

BioFiles

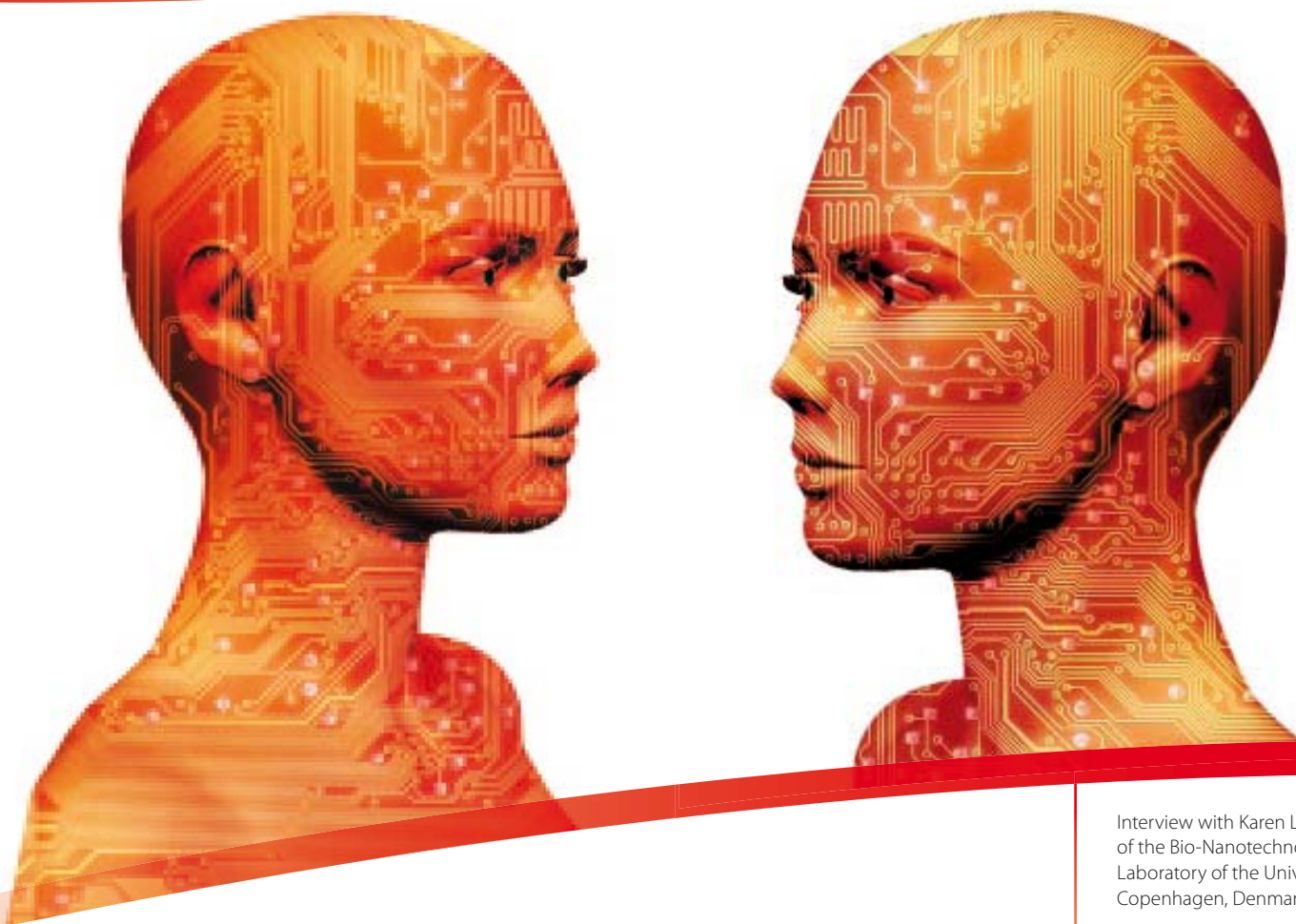
INVITRO

Issue 6 • 2009



Nano Technology for Molecular Diagnostics

An interview with Karen L. Martinez, Associate Professor in the Bio-Nanotechnology Laboratory, Department of Neuroscience and Pharmacology & Nano-Science Center, University of Copenhagen, Denmark



Interview with Karen L. Martinez
of the Bio-Nanotechnology
Laboratory of the University of
Copenhagen, Denmark

Screening and Profiling Protein
Expression in Human Cancer Serum
using Antibody Array Technologies

Staining Protocol Series:
ACCUSTAIN® Giemsa Stain and
Alkaline Phosphatase Stain

Cronobacter spp. Classic and
New Detection Methods

Reagents for Immunohistochemistry

Phosphate buffered saline, powder, pH 7.4

(Mat. No. P3813-10PAK)

Phosphate buffered saline, tablet

(Mat. No. P4417-50TAB; P4417-100TAB)

Phosphate buffered saline

(Mat. No. P3688-10PAK)

Albumin from bovine serum

(Mat. No. A9647-100G; A9647-500G)

Trypsin from porcine pancreas

(Mat. No. T7409-10G; T7409-100G)

Trypsin from porcine pancreas

(Mat. No. T7168-20TAB; T7168-50TAB)

Proteinase from *Bacillus licheniformis*

(Mat. No. P5380-100MG; P5380-250MG)

ExtrAvidin®-Peroxidase

(Mat. No. E2886-0.2ML; E2886-1ML)

ExtrAvidin®-Alkaline Phosphatase

(Mat. No. E2636-0.5ML; E2636-5 x 0.5ML)

SIGMAFAST™ 3,3'-Diaminobenzidine tablets

(Mat. No. D4418-5SET; D4418-50SET)

SIGMAFAST™ BCIP®/NBT, tablet

(Mat. No. B5655-5TAB; B5655-25TAB)

**50%
Discount**

- Consistent, reliable quality
- Application tested for formalin-fixed and paraffin-embedded tissue sections
- Best-in-class, together with IHC-validated Prestige Antibodies®

To order, please contact your local sales office and quote Promo Code: **V03**

Web ordering: sigma-aldrich.com/euvoucher

Promotion is valid until January 31st 2010.

Dear Researcher,



Sigma-Aldrich Chemie GmbH
Becki Davis

We are proud to release the latest In Vitro issue featuring an accomplished young scientist, Karen L. Martinez, an Associate Professor in the Nano-Science Center of the University of Copenhagen.

Karen L. Martinez is currently researching the development of new *in vitro* and *in vivo* biosensors suitable for diagnostics and drug screening. As a co-founder of two start-up companies, she bridges basic research with applied. Her newest endeavour is to bring a new generation of nano-sensors to the marketplace with increased sensitivity and specificity through collaborations with several biotechnology and pharmaceutical companies.

In this issue of Biofiles In Vitro our key focus is on the determination, screening and profiling of proteins in human plasma and serum. Through its superior Seppro™ depletion technology, Sigma offers a fast, robust system for the depletion of high-abundant and moderately abundant proteins in serum and plasma. In this article, Jiaxin Dong et al. developed two multiplex immunoassays and present a preliminary application in determining protein depletion for Seppro IgY14/SuperMix columns.

Our second article describes the screening and profiling of protein expression in human cancer serum, using antibody array technologies. This article details how our Panorama® Antibody Arrays Technology is a powerful tool for rapid protein expression profiling for biomarker discovery and validation in diseased serum samples.

Also featured in this issue, we continue our collection series of classical staining protocols for your daily laboratory work. This section includes a short introduction to the protocol with a condensed procedure including example images of the staining.

At the end of this issue, you will find an article about classical and new detection methods of *Cronobacter*, a bacteria known to cause neonatal infections.

Finally, the Biofiles In Vitro team would like to thank you for your input, to make this publication applicable to life science researchers. Please continue sending feedback and scientific content.

Kind regards,

Becki Davis
Product Manager Research Biotech

Table of contents

Interview with Karen L. Martinez	4
Cancer Serum Using Antibody Array Technologies	8
ACCUSTAIN® Giemsa Stain	11
Alkaline Phosphatase Stain	12
Development of Two Multiplex Immunoassays and Preliminary Application in Determining Protein Depletion	13
<i>Cronobacter spp.</i> Classic and New Detection Methods	17

Interview with Karen L. Martinez

Associate Professor in the Nano-Science Center of the University of Copenhagen
 martinez@nano.ku.dk; <http://www.nano.ku.dk/bionano>

CURRICULUM VITAE



Education at University Denis Diderot – Paris 7 – France

- 2000** Ph.D. in Molecular Biophysics – acknowledged with the highest mention
1996 Masters in Molecular Biophysics

Research Positions:

- since 2008** Associate Pr. Bio-Nanotechnology Laboratory, University of Copenhagen, DK
2005–2008 Assistant Pr. Bio-Nanotechnology Laboratory, University of Copenhagen, DK
2001–2005 Senior Scientist LCPPM – Pr. H. Vogel –, ETH Lausanne, CH
1996–2000 Ph.D. Student Lab. of Molecular Neurobiology – Pr. J.-P. Changeux – Pasteur Institute & LURE, FR

Present Research: Development of Biosensors for Nanomedicine

The understanding of the molecular basis of diseases revealed by Genomics and Proteomics, open perspectives in medicine, towards personalised medicine. Membrane proteins have a major potential since they are highly selective key players in cell signalling and major drug targets. We combine various surface-sensitive techniques and novel materials (polymer, semiconductor nanowires, etc.) with the high selectivity of membrane proteins (G protein coupled receptors and ligand gated ion channels) for the development of new in vitro biosensors suitable for diagnostics and drug screening. The development of biosensors field effect transistors (bioFET); the surface immobilisation of membrane proteins for reconstituted signalling platforms; and the interface of semiconductor nanowires in living cells, are our three major research axes.

Current Staff: 14 people

Funding: ~ 4 Mio. € of external funding (personal share)

Innovation and Technology – Valorisation of research activities:

Co-founder of InXell bionics ApS and Hafnia Nano Holding ApS – Biotech startup – 2009
 Member of the board of directors of InXell bionics ApS

Karen L. Martinez has made an impressive career in research. At the age of 35, she is associate professor at the Bionanotechnology Laboratory of the University of Copenhagen. Her present research deals with the development of new *in vitro* biosensors suitable for diagnostics and drug screening. The French researcher accomplished her Ph.D. in molecular biophysics in the laboratory of Pr. Jean-Pierre Changeux at the Pasteur Institute in Paris. After a 5-year post-doc in the laboratory of Pr. Horst Vogel at the ETH Lausanne in Switzerland, she was offered the chance to set up a research group interfacing biological samples with nanomaterials in Copenhagen. As a co-founder of a start-up company, she bridges basic and applied research. Karen L. Martinez is about to contribute to the field with a new generation of more sensitive and specific nanosensors and collaborates with several biotech and pharmaceutical companies.

IN VITRO: Could you please explain your field of research?

KM: We are developing biosensing platforms, interface proteins and cells one can find in human bodies, with materials adapted for highly sensitive detection. For example, electronic components present today in computers, mobile phones, etc.

The idea behind it is to exploit, in addition to the sensitivity of novel technologies, the intrinsic high sensitivity and selectivity of proteins, since they are the natural sensors in our body and trigger cell function, cell-cell communication, etc.

IN VITRO: For what applications can those biosensing platforms be used?

KM: Mainly for discovery of novel molecules to become future medicines. In this case, tools and assays to be used by the pharmaceutical industry at the early stage of drug discovery are developed. The effect of libraries of molecules on a particular protein involved in a target pathology can be tested.

Another field of application is diagnostics, e.g. monitoring the concentration of molecules in physiological liquids like blood, for example. They can then be used for clinical research, for hospital use during brain surgery in operational rooms, for example, for point of care measurements or for a regular check.



IN VITRO: Why do you find biosensors for Nanomedicine so fascinating?

KM: The new types of biosensors we are developing are meant to be more sensitive, more specific, more informative and less invasive. Why is it important? To provide treatments that are more appropriate and have limited side effects. For example, a biosensor for diagnostics with higher sensitivity will allow earlier detection of biomarkers. And early diagnostics will allow more efficient treatments. In the case of drug discovery, a more specific biosensing platform will allow the identification of less invasive drugs and limit side effects.

Contributing to the development of such new tools that can impact society at some point is a great motivation, even if we are sometimes still at an early stage of the development process.

IN VITRO: You are developing glutamate sensors. How can they be applied in drug development and diagnostics?

KM: Glutamate is the most abundant neurotransmitter in the brain. It is involved in numerous important brain functions such as learning, memory and cognition, as well as the development and plasticity of the central nervous system. Alterations in glutamate levels have been shown to be linked to several neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases, as well as ischemic stroke, to mention a few. Accurate measurement of glutamate levels has remained challenging, and different techniques have been developed to this end for clinical diagnostics.

Glutamate sensors exhibiting recognition elements coming from membrane proteins present in our body can be used for the discovery of new molecules interacting with the same receptors and thus with a potential

pharmacological effect. We recently wrote a review in *The Journal of Nanoneuroscience* regarding the existing glutamate biosensors and their performances.

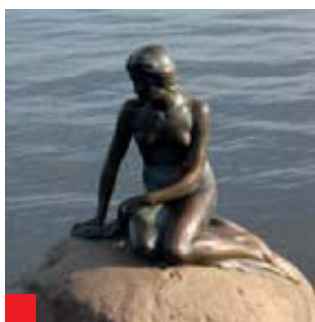
IN VITRO: What new perspectives can cells grown on nanowires open?

KM: This emerging field is very interesting. Carbon nanotubes, nanofibres and semiconductor nanowires can be interfaced in cell cultures with limited invasiveness. They can potentially be used for the delivery in cells of various compounds and also biosensing of cellular events. It is then possible that they impact diagnostics and drug discovery as we recently described in a paper.



IN VITRO: The safety aspect of nano technology is often discussed. What is your opinion about the potential risk involved in using nano structures in vivo and the environment?

KM: It is definitely a major concern at the moment and numerous investigations are currently in progress. The exploitation of nanomaterials in our group is exclusively meant for in vitro assays, with exclusive interface with cell cultures or physiological liquids. However, the toxicity of nanomaterials in vivo and their impact on the environment are some parameters we take into account when we manipulate the samples and analyse our data.



IN VITRO: Why are Denmark and The Nano-Science Center of the University of Copenhagen the right place for you to work?

KM: Four years ago, I was offered the opportunity by the University of Copenhagen to set up a research group interfacing biological samples with nanomaterials. At that time, this opportunity was quite unique in Europe and very attractive for several reasons. Denmark invests considerably in nanoscience and bionanotechnology per se; I therefore had extremely good conditions to start up my activities. This includes funding from the University of Copenhagen, the Danish Research Council and Lundbeckfonden, but also access to talented students educated in Nanoscience and having a strong interdisciplinary background.

IN VITRO: It sounds like an innovative environment.

KM: In my opinion, it is a fantastic incubator. It gathers in a single building research groups from different departments and faculties of the University. For example, the Bionanotechnology Laboratory is part of the Department of Neuroscience and Pharmacology of the Faculty of Health Science of the University. Some of my research projects are extremely interdisciplinary and require constant interaction with physicists. In this respect, the Nano-Science Center provides the perfect facilities to catalyse such synergies. I'm very grateful to the Department of Neuroscience and Pharmacology for giving me the opportunity to start up my research activities in this scientific environment.

IN VITRO: When you started studying, was it already your aim to stay in research and become a professor?

KM: No, ever since the beginning of my career I have had the chance to meet great people who contributed to catalysing my curiosity for novel scientific questions, while supporting me in most of my initiatives. It still remains a pleasure to meet my mentors and discuss with them my current research activities. Today, my continuous source of motivation comes from very enthusiastic members from my group, in addition to support from my colleagues and collaborators.



IN VITRO: In addition to your academic position at the University of Copenhagen, this year you became a co-founder and member of the board of directors of a biotech startup. Why is working in these two very different environments beneficial for you?

KM: Turning some of our fundamental results into products with a potential impact on drug discovery – and thus on society at a later stage – is an exciting, unique opportunity.

These two very different environments are quite complementary, and help provide me with a global picture of the impact of the research activities of my group: how to make our research useful for society, while exploring some visionary aspects.

IN VITRO: Fewer women choose academic or entrepreneurial careers than men. Why do you think this is, and how do you think it could be changed?

KM: I recognise this gender issue but can't really explain it. Indeed, my research group is composed of more than 60% female researchers. I guess it is partly a cultural issue, and that society has to evolve. For example, I often notice that women have to demonstrate that they have the qualifications to do the job, whereas men are assumed to have them. I hope that the situation will change, but don't have any real suggestions besides training women in leadership and management.

IN VITRO: How important is Sigma-Aldrich with its products and services to your work?

KM: We are regular users of Sigma-Aldrich products for life science and material science. We also always keep an eye on new products, especially for surface chemistry and fluorescence labelling.

Certified Stains for Histochemistry



Hematoxylin	<i>(Mat. No. H3136)</i>
Methylene blue	<i>(Mat. No. M9140)</i>
Alizarin Red S	<i>(Mat. No. A5533)</i>
Alcian Blue 8GX	<i>(Mat. No. A3157)</i>
Sudan Black B	<i>(Mat. No. 199664)</i>
Congo Red	<i>(Mat. No. 860956)</i>
Cresyl Violet acetate	<i>(Mat. No. C5042)</i>
Eosin Y disodium salt	<i>(Mat. No. E4382)</i>
Crystal Violet	<i>(Mat. No. C0775)</i>

**33%
Discount**

- 55 dyes with guaranteed performance
Certified by the independent Biological Stain Commission (BSC)
- Commission certification is reliability
Only those passing minimum assay limits, and who work in specified routine staining procedures, are certified
- Commission certification is cost-effective
Commission certified stains are often less expensive than non-certified

To order, please contact your local sales office and quote Promo Code: **Y42**
For a complete list, please go to sigma-aldrich.com/certified_stains

Promotion is valid until January 31st 2010.

Screening and Profiling Protein Expression in Human Cancer Serum using Antibody Array Technologies

By Angela S. Crawford, Beth K. Radwanski, Dian Er Chen

Abstract

There is a growing need for technologies that enable discovery and validation of protein biomarkers in human serum/plasma. Antibody microarrays have been used successfully to rapidly identify and characterise protein expression in a targeted approach. In this study, antibody arrays were used to interrogate proteome differences in whole serum and in serum that had been depleted of twenty high-abundance proteins. The depletion technology enhanced the identification of the lower abundance tissue leakage proteins, as compared to non-depleted serum samples. Antibody arrays were also used to profile differential protein expression between serum from normal and diseased patients. Proteins were identified which displayed significantly different expression levels between the samples. Results were validated with ELISA analysis. This study showed that antibody arrays are a powerful tool for rapid expression profiling of proteins and may potentially be applied to biomarker discovery and validation in diseased serum samples.

Introduction

Antibody arrays have been a promising and inexpensive tool for bulk analysis of protein level changes in human plasma and serum. These analyses have led to proteomic profiling of a number of disease states, as well as biomarker discovery. Here, we show the value of using a series of Panorama® antibody microarrays comparing normal and cancer serum samples to identify potential disease biomarkers. The arrays chosen for this work contained antibodies for proteins with known significance in intracellular processes. These arrays were used because it is believed that biomarkers exist in the low abundance tissue leakage proteins that make up approximately one-third to one-half of the thousands of proteins found in blood. In our study, we have also highlighted the contribution that depletion gives to the discovery/validation of the low abundance tissue leakage proteins on the antibody microarrays.

Figure 1: Microarray Analysis Workflow

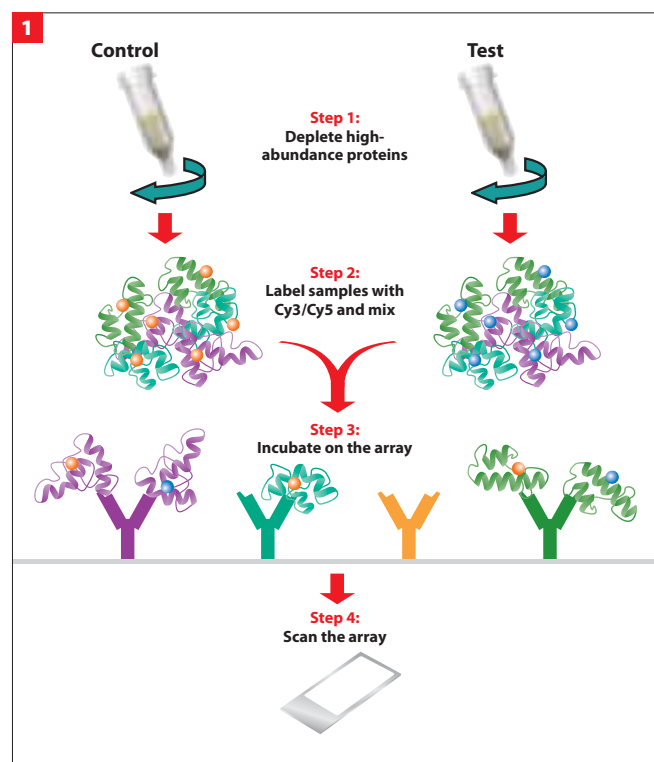
Serum samples were first depleted of twenty of the high-abundance proteins. The depleted samples were then labelled with either Cy3 or Cy5 (Amersham) and mixed at equal protein amounts to allow for parallel analysis. The labelled serum samples were incubated on both the Panorama Antibody Array – p53 Pathways (PPAA4) and the Panorama Antibody Array – Cell Signalling (CSAA1) for 30 minutes. Following incubation, the slides were scanned using a ScanArray Express (Perkin Elmer) and analysed using ImaGene 7.0 software (BioDiscovery).

Materials and Methods

Serum Samples Serum samples were obtained through Genomics Collaborative. Cancer samples were from either hepatocellular carcinoma (36-year-old Vietnamese male) or renal cell carcinoma (66-year-old Caucasian male) patients. Normal samples were from a 51-year-old Caucasian male.

Serum Depletion Depletion was completed using the ProteoPrep® 20 Immunoaffinity Depletion Kit (PROT20) and following the supplied protocol.

ELISA Analysis Serum samples were coated onto 96-well ELISA plates. Plates were incubated with purified primary antibodies corresponding to those on the arrays. Plates were washed and incubated with HRP-conjugated secondary antibodies. After a final wash, the plates were visualised using TMB substrate, and stopped with 1 M HCl. The absorbance was measured at 450 nm.



Results

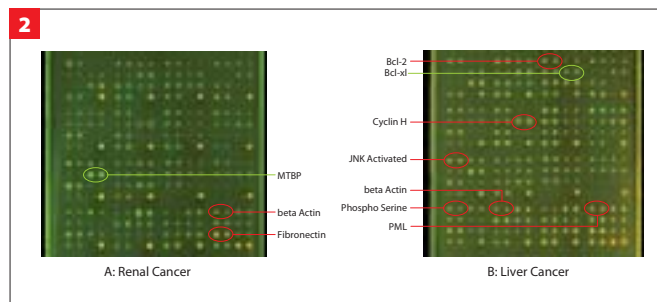


Figure 2: Benefits of Depletion by Comparison of Whole and Depleted

Normal Human Sera Using Panorama p53 (A) and Cell Signalling (B) Arrays. Each Panorama p53 array was incubated with 100 μ g of both depleted and whole (non-depleted) serum conjugated with either Cy3 or Cy5. A dye swap was performed to confirm results. In the comparisons, the whole serum is green and the depleted serum is red. Select proteins are identified. Note that only the top half of the Cell Signalling array slide is shown. As seen in the comparisons above, more proteins are visible in the depleted serum than in the whole serum. The depletion has made the less abundant proteins visible.

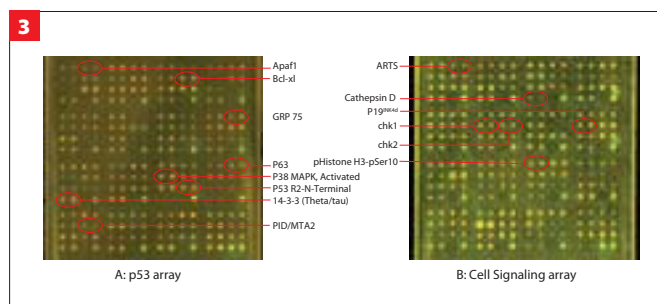


Figure 3: Differential Expression Levels of Various Tissue Leakage Normal Human Sera Using Panorama p53 (A) and Cell Signalling (B) Arrays

Following depletion, each Panorama p53 array was simultaneously incubated with a mixture containing equal amounts of normal and cancer serums (Renal cancer sample for slide A, Liver cancer for slide B) conjugated with either Cy3 or Cy5. A dye swap was performed to validate results. As seen in the slides, a number of spots were differentially expressed with the cancer samples when compared to the normal control. In both comparisons above, the normal serums are labelled red and the cancer serums are labelled green. Therefore, a red spot would indicate down-regulation in the cancer sample, and a green spot would indicate up-regulation in the cancer sample. Select proteins are identified.

Similar results were seen using the Cell Signalling array. Additional proteins found to be significantly different using the Cell Signalling array include, but are not limited to: alpha Catenin (up-regulated in both cancer samples), MAP Kinase (Erk1 + Erk2) (up-regulated in both cancer samples), Calmodulin (up-regulated in the liver cancer sample), Cyclin D1 (down-regulated in the liver cancer sample), DOPA Decarboxylase (down-regulated in both cancer samples), and Synculein (down-regulated in the renal cancer sample). Data not shown.

Learn more about the Panorama Antibody Array Technology at sigma.com/arrays

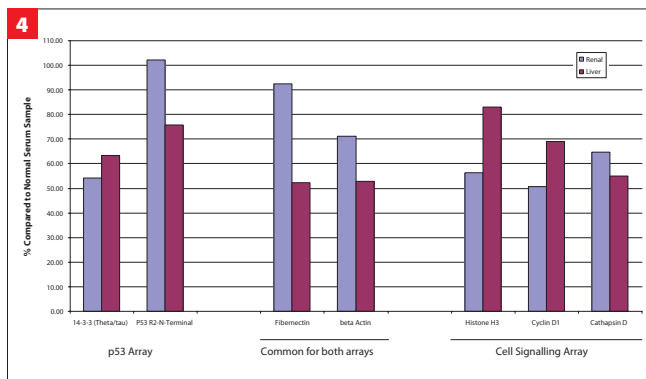


Figure 4: Validation of Results by ELISA Analysis

Changes in the expression of various tissue leakage proteins between the cancer and normal serum samples were validated by ELISA analysis. The proteins analysed were significantly different in the depleted vs. non-depleted and/or in the cancer vs. normal samples. An example of the results is illustrated above.

Conclusions

- Depletion allows for greater visibility of otherwise difficult to detect proteins on the antibody arrays.
- Multiplexed array assays (using two dyes on the same array) allow for a quick, direct, and inexpensive comparison of normal and diseased serum samples.
- As expected with the number of tissue leakage proteins present in blood serum, microarrays containing antibodies important to intracellular pathways are useful in the detection of potential biomarkers.
- This work shows proof-of-principle that the Panorama antibody microarrays can enable disease-state protein profiling.
- Due to the variability between individuals, future work would include the analysis of a large cancer sample population to determine protein profiles and validate biomarkers for a select type of cancer.

References

- 1] Haab, B. B. Antibody Arrays in Cancer Research. *Molecular & Cellular Proteomics*, 2005, 4, 377–383.
- 2] Miller, J. C. et al. Antibody microarray profiling of human prostate cancer sera: Antibody screening and identification of potential biomarkers. *Proteomics*, 2003, 3, 56–63.
- 3] Anderson, N. L.; Anderson, N. G. The Human Plasma Proteome. *Molecular & Cellular Proteomics*, 2002, 1, 845–867.
- 4] Celis, J. et al. Identification of Extracellular and Intracellular Signaling Components of the Mammary Adipose Tissue and Its Interstitial Fluid in High Risk Breast Cancer Patients, *Molecular & Cellular Proteomics*, 2005, 4, 492–522.

Prestige Antibodies®



Powered by  **ATLAS**
ANTIBODIES



Turn your research into a masterpiece with Prestige Antibodies®

The most highly characterised antibodies in the industry – powered by Atlas Antibodies.

- Over 6,100 antibodies, covering 5,300 human protein targets
- Validated by the Human Protein Atlas (HPA) Programme
- Standardised in universal protocols
- Over 700 IHC, IF and WB images per antibody
- All data conveniently searchable online

Visit sigma.com/prestige for more information.



Prestige Antibodies is a registered trademark of Sigma-Aldrich Biotechnology L.P. and Sigma-Aldrich Co.

Antibodies Catalogue



Request your copy at
sigma.com/reserve



Our Innovation, Your Research – Shaping the Future of Life Science

ACCUSTAIN® Giemsa Stain

Introduction

Giemsa stain is a Romanowsky-type stain that was first used in hematology and protozoology for staining *Plasmodium malariae*. The original formulation contained a blend of Eosin Y, Methylene Blue and its oxidation products, the methylene azures. Giemsa stain is considered a polychromatic stain in that the dyes produce multiple colours when applied to cells and cellular components. Sigma-Aldrich Giemsa stain is a buffered thiazine-eosinate solution designed to provide cytoplasmic (pink) staining with nuclear (blue) staining similar to the original product described by Giemsa. It may be used separately or in combination with May-Grünwald stain, also available from Sigma-Aldrich.

Reagents

Giemsa Stain Catalogue No. GS, modified, 0.4% w/v, in a buffered methanol solution, pH 6.9, with stabilizers.

Optional Reagents

May-Grünwald Stain Catalogue No. MG, 0.25% w/v, in methanol.

Reagent Preparation

Giemsa stain comes ready to use for the quick stain procedure. Follow stain procedure instructions for standard Giemsa and May-Grünwald stain preparation.

ACCUSTAIN® Giemsa Stain

Procedure

STANDARD GIEMSA

1. Fix slides in methanol 5–7 minutes.
2. Dilute Giemsa Stain 1:20 with deionised water. Colour can be varied by diluting in buffer.
3. Stain film for 15–60 minutes.
4. Rinse in deionised water.
5. Air dry and evaluate.

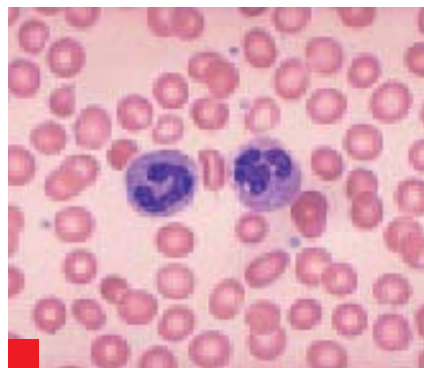
QUICK STAIN GIEMSA

1. Place dried blood film in undiluted Giemsa Stain for 1–2 minutes.
2. Place in deionised water for 2–4 minutes depending upon colour preference.
3. Rinse in deionised water.
4. Air dry and evaluate.

GIEMSA MAY-GRÜN WALD

1. Dilute Giemsa Stain 1:20 with deionised water. For bluer coloration, water buffered at pH 7.2 may be used in place of deionised water.
2. Place slides in May-Grünwald Stain for 5 minutes.
3. Place slides in phosphate or tris buffer (20–70 mmol/L), pH 7.2, for 1–5 minutes.
4. Place slides in dilute Giemsa solution from step 1 for 15–20 minutes.
5. Rinse slides briefly in deionised water.
6. Air dry and evaluate.

Results



Chromatin of white blood cells	Purple
Nuclei of parasitic protozoa	Red
Lymphocyte cytoplasm	Blue
Neutrophil cytoplasm	Light pink
Eosinophilic granules	Pink
Neutrophilic granules	Lilac-purple
Red blood cells	Pink to orange



Alkaline Phosphatase Stain

Introduction

Alkaline phosphatases are widely distributed enzymes found in kidney, jejunum, blood and bone marrow, bladder, adrenal glands, breast, ovary and liver cells. Intensely positive staining has been reported in certain bone cancers. The optimal pH for their activity lies between 9.0 and 9.6. In hematopoietic tissue, alkaline phosphatase appears restricted to band and segmented neutrophils. Its demonstration by simultaneous capture using substituted naphthols and diazonium salts is, perhaps, the earliest example of a cytochemical enzyme test with clinical significance. Most procedures, including those provided by Sigma-Aldrich, employ stable diazonium salts. These are formed by reacting an arylamine with sodium nitrite in an acid medium. The resulting diazonium chloride (usually unstable) can then be treated with compounds such as zinc chloride, zinc sulphate or naphthalene-1,6-disulphonate, forming stable salts. These stabilizers may exert marked inhibition on some enzymatic systems whereas the diazonium chlorides are less inhibitory. For this reason, Sigma-Aldrich now provides stable solutions of fast red violet LB base, fast blue BB base, and sodium nitrite for alkaline phosphatase cytochemistry. To further simplify these methods, a stable solution of naphthol AS-BI phosphate is included. To perform the test, fixed blood films are incubated at room temperature (18–26 °C) in a solution containing naphthol AS-BI phosphate and freshly prepared fast red violet LB salt or fast blue BB salt buffered at pH 9.5 with 2-amino-2-methyl-1,3-propanediol (AMPD). Sites of activity are either red or blue depending upon choice of diazonium salt. The procedure incorporating fast red violet LB is similar to a proposed NCCLS reference method.

Reagents

Naphthol AS-BI Alkaline Solution Catalogue No. 86-1, Naphthol AS-BI phosphate, 4 mg/mL in AMPD buffer, 2 mol/L, pH 9.5

FRV-Alkaline Solution Catalogue No. 86-2 Fast red violet LB base, 5 mg/mL, in hydrochloric acid, 0.4 mol/L with stabilizer.

FBB-Alkaline Solution Catalogue No. 86-3, Fast blue BB base, 5 mg/mL in hydrochloric acid, 0.4 mol/L with stabilizer

Sodium Nitrite Solution Catalogue No. 91-4, Sodium nitrite, 0.1 mol/L.

Citrate Solution Catalogue No. 91-5, Citric acid, 18 mmol/L, sodium citrate, 9 mmol/L, sodium chloride, 12 mmol/L with surfactant pH 3.6.

Hematoxylin Solution Gill. No. 3 Catalogue No. GHS-3, Hematoxylin, certified, 6.0 g/L, sodium iodate, 0.6 g/L and aluminum sulphate, 52.8 g/L with stabilizers.

Neutral Red Solution Catalogue No. N6264, Neutral red, certified, 0.5% w/v, in acetate buffer, pH 5.2

Reagent Preparation

Reagents are provided ready for use. Fixative: Take 25 mL Citrate solution and add 65 mL Acetone, and 8 mL formaldehyde.

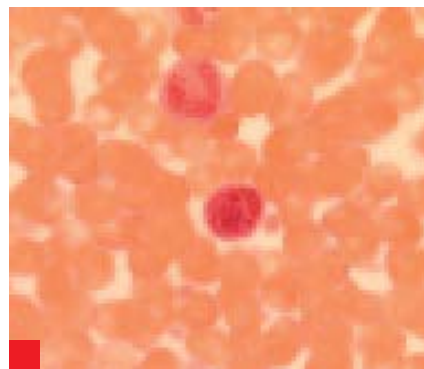
Alkaline Phosphatase Stain

Procedure

1. Add 1 mL Sodium Nitrite solution to 1 mL FRV-Alkaline solution OR add 1 mL Sodium Nitrite solution to 1 mL FBBAlkaline solution. Mix by gentle inversion and allow to stand for 2 minutes.
2. Add solution from step 1 to 45 mL of room temperature deionised water.
3. Add 1 mL Naphthol AS-BI Alkaline solution to diazonium salt solution (step 2). Mix thoroughly and pour into a Coplin jar.
4. Fix slides Citrate/Acetone fixative at room temperature for 30 seconds.
5. Rinse gently in deionised water.
6. Place slides in Naphthol/diazonium salt solution and incubate for 15 minutes protected from direct light.
7. Rinse slides for 2 minutes in deionised water.
8. Counterstain for 2 minutes.
If using FRV-Alkaline solution counterstain with Hematoxylin solution.
If using FBB-Alkaline solution counterstain with Neutral Red solution.
9. Rinse slides thoroughly in tap water and air dry.

Results

Sites of alkaline phosphatase activity will appear as blue or red granular staining.



SIGMA
Life Science

Development of Two Multiplex Immunoassays and Preliminary Application in Determining Protein Depletion for Seppro IgY14/SuperMix Depletion Columns

Jiaxin Dong, Chris Melm, Holly Chapman, Farrah Fan, Dian Er Chen and Henry Duewel
Protein Technology and Assay, Research Biotechnology, Sigma-Aldrich Corporation

Abstract

Depletion of Highly Abundant Protein (HAP) and Moderately Abundant Protein (MAP) from plasma or serum samples can be achieved through Sigma Seppro Tandem IgY14/ SuperMix columns. A range of 77–129 MAPs has been identified from the bound fraction of Seppro SuperMix resin by LC/MS-MS method. However, the efficiency and lot-to-lot consistency of MAP depletion using SuperMix resin has not been determined and requires a robust method to monitor. By taking advantage of the Luminex technology, we have developed two multiplex immunoassays to monitor protein targets of depletion or retention. The MAP10PLEX assay can simultaneously quantify 10 MAPs (Ceruloplasmin, C4, Plasminogen, IgD, C1q, Antithrombin III, Hemopexin, α -Antichymotrypsin, CRP and Prealbumin) from plasma or serum samples depleted utilizing IgY14/Supermix columns. The LAP4PLEX assay, which is used to track loss of low-abundant proteins (LAP) during depletion, is able to simultaneously quantify Adiponectin, soluble L-selectin, soluble ICAM-1 and TIMP-1. The preliminary assay data on Seppro IgY-SuperMix depleted samples has suggested the two assays to be accurate, fast and cost-saving methods to monitor depletion consistency from lot-to-lot of the SuperMix resin in the R & D, Operations and QC departments.

Introduction

An avian IgY based immunodepletion technology is used for Sigma Sepro depletion products. The Human IgY14 Columns can remove 14 highly-abundant proteins (HAP) and the unique Human SuperMix System is to remove moderately-abundant proteins (MAP). The combination of IgY14/Supermix columns can deplete approximately 96–99% of total protein mass from human serum or plasma (**Figure 3**). While the depletion of 14 HAPs has been well established, the further removal of MAPs is less well characterized. Previously, a range of 77–129 MAPs has been identified from the bound fraction of Seppro SuperMix resin by LC/MS-MS. However, the degree of specific depletion of MAPs as well as the non-specific impact on low-abundant proteins (LAP) is still unclear. This also makes tracking the lot-to-lot depletion consistency of SuperMix products a challenge. Regular ELISA methods are time-consuming and commercial ELISA kits are costly. Therefore, finding a fast and economical alternative analysis has appeared to be important.

Luminex xMAP technology is a widely used multiplex platform to quantify multiple analytes from biological fluid samples. It combines the accuracy of ELISA and multiplicity of antibody arrays through protein-antibody interaction on the surface of microspheres and is characterized by speed and minimal consumption of samples (**Figure 2**). In order to evaluate Seppro SuperMix resins in the R & D department and to monitor the lot-to-lot consistency of materials produced by Operations in the QC department, we developed two Luminex immunoassays to simultaneously quantify 10 representative MAPs (Ceruloplasmin, C4, Plasminogen, IgD, C1q, Antithrombin III, Hemopexin, α -Antichymotrypsin, CRP and Prealbumin) and 4 representative LAPs (Adiponectin, soluble L-selectin, soluble ICAM-1 and TIMP-1) from depleted plasma via Seppro SuperMix columns.

Materials and Methods

1. Instrument: Luminex® 100 (**Figure 2**, Luminex Corp., Austin, Texas, USA)
2. Vacuum pump, 96-well microtiter filter plates and filtration manifold (Millipore Corp)
3. Capture antibodies were immobilized on Luminex® beads (Luminex Corp., Austin, Texas, USA), detection antibodies were biotinylated before use, streptavidin-phycoerythrin conjugate (Sigma E4011)
4. Data were calculated using STATLIA® software with a 5-parameter logistic curve-fitting method (Brendan Scientific, CA)
5. Depletion procedure: 50 or 100 μ L human plasma (Sigma P9523) was loaded to Seppro IgY14-LC5/SuperMix-LC2 columns with tandem connection (**Figure 3**). Depletion was run in Waters 2695 HPLC System (Alliance). After collection of flowthrough from combined columns (SuperMix depleted sample), bound proteins were eluted from IgY14-LC5 and SuperMix LC2 column separately.
6. Multiplex assay protocol: 50 μ L standards or samples are mixed with 25 μ L antibody conjugated bead mixture in 96-well filter plate. Shake 2 hr at RT or overnight at 4 °C and wash plate twice. Add 50 μ L/well detection mixture, shake 1hr at RT and wash plate once. Add 50 μ L/well SAPE, shake 30 min at RT and wash plate twice. Add 100 μ L/well sheath fluid and shake 2–5 min at RT. Read plate on Luminex Instrumentation.

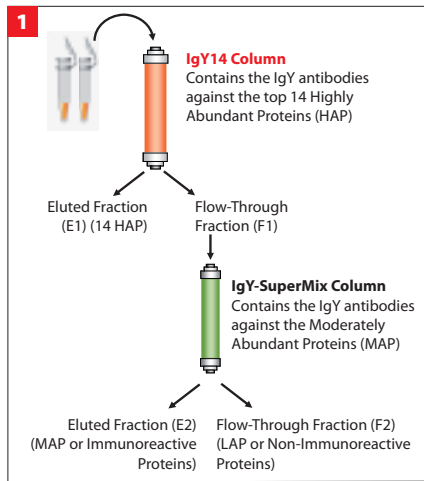


Figure 1: Seppro SuperMix Depletion System

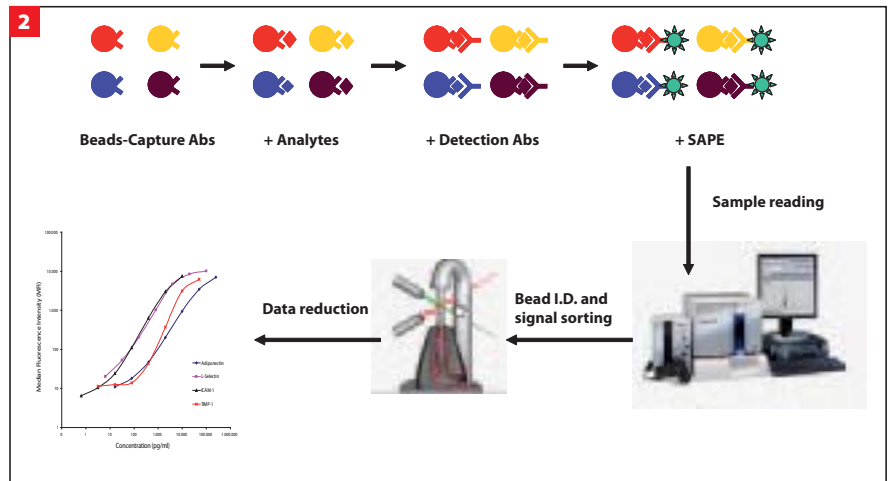


Figure 2: Principle of Luminex Multiplexing Assay

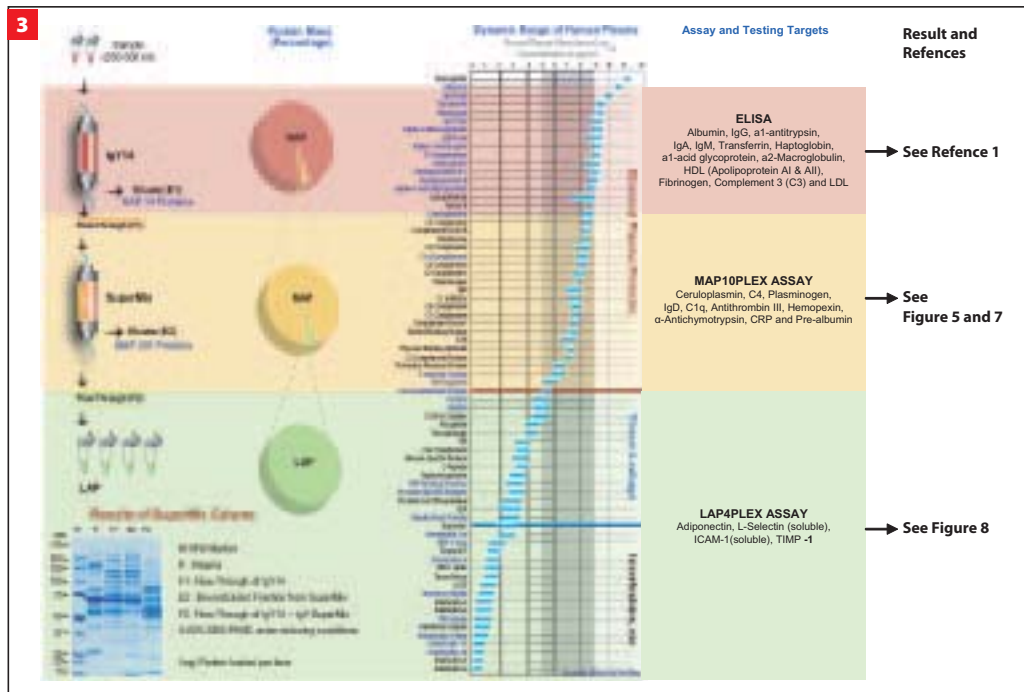


Figure 3: Flowchart of Seppro SuperMix Depletion Process and the Targets to Test

The top14 HAPs (plasma level ranges in 0.5–50 mg/ml) compose 95% of total plasma proteins. The 10 targets to test for SuperMix depleted plasma cover a wide range concentration (2.3 µg/ml for CRP, 0.5 mg/ml for Ceruloplasmin) of MAPs. The 4 LAP targets in normal human plasma ranges in 0.2–10 µg/ml.

Results

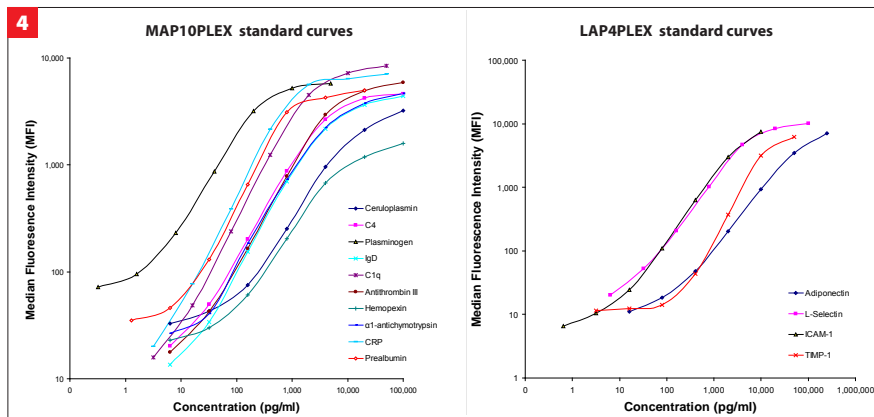


Figure 4: MAP10PLEX and LAP4PLEX assays are performed with protocol described above and standard curves are plotted for each assay.

MAP10PLEX

Final dilution of plasma (folds)	C4	C4	Plasminogen	IgD	C1q	Antithrombin III	Hemo- pexin	α 1-antichymo- trypsin	CRP
512000	103.73	74.21	153.77	112.98	125.17	127.80	104.88	115.24	86.50
256000	105.03	69.44	137.89	99.57	11.08	109.75	97.32	99.54	102.85
128000	105.01	68.59	120.09	114.09	103.57	101.52	104.10	93.74	110.09
64000 (start)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
AVG	104.59	70.74	137.25	108.88	113.27	113.02	102.10	102.84	99.81

LAP4PLEX

Table 1: Assay Accuracy – Dilution Linearity for Plasma Samples

Dilution linearity is commonly used to evaluate assay accuracy that is one of the most important requirements for immunoassays. A serial dilution of a pooled plasma was tested with MAP10PLEX and LAP4PLEX assay. The numbers in the table represent percentage of concentrations at each dilution point against start point. Both assays perform well. For normal human plasma samples, dilutions in the range from 5×10^4 to 5×10^5 for MAP10PLEX assay or 250 to 2000 for LAP4PLEX assay are suggested.

Final dilution of plasma (folds)	Adipo- nectin	L-Selectin	ICAM-1	TIMP-1
2000	140.59	98.95	107.32	87.11
1000	127.67	96.97	118.63	96.45
500	114.96	93.20	108.45	99.48
250 (start)	100.00	100.00	100.00	100.00
AVG	127.74	96.37	111.47	94.35

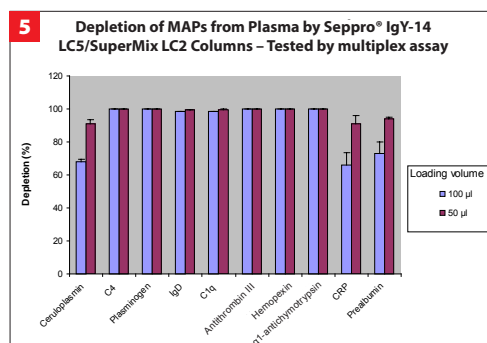


Figure 5: Depletion Efficiency of Seppro SuperMix Columns Tested by Luminex MAP10PLEX Assay

Depletion efficiency of 10 representative MAPs is related to loading volume of plasma samples. > 90% of all 10 MAPs can be depleted from 50µL human plasma.

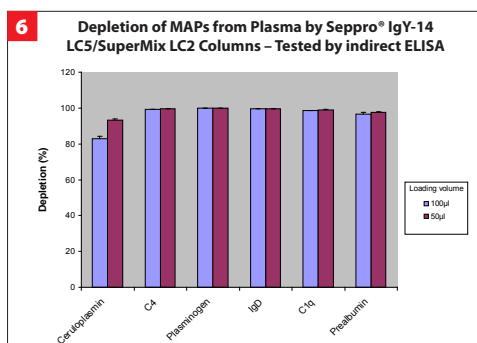


Figure 6: Depletion Efficiency of Seppro SuperMix Columns Tested by Indirect ELISA (for 6 targets only)

The same set of SuperMix depleted samples were tested by indirect ELISA and displayed a comparable result as Luminex MAP10PLEX assay.

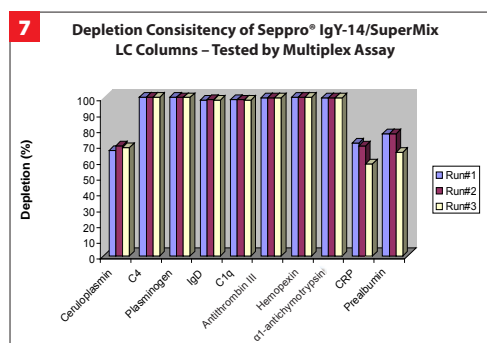


Figure 7: Run-to-Run Depletion Consistency of SuperMix Columns

Three depletion runs were performed with 100 µL plasma utilizing Seppro IgY14 LC5/SuperMix LC2 columns and the depletion efficiency was determined by Luminex MAP10PLEX assay. Depletion of all 10 targets is consistent (CV% < 15%)

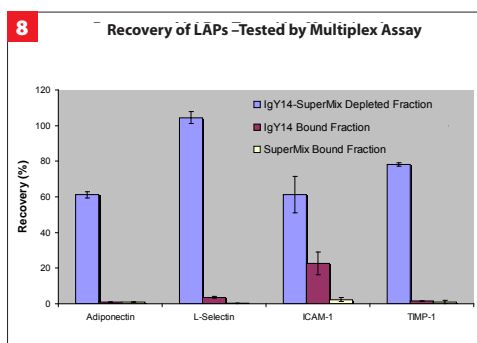


Figure 8: Impact of SuperMix Depletion Process on LAPs

Four representative LAPs were tested by Luminex LAP4PLEX assay to monitor the loss of LAPs during SuperMix depletion process. Depending on different biological properties of the individual target, approximately 1-40% loss has been observed from SuperMix depleted plasma (Note: some proteins in bound fraction are unstable).

Conclusions

1. The MAP10PLEX and LAP4PLEX assays are able to simultaneously measure 10 MAPs or 4 LAPs from human plasma or serum samples within 4 hours
2. These two multiplex assays can be used to replace regular ELISA methods to determine MAP depletion and LAP retention from Seppro IgY-14/SuperMix depleted samples
3. The preliminary test indicated that depletion of 10MAPs from human plasma through the tandem IgY14/SuperMix columns were reproducible and is consistent with Dr. Smith's result based on spectral count data (Ref. 3)
4. In order to remove > 90% of MAPs from plasma, columns with equal volume of IgY14 and SuperMix resins (IgY14/ SuperMix ratio = 1 : 1) are suggest to be combined

Summary

The newly developed MAP10PLEX and LAP4PLEX assays using Luminex technology have been proved to be both time and cost saving solutions to monitor the robustness of protein depletions from lot to lot and sample to sample.

References

- 1) Seppro® Depletion Product Info: <https://www.sigmaldrich.com>
- 2) Technical Bulletin, May 2001, Luminex Corp. <http://www.luminexcorp.com>
- 3) Qian WJ et. Al., Mol Cell Proteomics. 2008 Oct; 7(10):1963-73

NEW! HybridSPE™ – Precipitation Technology



Removal of Proteins and Phospholipids for LC-MS

Combines the simplicity of protein precipitation and the selectivity of SPE for the targeted removal of phospholipids and proteins from biological samples like plasma and serum

Facts, Features & Benefits:

Merges Protein Precipitation & SPE

- 100% removal of proteins & phospholipids in one simple 2 – 3 step procedure (96-well format)
- Minimal to no method development

No interference from phospholipids in LC-MS leads to

- Cleaner samples
- Less ion suppression
- More reproducible results & sensitivity
- Longer column life

Patent-pending and award-winning* technology based on zirconia-coated silica available in 96-well plates and 1 mL SPE tubes.

For further information on the HybridSPE™-ppt, please visit us at sigma-aldrich.com/hybridspe-ppt

If you would like to try the HybridSPE™-ppt, request your sample of the 1 mL HybridSPE™ cartridges from our technical service.

* Best New Separations Product
in 2008



Scientist Choice Awards 2009 of the selectscience.net community



Our Innovation, Your Research – Shaping the Future of Life Science

Cronobacter spp. Classic and New Detection Methods

Jvo Siegrist, Product Manager Microbiology ivo.siegrist@sial.com

Enterobacter sakazakii, now reclassified as a novel genus called *Cronobacter*, is known to cause neonatal infections.

Cronobacter is a rod-shaped, motile and facultatively anaerobic bacteria of the family Enterobacteriaceae. Originally *Cronobacter spp.* were listed as yellow-pigmented *Enterobacter cloacae* (see **Table 1**). The bacteria was then later on called *Enterobacter sakazakii*. Taxonomic studies have determined that *E. sakazakii* comprises a high genetic heterogeneity and should be reclassified as a novel genus, "*Cronobacter*".¹



Figure 1: Drinking baby

Kingdom: Bacteria
 Phylum: Proteobacteria
 Class: Gamma Proteobacteria
 Order: Enterobacteriales
 Family: Enterobacteriaceae
 Genus: *Cronobacter*

Did you know ...

the name *Cronobacter* comes from Cronus, leader of the Titans in greek mythology, who swallowed each of his children as soon as they were born. As *Cronobacter spp.* is harmful to neonates the name was thought to be appropriate.



Figure 2: Statue of Cronos

Cronobacter spp. are ubiquitous; frequently found on vegetables, meat, fermented bread, dairy products and especially in baby food. Consumption of contaminated powdered infant formula milk (IFM) can result in sepsis, infant meningitis and necrotising enterocolitis. In most cases the victims are pre-term, low birth weight infants or immunocompromised infants in the first weeks. Mechanisms are not fully understood but it is assumed that endotoxins are produced. The bacterium can adhere to and penetrate into various types of cells (e.g. endothelial cells). It also survives in macrophages.

As Enterobacteriaceae are susceptible to heat they do not survive production processes and it is most likely that confirmation happens after the process. The post process contamination can come from the addition of heat-sensitive additives such as vitamins or other micronutrients or incorrect handling while reconstituting or storage. It has been shown that stationary phase *Cronobacter spp.* are remarkably resistant to osmotic and dry stress compared with other species of the Enterobacteriaceae group. It is difficult to isolate *Cronobacter spp.* from samples as they are most likely stressed, unevenly distributed throughout the batch and also numbers will probably be fairly low, often lower than 1 CFU per g.

For the classical microbiological tests a pre-enrichment is used to recover the stressed cells followed by a selective enrichment step. The FDA method recommends Enterobacteriaceae enrichment (EE or Mossel) broth which is then streaked onto VRBG agar, and suspect colonies are subcultured onto TSA agar where the yellow pigmented colonies are confirmed by an oxidase test and other biochemical tests. See also **Table 1** with all kinds of biochemical reactions of *Cronobacter spp.* species (not only from FDA).

ISO/TS 22964:2008 method recommends buffered peptone water (BPW) as a pre-enrichment medium and modified lauryl sulphate broth with vancomycin (mLST) incubated at 44 °C for the secondary selective enrichment step. The next is a chromogenic agar isolation and identification (see **Table 2**).

There are various chromogenic agars available, which help to save work and time to result and are more reliable than traditional media. The detection principle is based on the alpha-glucosidase possessed by *Cronobacter spp.* (not by most other Enterobacteriaceae) which cleaves the 5-bromo-4-chloro-3-indolyl-a-D-glucopyranoside or similar substrates. The result is a plate with, for example, blue colonies in the case of *Cronobacter spp.* (see **Figure 3 and 4**) but biochemical confirmation is still required.

Today there are also studies which show that not all *Cronobacter spp.* give yellow pigmented colonies on tryptic soy agar, and it was shown that some types of strains did not grow at 44 – 45 °C. Also some selective media may be too selective to recover all the *Cronobacter* species. There is still some work to do to improve the official methods.



Figure 3: HiCrome™ *Cronobacter spp.* Agar (Fluka 92324); *Cronobacter spp.* (blue), *E. aerogenes* (green) *K. pneumoniae* (yellow)

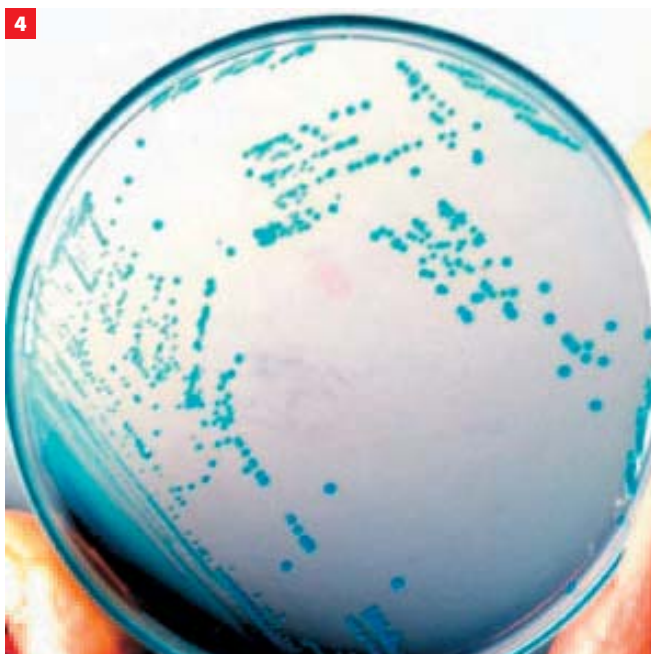


Figure 4: HiCrome™ *Cronobacter spp.* Agar, Modified (Fluka 14703), with *Cronobacter spp.* colonies

Biochemical Test	Reaction	of <i>Cronobacter spp.</i>
Gram	-	
Oxidase	-	
Catalase	+	
H ₂ S production	-	
Nitrate reduction	+	
Citrate utilisation	+	
Esculin hydrolysis	+	
Arginine hydrolysis	+	
Lysine	-	
L-ornithine decarboxylation	+	
Urease	-	
Indole	-	
ONPG	+	
D-adonitol	-	
L-arabinose	+	
D-arabitol	-	
D-cellobiose	+	
Dulcitol	-	
D-fructose	+	
D-glucose	+	
D-galactose	+	
Galacturonate	+	
Inositol	+(75%)/-	
Inulin	+	
Lactose	+	
Malonate	+/-	
D-maltose	+	
D-mannitol	+	
D-mannose	+	
D-melibiose	+	
X-methyl-D-glucoside	+	
D-raffinose	+	
L-rhamnose	+	
Salicin	+	
Sorbitol	-	
D-sucrose	+	
D-trehalose	+	
Xylose	+	
Acetoin production (VP test)	+	
Methyl red test	-	
Tryptic soy agar at 25 °C	yellow pigmented	

Table 1: Biochemical reactions of *Cronobacter spp.*

Brand	Cat. no.	Medium	Description
Fluka	92324	HiCrome™ <i>Cronobacter</i> spp. Agar	chromogenic media
Fluka	14703	HiCrome™ <i>Cronobacter</i> spp. Agar, Modified	chromogenic media according ISO
Fluka	69965	Mosel Broth (E.E. broth)	for pre-enrichment
Fluka	77187	Peptone Water, phosphate-buffered	for pre-enrichment
Fluka	22091	Tryptic Soy Agar	for isolation and differentiation
Fluka	79872	Tryptic Soy Agar, ready to use	for isolation and differentiation

Table 2: Media for detection of *Cronobacter* spp.

A New Molecular Biology Method

Rapid detection and identification of *Cronobacter* spp. is required, since even low cell numbers have been reported to cause a disease. HybriScan®D *Cronobacter* spp. is a new rapid molecular test system for detection of bacteria of the genus *Cronobacter* in food, especially in dried infant formula milk and its production environment. It is based on the detection of rRNA by sandwich hybridisation and so no PCR is needed. It is a 96 well microplate format and the workflow is very similar to an ELISA test.

Figure 5 shows the specificity of HybriScan®-*Cronobacter* spp. Different cell amounts and related Enterobacteriaceae were tested within a validation study. No signals were observed using $2,3 \times 10^8$ *Enterobacter cloacae* cells or 7×10^8 *Citrobacter freundii* cells per assay, whereas clear specific signals were detectable using 2.6×10^3 , 1.3×10^4 , and 2.6×10^4 cells of *Cronobacter* spp., respectively. These results demonstrate that the HybriScan® system is highly specific for *Cronobacter* spp.

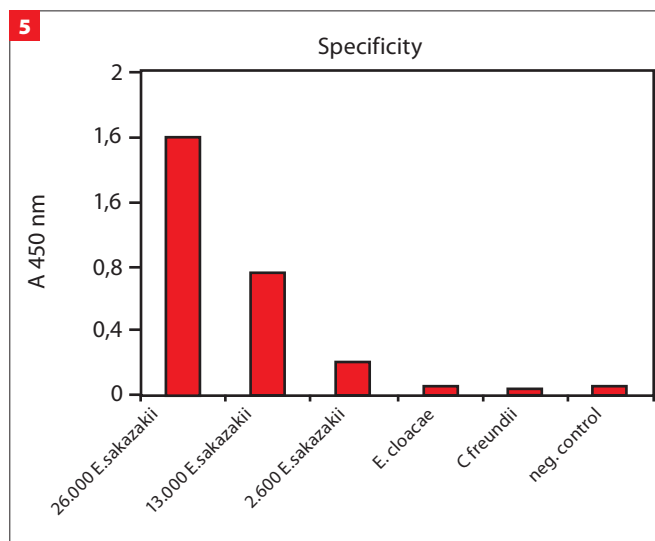


Figure 5: Specificity of HybriScan®-*Cronobacter* spp.

Different cell numbers of *Cronobacter* spp. and related Enterobacteriaceae like *E. cloacae* and *Citrobacter freundii* were tested. Measurement data for HybriScan® analyses represent absorbance at 450 nm.

A validation study of HybriScan®-*Cronobacter* spp. was performed using two different enrichment procedures: (1) single-step enrichment for 24–26 hours at 37 °C in ESSB broth (*Enterobacter* spp. selective broth) and (2) two-step enrichment starting with a pre-enrichment for 18–20 hours at 37 °C in buffered peptone water and followed by a selective enrichment for 24–26 hours at 45 °C in mLST selective broth. The results of the above-mentioned validation study are presented in **Figure 5**.

Brand	Cat. no.	Name	Description
Fluka	12838	HybriScan® <i>Cronobacter</i> spp.	96

Table 3: Ordering Information

References

- 1] Cawthorn, D.M.; Botha, S.; Witthuhn, R.S. Evaluation of different methods for the detection and identification of *Enterobacter* spp. isolated from South African infant formula milks and the processing environment. *International Journal of Food Microbiology*, 127:129–138 (2008).
- 2] Isolation and Enumeration of *Enterobacter* spp. from Dehydrated Powdered Infant Formula, U.S. Food and Drug Administration Center for Food Safety and Applied Nutrition (2002).
- 3] Color Atlas and Textbook of Diagnostic Microbiology, 5th edition, Lippincott Williams & Wilkins (1997).
- 4] Iversen et al., The taxonomy of *Enterobacter* spp. proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter* spp. comb. nov. *Cronobacter* spp. subsp. spp., comb. nov., *Cronobacter* spp. subsp. malonicus subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov. and *Cronobacter* genomospecies 1, *BMC Evolutionary Biology*, 7:64 (2007).
- 5] Bergey's manual of determinative bacteriology, D.H. Bergey, J.G. Holt, 9th Edition, Lippincott Williams & Wilkins (1994).
- 6] M.B. Kleiman et al., Meningoencephalitis and Compartmentalization of the Cerebral Ventricles Caused by *Enterobacter* spp., *Journal of Clinical Microbiology*, p. 352–354 (1981).
- 7] ISO/TS 22964:2006 Milk and milk products detection of *Enterobacter* spp.
- 8] O. Guillaume-Gentil, V. Sonnard, M.CRONOBACTER Kandahai, J.D. Mauragg, H. Jootsen, A Simple and Rapid Cultural Method for Detection of *Enterobacter* spp. in environmental samples. *Journal of Food Protection*, 68 (1), 2005, pp. 64–69.
- 9] K. Riedel, A. Lehner, Identification of proteins involved in osmotic stress response in *Enterobacter* spp. by proteomics. *Proteomics* 7, 1217–1231 (2007).
- 10] F.J. Pagotto, M. Nazarowec, S. Bidawid, J.M.Farber, *Enterobacter* spp.: Infectivity and enterotoxin production in vitro and in vivo; *J. of Food Protection*, Vol. 66, 3, p. 370–375 (2003).

Argentina

SIGMA-ALDRICH DE ARGENTINA S.A.
 Free Tel: 0810 888 7446
 Tel: (+54) 11 4556 1472
 Fax: (+54) 11 4552 1698

Australia

SIGMA-ALDRICH PTY LTD.
 Free Tel: 1800 800 097
 Free Fax: 1800 800 096
 Tel: (+61) 2 9841 0555
 Fax: (+61) 2 9841 0500

Austria

SIGMA-ALDRICH HANDELS GmbH
 Tel: (+43) 1 605 81 10
 Fax: (+43) 1 605 81 20

Belgium

SIGMA-ALDRICH NV/SA.
 Free Tel: 0800 14747
 Free Fax: 0800 14745
 Tel: (+32) 3 899 13 01
 Fax: (+32) 3 899 13 11

Brazil

SIGMA-ALDRICH BRASIL LTDA.
 Free Tel: 0800 701 7425
 Tel: (+55) 11 3732 3100
 Fax: (+55) 11 5522 9895

Canada

SIGMA-ALDRICH CANADA LTD.
 Free Tel: 1800 565 1400
 Free Fax: 1800 265 3858
 Tel: (+1) 905 829 9500
 Fax: (+1) 905 829 9292

China

SIGMA-ALDRICH (SHANGHAI)
 TRADING CO. LTD.
 Free Tel: 800 819 3336
 Tel: (+86) 21 6141 5566
 Fax: (+86) 21 6141 5567

Czech Republic

SIGMA-ALDRICH spol. s r. o.
 Tel: (+420) 246 003 200
 Fax: (+420) 246 003 291

Denmark

SIGMA-ALDRICH DENMARK A/S
 Tel: (+45) 43 56 59 10
 Fax: (+45) 43 56 59 05

Finland

SIGMA-ALDRICH FINLAND OY
 Tel: (+358) 9 350 9250
 Fax: (+358) 9 350 92555

France

SIGMA-ALDRICH CHIMIE S.à.r.l.
 Free Tel: 0800 211 408
 Free Fax: 0800 031 052
 Tel: (+33) 474 82 28 00
 Fax: (+33) 474 95 68 08

Germany

SIGMA-ALDRICH CHEMIE GmbH
 Free Tel: 0800 51 55 000
 Free Fax: 0800 64 90 000
 Tel: (+49) 89 6513 0
 Fax: (+49) 89 6513 1160

Greece

SIGMA-ALDRICH (O.M.) LTD.
 Tel: (+30) 210 994 8010
 Fax: (+30) 210 994 3831

Hungary

SIGMA-ALDRICH Kft
 Ingyenes zold telefon: 06 80 355 355
 Ingyenes zold fax: 06 80 344 344
 Tel: (+36) 1 235 9055
 Fax: (+36) 1 235 9050

India

SIGMA-ALDRICH CHEMICALS
 PRIVATE LIMITED
 Telephone
 Bangalore: (+91) 80 6621 9600
 New Delhi: (+91) 11 4165 4255
 Mumbai: (+91) 22 2570 2364
 Hyderabad: (+91) 40 4015 5488
 Fax
 Bangalore: (+91) 80 6621 9650
 New Delhi: (+91) 11 4165 4266
 Mumbai: (+91) 22 2579 7589
 Hyderabad: (+91) 40 4015 5466

Ireland

SIGMA-ALDRICH IRELAND LTD.
 Free Tel: 1800 200 888
 Free Fax: 1800 600 222
 Tel: (+353) 1 404 1900
 Fax: (+353) 1 404 1910

Israel

SIGMA-ALDRICH ISRAEL LTD.
 Free Tel: 1 800 70 2222
 Tel: (+972) 8 948 4100
 Fax: (+972) 8 948 4200

Italy

SIGMA-ALDRICH S.r.l.
 Numero Verde: 800 827018
 Tel: (+39) 02 3341 7310
 Fax: (+39) 02 3801 0737

Japan

SIGMA-ALDRICH JAPAN K.K.
 Tel: (+81) 3 5796 7300
 Fax: (+81) 3 5796 7315

Korea

SIGMA-ALDRICH KOREA
 Free Tel: (+82) 80 023 7111
 Free Fax: (+82) 80 023 8111
 Tel: (+82) 31 329 9000
 Fax: (+82) 31 329 9090

Malaysia

SIGMA-ALDRICH (M) SDN. BHD
 Tel: (+60) 3 5635 3321
 Fax: (+60) 3 5635 4116

Mexico

SIGMA-ALDRICH QUÍMICA, S.A. de C.V.
 Free Tel: 01 800 007 5300
 Free Fax: 01 800 712 9920
 Tel: (52) 722 276 1600
 Fax: (52) 722 276 1601

The Netherlands

SIGMA-ALDRICH CHEMIE BV
 Free Tel: 0800 022 9088
 Free Fax: 0800 022 9089
 Tel: (+31) 78 620 5411
 Fax: (+31) 78 620 5421

New Zealand

SIGMA-ALDRICH NEW ZEALAND LTD.
 Free Tel: 0800 936 666
 Free Fax: 0800 937 777
 Tel: (+61) 2 9841 0555
 Fax: (+61) 2 9841 0500

Norway

SIGMA-ALDRICH NORWAY AS
 Tel: (+47) 23 17 60 60
 Fax: (+47) 23 17 60 50

Poland

SIGMA-ALDRICH Sp. z o.o.
 Tel: (+48) 61 829 01 00
 Fax: (+48) 61 829 01 20

Portugal

SIGMA-ALDRICH QUÍMICA, S.A.
 Free Tel: 800 202 180
 Free Fax: 800 202 178
 Tel: (+351) 21 924 2555
 Fax: (+351) 21 924 2610

Russia

SIGMA-ALDRICH RUS, LLC
 Tel: (+7) 495 621 6037
 (+7) 495 621 5828
 Fax: (+7) 495 621 5923

Singapore

SIGMA-ALDRICH PTE. LTD.
 Tel: (+65) 6779 1200
 Fax: (+65) 6779 1822

Slovakia

SIGMA-ALDRICH spol. s r.o.
 Tel: (+421) 255 571 562
 Fax: (+421) 255 571 564

South Africa

SIGMA-ALDRICH
 SOUTH AFRICA (PTY) LTD.
 Free Tel: 0800 1100 75
 Free Fax: 0800 1100 79
 Tel: (+27) 11 979 1188
 Fax: (+27) 11 979 1119

Spain

SIGMA-ALDRICH QUÍMICA, S.A.
 Free Tel: 900 101 376
 Free Fax: 900 102 028
 Tel: (+34) 91 661 99 77
 Fax: (+34) 91 661 96 42

Sweden

SIGMA-ALDRICH SWEDEN AB
 Tel: (+46) 8 742 4200
 Fax: (+46) 8 742 4243

Switzerland

SIGMA-ALDRICH CHEMIE GmbH
 Free Tel: 0800 80 00 80
 Free Fax: 0800 80 00 81
 Tel: (+41) 81 755 2828
 Fax: (+41) 81 755 2815

United Kingdom

SIGMA-ALDRICH COMPANY LTD.
 Free Tel: 0800 717 181
 Free Fax: 0800 378 785
 Tel: (+44) 1747 833 000
 Fax: (+44) 1747 833 313
 SAFC (UK) Free Tel: 01202 712305

United States

SIGMA-ALDRICH
 P.O. Box 14508
 St. Louis, Missouri 63178
 Toll-Free: (+1) 800 325 3010
 Toll-Free Fax: (+1) 800 325 5052
 Call Collect: (+1) 314 771 5750
 Tel: (+1) 314 771 5765
 Fax: (+1) 314 771 5757

Vietnam

SIGMA-ALDRICH Pte Ltd. VN R.O.
 133/27N Ngo Duc Ke Street
 Ward 12 Binh Thanh District
 Ho Chi Minh City
 Tel: (+84) 8 3516 2810
 Fax: (+84) 8 6258 4238

Internet

sigma-aldrich.com

**World Headquarters**

3050 Spruce St., St. Louis, MO 63103
 (+1) 314 771 5765
sigma-aldrich.com

Technical Service EUR: EurTechServ@sial.com

Technical Service US: techservice@sial.com

Development/Bulk Manufacturing Inquiries SAFC™ (800) 244-1173

*Accelerating Customers' Success
 through Innovation and
 Leadership in Life Science, High
 Technology and Service*

© 2009 Sigma-Aldrich Co. All rights reserved. SIGMA, SAFC, SAFC™, SIGMA-ALDRICH, ALDRICH, FLUKA, and SUPELCO are trademarks belonging to Sigma-Aldrich Co. and its affiliate Sigma-Aldrich Biotechnology, LP. Sigma brand products are sold through Sigma-Aldrich, Inc. Sigma-Aldrich, Inc. warrants that its products conform to the information contained in this and other Sigma-Aldrich publications. Purchaser must determine the suitability of the product(s) for their particular use. Additional terms and conditions may apply. Please see reverse side of the invoice or packing slip.

Date: 11/2009;
 SAMS Code: LWK

SIGMA-ALDRICH®