SsoFast EvaGreen supermix is the first member of a next-generation family of high-performance, real-time PCR reagents. This supermix uses patented* Sso7d fusion protein technology to deliver excellent performance in a wide range of qPCR applications. By combining a novel engineered hot-start fusion polymerase with optimized buffer and EvaGreen dye, robust qPCR results can be generated in less time and with increased reliability and sensitivity. Benefits include:

- Minimal inhibition of PCR — ensures maximum efficiency, sensitivity, and reproducibility, while providing higher fluorescence compared to SYBR® Green
- Optimal primer binding and extreme sensitivity for detection of a single-copy target molecule
- Guaranteed high-resolution melt compatibility for genotyping studies



The unique fusion polymerase in SsoFast EvaGreen supermix delivers extreme speed and generates exceptional qPCR results in under 30 minutes. Serial dilutions (10-fold) from 10 to 100 ng of cDNA from human spleen were used in each 20 µl reaction to detect 18S RNA; 18S efficiency = 101.8%, r = 0.997. Total qPCR run time = 29 minutes.



SsoFast EvaGreen supermix provides extreme sensitivity in detection of a single-copy target gene. The ZAP-70 gene was amplified and detected from 5-fold serial dilutions of 10 ng to 80 pg (—) and 3.2 pg (—) from human genomic DNA; ZAP-70 efficiency = 102.7%, r = 0.991. Inset shows the standard curve for the various dilutions. The C_T variation (±1 cycle) is within the expected range for detection of a single-copy target gene.

Ordering Information

Catalog #	Description
172-5200	SsoFast EvaGreen Supermix, 200 x 20 µl reactions, 2x mix contains dNTPs, Sso7d fusion polymerase, $MgCl_2$, EvaGreen dye, stabilizers
172-5201	SsoFast EvaGreen Supermix, 500 x 20 µl
172-5202	SsoFast EvaGreen Supermix, 1,000 x 20 µl
172-5203	SsoFast EvaGreen Supermix, 20 ml bottle, 2,000 x 20 µl

* U.S. patent 6,627,424.

CFX384™ Real-Time PCR Detection System

The CFX384 real-time PCR detection system brings flexibility and simplicity to researchers performing high-throughput real-time PCR in a 384-well format, with up to four-target detection and powerful data analysis. With the precise thermal control of the C1000™ thermal cycler, the CFX384 system delivers sensitive, reliable detection for real-time PCR applications, including absolute quantitation, genetic variation analysis, and gene expression. CFX Manager™ software, which runs on a PC, provides numerous features and tools to streamline experiment setup and data analysis.

The CFX384 system maximizes fluorescence detection for specific dyes in specific channels — the optics shuttle individually illuminates and detects fluorescence from each well with high sensitivity and no cross talk. At every position and with every scan, the optics shuttle is reproducibly centered above each well, so the light path is always optimal and there is no need to sacrifice data collection in one of the channels to normalize to a passive reference.

Key features:

- Optical system uses long-lasting solid-state technology with 5 filtered LEDs and 5 filtered photodiodes for precise quantitation and target discrimination
- Factory calibration minimizes set-up time, so you can start doing experiments right away
- Multiple data acquisition modes let you tailor a run to suit your application
- Minimized sample and reagent usage allows you to obtain accurate results with low sample volumes
- Runs can be performed in several control configurations, including stand-alone with no computer attached
- Optimized system design makes throughput expansion easy, with the ability to control up to 4 instruments by a single computer
- CFX Manager software, Security Edition, integrates the power of the CFX384 real-time PCR detection system with good laboratory practice standards for data collection and analysis

For more information, request bulletin 5637, or visit www.bio-rad.com/realtime/.

Ordering Information

Catalog #	Description
CFX384 Real-Time PCR Detection System	
184-5384	CFX384 Optical Reaction Module, includes CFX Manager software, communication cable, reagent and consumable samples, instructions
185-5384	CFX384 Real-Time PCR Detection System, includes C1000 thermal cycler chassis, CFX384 optical reaction module, CFX Manager software, communication cable, power cord, reagent and consumable samples, instructions
Accessories	
170-9799	Real-Time PCR Applications Guide
HSP-3805	Hard-Shell Thin-Wall 384-Well Skirted PCR Plates, clear shell, white well, 50
MSB-1001	Microseal 'B' Adhesive Seals, 100

Amplification Software Releases

CFX Manager™ Software, Security Edition

CFX Manager software, Security Edition, offers all the features of CFX Manager software, plus important tools for compliance with U.S. FDA 21 CFR Part 11 regulations. The Security Edition works with the security features of the Windows XP and Windows Vista operating systems to provide a secure environment for the maintenance, verification, and tracking of electronic records and signatures generated by the software. The Security Edition adds the following features to CFX Manager software:

- Mandatory password-protected log-in — valid Windows XP or Windows Vista username and password are required for all users
- Hardware protection — HASP hardware license (HL)-based key must be attached to a USB port on the computer to use the software
- File encryption — files cannot be opened or edited using other programs
- Automatic file checking — integrity and validity are checked each time a file is opened
- Electronic signatures — multiple electronic signatures can be applied to any file that can be opened within Security Edition software
- Time- and date-stamped audit trails — read-only information displayed in the audit trail can be viewed only while the data file of interest is open

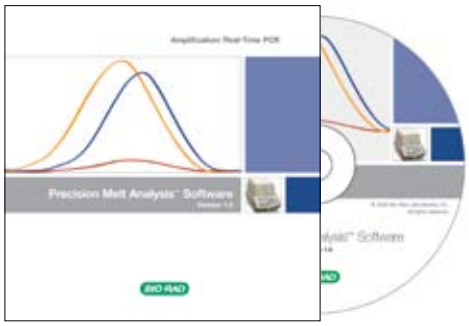
C1000 Manager™ Software

C1000 Manager software, when installed on a PC, can be used to control multiple connected 1000-series thermal cyclers. The software operates on Windows XP and Windows Vista operating systems and allows users to:

- Write and edit protocols on a PC, then transfer them to a C1000™ thermal cycler using a USB portable memory device
- Control up to thirty-two 1000-series cyclers from a single computer (eight C1000 thermal cyclers, each connected to three S1000™ thermal cyclers)
- Send protocols to connected thermal cyclers and start runs on individual or multiple blocks, either independently or simultaneously
- Monitor the progress of individual blocks and instruments from a central screen
- Create and manage user profiles, preferences, and access privileges
- View, zip, and send report logs
- Receive email notification of run completion

Precision Melt Analysis™ Software

Precision Melt Analysis software imports and analyzes data files generated from the CFX96™ or CFX384™ real-time PCR detection system to genotype samples based on the thermal denaturation profiles of double-stranded DNA. The software can be used for a variety of genotyping applications, including scanning for new gene variants, screening DNA samples for SNPs, identifying insertions/deletions or other unknown mutations, and determining the percentage of methylated DNA in unknown samples.



Precision Melt Analysis software makes it easy for you to:

- Streamline data analysis using the customizable default analysis settings
- Utilize the multiple data view options to manually assign sample genotypes by tailoring the software to the appropriate analysis
- Get consistent results by sharing your individual assay analysis settings across multiple melt files
- Analyze multiple experiments in a single plate using the Well Groups feature
- Publish your data in multiple formats by easily exporting to Microsoft Excel, to Microsoft PowerPoint, or as an image

System Requirements*

	Minimum	Recommended
Operating system	Windows XP Professional SP2, Windows Vista Home Premium	Windows XP Professional SP2, Windows Vista Home Premium
Processor	1 GHz	2 GHz
RAM	1 GB (2 GB for Windows Vista)	2 GB
Hard drive space	10 GB	20 GB
Screen resolution	1,024 x 768 with true-color mode	1,280 x 1,024 with true-color mode
Drive	CD-ROM	CD-RW
USB port	2.0 Hi-Speed	2.0 Hi-Speed
Internet browser	Internet Explorer	Internet Explorer
Additional software	—	Microsoft Office Suite

* Applicable to CFX Manager, C1000 Manager, and Precision Melt Analysis software packages.

Ordering Information

Catalog #	Description
CFX Manager Software, Security Edition	
184-5001	CFX Manager Software, Security Edition, includes 1-user license, installation CD, HASP HL key, instructions
184-5005	CFX Manager Software, Security Edition, includes 5 user licenses, 5 installation CDs, 5 HASP HL keys, instructions
184-5010	CFX Manager Software, Security Edition, includes 10 user licenses, 10 installation CDs, 10 HASP HL keys, instructions
C1000 Manager Software	
184-4000	C1000 Manager Software, version 1.0, includes installation CD, instructions
Precision Melt Analysis Software	
184-5025	Precision Melt Analysis Software, includes 2 user licenses, installation CD, HASP HL key, calibration kit, instructions

Selecting the Right Agarose for Your Laboratory Application

Agarose gels are commonly used tools in many biotechnology laboratories for applications that incorporate routine nucleic acid electrophoresis. Although a wide selection of agarose powders are commercially available, optimal results are achieved by using the appropriate agarose powder designed for the application of interest. The most important factor to consider when choosing agarose is the size of the DNA fragment being separated. The range of fragment separation and resolution of fragments into distinct, sharp bands should also be taken into consideration, as should mechanical strength and ease of gel handling. Using the correct percentage of agarose in the gel is crucial, since it often leads to better resolution of the fragments of interest and can produce a gel that yields a publication-quality image. Higher percentage agarose gels resolve smaller DNA fragments, while lower percentage gels resolve large fragments. The resolution characteristics of Bio-Rad Certified™ agaroses are summarized in Table 1.

Each agarose in the Bio-Rad Certified agarose product line is pure and genomic quality tested (GQT), guaranteeing the absence of inhibitors, DNases, and RNases. Bio-Rad agaroses are available in a wide range of formulations to suit most routine nucleic acid electrophoresis and downstream molecular biology applications.

Routine DNA separations — Certified molecular biology agarose is a general-purpose agarose for routine separations of DNA fragments from 100 bp to 20 kb (20 kb fragments can be resolved using lower gel percentages, 0.3–0.5%). This agarose offers rapid migration rates, produces easy-to-manipulate gels, and yields high gel strength even at low agarose percentages.

Separation of small fragments — For separations of small fragments such as PCR products, Certified PCR agarose is recommended. Depending on the gel percentage, the Certified PCR agarose can resolve fragments from 20 bp–2.5 kb. The standard gelling temperature makes preparation simple, resulting in gels that are easy to manipulate and are flexible even at high gel percentages.

Separation of very small DNA fragments — For separation of extremely small PCR fragments and primers (~10–200 bp), Certified low range ultra agarose is recommended. This agarose provides exceptional resolution at lower concentrations than standard gels, allowing visualization of differences of ~5 bp in the 10–100 bp range.

Low-melt applications for DNA fragments <1 kb — Certified PCR low-melt agarose is designed for applications such as cloning, in-gel applications, or DNA and RNA fragment recovery. This agarose has high-sieving capacity and offers excellent resolution of fragments <1 kb in low-melt applications.

Low-melt applications for DNA fragments >1 kb — For preparing samples for CHEF separations, recovery of larger fragments, or sealing IPG strips in 2-D SDS-PAGE gels, Certified low-melt agarose is recommended. This agarose is used for separations of molecules >1 kb.

Separation of large DNA fragments for PFGE — For analytical separation of large DNA fragments using pulsed field gel electrophoresis (PFGE), pulsed field Certified agarose is recommended. This agarose has an optimal separation range of 1 kb to 2 Mb and can be used as a preset selectable method of the CHEF Mapper® XA system algorithm. This agarose can also be used for blotting.

Faster migration rates for CHEF and FIGE — Certified megabase agarose is suitable for CHEF and FIGE applications for separation of megabase DNA fragments or analysis of other large DNA fragments. This agarose has a high exclusion limit, electrophoretic mobility, and gel strength. Gels are easy to handle even at 0.3%, while offering shorter run times. The separation range for this agarose is 1 kb–5 Mb.

Table 1. Influence of agarose gel percentage on optimal DNA fragment resolution.

Agarose	Gel Percentage	Optimal Resolution Range
Certified molecular biology	0.75%	0.5–10 kb
	1%	0.3–9 kb
	1.25%	0.1–8 kb
Certified PCR agarose	2%	100 bp–2.5 kb
	3%	40 bp–2 kb
	4%	20 bp–1 kb
Certified low range ultra agarose	3%	10 bp–1 kb
	4%	10–400 bp
Certified PCR low-melt agarose	2%	40 bp–2 kb
	3%	20 bp–1 kb
	4%	10–600 bp
Certified low-melt agarose	1%	100 bp–5 kb
	1.25%	100 bp–3 kb

For more information, request bulletin 2755.

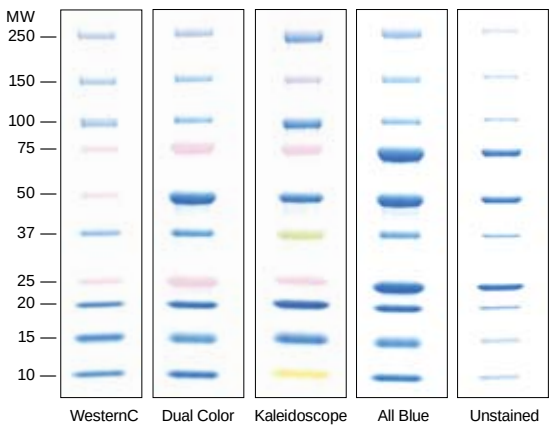
Protein Standards: How to Choose?

Introduction

Protein standards are used in protein gel electrophoresis and western blotting applications to help identify and characterize the separated proteins. Bio-Rad Precision Plus Protein™ standards contain a blend of ten highly purified recombinant proteins with a molecular weight (MW) range of 10–250 kD. These standards offer unsurpassed band sharpness, accurate MW estimation, and lot-to-lot consistency. All exhibit even, ladder-like band separation and convenient reference bands that allow you to quickly estimate the MW of your protein and monitor its transfer (in western blotting applications). This article provides a guide to selecting the standards most appropriate for your application.

Gel Electrophoresis

In gel electrophoresis, protein standards offer a means to evaluate the MW of separated proteins, and either unstained or prestained standards can be used. Whereas prestained standards can be used to estimate MW and are useful for monitoring the progress of electrophoresis, unstained standards are the first choice for the most accurate MW estimation possible. Unstained standards are required when a fluorescent dye is used to stain the gel or blot to visualize total protein.



Precision Plus Protein standards offer accurate and consistent separation. Available in prestained and unstained formats, these standards offer convenient reference bands (distinguished by color or intensity) for easy estimation of MW.

To estimate MW, calculate the relative mobility (R_f) of each protein standard and plot the R_f of each band against the $\log_{10}$ of its MW to generate a standard curve. Then, use this curve to estimate the MW of the unknown proteins (see bulletins 3133 and 3144).

Western Blotting

When doing western blotting, protein standards are used for MW estimation as well as for determining the efficiency of protein transfer. While unstained standards enable the most accurate MW estimation (see bulletins 3133 and 3144), prestained standards offer the benefit of allowing you to monitor both electrophoresis and transfer. The ultimate choice, though, depends on the method you will use for visualization:

- Colorimetric stains (such as Coomassie) — unstained or prestained standards
- Fluorescent stains — unstained standards
- Chemiluminescent detection — either unstained or Precision Plus Protein WesternC™ standards. These standards both contain a *Strep*-tag affinity peptide that has intrinsic binding affinity for both native streptavidin and StrepTactin. For detection, streptavidin or StrepTactin is added along with the secondary antibody, both of which contain the same enzyme conjugate. The standards, along with the antigen of interest, are visualized by the addition of the appropriate chemiluminescent substrate. WesternC standards also have stained bands, allowing you to monitor electrophoresis and transfer efficiency
- Fluorescently labeled antibodies — In the line of Precision Plus Protein standards, the dual color, Kaleidoscope™, and WesternC prestained standards include bands that fluoresce when excited by different wavelengths of light. This can be useful for multiplex detection when multicolored antibodies are chosen (see bulletins 5686 and 5723). Blue bands are excited by light in the red range of the spectrum, pink bands by light in the green range, and yellow and green bands by light in the blue range

For more information, request bulletins 3133, 3144, 5686, and 5723.

Selection Guide

		Gel Electrophoresis Detection		Western Blotting Detection			
Precision Plus Protein Standards	Catalog No.	Colorimetric	Fluorescent Stain	Colorimetric	Fluorescent Stain	Chemiluminescent	Fluorescently Labeled Ab (Multiplex)
Prestained							
WesternC	161-0376	•		•		•	•
Dual color	161-0374	•		•			•
Kaleidoscope	161-0375	•		•			•
All blue	161-0373	•		•			
Unstained	161-0363	•	•	•	•	•	

Experion™ Automated Electrophoresis System vs. SDS-PAGE: Molecular Weight Estimation

In addition to concentration and purity, molecular weight (MW) is a parameter commonly analyzed in the study of proteins. Mass spectrometry is the most accurate method used for size determination, but for size estimation, both SDS-PAGE and the Experion automated electrophoresis systems yield comparable results in terms of accuracy and reproducibility (Zhu et al. 2005). Estimations from SDS-PAGE and the Experion system may differ from mass spectrometry MW determinations, but these tools are faster and more economical than mass spectrometry, and provide sufficiently accurate size estimations for most applications. However, several differences between how SDS-PAGE and the Experion system work can lead to differences in how some proteins separate, resulting in a disparity in size estimations between these two methods (Table 1). This is particularly true when comparing proteins that have been posttranslationally modified such as glycosylated proteins (Nguyen and Strong 2007).

In SDS-PAGE, proteins are denatured in the presence of SDS, separated by electrophoresis through a polyacrylamide matrix, stained (after the removal of SDS), destained, and then analyzed for MW, using a protein standard (ladder) as a reference.

With the Experion electrophoresis system, proteins are denatured in lithium dodecyl sulfate (LDS) and separated by electrophoresis within a microfluidic chip. For separation, microchannels within the chip are filled with a proprietary gel-stain solution that acts as a sieving matrix and that includes the protein stain required for detection. Protein separation can be affected by differences in pore structure of the sieving matrices used. SDS-PAGE uses a cross-linked polymer; the Experion system uses an entangled linear polymer. Both methods determine size using a ladder; however, if the ladder migrates through the sieving matrix differently than a particular protein sample, size estimations can be affected.

Another difference between the two methods lies in how sample fragments are detected: the stain used in SDS-PAGE binds directly to proteins, while the stain used in the Experion system binds to LDS micelles coating the proteins. In SDS-PAGE, samples are generally stained in the gel once separation is complete, with the proteins often immobilized (fixed) in the gel. In contrast, proteins, LDS, and fluorescent stain all interact dynamically during separation using the Experion system.

The majority of proteins however, show similar separation characteristics with both methods (Table 1), and both techniques accommodate differences by using a protein standard as the reference for sizing.

SDS-PAGE and the Experion automated electrophoresis system represent different technologies that provide size estimations. Obtaining the most accurate MW data requires the use of mass spectrometry. The Experion system yields comparable or better reproducibility of molecular weight estimations than traditional SDS-PAGE, as indicated by Experion system %CVs under 1% compared to SDS-PAGE %CVs ranging from 2.67% to 3.78% (Table 1). Additionally, the Experion system provides walk-away convenience, requires less sample for analysis, and provides analyzed quantitation and MW results in 30 minutes, making the Experion system a logical — and accurate — choice for protein sample analysis.

References

Nguyen M and Strong W (2007). Application of the Experion automated electrophoresis system to glycoprotein visualization and analysis, Bio-Rad Bulletin 5453.

Zhu K et al. (2005). Performance comparison of the Experion automated electrophoresis system and SDS-PAGE for protein analysis, Bio-Rad Bulletin 5299.

Table 1. Comparison of protein sizing accuracy* and reproducibility using the Experion system*** and SDS-PAGE***.**

		Experion System			SDS-PAGE		
Protein	Expected MW†	MW	Accuracy	Reproducibility	MW	Accuracy	Reproducibility
Lysozyme	14.3	14.23 ± 0.12	–0.49%	0.82%	12.10 ± 0.42	–15.38%	3.47%
β-Lactoglobulin	18.4	18.82 ± 0.13	2.26%	0.69%	14.70 ± 0.50	–20.11%	3.40%
Triosephosphate isomerase	26.6	26.10 ± 0.27	–1.86%	1.05%	24.00 ± 0.69	–9.77%	2.88%
Lactate dehydrogenase	36.5	33.37 ± 0.29	–8.56%	0.86%	32.20 ± 1.00	–11.78%	3.11%
Ovalbumin	45	44.43 ± 0.34	–1.26%	0.77%	42.30 ± 1.60	–6.00%	3.78%
Glutamate dehydrogenase	55	56.32 ± 0.47	2.39%	0.84%	51.40 ± 1.37	–6.55%	2.67%
Bovine serum albumin	66	71.60 ± 0.70	8.49%	0.97%	66.70 ± 1.78	1.06%	2.67%
Phosphorylase b	97	95.44 ± 0.67	–1.61%	0.71%	96.20 ± 2.75	–0.82%	2.86%
β-Galactosidase	116	123.00 ± 0.55	6.02%	0.45%	110.40 ± 3.06	–4.83%	2.77%

* Calculated as % difference relative to expected.

** Calculated as % CV.

*** Proteins were separated using the Experion Pro260 analysis kit (n = 25) or SDS-PAGE (n = 25). MW values shown are mean ± SD.

† Expected MWs obtained from manufacturer labels (Sigma-Aldrich Company, NIST, and Boehringer Mannheim).

— Ruthellen Miller, PhD, senior technical support consultant, Bio-Rad Laboratories

Development and Validation of Two Human Cytokine Assay Panels on Magnetic Microspheres

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Introduction

Cytokines, chemokines, and growth factors play an important role in a wide range of physiological responses, including immune response, inflammation, hematopoeisis, and carcinogenesis. Changes in the circulating levels of these proteins have been linked to a spectrum of disease states, making them valuable diagnostic and therapeutic biomarkers. However, it is often necessary to measure the levels of multiple biomarkers in a single sample of small quantity. Multiplex analysis greatly facilitates analyzing multiple biomarkers by reducing the sample requirement, reagent requirement, and time to results.

Now that multiplex analysis has been widely adopted, new technologies have been developed to further improve the workflow. For multiplex suspension bead arrays, the advent of magnetic bead-based assays has enabled automated wash steps during assay preparation. This improvement has led to less hands-on time, and many users have experienced better assay performance as a result of eliminating the manual vacuum manifold from the procedure. In addition, the steps to building a fully automated assay preparation platform are simplified when using magnetic-based separation.

We have developed two multiplex immunoassay panels on magnetic beads that measure a total of 50 human cytokines, chemokines, and growth factors in the amount of time it takes to perform a typical ELISA. This article summarizes the performance of these panels, the Bio-Plex Pro™ human cytokine 27-plex group I panel and the Bio-Plex Pro human cytokine 21-plex group II panel.

Methods

The Bio-Plex Pro human group I panel was analyzed in 27-plex. The Bio-Plex Pro human group II panel is now available as 21 premixed assays plus two separate assays for soluble ICAM-1 and VCAM-1, which require a different sample dilution than the other 21 targets. The lyophilized antigen mixture includes all 23 targets and the group II panel is evaluated here as a 23-plex.

Evaluation of Assay Sensitivity (Limit of Detection)

The limit of detection (LOD) was calculated as the concentration of analyte on the standard curve for which the corresponding MFI value is two standard deviations above the background measured in the blank. The mean of five independent assays was calculated using standard diluent or culture media as a matrix.

Evaluation of Assay Precision

Intra-assay precision was calculated as the coefficient of variation (%CV) among MFI values of six replicate wells of standard curve points on a single assay plate.

Inter-assay precision was calculated as the %CV of the observed concentration of spike controls from five independent assays.

Evaluation of Assay Accuracy

Assay accuracy (% recovery) was calculated as the percentage of the observed value of a spiked standard of known concentration relative to the expected value. Spike concentrations were measured at six different points within the assay range.

Determination of Assay Working Ranges

Working ranges for Bio-Plex assays are determined based on standard and spike recovery and assay precision. The assay working range is the range of concentrations in which the assay is both precise (intra-assay %CV <20% and inter-assay %CV <30%) and accurate (70–130% recovery). Assay working range is described as the lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ).

Evaluation of Assay Linearity of Dilution

Linearity of dilution ensures that analytes present in concentrations above the LLOQ can be diluted and measured accurately within the assay working ranges. Human serum/plasma diluent was prepared from 4-fold dilution of pooled human serum/plasma with sample diluent. Standard antigens were reconstituted at the highest concentration point of the standard curve (S1), followed by 3-fold serial dilutions using the prepared human serum/plasma diluent for a series of six dilution samples. The highest concentration of spiked multiplexed human cytokines (dilution 1) is one-third that of S1. The linear relationship of observed concentration and expected concentration of each dilution point within assay working range is plotted for each target. The R^2 value of each linear plot reflects the linearity of sample dilution for that assay.

Evaluation of Assay Parallelism

Assay parallelism is a measure of the impact of the matrix effect on the binding characteristics of an assay. This was investigated by comparing slopes of spiked standard concentration-response curves in human serum or plasma with those of standard concentration-response curves in standard diluent. The concentration-response curves were prepared with six points of 3-fold serial dilutions of standards in either standard diluent or in human serum or plasma diluent. The difference of the curve slopes (slope of the tangent at midpoint) between the two types of curves demonstrates the assay parallelism between standard diluent and natural matrices such as human serum and plasma.

Determination of Assay Specificity (% Cross-Reactivity)

The group I and group II assay panels were tested on different plates with the recommended concentration of 27-plex capture beads or 23-plex beads (21-plex plus ICAM-1 and VCAM-1), and with the concentration of 27-plex or 23-plex antigen standards at the second dilution point. Detection antibodies were added individually. Nonspecific, cross-reacting signal was defined as the percentage of signal detected relative to the specific signal for that analyte.

Data Analysis

Bio-Plex Pro human cytokine assays were analyzed using Bio-Plex Manager™ software, version 5.0.

Results

Assay Sensitivity, Working Ranges, and Precision

Assay sensitivity for each target is reflected by the LOD. Assay precision is measured by intra- and inter-assay %CV. Assay accuracy is determined by spike recovery. Assay working ranges (LLOQ–ULOQ) are defined as the concentration ranges in which the assays are both precise and accurate.

The assay working ranges, LOD, intra- and inter-assay %CV data for all targets of Bio-Plex Pro human cytokine assays in 27-plex group I and 21-plex group II (plus ICAM-1 and VCAM-1) in serum-based matrix are summarized in Table 1A and Table 1B. Similar results were obtained in RPMI cell culture media matrix (not shown).

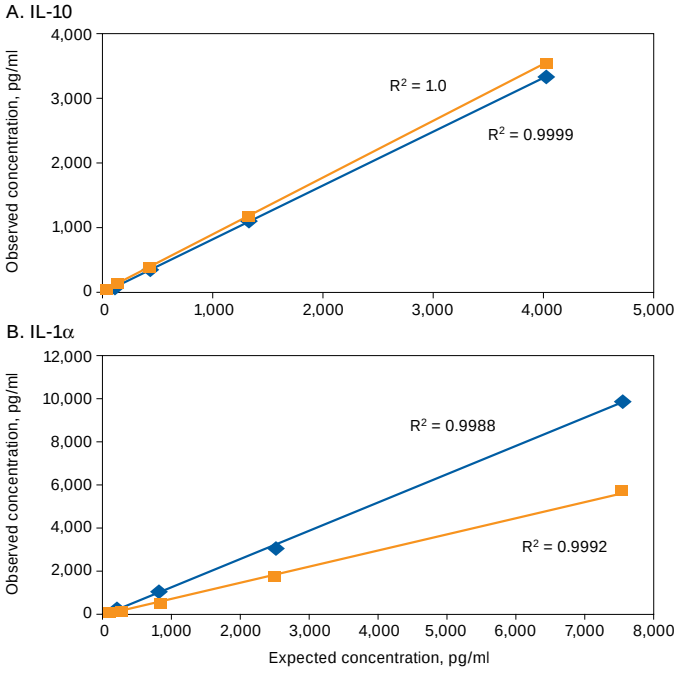


Fig. 1. Representative assay linearity of dilution plots in human serum and plasma using 6-point spike concentrations. A, IL-10 as an example of 27-plex group I panel; B, IL-1α as an example of 21-plex group II panel. Human serum matrix (■); human plasma matrix (■).

Table 1A. Representative assay working range, sensitivity, and precision of the Bio-Plex Pro human cytokine 27-plex group I panel. The LLOQ, ULOQ, LOD, and intra-/inter-assay %CV are data determined from five assays in serum-based matrix.

	Assay Working Range, pg/ml		Assay Sensitivity, pg/ml	Assay Precision*	
Target	LLOQ	ULOQ	LOD	Intra-Assay %CV	Inter-Assay %CV
IL-1β	3.2	3,261	0.6	6	8
IL-1Rα	81.1	70,487	5.5	9	8
IL-2	2.1	17,772	1.6	7	9
IL-4	2.2	3,467	0.7	9	8
IL-5	3.1	7,380	0.6	8	10
IL-6	2.3	18,880	2.6	7	11
IL-7	3.1	6,001	1.1	6	8
IL-8	1.9	26,403	1.0	9	4
IL-9	2.1	7,989	2.5	8	9
IL-10	2.2	8,840	0.3	5	6
IL-12 (p70)	3.3	13,099	3.5	6	6
IL-13	3.7	3,137	0.7	8	7
IL-15	2.1	2,799	2.4	5	6
IL-17	4.9	12,235	3.3	8	6
Eotaxin	40.9	5,824	2.5	8	11
Basic FGF	27.2	7,581	1.9	8	8
G-CSF	2.4	11,565	1.7	10	5
GM-CSF	63.3	6,039	2.2	12	6
IFN-γ	92.6	52,719	6.4	15	9
IP-10	18.8	26,867	6.1	11	9
MCP-1	2.1	1,820	1.1	9	7
MIP-1α	1.4	836	1.6	7	8
MIP-1β	2.0	1,726	2.4	8	8
PDGF-BB	7.0	51,933	2.9	9	8
RANTES	2.2	8,617	1.8	9	6
TNF-α	5.8	95,484	6.0	8	6
VEGF	5.5	56,237	3.1	9	7

Table 1B. Representative assay working range, sensitivity, and precision of the Bio-Plex Pro human cytokine 21-plex group II panel and of ICAM-1 and VCAM-1. The LLOQ, ULOQ, LOD, and intra-/inter-assay %CV are mean data determined from five assays in serum-based matrix.

	Assay Working Range, pg/ml		Assay Sensitivity, pg/ml	Assay Precision*	
Target	LLOQ	ULOQ	LOD	Intra-Assay %CV	Inter-Assay %CV
CTACK	13	16,874	3.4	5	6
GRO-α	19	2,711	6.3	5	8
HGF	9.5	25,357	4.9	5	6
IFN-α2	307	3,438	4.3	7	3
IL-1α	1.4	22,569	0.5	4	4
IL-2Rα	299	7,338	2.1	6	4
IL-3	12	22,803	4.8	7	4
IL-12 (p40)	41	28,662	23.3	5	8
IL-16	190	12,133	0.4	6	4
IL-18	1.8	28,677	0.2	4	5
LIF	12	22,184	5.5	4	3
MCP-3	79	11,234	1.0	7	8
M-CSF	2.0	32,200	0.9	4	5
MIF	231	24,373	1.5	5	8
MIG	2.7	8,584	1.2	6	6
β-NGF	1.0	4,247	0.2	4	7
SCF	1.5	25,078	1.0	5	4
SCGF-β	66	47,862	45.4	6	8
SDF-1α	10	10,391	8.7	6	6
TNF-β	1.5	24,505	0.3	4	4
TRAIL	4.4	44,769	2.1	4	8
ICAM-1	13	26,368	2.4	4.3	3.8
VCAM-1	38	21,430	0.6	6.7	5.5

* These assays were performed using the vacuum-based wash method; %CV is expected to be comparable or lower with the magnet-based wash method.

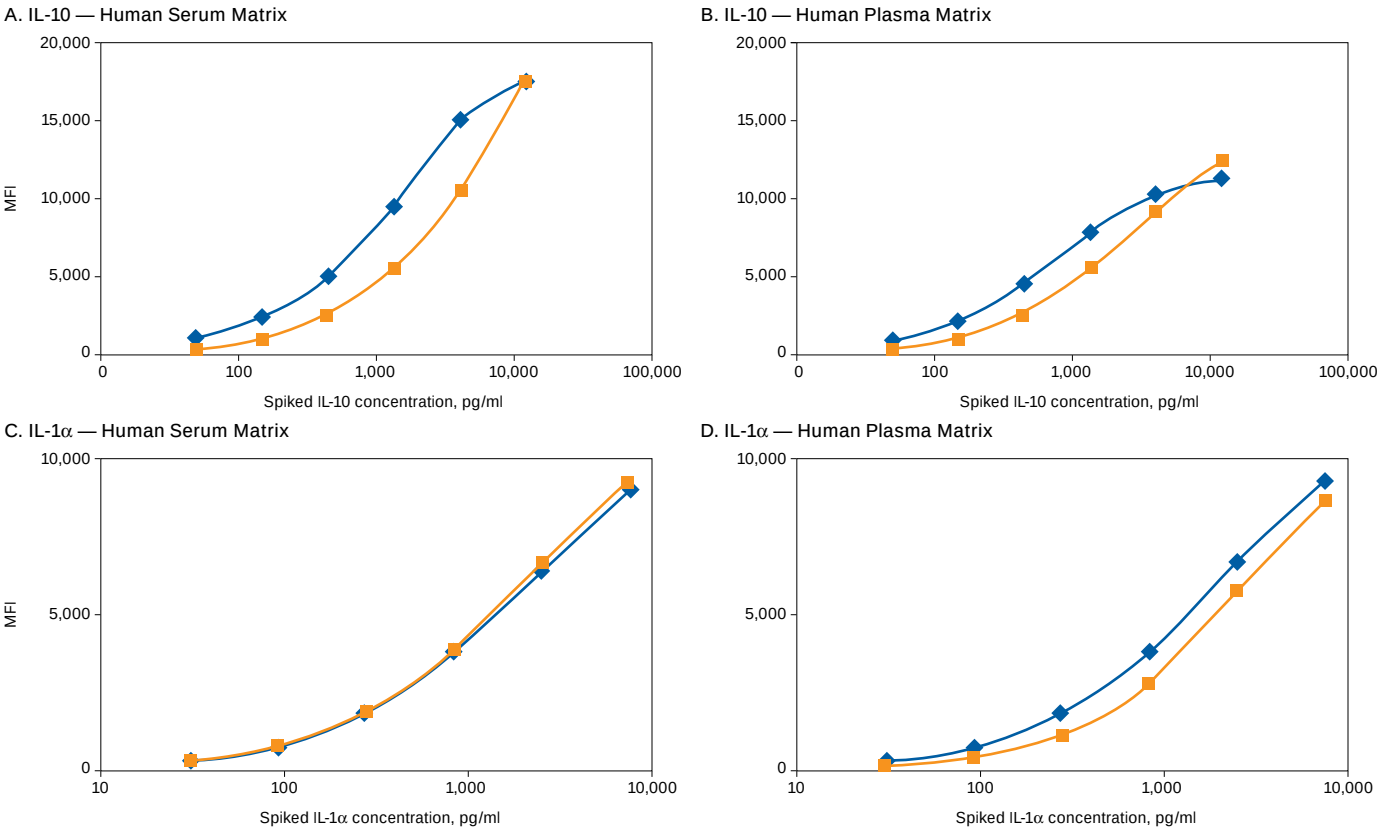


Fig. 2. Representative assay parallelism assessed by comparisons between concentration response curves in standard diluent (■) and in human serum or plasma samples (■). Parallelism analysis of IL-10 from the group I panel (A and B) and IL-1α from the group II panel (C and D) was conducted using six-point concentration-response curves with 4PL curve fitting (4PL curve fitting is used with comparing slopes between two different curves). The curve slope difference between standard diluent and human serum is 3.2% for IL-10 (A) and 1.5% for IL-1α (C). The curve slope difference between standard diluent and human plasma is 2.7% for IL-10 (B) and 12% for IL-1α (D).

Assay Linearity of Dilution

The linear relationship between observed concentration and expected concentration of each dilution point within assay working range is plotted for each target. The R^2 value of each plot reflects the linearity of sample dilution for that assay. The R^2 values for 27 targets in group I and 21 targets in group II are all above 0.95. Figure 1 shows representative assay linearity of dilution plots for IL-10 of 27-plex group I and IL-1α of 21-plex group II.

Assay Parallelism

The assay parallelism is investigated by comparing the spiked standard concentration-response curve in human serum or plasma with the standard concentration-response curve in standard diluent. The difference (% values) of the curve slopes between the concentration-response curves in standard diluent and in human serum is <15% for all target assays in group I and II panels. The difference (% values) of the curve slopes between the concentration-response curves in standard diluent and in human plasma is ≤25% for all assays in the group I and group II panels, except for RANTES (56%) due to high endogenous levels in human plasma sample. Figure 2 demonstrates the parallelism of spike concentration-response curves, IL-10 of the 27-plex group I and IL-1α of the 21-plex group II panels.

Conclusions

This report demonstrates the development and validation of two magnetic bead-based cytokine assay panels, Bio-Plex Pro human cytokine group I (27-plex) and group II (21-plex plus ICAM-1 and VCAM-1). These panels allow simultaneous measurement of multiple cytokine, chemokine, and growth factors in a single sample in serum, plasma, and cell culture-based matrices, thus significantly reducing the time and cost required to measure samples for these biomarkers. In addition, the implementation of magnetic bead-based assays allows automation of assay wash steps (using the Bio-Plex Pro wash stations), which helps reduce inter-assay variations by eliminating manual vacuum manifold washes. These assays have been shown to achieve a working range that is sufficiently broad for a variety of applications. The performance is comparable or superior to the original Bio-Plex nonmagnetic bead-based assays.

For an extended version of this article, request bulletin 5803.

The field of genomics is rapidly expanding beyond merely identifying the genetic makeup of biological organisms to encompass studies of how variations in genotype impact physiological function. Applications involving analysis of the genome include mutation discovery, single nucleotide polymorphism (SNP) genotyping, DNA mapping, genetic screening, and population studies. It is hoped that better insight into how changes in genotype translate into effect upon phenotypic expression will lead to earlier disease diagnosis as well as therapies targeted to particular genetic profiles.

Two primary tools engaged in pursuit of genomic discovery are PCR and DNA sequencing. Traditional methods used in post-PCR analysis include denaturing high-performance liquid chromatography (dHPLC), single-stranded conformation polymorphism (SSCP), temperature gradient capillary electrophoresis (TGCE), and restriction fragment length polymorphism (RFLP). Though precise and highly sensitive, these techniques require screening protocols and use of specially labeled probes — both of which are expensive and can consume significant time and labor resources.

Until recently, what has been lacking is a low-cost-per-sample method that also enables high-throughput sample processing. High-resolution melt (HRM) analysis is a tool recently developed as an alternative to probe-based genotyping assays that overcomes the cost, time, and labor-intensity challenges. This simple, yet highly sensitive and accurate tool is quickly becoming a mainstream part of the genetic analysis workflow. This article defines HRM analysis and describes how it is impacting research in breast cancer, SNP genotyping, and forensics in laboratories around the world.

Authors: Viresh Patel, Marwan Alsarraj, and Shawn Miller

EXPLORING THE POSSIBILITIES

Of High-Resolution Melt Analysis

HRM Analysis: The Basics

DNA melt-curve analysis — applying temperature to melt and characterize the resulting curve profiles of double-stranded (ds)DNA samples — has proven useful for scanning for sequence variations, primarily to confirm the specificity of primers as well as reveal the presence of primer-dimers in quantitative PCR (Brisson et al. 2002). The goal of this well-established method has been to prevent nonspecific amplification and improve data accuracy. Recent advances in dye chemistry, instrument sensitivity, and data acquisition rates have led to the next generation of this technique — HRM analysis. A closed-tube, post-PCR analysis method that requires no post-PCR handling, HRM analysis generates DNA melt-curve profiles sufficiently specific and sensitive to be used for mutation scanning, methylation analysis, and genotyping.

HRM analysis is performed to discriminate nucleotide sequence differences between samples (see How HRM Analysis Works sidebar). HRM analysis also enables mixed DNA fragments to be distinguished from each other — important for SNP genotyping of wild-type, heterozygous (mixed), and homozygous mutant individuals.

Three basic tools are used in HRM analysis:

- Standard PCR reagents and DNA binding dye — third-generation saturating, low-toxicity dye that can be used in high concentrations to yield strong melt curves, but does not interfere with amplification during PCR (Wittwer et al. 2003)
- Real-time PCR instrumentation — system should offer sensitive detection for accurate quantitation and target discrimination, as well as precise thermal control
- HRM analysis-compatible software — generates and analyzes melt-curve profiles, and clusters samples with similar properties

Because of its simplicity and abbreviated workflow, HRM analysis offers a cost-effective, yet accurate alternative to probe-based genotyping assays such as SSCP, RFLP, and DNA sequencing (White and Potts 2006).

HRM Analysis: The Workflow

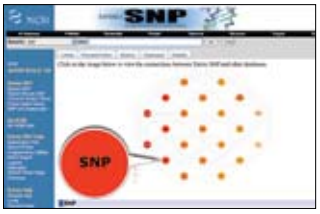
Figure 1 shows the HRM analysis workflow. After a DNA sample is amplified in a real-time PCR instrument using a saturating dye-based master mix, the PCR product is melted using high data acquisition rates to generate melt curves. Software developed specifically for HRM analysis (such as Precision Melt Analysis™ software, see page 9) enables analysis of the melt curves for genotyping and mutation scanning. Melt curves are analyzed in the software using three basic steps (Figure 1C):

- Normalization — all samples are normalized along the fluorescence axis such that their average relative fluorescence value at the pre-melt (initial fluorescence) signal is set to 100% and post-melt (final fluorescence) signal is set to 0%. This serves as a visual aid to interpret the data
- Difference plotting — to magnify the differences in the melting curves between samples, each melt curve is subtracted from a user-defined reference melt curve and the fluorescence differences between samples are plotted. Similarly curved shapes will be clustered automatically into groups representing different genotypes/sequences
- Temperature shifting (optional) — makes it easier to distinguish heterozygous from wild-type homozygous samples. Curves can be shifted along the high end of the temperature axis to all meet at the same specific temperature so that curve shapes are more accurately compared

This simple post-PCR workflow has far fewer steps than traditional screening applications, significantly reducing the resources required to compare DNA samples by sequence, length, content, or complementarity.

A. Design

Identify target sequence. | **Design amplicon.** | **Check specificity.**

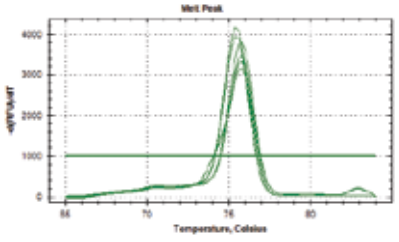


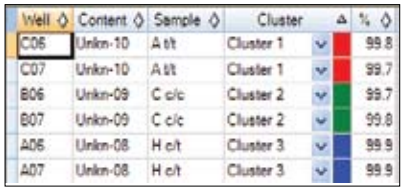
B. Run

Set up reactions. | **Run PCR and HRM analysis.**

C. Analyze

Analyze results and auto-call genotypes.





Well	Content	Sample	Cluster	%
C06	Unkn-10	A t/t	Cluster 1	99.8
C07	Unkn-10	A t/t	Cluster 1	99.7
B06	Unkn-09	C c/c	Cluster 2	99.7
B07	Unkn-09	C c/c	Cluster 2	99.8
A06	Unkn-08	H c/t	Cluster 3	99.9
A07	Unkn-08	H c/t	Cluster 3	99.9

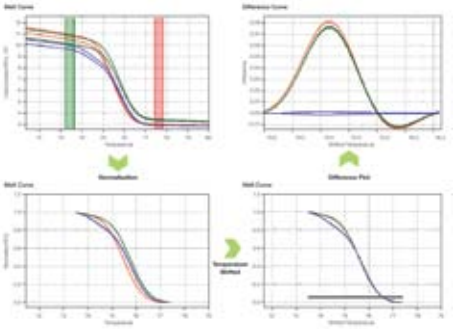


Figure 1. HRM analysis workflow, from experiment design (A) and setup/experiment run (B), to analysis (C).

HRM Analysis In Action 1 » Breast Cancer Research

Kim de Leeneer » Center for Medical Genetics Ghent, Ghent University Hospital, Ghent Belgium

Perhaps the most challenging issue facing researchers seeking to identify the genetic factors involved in the proliferation of breast cancer is the polymorphic nature of genes commonly involved in expression of the disease: *BRCA1* and *BRCA2*. In Belgium, Kim de Leeneer is a PhD student at the Center for Medical Genetics Ghent (CMGG) focused on researching the genetics of breast cancer — specifically the *BRCA1* and *BRCA2* genes. The diagnostic and research aspects of disease discovery are intertwined in this laboratory; for example, part of de Leeneer's research thesis (in progress) explores research techniques used in the diagnostic field.

CMGG acquired its first HRM instrument at the same time de Leeneer started her graduate work. De Leeneer conducted her first HRM experiments in January 2007, screening 212 positive control samples for breast cancer. Until these experiments, the primary tools used in CMGG to screen for the genetic inheritance of breast cancer were denaturing gradient gel electrophoresis (DGGE) and direct sequencing of both large exons 11 of *BRCA1* and *BRCA2*. Traditional sequencing experiments were conducted in parallel to compare results and verify accuracy of the new technique. All controls were recognized, so de Leeneer and colleagues began converting traditional assays to HRM analysis.

De Leeneer estimates that her laboratory currently processes 600 HRM samples per week (450 of which are breast cancer-related). Initially, all HRM analysis results were confirmed by sequencing; results demonstrated 100% sensitivity and 98.7% specificity of HRM analysis, with very few false positives. Because they have developed confidence in HRM analysis and the diagnostic aspects of their work require a high degree of throughput, they are now processing sample assays in single replicates. Only aberrant melting curves get sequenced to confirm the presence of a genetic variant.

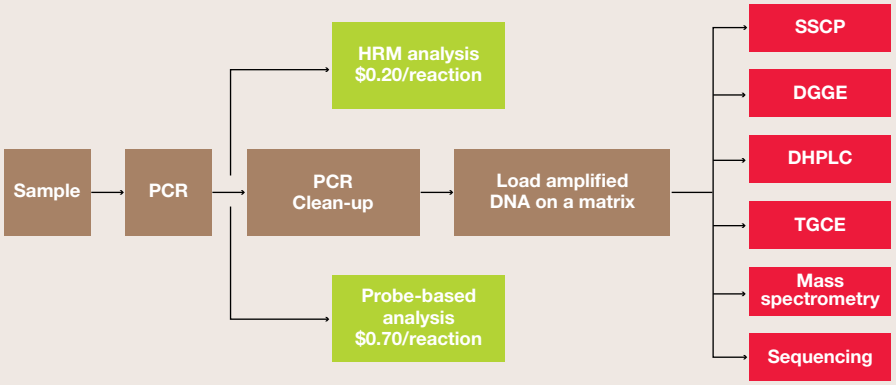
For HRM analysis, "Everything that is assigned to an aberrant melting curve cluster [by the software] has to be sequenced," says de Leeneer. In fact, a big lesson they've learned is: "Don't trust that because curves look similar, that results are also similar;" referring to the fact that samples exhibiting similar curves don't necessarily exhibit the same genetic variants. This phenomenon is inherent in any technique based on melting of amplicons. Also, de Leeneer explains that as a diagnostic laboratory, CMGG receives samples from other laboratories. Differences in sample handling can contribute to aberrant melting curves, possibly generating false positives.

To overcome challenges intrinsic in the nature of their work, de Leeneer explains that they've conducted experiments to optimize all factors in the HRM workflow — from the use of positive controls and amplicon design (the best results are obtained with amplicons <400 bp), to standardization of buffers and reagents. Identifying the ideal number of samples per amplicon in each experiment is also important. Software normalization calculations for each sample affect the shape of the melt curve generated; experiments with fewer than five samples per amplicon make it difficult for HRM software to distinguish wild-types. De Leeneer explains that 11 samples per amplicon has proven ideal for their protocols.

De Leeneer believes that the minimal amount of optimization required to modify protocols for HRM analysis has been well worth it. As a prescreening tool, HRM analysis makes it easy to identify samples with genetic variants — and with a significant reduction in cost and time required over traditional methods. "HRM has reduced by approximately one-third the costs and workload compared to DGGE and direct sequencing," says de Leeneer. She believes that HRM will eventually replace other prescreening techniques (such as dHPLC and DGGE) for other disorders and in other laboratories.

What is de Leeneer's bottom-line advice for other researchers considering the switch to HRM analysis? It's a recommendation that makes sense with practically any new technology in any field of research: "Before you go to the bench, do your homework."

HRM Analysis vs. Existing Genotype Analysis Approaches



HRM analysis requires fewer steps and less time than other post-PCR genetic analysis techniques such as SSCP, mass spectrometry, and sequencing. Post-PCR sample handling is eliminated, reducing the potential for contaminant introduction and eliminating separation steps. At a typical cost of ~$0.20 per reaction, HRM is significantly more cost-effective than probe-based analyses, which typically cost ~$0.70 per reaction.

HRM Analysis In Action 2 » Genetics of Disease-Related Pathways (SNP Genotyping)

Dr. Alessandro Martino » Department of Biology, Pisa University, Pisa, Italy

Alessandro Martino is a PhD student working in the Department of Biology at Pisa University. The main focus of his studies is on SNP-based pharmacogenetics of multiple myeloma (MM), but he is also interested in gene expression in both humans and closely related species. Currently, he is engaged in the study of rat gene expression that triggers repair responses in heart perfusion following heart failure. Martino's experiments mainly center on membrane transporters (ABCD1, MDR1, and SLC19A1), cytokines (interleukins 1, 2, 4, 6, and TNF-α and their receptors), and other pathways that potentially modulate drug response and survival rates after chemotherapy. Studies involve analyzing SNP mutations — primarily class I and II (C:T, G:T, or A:G), but also some class IV (A:T) — in MM patient blood samples.

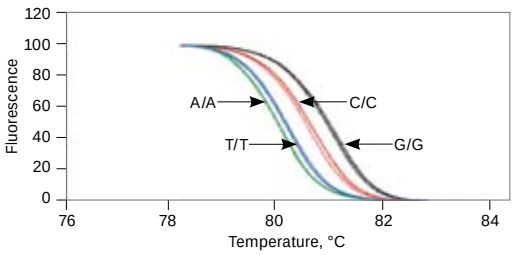
Until recently, the primary techniques used for SNP analysis were TaqMan assays, but Martino has tested HRM analysis with the aim to replace probe-based screening methods. In initial HRM experiments, researchers used the same primer pairs and reagents as with TaqMan assays. These early HRM experiments were run in parallel with probe-based assays, and Martino observed "good correlation between TaqMan assays and HRM." These studies did, however, demonstrate that melting temperature of the amplicon and primer pair specificity are influenced by the primer pairs used, so Martino began to develop primer pairs specifically for HRM studies. "Theoretically, we can assume that the shorter the amplicon, the more sensitive to the SNPs. However, the higher the melting temperature, the greater the difference in the melting profiles of different genotypes. The really critical point in the choice of primers pairs is their specificity — a primer pair has to be designed to optimize these factors," explains Martino.

Experiments were further optimized by replacing a self-made reagent mix with SsoFast™ EvaGreen® supermix (developed by Bio-Rad to optimize HRM experimentation). The new reagent enabled amplification of targets that were previously problematic. HRM analysis also improved their results with allelic discrimination experiments over probe-based assays. Because allelic groups cluster using HRM-based methods, they can be identified via melting curves generated by the software.

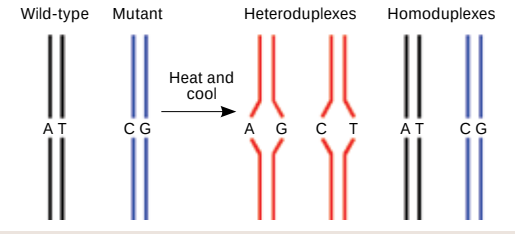
Currently, the laboratory conducts ~200 (two 96-well-plate) HRM experiments per week, although up to 200 experiments can be run in a single day. As more of the laboratory's key instrumentation is replaced with HRM-compatible models, Martino believes that HRM has the potential to replace many probe-based assays. Cost is a crucial motivation for this conversion, and the savings can be dramatic. Martino states that a typical study of 3–4 SNPs on 250 samples that used to cost the laboratory €2,000 (for probes alone), can now be conducted using HRM analysis for only €200 (the primer pair cost) — with good levels of sensitivity.

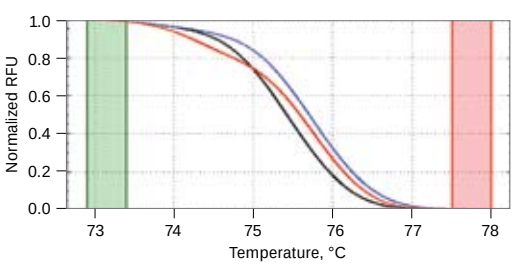
How HRM Analysis Works

PCR fragments containing one of the four nucleotides at a given position can be distinguished through HRM analysis. Given that C:G base pairs have greater thermostability (higher melting temperature) than A:T base pairs, GC-rich fragments will exhibit shifted/elevated melt curve profiles with a similar shape (compare black and red traces with blue and green traces below).



Amplification of a heterozygote sample (depicted as a mixture of wild-type/black and mutant/blue DNA) results in the formation of heteroduplexes after the plateau phase of PCR. The resulting DNA complexes can be distinguished by a temperature shift and shape difference in the melt curve. Note the shape difference of the melting heteroduplexes (red traces) from the homoduplexes (blue and black).





When asked whether there is any advice for laboratories considering the conversion to HRM technology, Martino's primary recommendation is to "dedicate particular attention to the design of primer pairs and to try to reduce user influence on software-based normalization of the results." Specifically, he is referring to the fact that user-defined normalization conditions in the software can affect HRM analysis. He also recommends that researchers standardize processing methods, such as starting sample amount and those steps intrinsic in any experimental setup — all of which can be affected by normalization. But once these parameters are defined and optimized, Martino predicts that, "of course, the use of HRM will continue to grow."

HRM Analysis In Action 3 » Forensic Potential

Dr. Hildegard Haas » Genedia AG,
Biotech Services & Products, Munich, Germany

Dr. Hildegard Haas was instrumental in setting up the first DNA testing laboratory in Giessen, Germany approximately 15 years ago. Today, she runs a private forensics laboratory in Germany that processes 1,000 samples per month from private laboratories, police laboratories, and government agencies throughout the country. The primary focus of Genedia scientists is answering questions related to breeding (agriculture), heredity (paternity testing), and criminology. The primary techniques used are DNA fingerprinting, SNP genotyping, and short tandem repeat (STR) typing using TaqMan probe-based assays. Typically, the laboratory conducts real-time PCR for both absolute and relative quantitation, then sequences for SNP analysis and genotyping. With experiments involving mitochondrial DNA, the primary tool used is minisequencing — clusters of genes with 1 base pair differences are amplified then minisequenced to identify the single base pair difference.

When asked what changes she has observed in the forensic sample testing workflow over the past decade, Haas says that there have been very few. Not because of a lack of innovation, but because in the forensic environment — where accuracy, specificity, and reproducibility are critical — movement to adopt changes in methodology is often slow. “We need to be 100% precise, 100% of the time,” explains Haas. She does cite the introduction of fluorescence and multiplexing technologies as having “made results more accurate, samples easier to handle, and overall experiments faster.”

The next evolution in their DNA analysis workflow appears to be the adoption of HRM analysis, which Haas and her team are hoping will replace probe-based assays and eliminate the need for minisequencing. Beginning in January 2009, this cost-reduction–based initiative launched with a series of sample tests for species identification using both HRM analysis and minisequencing. Results of these tests — determining whether HRM analysis is as sensitive and accurate as current protocols — will dictate whether the laboratory will move forward in their efforts to replace STR typing and minisequencing. The new technology must enable researchers to identify, with precision, single base pair differences in fragments typically 88 to 100 base pairs in length. Ultimately, as Haas states, it is “HRM that must show whether or not it will be a replacement technology.”

Early results of Haas’s testing combined with the experiences of other researchers (Erali et al. 2008) point to HRM analysis as an accurate, cost-cutting tool for DNA analysis — even down to identifying single base pair mutations — in the highly precise forensic environment.

HRM Analysis in the Literature

Basics of the Technology

Liew M et al. (2004). Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. Clin Chem 50, 1156-1164.

Wittwer CT, et al. (2003). High-resolution genotyping by amplicon melting analysis using LCGreen. Clin Chem 49, 853-860.

Advanced Techniques

Dames S et al. (2007). Characterization of aberrant melting peaks in unlabeled probe assays. J Mol Diagn 9, 290-296.

Seipp MT et al. (2007). Unlabeled oligonucleotides as internal temperature controls for genotyping by amplicon melting. J Mol Diagn 9, 284-298.

Oncology (Heterozygous Dominant Mutations and Allele Fractions)

Margraf RL et al. (2008). Rapid diagnosis of MEN2B using unlabeled probe melting analysis and the LightCycler 480 instrument. J Mol Diagn 10, 123-128.

Willmore-Payne C et al. (2006). Detection of *c-kit* exons 11- and 17-activating mutations in testicular seminomas by high-resolution melting amplicon analysis. Mod Pathol 19, 1164-1169.

Margraf RL et al. (2007). RET proto-oncogene genotyping using unlabeled probes, the masking technique, and amplicon high-resolution melting analysis. J Mol Diagn 9, 184-196.

Infectious Disease (Haploid Genomes)

Dames S et al. (2007). Unlabeled probes for the detection and typing of herpes simplex virus. Clin Chem 53, 1847-1854.

Slinger R et al. (2007). High-resolution melting assay for the detection of *gyrA* mutations causing quinolone resistance in *Salmonella enterica* serovars Typhi and Paratyphi. Diagn Microbiol Infect Dis 57, 455-458.

Cheng JC et al. (2006). Rapid detection and identification of clinically important bacteria by high-resolution melting analysis after broad-range ribosomal RNA real-time PCR. Clin Chem 52, 1997-2004.

HLA Matching

Zhou L et al. (2004). High-resolution DNA melting curve analysis to establish HLA genotypic identity. Tissue Antigens 64, 156-164.

Genotyping for Genetic disorders

Poulson MD and Wittwer CT (2007). Closed-tube genotyping of apolipoprotein E by isolated-probe PCR with multiple unlabeled probes and high-resolution DNA melting analysis. Biotechniques 43, 87-91.

Seipp MT et al. Quadruplex genotyping of *F5*, *F2*, and *MTHFR* variants in a single closed tube by high-resolution amplicon melting. Clin Chem 54, 108-115.

Hill CE et al. (2006). Detection and identification of cytochrome P-450 2C9 alleles *1, *2, and *3 by high-resolution melting curve analysis of PCR amplicons. Am J Clin Pathol 125, 584-591.

Mutation Scanning for Genetic Disorders

Dobrowolski SF et al. (2005). Validation of dye-binding/high-resolution thermal denaturation for the identification of mutations in the SLC22A5 gene. Hum Mutat 25, 306-313.

McKinney JT et al. (2004). Rapid, comprehensive screening of the human medium chain acyl-CoA dehydrogenase gene. Mol Genet Metab 82, 112-120.

Montgomery J et al. (2007). Scanning the cystic fibrosis transmembrane conductance regulator gene using high-resolution DNA melting analysis. Clin Chem 53, 1891-1898.

References

Brisson M et al. (2002). Identification of nonspecific products using melt-curve analysis in the iCycler iQ detection system. Bio-Rad bulletin 2684.

Erali M et al. (2008). High resolution melting applications for clinical laboratory medicine. Exper and Mol Pathol 85, 50-58.

White H and Potts G (2006). Mutation scanning by high resolution melt analysis. Evaluation of Rotor-Gene 6000 (Corbett Life Science), HR-1, and 384-well LightScanner (Idaho Technology). Natl Gen Ref Lab - Wessex.

Wittwer CT et al. (2003). High resolution genotyping by amplicon melting analysis using LCGreen. Clin Chem 49, 853-860.

Electroporation of Primary Murine Mast Cells Using the Gene Pulser MXcell™ Electroporation System

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Introduction

The use of primary cells — cells isolated directly from tissues or blood — is rapidly becoming the desired model system for examining physiological processes. Transfection of these cells provides researchers with a powerful means of examining a multitude of cellular processes in vitro, as well as allowing ex vivo studies such as cell tracking analyses. In contrast to immortalized cell lines, these cells typically undergo minimal cell division and have a finite lifespan in culture, making gene transfer and expression a challenge.

In order to study primary cells, suitable tissue culture conditions to generate and/or maintain the cell of interest must first be developed. Typically, mature primary cells, or immature precursors, are harvested from an animal and then grown ex vivo as a cell culture. Hematopoietic cells are particularly well suited to growth and differentiation by culturing them in vitro and many different types of blood cells can be generated from bone marrow. One such hematopoietic cell, the mast cell, is particularly amenable to ex vivo differentiation and is comparatively hardy and long-lived (Itakura et al. 2001, Razin et al. 1984). Mast cells play a fundamental role in asthma and allergy, and as such, these cells represent an excellent primary cell model.

Electroporation is a fast and adaptable method to introduce exogenous nucleic acid into primary cells. Here, we report the use of the Bio-Rad Gene Pulser MXcell electroporation system to successfully transfect mast cells. Results shown here demonstrate that using this approach, mast cells can be transfected with high efficiency and low cytotoxicity.

Methods

Cell Culture

Primary mast cell cultures were established from the bone marrow of 8–12 week BALB/c mice (The Jackson Laboratory). Bone marrow was flushed from the femurs and tibiae and cells were cultured at 1 x 10^6 cells/ml in RPMI media (Invitrogen Corporation) supplemented with 10% fetal calf serum, 1% L-glutamine, 1% penicillin/streptomycin, and β-mercaptoethanol. Interleukin-3 (IL-3) (10 ng/ml, BD Biosciences) was added at the initial plating, and every seven days thereafter to promote differentiation to mast cells. Stem cell factor (20 ng/ml, PeproTech, Inc.) was added after 14 days, and every 7 days thereafter. The medium was changed weekly. After 5 weeks in culture, mature mast cells were fully differentiated and ready for transfection.

Electroporation

Mast cells were washed once with PBS, counted, and suspended at 1 x 10^7 cells/ml in Gene Pulser® electroporation buffer (Bio-Rad Laboratories, Inc.). Plasmid DNA with the Green Fluorescent Protein (GFP) reporter gene (gWIZ GFP mammalian expression vector, Genlantis, Inc.) was added to the cells at a final concentration of 20 µg/ml. Subsequently, the cell suspension was transferred into the wells of a 96-well electroporation plate (Bio-Rad) and pulsed using the Gene Pulser MXcell electroporation system. To determine the optimal electroporation conditions for mast cells, both exponential and square-wave pulses were tested using a variety of settings.

After electroporation, the cells were transferred into tissue culture plates containing prewarmed RPMI media with 10 ng/ml IL-3 and incubated for 24 hr at 37°C. Transfection efficiency and cell viability were determined by flow cytometric analysis 24 hr postelectroporation. The cells were stained with propidium iodide (PI) prior to flow cytometry analysis. Transfection efficiency was expressed as the number of cells expressing GFP relative to the total number of live cells. Cell viability was assessed using PI staining.

Results and Discussion

Square-wave pulses were found to be more effective than exponential-decay wave pulses at delivering plasmid DNA to mast cells while maintaining cell viability (Figure 1).The highest transfection efficiencies were obtained with a square-wave protocol delivering a 15 ms pulse at 350 V; this reproducibly gave ~30% transfection rates with ~50% cell viability. As illustrated in Figure 1, higher cell viabilities can be obtained; however, this is at the cost of reduced transfection efficiency.

We tested the effect of cell density on electroporation efficiency. Cell densities of 1 x 10^6, 5 x 10^6, and 1 x 10^7 cells/ml yielded similar transfection efficiencies and cell viabilities (Figure 2).

Expression of GFP was examined at 24 hr and 48 hr postelectroporation. At both time points, comparable levels of GFP expression relative to living cells were observed, thus illustrating that these electroporation conditions are suitable for extended time point cell analyses (Figure 3).

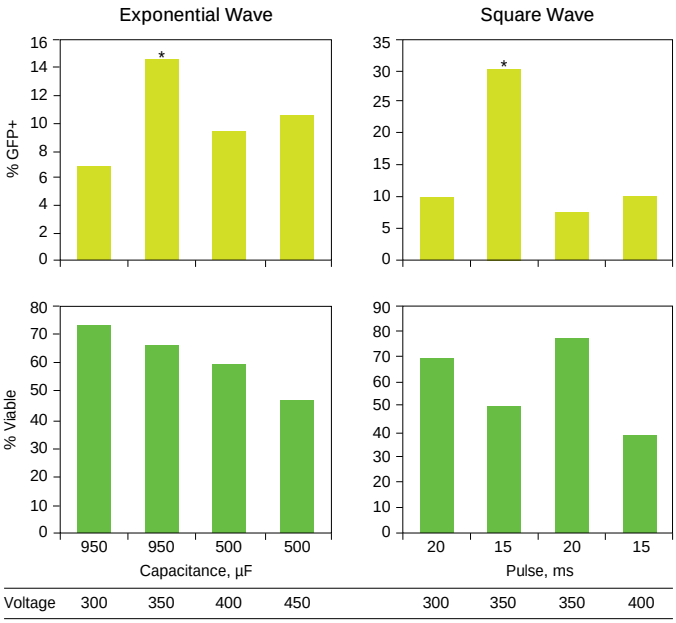

Fig. 1. Transfection efficiency and viability of mast cells after electroporation. Cells were electroporated using exponential and square wave electric pulses with different parameters. Expression of the reporter GFP protein (top charts) and cell survival (bottom charts) was monitored 24 hr postelectroporation. The best parameters are indicated by an asterisk. Values are the mean of three replicate experiments.

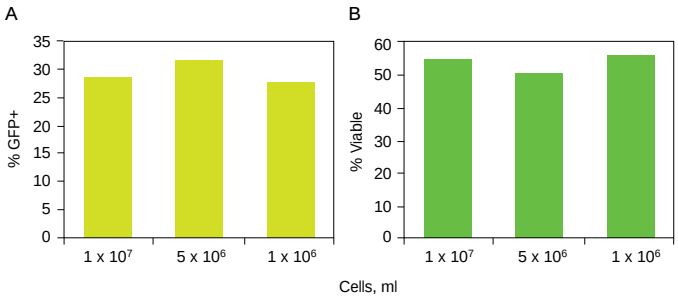

Fig. 2. Transfection efficiency (A) and cell viability (B) of electroporated mast cells at three cell densities. All experiments were carried out at 350 V, with a pulse length of 15 ms. Values are the mean of three replicate experiments.

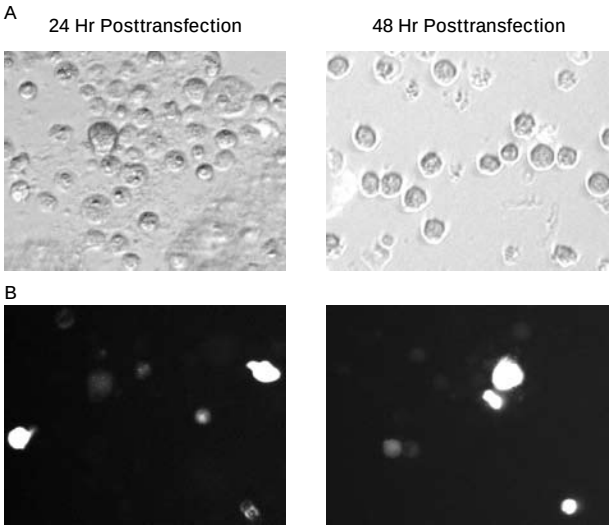

Fig. 3. Microscopic observation of mast cells after transfection. Contrast phase microscopy images of cells (A) and corresponding fluorescence microscopy images of cells expressing GFP (B).

Conclusions

Using the Gene Pulser MXcell electroporation system we have been able to rapidly optimize electroporation conditions for primary mast cells. Our results indicate that after a 15 ms pulse at 350 V using a square-wave protocol, roughly one-third of the cells express the reporter gene (GFP). Depending upon experimental needs, electroporation conditions can be modified to increase either cell viability or transfection efficiency.

This work demonstrates the utility of the Gene Pulser MXcell electroporation system in the optimization of electroporation conditions for primary cells. Rapid optimization is particularly valuable when working with primary cells, as conditions may vary substantially for each cell type due to differences in size, granularity, and replicative state. Furthermore, primary cells are often not available in large numbers and are not long-lived. Therefore, simultaneous examination of numerous electroporation conditions using small numbers of cells greatly reduces the amount of cell culture required to obtain sufficient cell numbers for analysis.

References

Itakura A et al. (2001). Interluekin-3 and stem cell factor modulate cell cycle regulatory factors in mast cells: negative regulation of p27kip1 in proliferation of mast cells induced by interleukin-3 but not stem cell factor. Exp Hematol 29(7), 803-11.

Razin E et al. (1984). Interleukin-3: a differentiation and growth factor for the mouse mast cell that contains chondroitin sulfate *E proteglycan*. Journal Immunol 132(3), 1479-86.

Periplasmic Expression and Purification of Recombinant Proteins From *E. coli* Using the Profinity eXact™ Protein Purification System

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Introduction

The Profinity eXact fusion-tag purification system offered by Bio-Rad provides an efficient one-step purification-cleavage protocol for bacterial recombinant protein production. The Profinity eXact affinity tag, in an N-terminal fusion with a target protein, binds to the Profinity eXact resin and, upon on-column cleavage, releases the purified target protein in the elution step. The highly specific, controlled cleavage generates a recombinant protein containing the desired native N-terminal amino acid sequence with the tag still attached to the resin, eliminating the need for tag and protease removal. A protein of interest can be cloned into the pPAL7 vector and expressed in *E. coli* as a fusion protein under control of the T7 promoter.

Although expression of recombinant proteins in the *E. coli* cytoplasm is widely used, improper folding of many target proteins may occur. Improper folding often results in the formation of inclusion bodies despite attempts to optimize growth conditions. One possible solution for obtaining a correctly folded recombinant protein is to export the protein into the *E. coli* periplasm. Some successful examples of this approach are production of soluble and functional human growth hormone (Soares et al. 2003), human interferon-γ (Balderas Hernández et al. 2008), and murine CMP-sialic acid transporter (Maggioni et al. 2007). Secretion of recombinant proteins to the periplasm of *E. coli* has several advantages over intracellular production. These advantages include simplified downstream processing, enhanced biological activity, higher product stability and solubility, and N-terminal authenticity of the expressed protein (Mergulhão et al. 2005).

In order to utilize the Profinity eXact fusion-tag system for expression of recombinant proteins in the *E. coli* periplasm, we constructed a new expression vector by adding the OmpA signal peptide to the Profinity eXact tag and report successful periplasmic expression and purification of human interferon-α 2a (hIFNα2a).

Methods

Plasmid Construction

The OmpA signal sequence was constructed by annealing two phosphorylated primers encoding all 21 amino acids of the signal peptide (underlined): forward 5'-TATG ATG AAA AAG ACA GCT ATC GCG ATT GCA GTG GCA CTG GCT GGT TTC GCT ACC GTA GCG CAG GCC CA-3' and reverse 5'-TATG GGC CTG CGC TAC GGT AGC GAA ACC AGC CAG TGC CAC TGC AAT CGC GAT AGC TGT CTT TTT CAT CA-3'. A mixture of 10 µl (0.5 µg/µl) of each primer was incubated for 5 min at 75°C. The mixture was then slowly cooled at room temperature. Annealed product containing NdeI cohesive ends was ligated into the NdeI site of the pPAL7 vector (Bio-Rad Laboratories, Inc.), incorporating the OmpA signal peptide at the Profinity eXact tag's N-terminus (Figure 1) to generate plasmid pOPAL7.

The cDNA encoding hIFNα2a was amplified using restriction-independent cloning (RIC) as described in the Profinity eXact system instruction manual (bulletin 10011260). Then the RIC hIFNα2a fragment was cloned into the SapI and EcoRI sites of pOPAL7. The resulting pOPAL-IFNα2a plasmid generates an OmpA signal-Profinity eXact tag-IFNα2a fusion with a Thr-Ser linker to minimize premature elution of the target due to the natural N-terminal Cys residue present in the mature interferon (see bulletin 10011260).

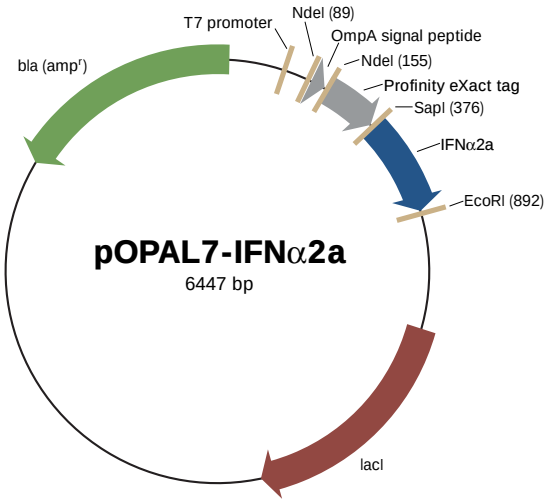

Fig. 1. Map of pOPAL7-IFNα2a plasmid.

Bacterial Strains and Cultivation

E. coli C-Max™ 5α competent cells (Bio-Rad) were used as a primary host for transformation and propagation of plasmids. *E. coli* BL21(DE3) (Bio-Rad) were used for protein expression. A noninducing medium (Studier 2005) containing glucose, salts ($MgSO_4$, $(NH_4)_2SO_4$, KH_2PO_4, Na_2HPO_4, $CaCl_2$), and trace metals (Mn, Fe, Zn, Co, Cu, Ni, Mo, Se, B) was used for overnight starter culture. Expression was carried out in M9 minimal medium containing 3 g/L Na_2HPO_4, 1.5 g/L KH_2PO_4, 0.5 g/L NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.4% glucose. Both media were supplemented with 100 µg ampicillin/ml.

Protein Expression and Purification

Expression plasmids were transformed into *E. coli* BL21(DE3) cells, and the resulting transformants were grown overnight at 37°C in 5 ml noninducing medium. From this overnight culture, 2.5 ml was used to inoculate 250 ml M9 medium; the cell culture was grown at 37°C until OD_{600} reached 0.5. Protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. The induced culture was grown for 16 hr at 20°C and 100 rpm. Periplasmic proteins were released by osmotic shock (Thorstenson et al. 1997). Briefly, the cell pellet was resuspended with 10 ml of 0.1 M Tris-acetate buffer (pH 8.0) containing 20% sucrose, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and 0.5 mM EDTA and incubated on ice for 15 min. The cell pellet was collected by centrifugation at 6,000 × g for 10 min and the supernatant was stored on ice. Cells were resuspended in 10 ml of ice-cold deionized water and incubated on ice for 15 min. After centrifugation at 6,000 × g for 10 min, the recovered supernatant was combined with supernatant from the sucrose wash. The resulting periplasmic fraction was purified using Bio-Scale™ Mini Profinity eXact™ cartridges (Bio-Rad). After purification, 500 µl of periplasmic solution was loaded onto prepacked mini spin columns pre-equilibrated with ice-cold 0.1 M Tris-acetate buffer (pH 8.0). The resin was washed three times with 500 µl of the same buffer and incubated at room temperature in 500 µl of cleavage buffer (0.1 M Tris-acetate, pH 8.0, and 10 mM NaN_3). After incubation for 30 min, the eluate containing tag-free target protein was collected.

SDS-PAGE Analysis

Protein samples were analyzed on 4–20% Criterion™ Tris-HCl gels followed by staining with Bio-Safe™ Coomassie stain (Bio-Rad). Image acquisition and analysis were done using a Molecular Imager® GS-800™ calibrated densitometer and Quantity One® 1-D analysis software (Bio-Rad).

Results and Discussion

Periplasmic Expression of the Profinity eXact Tag in *E. coli*

Periplasmic translocation is one of the widely employed approaches to improve soluble recombinant protein expression in *E. coli*. The oxidizing environment of the periplasmic space facilitates the formation of disulfide bonds, promoting correct folding and protein stability. Purification of periplasmically expressed recombinant proteins benefits from the lower content of bacterial proteins in the periplasm and easy osmotic shock extraction protocols.

In order to develop an alternative strategy for expression of Profinity eXact fusion-tagged recombinant proteins in *E. coli*, we investigated the incorporation of an N-terminal OmpA signal sequence as a means of targeting fusion proteins into the periplasmic space. Many soluble and active proteins have been successfully produced using the OmpA signal sequence (Mergulhão et al. 2005).

The vast majority of *E. coli* secreted proteins use the SecB-dependent pathway for translocation across the inner membrane (Mergulhão et al. 2005). These proteins contain an amino-terminal signal peptide that functions as a targeting and recognition signal. The OmpA signal peptide targets the *E. coli* outer membrane protein A to the Sec translocase (the *E. coli* translocation machinery in the inner membrane), permitting the translocation of OmpA into the periplasm, where it is removed by a signal peptidase (Binet et al. 1997).

To confirm that the OmpA signal peptide can direct the Profinity eXact tag into the *E. coli* periplasm, we expressed the tag fused with and without the signal peptide. The periplasmic deposition was optimal at the following conditions: 20°C culturing, minimal expression medium, gentle shaking of induced cell culture, and induction with 0.1 mM IPTG. We speculate that using less than optimal expression conditions favor transportation of a target protein directed by the signal peptide into the periplasm where it could be correctly folded.

After induction with IPTG and expression, the harvested cell pellet was subjected to osmotic shock in order to release periplasmic proteins. All fractions were analyzed by SDS-PAGE as shown in Figure 2.

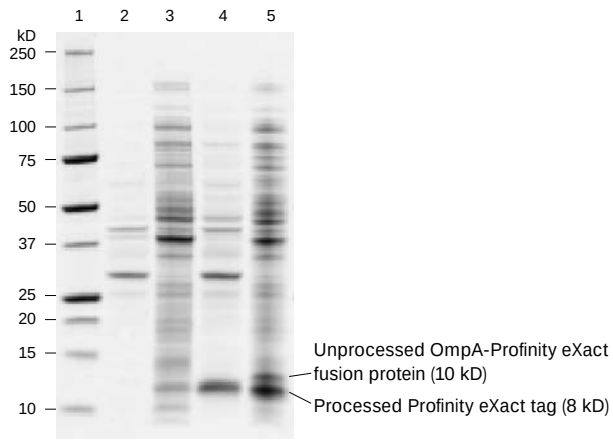

Fig. 2. SDS-PAGE analysis of the Profinity eXact tag distribution between periplasmic and intracellular fractions. Profinity eXact tag (8 kD) was expressed in *E. coli* alone (Profinity eXact tag) or fused with the OmpA signal peptide (OmpA-Profinity eXact fusion protein). Lane 1, Precision Plus Protein™ unstained standards; lanes 2 and 4, periplasmic fraction (supernatant) of Profinity eXact tag and OmpA-Profinity eXact fusion protein, respectively; lanes 3 and 5, intracellular fraction (pellet) of Profinity eXact tag and OmpA-Profinity eXact fusion protein, respectively. The 8 kD mass of the Profinity eXact tag has been confirmed by mass spectrometry (data not shown) and has an apparent electrophoretic mass of approximately 12 kD.

The Profinity eXact tag, as expressed without the signal peptide using the unmodified pPAL7 vector, accumulates in the cytoplasm and cannot be released by osmotic shock (Figure 2, lanes 2 and 3). The presence of the OmpA signal peptide (pOPAL7 vector) changes the localization of the Profinity eXact tag, targeting it to the periplasm (Figure 2, lane 4). Similar mobility of the Profinity eXact tag from the periplasmic fraction (Figure 2, lane 4) and intracellularly expressed tag without OmpA signal peptide (Figure 2, lane 3) suggests that the OmpA signal peptide was processed by the signal peptidase. The presence of processed Profinity eXact tag in the pellet fraction may be

explained by incomplete periplasmic extraction (Figure 2, lane 5). Efficiency of periplasmic extraction can be improved by performing additional osmotic shock (Sletta et al. 2007).

Judging from gel mobility, some of the Profinity eXact tag encoded by pOPAL7 in the pellet fraction is not proteolytically processed. This can be explained by cytoplasmic retention of the OmpA-fused Profinity eXact tag.

Comparing band intensities on the gel, the amount of Profinity eXact tag released by osmotic shock under the described conditions is 15 mg/L. Expression yield of the Profinity eXact tag is significantly higher when it is expressed with the OmpA signal peptide. This phenomenon needs further investigation, but similar observations have been made by other researchers (Sletta et al. 2007).

Expression and Purification of Human Interferon-α 2A

Interferon-α 2A belongs to the IFN family of cytokines, which can induce antiproliferative, immunomodulatory, and potent antiviral activities against a wide range of mammalian viruses (Pestka et al. 1987). It has pharmaceutical value and is used to treat several diseases, including some types of cancer and hepatitis, in particular hepatitis C.

Expression of human IFNα2a either untagged or with the His6 tag usually results in insoluble protein accumulation and inclusion body formation (Beldarraín et al. 2001, Piehler and Schreiber 1999). We used hIFNα2a as a model protein to examine targeting of Profinity eXact-tag fusion protein into the periplasm and subsequent purification.

After expression, periplasmic proteins were extracted by osmotic shock. In this experiment, we also included a wash step prior to periplasmic extraction to check for periplasmic leakage. A portion of the cell pellet was washed twice with cold 0.1 M Tris-acetate buffer (pH 8.0). All fractions were analyzed separately by SDS-PAGE, including sucrose wash and ice-cold water extraction (Figure 4).

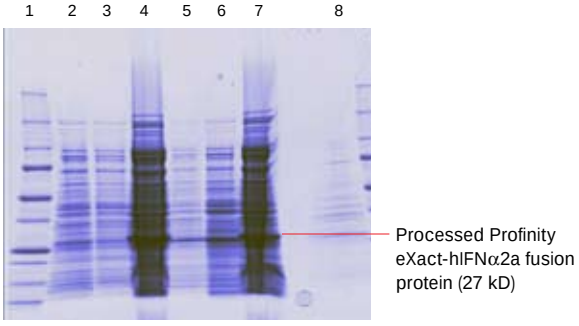

Fig. 3. SDS-PAGE analysis of the periplasmic secretion of the Profinity eXact-hIFNα2a fusion protein. Lane 1, Precision Plus Protein unstained standard; lanes 2 and 3, proteins extracted by a 0.1 M Tris-acetate buffer (pH 8.0) wash; lane 4, total cell proteins remaining after Tris-buffer wash; lane 5, proteins extracted with 0.1 M Tris-acetate buffer (pH 8.0), 20% sucrose, 0.1 mM PMSF, and 0.5 mM EDTA; lane 6, proteins extracted with ice-cold water; lane 7, total proteins after osmotic shock extraction; lane 8, spent culture medium.

Based on the results shown in Figure 3, it is believed that the OmpA signal peptide was processed off the initial OmpA-Profinity eXact-hIFNα2a fusion protein. The gel image shows soluble Profinity eXact-hIFNα2a can be released by osmotic shock (lanes 4 and 5). The presence of Profinity eXact-hIFNα2a and other bacterial proteins in the Tris-buffer wash fractions (lanes 2 and 3) as well as in the growth medium (lane 8) suggests periplasmic leakage. The periplasmic leakage may have several causes. During cell division, leakage of periplasmic contents can happen prior to the formation of the individual outer membrane (Mergulhão et al. 2004). The accumulation of recombinant protein in the periplasm may cause an osmotic pressure buildup, which can be a driving force for transport across the outer membrane (Hasenwinkle et al. 1997). Periplasmic secretion may also induce cell lysis, resulting in the release of periplasmic content (Lee and Bernstein 2001).

About 40% of the expressed Profinity eXact-hIFNα2a was released by the osmotic shock and 20% was secreted into the culture medium. The amount of secreted Profinity eXact-tagged hIFNα2a estimated from the gel is 10 mg/L.

hIFNα2a encoded by pPAL7 vector lacking signal peptide was used as a negative control for periplasmic targeting. No band corresponding to Profinity eXact-hIFNα2a can be detected in the periplasmic fraction of the cell expressing interferon without signal peptide (Figure 4).

Periplasmic hIFNα2a was purified using Bio-Scale Mini Profinity eXact cartridges (Figure 5). The detailed analysis of recombinant protein purification using the Profinity eXact system is described elsewhere (see bulletin 5652). On-column cleavage releases tag-free hIFNα2a and presents a great advantage in periplasmic protein purification. Since specific cleavage occurs between the last amino acid of the Profinity eXact tag and the N-terminal amino acid of the target protein, N-terminal heterogeneity due to possible errors in signal peptide processing of the target is eliminated.

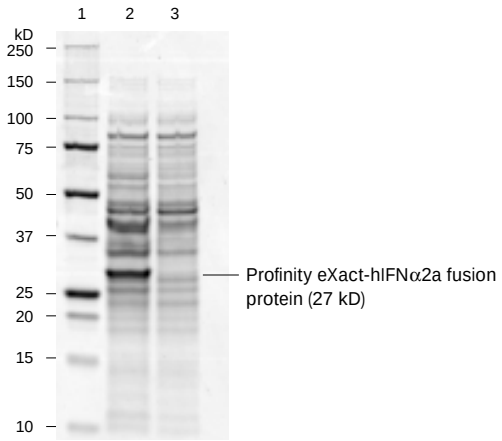

Fig. 4. SDS-PAGE analysis of the periplasmic proteins extracted from the cells expressing Profinity eXact-hIFNα2a with and without OmpA signal peptide. Lane 1, Precision Plus Protein unstained standard; lane 2, Profinity eXact-hIFNα2a (27 kD) with OmpA signal peptide; lane 3, Profinity eXact-hIFNα2a (27 kD) without OmpA signal peptide.

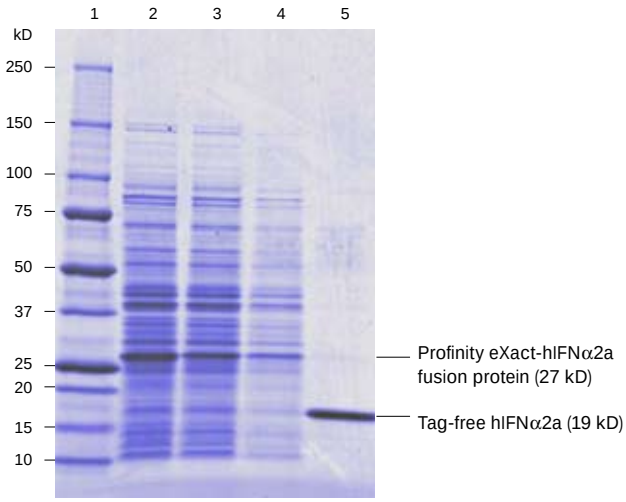

Fig. 5. Bio-Scale Mini Profinity eXact cartridge purification of periplasm targeted Profinity eXact-tagged hIFNα2a. Lane 1, Precision Plus Protein unstained standard; lane 2, periplasmic proteins extracted by osmotic shock, containing Profinity eXact-hIFNα2a fusion protein (27 kD); lane 3, flowthrough from spin column; lane 4, wash; lane 5, eluted tag-free hIFNα2a (19 kD).

Conclusions

In this paper we report the applicability of the Profinity eXact fusion-tag system for purification of periplasmic proteins. Results show that hIFNα2a in fusion with the Profinity eXact tag and with an N-terminal OmpA signal peptide can be successfully translocated into the periplasm, suggesting utility with other proteins of interest. Fusion protein can then be natively purified using the Profinity eXact purification resin. Precise on-column cleavage of the Profinity eXact tag facilitates affinity purification of the target and eliminates the need to maintain compatibility of the leader peptide with target proteins for periplasmic targeting and correct signal peptide processing.

The secretory production of recombinant proteins by *E. coli* has been proven to be a successful method to facilitate downstream processing, folding, and in vivo stabilization, enabling the production of soluble and biologically active proteins. Our interest in developing new protein purification techniques and generating native soluble proteins of interest led us to explore the possibility of targeting Profinity eXact fusion-tagged proteins into the *E. coli* periplasmic space.

References

Balderas Hernández VE et al. (2008). Periplasmic expression and recovery of human interferon gamma in *Escherichia coli*. Protein Expr Purif 59, 169-174.

Beldarraín A et al. (2001). Purification and conformational properties of a human interferon alpha2b produced in *Escherichia coli*. Biotechnol Appl Biochem 33, 173-182.

Binet R et al. (1997). Protein secretion by gram-negative bacterial ABC exporters — a review. Gene 192, 7-11.

Hasenwinkle D et al. (1997). Very high-level production and export in *Escherichia coli* of a cellulose binding domain for use in a generic secretion-affinity fusion system. Biotechnol Bioengin 55, 854-863.

Lee HC and Bernstein HD (2001). The targeting pathway of *Escherichia coli* presecretory and integral membrane proteins is specified by the hydrophobicity of the targeting signal. Proc Natl Acad Sci USA 98, 3471-3476.

Maggioni A et al. (2007). Targeting the expression of functional murine CMP-sialic acid transporter to the *E. coli* inner membrane. Biochem Biophys Res Comm 362, 779-784.

Mergulhão FJ et al. (2005). Recombinant protein secretion in *Escherichia coli*. Biotechnol Adv 23, 177-202.

Mergulhão FJ et al. (2004). Evaluation of bottlenecks in proinsulin secretion by *Escherichia coli*. J Biotechnol 109, 31-43.

Pestka S et al. (1987). Interferons and their actions. Annu Rev Biochem 56, 727-777.

Piehler J and Schreiber G (1999). Biophysical analysis of the interaction of human Ifnar2 expressed in *E. coli* with IFNα2. J Mol Biol 289, 57-67.

Sletta H et al. (2007). The presence of N-terminal secretion signal sequences leads to strong stimulation of the total expression levels of three tested medically important proteins during high-cell-density cultivations of *Escherichia coli*. Appl Environ Microbiol 73, 906-912.

Soares CR et al. (2003). Periplasmic expression of human growth hormone via plasmid vectors containing the λPL promoter: use of HPLC for product quantification. Protein Eng 16, 1131-1138.

Studier FW (2005). Protein production by auto-induction in high density shaking cultures. Protein Expr Purif 41, 207-234.

Thorstenson YR et al. (1997). Leaderless polypeptides efficiently extracted from whole cells by osmotic shock. J Bacteriol 179, 5333-5339.

In-Gel Protein Quantitation Using the Criterion Stain Free™ Gel Imaging System

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Introduction

The Criterion Stain Free gel imaging system is an alternative to Coomassie staining for protein visualization on SDS polyacrylamide gels after electrophoresis. The system consists of three components: Criterion Stain Free gels, the Criterion Stain Free imager, and Image Lab™ software. Criterion Stain Free technology is based on a UV-induced trihalocompound modification of tryptophan residues contained in proteins after separation by electrophoresis (Kazmin et al. 2002) on Criterion Stain Free gels. The system uses standard sample preparation reagents and electrophoresis protocols. After electrophoresis, the gel is removed from the cassette and placed into the Criterion Stain Free imager where the separated proteins are activated by UV irradiation and produce a fluorescent signal that is detected and captured by a CCD camera. In 2.5–5 min, the system provides an image of the proteins, and using the molecular weight standard lane, it automatically estimates the molecular weight and quantity for each detected protein band. After activation, the gel remains compatible with downstream applications such as western blotting (Elbaggari et al. 2008; Ladner et al. 2004) or staining with Coomassie stain, silver stain, or fluorescent dyes.

A comparison of standard SDS-PAGE/Coomassie staining and Criterion Stain Free system workflows shows a 70% reduction in experiment time with the Criterion Stain Free system (Figure 1). Rinsing, staining, and destaining steps are eliminated from the workflow when using the Criterion Stain Free system, and the imaging and analysis steps are automated, thus reducing post-electrophoresis time from more than 2 hr to about 2.5 min.

This study compares the reproducibility of protein quantitation results and sensitivity of the Criterion Stain Free system to Bio-Safe™ colloidal Coomassie Brilliant Blue G-250 stain (Bio-Rad Laboratories, Inc.). Results indicate superior to equivalent performance of the Criterion Stain Free system compared with traditional Coomassie staining.

Methods

Electrophoresis was performed on 4–20% gradient Criterion Stain Free Tris-HCl gels (Bio-Rad). Samples were prepared in Laemmli buffer containing 5% β-mercaptoethanol and heated at 95ºC for 5 min.

The broad range unstained SDS-PAGE molecular weight standards (Bio-Rad) were diluted 1:40 in Laemmli sample buffer containing 5% β-mercaptoethanol, and 2-fold serial dilutions were made in the same sample buffer for a total of 13 protein concentrations. A volume of 10 µl of each dilution was loaded onto 26-well gels and electrophoresed for 55 min at 200 V.

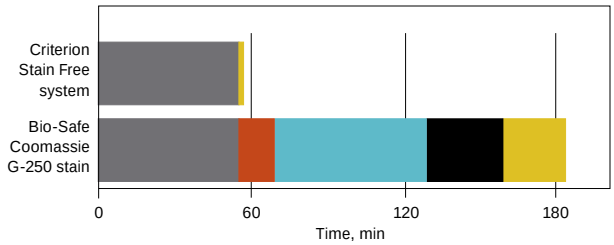

Fig. 1. Comparison of Criterion Stain Free system and Bio-Safe Coomassie stain workflows. The electrophoresis run time for both Criterion Stain Free system and Coomassie-stained gels is the same at 55 min. After electrophoresis, Criterion Stain Free gels take 2.5–5 min to generate results, while Coomassie staining takes at least 2 hr to generate the same level of sensitivity (the graph does not includes times for changing solutions). Separate proteins (■), rinse gel (■), stain gel (■), destain gel (■), image/analyze gel (■).

Coomassie staining was performed with Bio-Safe colloidal Coomassie Brilliant Blue G-250 stain. Bio-Safe G-250 staining was performed following manufacturer's instructions. Gels were rinsed 3 x 5 min in deionized water before being placed in 200 ml of Bio-Safe G-250 stain for 1 hr under agitation. The gels were rinsed 3x with 200 ml water and destained for 3 x 30 min in 200 ml water with gentle rocking at room temperature.

Imaging and Analysis

Criterion Stain Free gels were placed in the Criterion Stain Free imager, activated for 5 min, and imaged for 1.1 sec. Gel images were analyzed using Image Lab software. Gels stained with Coomassie stain were imaged on the Molecular Imager® GS-800™ calibrated densitometer (Bio-Rad) at 95 µm resolution to achieve resolution equivalent to that of the Criterion Stain Free imager and analyzed using Quantity One® 1-D analysis software (Bio-Rad).

Results and Discussion

Reproducibility of Protein Quantitation on Polyacrylamide Gels

Quantitation of protein bands on a polyacrylamide gel depends on the quality of the staining used to visualize the protein. Variables in staining/destaining conditions such as agitation times, volumes, solution changes, and temperature affect reproducibility of results. Uneven staining of the gels (Figure 2) may result in erroneous quantitation of the protein bands. In contrast, because they do not require staining and destaining steps, Criterion Stain Free gels have a uniform and low background level (Figure 2) and yield consistent and reproducible results.

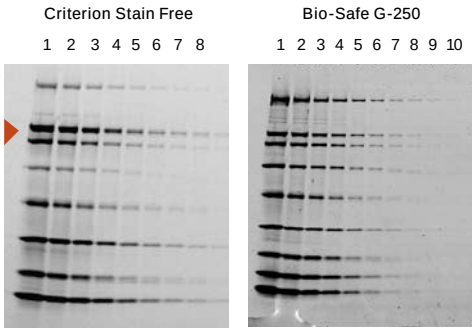

Fig. 2. Comparison of a Criterion Stain Free gel image and a Bio-Safe G-250–stained gel image. Serial 1:2 dilutions of broad range unstained molecular weight standards were separated on a 4–20% Criterion Stain Free Tris-HCl gel. The gel was imaged with the Criterion Stain Free system, then stained with Bio-Safe G-250 stain and imaged on a Molecular Imager GS-800 calibrated densitometer. Arrowhead indicates β-galactosidase.

To assess reproducibility of the Criterion Stain Free system compared with Bio-Safe G-250 staining, we quantitated the band corresponding to β-galactosidase in serial dilutions of the broad range SDS-PAGE standards. Thirteen serial dilutions of the standard, ranging from 500 ng to 0.12 ng per band, were run in triplicate on Criterion Stain Free gels. The gels were imaged using the Criterion Stain Free imager and subsequently stained using Bio-Safe G-250 stain. Figure 2 shows representative images of a Criterion Stain Free gel and a Bio-Safe G-250–stained gel. The quantity of protein present in the band corresponding to β-galactosidase (MW 116.000) was estimated in the four replicates using Image Lab software and Quantity One software after scanning the Bio-Safe G-250–stained gels using a densitometer. Only the first ten dilutions were compared, because they were detected in all of the images. Figure 3 shows a greater variability in the quantitation results when Coomassie staining was used. The average %CV across all concentrations for the samples quantified with the Criterion Stain Free imager was 7.8%, while the average for the gels stained with Bio-Safe G-250 stain was 19.67%. A similar trend was observed for all other proteins (data not shown).

Our results show that Coomassie staining yields less consistent quantitation results than the Criterion Stain Free system. There is some difference in %CV between the Criterion Stain Free system data sets, but it is significantly less than the 20% range seen with the Coomassie stain.

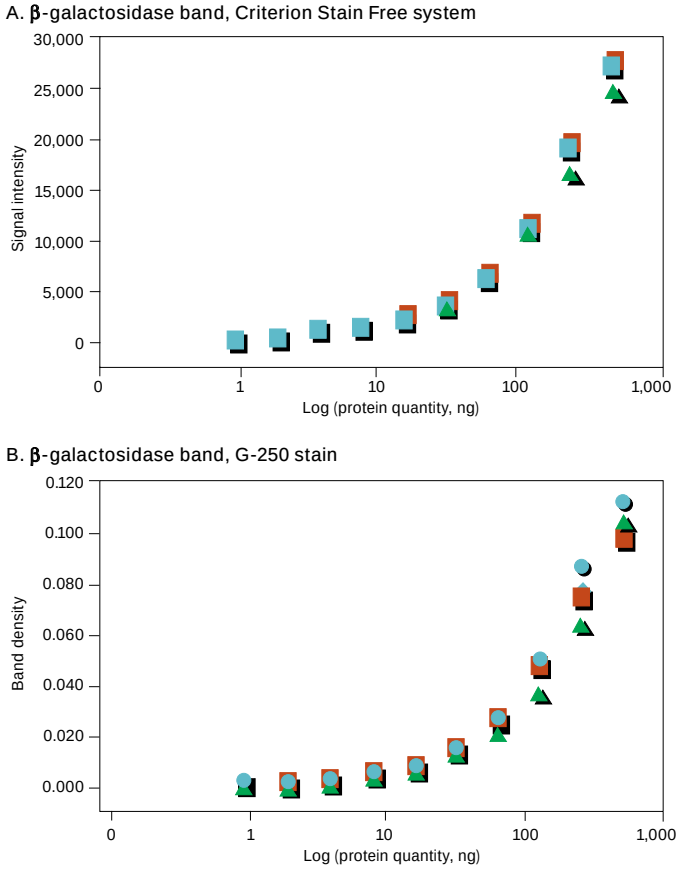

Fig. 3. Reproducibility of protein quantitation in Criterion Stain Free gels (A) and Bio-Safe G-250–stained gels (B). Quantitation of the β-galactosidase bands of the broad range standards was performed for different dilutions of the protein in four replicate gels. Higher reproducibility is observed with the Criterion Stain Free system compared with Coomassie staining. Average %CV is 7.8% for Criterion Stain Free gels and 19.67% for Bio-Safe G-250 staining. The band detection sensitivity of the Image Lab software was set to high.

Conclusions

The Criterion Stain Free system is a fast and easy-to-use protein visualization system that utilizes the standard conventions of SDS-PAGE. Consisting of a new formulation of Bio-Rad's Criterion™ precast gels, a Criterion Stain Free imager, and Image Lab software, the system triggers protein fluorescence and generates a digital image of the gel after electrophoresis in as little as 2.5 min. Maximum sensitivity is reached in 5 min, with equal or better sensitivity than that of Coomassie staining.

The Criterion Stain Free gel imaging system is a tool for reproducible, fast, and environmentally friendly SDS-PAGE analysis that enables efficient protein purification workflows. Because of its ease of use and its protein quantification and data analysis capabilities, this system is the first serious alternative to the decades-old Coomassie Blue gel staining technique.

References

Elbaggari A et al. (2008). Evaluation of the Criterion Stain Free™ gel imaging system for use in western blotting applications. Bio-Rad bulletin 5781.

Kazmin D et al. (2002). Visualization of proteins in acrylamide gels using ultraviolet illumination. Anal Biochem 301, 91–96.

Ladner et al. (2004). Visible fluorescent detection of proteins in polyacrylamide gels without staining. Anal Biochem 326, 13–20.

Rapid Assay Development and Optimization for Small Molecule Drug Discovery

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Introduction

The drug discovery and development process requires assays amenable to high throughput, where large libraries of small molecules are screened to identify those that interact with their protein target with high affinity. Being able to perform this type of analysis with an assay that has a short development time is key. At later stages in the drug development process, the affinity of the small molecule lead compound is evaluated against human serum albumin and other relevant serum proteins to fully understand its absorption, distribution, metabolism, and excretion (ADME). The affinity and specificity of the lead compound must also be tested in several animal models in order to choose the most appropriate one for initial toxicity studies. High sensitivity and reproducibility for these binding assays, while maintaining throughput, is an absolute requirement in this process.

Surface plasmon resonance (SPR) can give detailed information on the binding affinity and kinetics of an interaction, without the need for a molecular tag or label. Labeling adds extra time and cost to assay development, and can in some cases interfere with the molecular interaction by occluding a binding site (Cooper 2002). SPR technology can be used to design information-rich assays that provide a quantitative ranking of interaction affinities and the active concentration of protein ligand, which can be extremely valuable in the early stages of drug discovery (Huber and Mueller 2006). However, to be useful to the drug discovery process, interaction studies using SPR must be fast and cost effective to develop and be amenable to medium to high throughput.

The ProteOn™ XPR36 protein interaction array system and the One-shot Kinetics™ approach (Bravman et al. 2006) can provide the rapid assay development and high throughput required in a drug discovery environment. This multiplexed SPR device integrates a 6 x 6 interaction array for the analysis of up to six ligands with panels of up to six analytes, producing 36 data points in a single injection. Multiplexing enables several quantitative binding experiments, using multiple conditions, to be performed in parallel, so that robust interaction assays can be developed and optimized very quickly. This one-shot approach reduces assay costs and time and will generate a complete kinetic profile for a biomolecular interaction in a single experiment using a single sensor chip, without the need for regeneration.

In this report, we demonstrate the application of the ProteOn XPR36 interaction array system and the One-shot Kinetics approach to the rapid development of a small molecule screening and characterization assay. The model system described in this study is composed of a putative lead compound (Y) and a protein domain of its target protein X (PDX). The inhibition of PDX is assumed to be efficacious in the treatment of cancer.

Methods

Instrument and Reagents

Experiments were performed using the ProteOn XPR36 system with ProteOn GLM sensor chips. ProteOn PBST running buffer (phosphate buffered saline, pH 7.4 with 0.005% Tween 20) containing 2% DMSO was used as running buffer throughout, and all experiments were performed at 25°C. The PDX (N-terminal histidine His6-tagged protein construct of around 240 amino acids; MW 30.9 kD), the small molecule inhibitor, and the putative lead compound Y (439 Da) were obtained from Merck KGaA.

PDX Immobilization Conditions

The PDX was preincubated either in the presence or absence of 50 µM inhibitor and then immobilized in all six vertical channels. Immobilization was performed at three different pH values: 4.0, 4.5, and 5.0 in 10 mM acetate buffer.

Kinetic Binding Analysis

After deactivation, five different concentrations of compound Y were injected in the horizontal direction. Running buffer was injected in the sixth channel as a reference. Dissociation was monitored for 10 min. Regeneration of the GLM sensor chip between the two injections was not required as Y had a relatively fast dissociation time from PDX.

Data Analysis

The data were analyzed using ProteOn Manager™ 2.0 software. Binding curves were processed for baseline and start injection alignment and interspot reference subtraction was used. Excluded volume correction was also performed because DMSO was present in the running buffer. Each set of six reference-subtracted sensorgrams was fitted globally to curves describing a homogeneous 1:1 biomolecular reaction model. Data from the six ligand surfaces were grouped together to fit the k_a, k_d, and R_{max} parameters. The equilibrium dissociation constant, K_D, was calculated using the equation $K_D = k_d / k_a$.

Results and Discussion

Optimization of PDX Binding to the Sensor Chip

Immobilization of PDX to the sensor chip surface was optimized by altering the binding buffer conditions. Three sodium acetate buffers with different pH values were tested. To prevent ligand inactivation as a result of crosslinking to the sensor chip at the binding site, the protein was preincubated with 50 µM inhibitor at each pH value tested. This way, six different immobilization conditions (three different pH values; with or without preincubation with the inhibitor) were tested in parallel on one sensor chip.

Figure 1 shows the immobilization levels for the six different conditions. The final immobilization levels were significantly higher for all three buffer types when the protein was preincubated with the protecting inhibitor molecule. However, the final immobilization level of PDX may be lower as the presence of the inhibitor seems to increase protein stability in the acidic environment. The highest immobilization level was obtained using the pH 4.5 buffer (~9,300 RU) although the two other buffers, pH 4.0 and 5.0, also yielded relatively high immobilization levels. All six binding curves in each vertical channel are superimposed, illustrating identical binding of the ligand across all six spots within the channels (Figure 1).

The activity of the bound PDX ligand was then determined for each immobilization condition. High activity levels are indicative of a more sensitive assay for the analyte, compound Y. The interaction of PDX with 10 different concentrations of compound Y was determined using the One-shot Kinetics approach. Two analyte injections were performed, each containing five channels with different concentrations of the analyte and one channel containing only running buffer (no analyte), which is used for double referencing. The results shown in Figure 2 clearly demonstrate that PDX ligand immobilized without preincubation with the inhibitor is essentially inactive, regardless of the pH used during immobilization. However, when preincubation with the inhibitor was used, the protein maintained its activity regardless of the pH used during immobilization, suggesting that the binding site on the target protein was protected by the small molecule inhibitor.

Evaluation of the Kinetic Data

The kinetic binding constants data are summarized in Table 1. The experimental data and calculated binding model are almost superimposable, as shown in Figure 2, and all binding curves for the three active ligand surfaces could be fit to a homogeneous 1:1 binding model. The k_a and k_d could be fit grouped, whereby each ligand surface has a separate k_a and k_d, or globally.

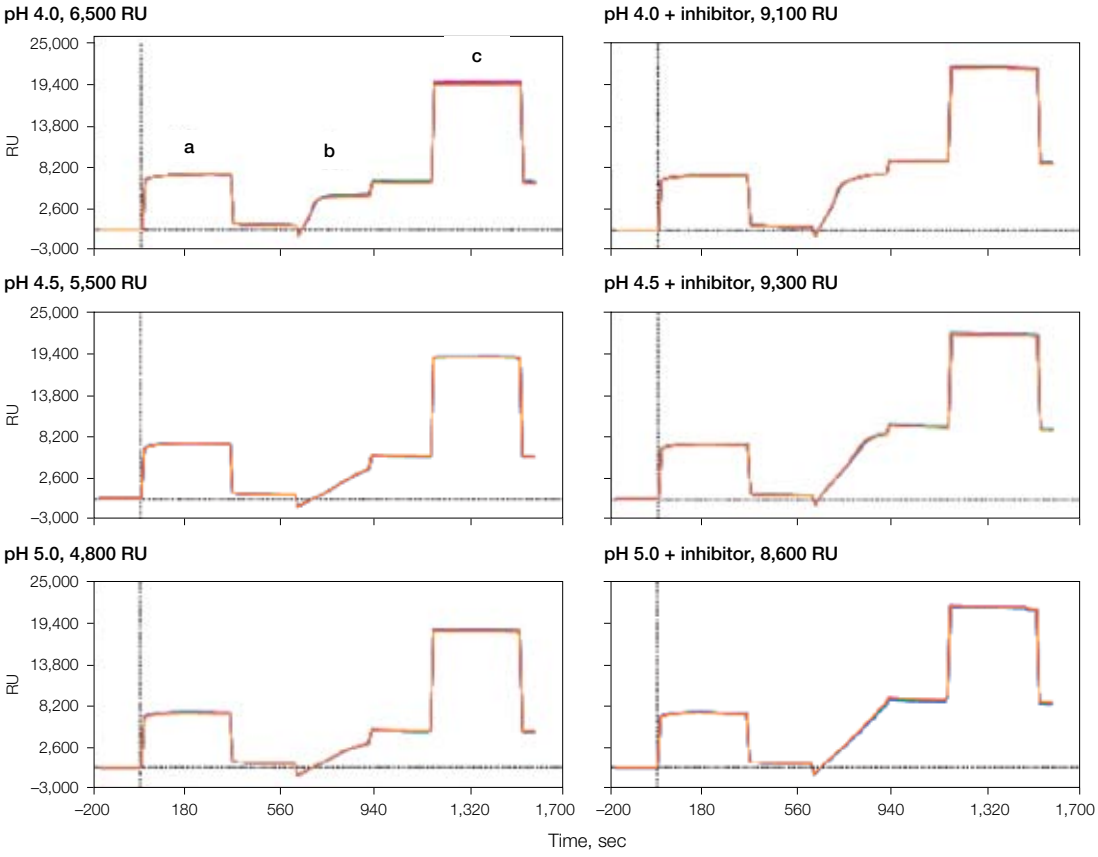

Fig. 1. Immobilization of the target protein PDX on the ProteOn GLM sensor chip. Each panel shows the sensorgrams representing the six vertical channels on the sensor chip at a given pH and with or without the PDX inhibitor present. A1 through A6 represent the six spots in each vertical channel at which the target protein ligand binds. Sections a, b, and c represent successive injections of the EDAC/sulfo-NHS activator, the target protein, and the ethanolamine inactivator, respectively. The height of the sensorgram at the end of section c indicates the final amount of target protein PDX bound to the sensor chip. A1 (—), A2 (—), A3 (—), A4 (—), A5 (—), A6 (—).

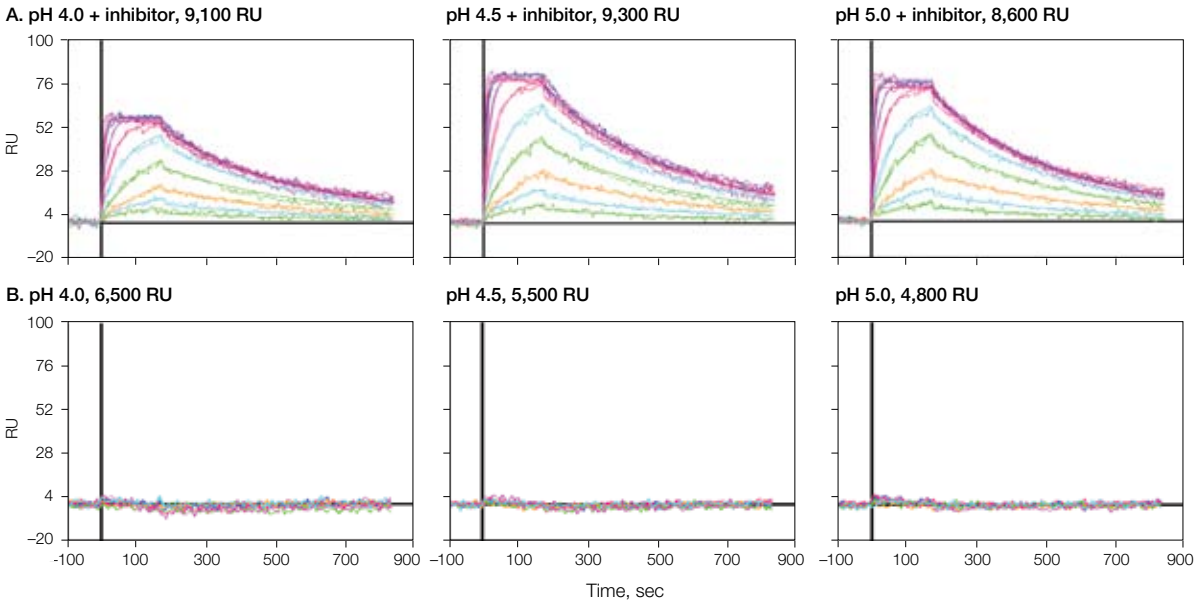

Fig. 2. Activity of the target protein PDX for the putative lead compound Y. PDX was immobilized in all six vertical channels, under six different binding conditions, using three acetate buffers of different pH (4.0, 4.5, and 5.0), and in the presence (**A**) or absence (**B**) of 50 µM inhibitor. A flow rate of 25 µl /min was used at 25°C for 5 min. Multiple concentrations of analyte were injected in the horizontal direction at 100 µl/min for 3 min. Dissociation was monitored for 10 min. In the upper three sensorgrams (**A**) the calculated binding model is visible overlaid on the experimental data for each analyte concentration.

Table 1. Kinetic data* for the interaction of the target protein PDX with the putative lead compound Y.

	Global Fitting					Individual Fitting				
	k_a 1/msec	k_d 1/sec	K_D	R_{max} RU	Chi^2 RU	k_a 1/msec	k_d 1/sec	K_D	R_{max} RU	Chi^2 RU
pH	Global	Global	Auto Defined	Grouped		Global	Global	Auto Defined	Grouped	
4.0	2.42E+04	2.69E–03	1.12E–07	54.1	4.46	2.61E+04	2.82E–03	1.08E–07	53.35	3.76
4.5				75.37	4.46	2.31E+04	2.69E–03	1.17E–07	75.88	5.42
5.0				72.4	4.46	2.65E+04	2.89E–03	1.09E–07	73.3	3.73

* Data were fitted to a 1:1 binding model using ProteOn Manger 2.0 software. For both methods, the R_{max} value was fitted as a grouped parameter, as it is specifically dependent on the level of immobilization.

The kinetic values obtained from either method are almost identical, and when comparing data for the three different surfaces determined independently, there is also a close correlation.

The low chi^2 values obtained for each surface individually, shown in Table 1, are also an indicator of the confidence of the binding model generated in response to the experimental data collected (1 RU is the lowest theoretical chi^2 attainable, since it is at the noise level of the system). The chi^2 values were also very low when the data were fitted globally, giving increased confidence in the quality of the data, as it is an indicator that the data are robust and of the highest quality. They also indicate an absence of systematic errors from the instrumentation, reagents, or experimental design.

Conclusions

The ProteOn XPR36 protein interaction array system and the One-shot Kinetics approach are powerful tools for rapid, high throughput assay development in the drug discovery environment. Assay development times are reduced from days to hours because up to 36 biomolecular interactions can be assayed simultaneously, yielding valuable kinetic, equilibrium, and concentration data. The ProteOn XPR36 system has been used to rapidly screen and accurately characterize the affinity of small molecule drugs for human serum albumin (Bronner et al. 2008). It is also an effective tool for the rapid screening of monoclonal antibody supernatants to identify high affinity candidates for potential drug development (Yousef et al. 2007). Multiplexed SPR analysis is rapidly becoming an indispensable asset across the drug discovery workflow.

References

Bravman T et al. (2006). Exploring “One-shot” Kinetics and small molecule analysis using the ProteOn XPR36 array biosensor. Anal Biochem 358, 281-288.

Bronner V et al. (2008). Evaluating candidate lead compounds by rapid analysis of drug interactions with human serum albumin. Am Biotechnol Lab 26, 14-16.

Cooper MA (2002). Optical biosensors in drug discovery. Nat Rev Drug Discov 1, 515-528.

Huber W and Mueller F (2006). Biomolecular interaction analysis in drug discovery using surface plasmon resonance technology. Curr Pharm Des 12, 3999-4021.

Yousef M (2007). Advances in rapid monoclonal antibody screening. Am Biotechnol Lab 25, 26-28.

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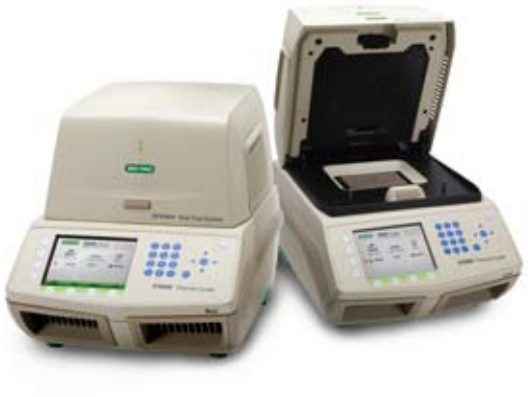
For 10 years, Bio-Rad has been innovating real-time PCR by listening to the experts: you.

Our CFX96™ and CFX384™ real-time PCR detection systems give you the best value in real-time PCR because you've helped us rethink what you should expect. Match your throughput to your needs with the performance and reliability in PCR that Bio-Rad has offered for more than two decades.



Bio-Rad's real-time PCR systems deliver what you asked for:

- Convenient, compact, and customizable systems support low sample volumes and multiplexing
- Advanced optical design and unparalleled thermal uniformity provide maximal assay reproducibility and worry-free operation
- Easy-to-use software packages offer more analysis tools with convenience and flexibility

Together, we've rethought real-time PCR. Find the CFX system that fits your needs by visiting **www.bio-rad.com/amplification/** or contacting your Bio-Rad sales representative.

Research. Together.