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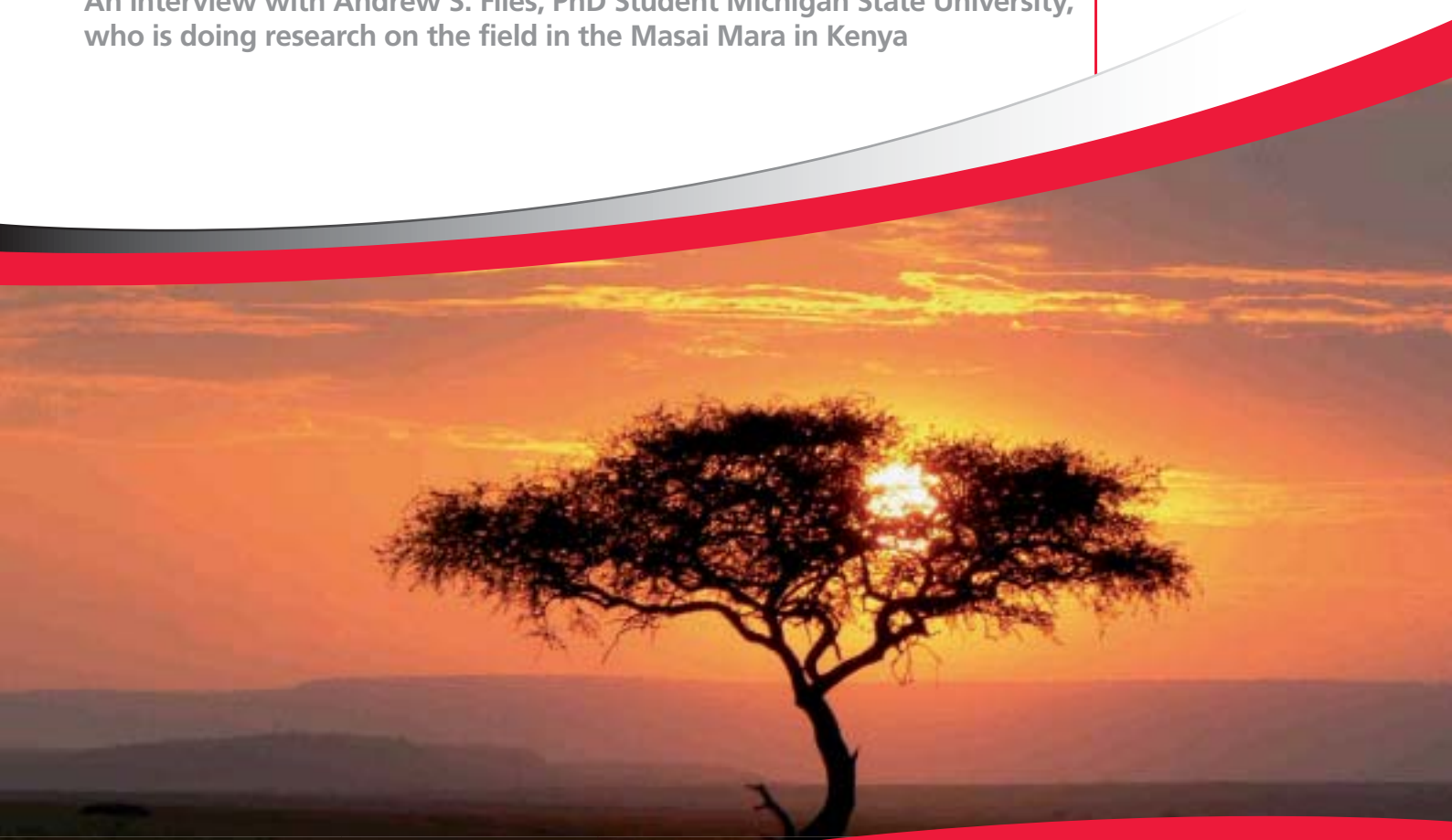
INVITRO

Issue 3 • 2009



The Role of Hyena within the Ecology of Disease

An interview with Andrew S. Flies, PhD Student Michigan State University, who is doing research on the field in the Masai Mara in Kenya



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Dear Colleague,



Sigma-Aldrich Chemie GmbH
Anne-Cathrin Burkert

Welcome to the first issue of our IN VITRO Journal for the year 2009, focusing on the clinical medicine market.

A big thank you for all the positive feedback that we received in our first year. We would like to wish our customers a prosperous and healthy New Year, with continued collaborative success in 2009.

The European Marketing Communication Department publishes this quarterly newsletter and we are always happy to receive news items and articles for inclusion in this publication or

for general promotion purposes from any member of Europe's life science community. Comments and suggestions are also welcome!

Our mission statement: to give our customers the possibility to present their daily scientific work as a scientific article in this journal.

In addition, we aim to support you with material which provides an excellent opportunity to have an inside view of scientific research. In every issue we present a portrait of a person and/or the environment that they are working in. Featured in the magazine is an interview with Andrew Flies, a PhD student, who is researching hyenas in the Masai Mara National Reserve in Kenya. Turn to page 4 to read his fascinating account about working in a scientific camp in Africa, and details about his dissertation.

Also in this issue: what is the connection between an artist in the Renaissance and a skilled histotechnologist? Have a look at page 8 to find out.

Finally, Sigma-Aldrich would like to introduce a new sample prep platform called HybridSPE™ – Precipitation Technology for Pharmaceutical Bioanalytical Sample Preparation. HybridSPE™ combines the simplicity of protein precipitation (2–3 steps) with the selectivity of SPE towards the targeted removal of proteins and phospholipids. To learn more, please turn to page 14 of this issue.

I hope you find this edition of IN VITRO interesting and beneficial.

Kind regards,

Anne-Cathrin Burkert
Project Leader European Marketing Communication
anne-cathrin.burkert@sial.com

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Interview with Andrew S. Flies

PhD Student Michigan State University (MSU), who is doing research on the field in the Masai Mara in Kenya



CURRICULUM VITAE



Education

Aug. 2006 – present

Michigan State University, East Lansing, MI1

Dual Ph.D. program for: Zoology Ecology, Evolutionary Biology, and Behavior (EEBB)

Concentration: Disease Ecology and Conservation Medicine

Research: Disease ecology and immune response in spotted hyenas (*Crocuta crocuta*)

Advisors: Kay Holekamp and Jean Tsao

Sept. 2004 – May 2006

Johns Hopkins University, Baltimore, MD

Advanced Academic Programs – Environmental Sciences

Sept. 1997 – May 2002

Minnesota State University, Mankato, MN

Computer Science, Bachelor of Science

Minors: Math, Chemistry

Membership

- American Society of Mammalogists (ASM)
- National Geographic Society
- Sierra Club
- National Wildlife Federation

Awards Received

- College of Natural Science Summer Support Fellowship 2008
- MSU EEBB Travel Funding 2008
- Sigma Xi Grants-in-Aid of Research 2008
- National Science Foundation (NSF) Graduate Research Fellowship 2007
- American Society of Mammalogists (ASM) Grants-in-Aid of Research 2007
- MSU EEBB Summer Research Fellowship 2007
- MSU Graduate Research Enhancement Award 2007
- MSU EEBB Wilderness Medicine Fellowship 2007
- MSU Zoology general departmental funds 2007, 2008
- Mayo Foundation Scholarship 1997–2001

The Kay E. Holekamp Lab has its headquarters on the campus of Michigan State University. And: the lab has a second field office on the other side of the world, in the Masai Mara National Reserve in Kenya, for studying hyenas. Andy S. Flies spent the first two years of his PhD at the MSU and lived in the Masai Mara for 2.5 months in 2007. Right now he is back in Kenya until March 2009 and over the next three years will be roughly split between Africa and the States. After his bachelor degree in computer science, Andy Flies worked as a lab manager in an immunology lab at Johns Hopkins University in Baltimore, Maryland and the Mayo Clinic in Rochester, Minnesota. That was where he realised that he wanted to pursue a different career, more exciting and fulfilling. With the PhD at the MSU he got the opportunity to study the role of the hyena within the ecology of disease in large carnivores of the Masai Mara. That was also when he set his sights on Africa.

IN VITRO: Why are the hyenas in the Masai Mara an interesting model to study?

AF: In 1994 nearly one third of the lions in the Serengeti-Mara ecosystem died from canine distemper virus (CDV). Nearly half of the hyenas in our study clans tested positive for CDV by serum neutralisation assay, but few, if any, actually died from the virus. CDV has also caused large die-offs in wild dogs in the ecosystem. Rabies has caused similar problems, contribut-



ing to the extirpation of wild dogs in the Mara. Through all these outbreaks, the hyenas in Dr. Holekamp's study clans showed few signs of disease.

IN VITRO: What exactly is your area of research?

AF: To study the reasons for the apparent resilience of hyenas to disease. Making a list of possibilities and trying to exclude some of the possibilities is the foundation of my research. In my dissertation I address two broad areas of research. The first being, what is the relationship between hyenas, disease and the general health of the ecosystem? If they are reservoirs of disease that infect other animals, it is important that we know this in order to understand the disease dynamics in this amazing ecosystem. Second, by gaining insight into how the hyena immune system functions, I hope it will be useful for both human and animal health.

IN VITRO: What do you think about hyenas? Why do they have a bad image as scavengers?

AF: Hyenas are one of the most interesting animals in the Serengeti-Mara ecosystem. Most large carnivores are not highly social. Wild dogs and lions live in groups, but not nearly as large as hyena clans. A large clan can have up to 80 hyenas, so the social interaction among all the members provides countless opportunities to study behavioural interaction. Hyenas have a strict hierarchy that is dominated by females. The ladies are larger and generally more aggressive than males. Hyenas are commonly thought of as scavengers, but they are actually one of the most efficient hunters in the ecosystem. Lions, cheetahs and leopards rely on stealth and short bursts of speed to catch their prey. Hyenas are capable of running at high speed for very long distances,



eventually wearing down the animal they are chasing. When a hyena decides to hunt, there is a high probability they will get a meal.

IN VITRO: Can you remember your first face-to-face meeting with hyenas?

AF: We left camp around 5:30 am for our morning observations. We had been driving for about ten minutes when I saw my first hyena. The name was Morpheus, an adult female from our primary study clan. She was chewing on a scrap of food and would occasionally glance at our research vehicle and scan for other animals. Seeing large carnivores like hyenas and lions in the dark is quite a sight. Their eyes flash brightly when the light hits them, and you realise that these animals own the night and have been finely tuned by evolution to thrive in this setting. I spend a lot of time reading about animals in the wild and watching documentaries about wild animals, so actually witnessing a hyena feeding in the wild for the first time was quite surreal.

IN VITRO: What does the scientific equipment look like at your camp in Africa?

AF: The "lab" at our research camp in the bush is primitive. In the main camp we have some basic equipment. We extract DNA in the field, separate plasma and serum from whole blood, make blood smears, measure blood glucose, quantify total solids in serum.



This is all done using blood we take from anaesthetised hyenas. While the hyena is anaesthetised we take a plethora of morphological measurements. We also collect ectoparasites, take blood pressure, temperature and weigh the animal.

IN VITRO: Which experiments can be carried out onsite?

AF: The DNA extraction. Most of the other work on the blood and DNA is done back in the lab at Michigan State. I am working on a pathogen transmission project that will be done onsite. Other graduate students are testing hypotheses about mate choice, cognitive ability of social animals, maternal behaviour and genetic diversity. Most of the research avenues involve a field component, some lab analysis and a heavy statistical aspect.

IN VITRO: Which molecular methods are available to a scientist who is doing research with exotic animals?

AF: The amount of work you can get done in the field is limited by budget/equipment and creativity. The only real molecular meth-





ods we do are to extract DNA from whole blood. I will be doing a lot of polymerase chain reactions (PCR) when I get back to the lab. Most of the genes I am interested in have not been studied before in hyenas, so one of my first projects was to find primers for that work. I recently finished analysing my sequencing data and now have the primers I need. Doing analysis of data and statistics in the field is actually a good way to keep my lab work fresh in my mind.

IN VITRO: Which problems can occur with the methods/reagents that you're using?

AF: One major obstacle is trying to keep reagents at the appropriate temperature. The temperature ranges from about 14 Celsius up to nearly 30 Celsius on some days. Our camp is run entirely on a few solar panels. The system is not large enough for a refrigeration unit, so reagents that need to be kept cool present a problem. For a while, I used the liquid nitrogen to make ice, but this depletes the liquid nitrogen stock rapidly. I also have some cold packs that can keep things cool for a short time. My latest attempt is to use an electric cooler that plugs into the car. We do not have sterile facilities or laminar air flow hoods to work in either, so contamination is always a worry. This is another reason why most of the work is done back in the lab.

IN VITRO: How is the supply of lab reagents and especially liquid nitrogen secured?

AF: Samples in the field are stored in liquid nitrogen until they are taken to the lab at Michigan State University. There is risk associated with this, since running out of liquid nitrogen is a possibility and the experiments may not work. We have recently begun a collaboration with the International Livestock Research Institute (ILRI) and they have been very helpful in procuring our reagents. Our sales agent from "Kobian Kenya" (editor's note: the local distributor of Sigma-Aldrich products) has also volunteered to deliver our next product order to the Mara



when he makes a trip down here. We make a trip to Nairobi every six to eight weeks in order to replenish our liquid nitrogen stock. We have two liquid nitrogen dewars and transfer the LN2 between the them, then take the empty one to Nairobi. It takes about six hours to get there and the roads really wear down our vehicles, so we try to minimise these trips.



IN VITRO: What kind of new scientific things did you learn in Africa?

AF: Being in the field and watching hyenas every day constantly leads me to ask new questions. Before my first visit to the field site, I had many research objectives I was interested in pursuing, but many turned out not to be feasible. There is no substitute for actually spending time in the field. Even the best laid-out plans on paper may turn out to be ineffective in the field. Wild animals can be very uncooperative. If they are not comfortable with our research vehicle, we have no chance of getting blood samples from the hyena. What I have learned is that research protocols and objectives need to be meticulously prepared, but you need to be able to adjust to rapidly changing conditions and always be prepared for the unexpected. I have also learned to use hypothesis testing in everyday life, for things like trying to figure out why our Land Cruiser is making strange noises.

IN VITRO: What personal experiences have you had?

AF: The experience of living in the bush has really altered my perception of the world. You can really do without a lot of the things most people would consider necessities. Living out here requires a lot of self-reliance, so most people find they are capable of a lot of things they may not have previously thought they could do.



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The Art & Mosaic of Special Stains

Herbert Skip Brown, Lab Management Consultants

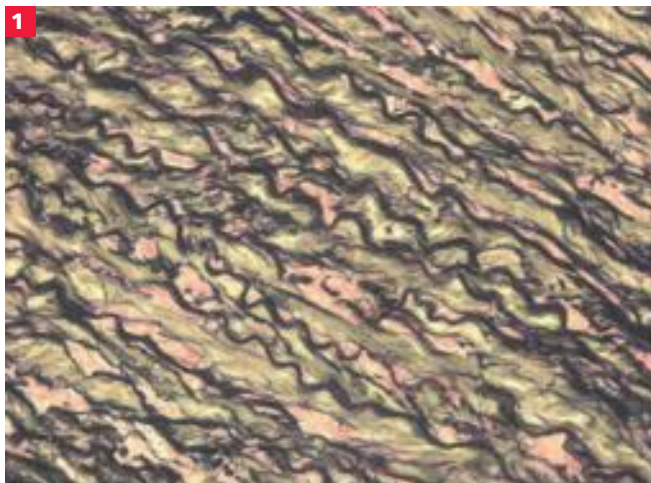


Figure 1: The Art of an Elastic Stain

The Renaissance Age is documented as being a period of transition from medieval to modern times in which there was a new birthing of creativity and uniqueness in classical art and literature. Webster describes this period as one that expressed ‘a flowering of the arts’, and was characterized particularly in paintings by an explosion of colors, contrast, and imagery. When we speak of special stains in the field of histotechnology it is easy to think along the lines of an artful mosaic, i.e., a unique creation made by inlaying small pieces of variously colored material to form a pattern or picture. Although patterns can be copied, mosaics are rarely reproducible in exactness because of the uniqueness of each elemental piece. Such is the nature of how dyes and stains are blended and used to produce a kaleidoscope of visual colors.

In as much as special stains hold a more practical application as a diagnostic tool for physicians; one where critical decisions are made concerning the identification of pathologic conditions, we cannot help from time to time to stop and marvel at the explosion of colors. I am reminded, as many in the field can attest, of the first time that we successfully performed a Masson’s Trichrome stain. The brilliance of blue collagen and red muscle fibers in contrast demonstrated that ours is a field that possesses hidden wonders beyond



Figure 2: Gaudi, casa batllo

the science. An artist takes a blank canvas and visualizes a form then an image. Then in applying various colors and blending of colors he or she creates something beyond the initial vision. Upon looking at the finished product it becomes apparent that the initial vision was only a naïve concept of what was to be created. It is also apparent that the beauty of this new creation is something that evolved during the process of being created. This is also true with many special stains that are routinely performed in histology. While it is true that in a clinical setting, when workloads are high and the physicians are waiting for their results, there is little time to look at the finished slide with more than a glance. Too often do we jet rocket past our visual perception as we look through the microscope to make sure the stain and procedure worked effectively. We miss the fact that a wonderful creation has happened. At first it may seem that a prolonged look is a waste of time, and that it has no significant value in the context of patient care. This is predominantly because we as laboratorians have conditioned ourselves to look only for a quick verification that the stain worked. The intricate details of how the dyes bind to certain tissue elements are often missed or irrelevant to us. Research in special stains has demonstrated through taking a more critical look, that we learn more about the human anatomy, organs, and discrete cells when we visually focus on the affinity of dyes to specific tissue. We begin to understand that there is still science involved in taking an artistic look at histological stains. When we look closely at the contour and texture of a mucicarmine dye as it captures the pressed cellophane appearance of goblet cells, we not only notice their colorful beauty in red, but we also see how they function to secrete mucoid substances into the small intestine. We also see in brilliant yellow contrast, the supportive cell structures with their respective nuclei.

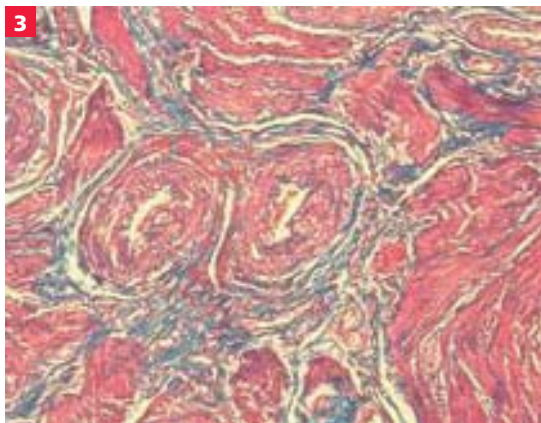


Figure 3: The Art of Masson's Trichrome Stain

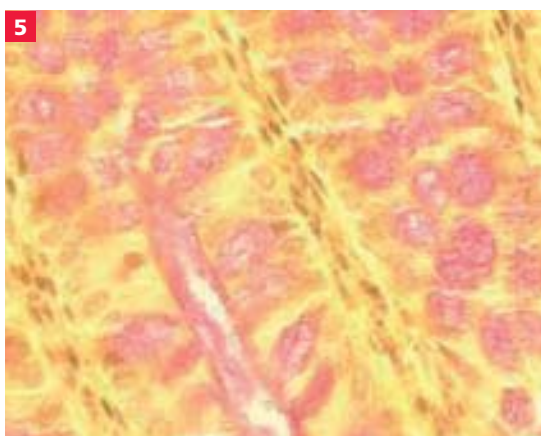


Figure 5: Mucicarmine Stain

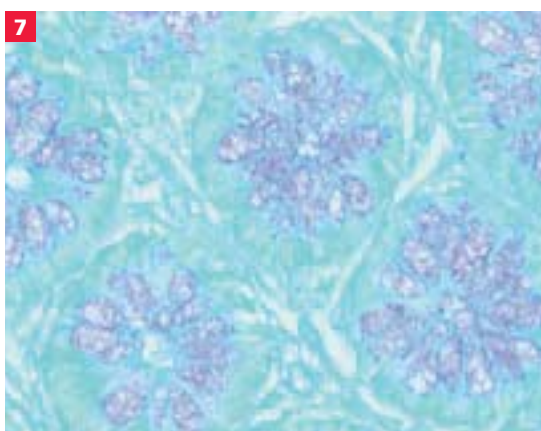


Figure 7: A Periodic Acid-Schiff's Reagent Stain



Figure 4: Close up photograph of a Stained Glass Window



Figure 6: Mosaic in the dome of St. Peter's Basilica in the Vatican



Figure 8: Bench at the park guell at Barcelona.

The skilled histotechnologist is in fact an artist when it comes to special stains because they have the knowledge and ability to change the elements of color just as an artist would. Through knowledge of histochemistry we understand that this very same tissue stained for mucoid substances, can be given an alternative artistic look creating a whole new work of art. This simple change of primary solutions from mucicarmine to periodic acid-schiff's reagent, along with a light green background counterstain, has produced a work of art that separates it from the original, and establishes its own uniqueness as a new product. This artistic ability to play with colors also allows the technologist to create stains that are more appealing to the eyes of their pathologist. Just as an artist has a choice of what he/she would try to create, so does the histotechnologist. And just as a mosaic can never be reproduced exactly as the original work, this new stain has an identity, character, and even desired application different from the first.

Serendipity can be described as the sudden and unanticipated discovery of treasures previously not known. We can apply this same discovery to special stains when we stop and look closely at the finished stain. The serendipity of stains is something that is capitalised on in histology schools where meticulous observation and interest have not become secondary to workflow demands. In our training schools it is important to spend time to observe the different tissues as they are brought into focus by the contrasting and blending of colors. In the daily routine of the laboratory we rarely have time to appreciate this, but we forget that for many of us it was one of the hooks that fascinated us and captivated our interest in this profession. As one who loves the beauty of what we do and what we create, I urge you from time to time to stop and rediscover not only the science of our profession, but the art as well. Special stains allow us to create a museum of colors and a library of knowledge about the human body.

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■ Introduction

The Sigma-Aldrich ACCUSTAIN® Papanicolaou Staining system is intended for staining exfoliative cells in cytologic specimens. Papanicolaou staining reagents are for "In Vitro Diagnostic Use".

Papanicolaou staining techniques, reviewed in a concise report by Street¹, have changed little in the past 40 years. The stain is used for examining exfoliative cells of sputum as well as vaginal, cervical, and other body secretions. In general, cells are fixed to a slide, treated with a hematoxylin nuclear stain and counterstained with a mixture of orange G, eosin Y and fast green FCF (a replacement for light green SF yellowish). These treatments impart characteristic colour to nuclei and cytoplasmic components.²

Gill No. 1 formulation is used as a progressive cytology stain; Gill formulations No. 2 and No. 3 may be used as progressive or regressive stains depending on length of staining time. These hematoxylin solutions are manufactured as half-oxidised hematoxylin; mordanted with aluminium and stabilised with glycols. The positively charged aluminumhematein complex combines with negatively charged phosphate groups of nuclear DNA, forming the blue-purple colour characteristic of hematoxylin stains.

■ Reagents

Papanicolaou Stain OG-6

Catalogue No. HT40-1, certified orange G, 0.3 % w/v, phosphotungstic acid, 0.015 % w/v, in denatured alcohol.

EA-50 Papanicolaou Stain

Catalogue HT40-3, certified eosin Y, 0.23 % w/v, certified fast green FCF, 0.08 % w/v, certified bismark brown, 0.05 %, phosphotungstic acid, 0.2 % w/v, in denatured alcohol.

Gill's 2 Hematoxylin Solution

Catalogue No. GHS-2, certified hematoxylin, 4g/L, sodium iodate, 0.4 g/L, aluminium sulphate, 35.2 g/L and stabiliser.

Scott's Tap Water Substitute

Catalogue No. S 5134

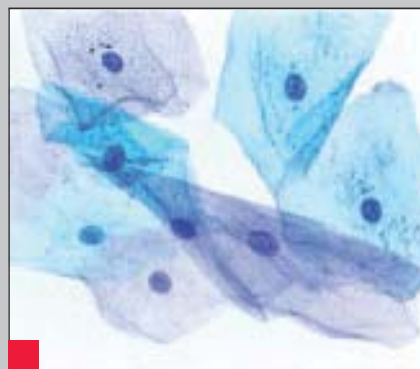
■ Reagent Preparation

Mix one part Scott's Tap Water Substitute with 9 parts deionised water (i.e. one bottle with 900 mL deionised water).

Procedure

1. Fix slides in 95 % alcohol for 15 minutes.
2. Rinse in tap water.
3. Stain in Gill's Hematoxylin No. 2 solution for 1 to 3 minutes.
4. Rinse in tap water.
5. Dip slides 10 times in working Scott's Tap Water Substitute.
6. Rinse in tap water.
7. Dip slides 10 times in 95 % alcohol.
8. Stain in OG-6 solution for 1.5 minutes.
9. Dip slides 10 times in 95 % alcohol.
10. Stain in EA-50 solution for 2.5 minutes.
11. Dehydrate in 2 changes of 95 % alcohol.
12. Dehydrate in 100 % alcohol for 1 minute.
13. Clear in 2 changes of xylene and coverslip with mounting media.

Results



Nuclei are stained blue while cytoplasm displays varying shades of blue, orange, pink, and red.

References

- 1] Street CM: Papanicolaou Techniques in Exfoliative Cytology. IN Laboratory Technique in Biology and Medicine, 3rd ed. EV Cowdry Editor, Williams & Wilkins, Baltimore, 1952, p.253.
- 2] Johnson PL, Klein MN: Application of Papanicolaou stain to paraffin sections. Stain Technol 31:223, 1956.

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Hematoxylin, a common nuclear stain, is isolated from an extract of logwood (*Haematoxylon campechianum*).³ The first successful biologic application of hematoxylin was described by Bohmer³ in 1865. Before hematoxylin can be used as a nuclear stain, it must be oxidised to hematein and combined with a metallic ion (mordant). Most successful mordants have been salts of aluminium or iron.

Hematoxylin Solutions are regressive stains for use in routine histology and cytology. The positively charged aluminium-hematein complex combines with negatively charged phosphatase of nuclear DNA, forming the blue purple colour characteristic of hematoxylin stains.

■ Reagents

Papanicolaou Stain OG-6

Catalogue No. HT40-1, certified orange G, 0.3 % w/v, phosphotungstic acid, 0.015 % w/v, in denatured alcohol.

EA-50 Papanicolaou Stain

Catalogue HT40-3, certified eosin Y, 0.23 % w/v, certified fast green FCF, 0.08 % w/v, certified bismark brown, 0.05 % phosphotungstic acid, 0.2 % w/v, in denatured alcohol.

Harris' Hematoxylin Solution

Catalogue HHS, certified hematoxylin, 7.0g/L, sodium iodate, aluminium ammonium sulphate 12 H₂O, preservative and stabilisers.

Scott's Tap Water Substitute

Catalogue No. S 5134

ACCUMATE Differentiating Solution

Catalogue No. A3179

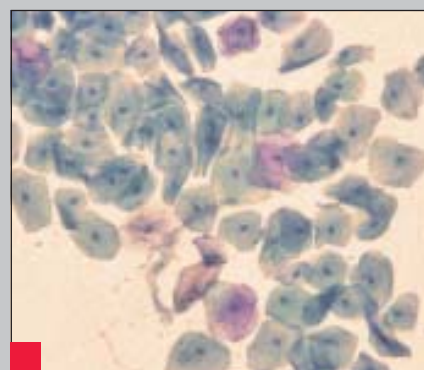
■ Reagent Preparation

Mix one part Scott's Tap Water Substitute with 9 parts deionised water (i.e. one bottle with 900 mL deionised water).

Procedure

1. Fix slides in 95 % alcohol for 15 minutes.
2. Rinse in tap water.
3. Stain in Harris' Hematoxylin solution for 1 to 3 minutes.
4. Rinse in tap water.
5. Dip slides in differentiating solution for 20 to 60 seconds.
6. Rinse in tap water.
7. Dip slides 10 times in working Scott's Tap Water Substitute.
8. Rinse in tap water.
9. Dip slides 10 times in 95 % alcohol.
10. Stain in OG-6 solution for 1.5 minutes.
11. Dip slides 10 times in 95 % alcohol.
12. Stain in EA-50 solution for 2.5 minutes.
13. Dehydrate in 2 changes of 95 % alcohol.
14. Dehydrate in 100 % alcohol for 1 minute.
15. Clear in 2 changes of xylene and coverslip with mounting media.

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References

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- 2] Johnson PL, Klein MN: Application of Papanicolaou stain to paraffin sections. Stain Technol 31:223, 1956
- 3] Natural Dyes. IN HJ Conn's Biological Stains, 9th ed., RD Lillie, Editor, Williams and Wilkins Co., Baltimore, MD, 1977, pp 468, 472.

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Introducing HybridSPE™-Precipitation Technology for Pharmaceutical Bioanalytical Sample Preparation

Craig Aurand, An Trinh, Michael Ye and Charles Mi an.trinh@sial.com

In pharmaceutical bioanalysis, researchers develop and run various assays to quantitate drugs, pharmaceutical candidates and their metabolites in biological fluids such as serum and plasma. The data resulting from these assays is used to help determine the pharmacodynamic and pharmacokinetic properties as well as the toxic and therapeutic concentrations of existing and emerging pharmaceutical compounds in living cells, tissues and animals. Although advances in Liquid Chromatography-Mass Spectrometry (LC-MS) technology have reaped overwhelming benefits in terms of increased throughput and sensitivity, good sample preparation continues to be a critical component of bioanalysis.

■ Features & Benefits:

- Merges both protein PPT & SPE
 - Offers simplicity & generic nature of protein PPT PLUS
 - Selectivity approaching SPE via the targeted removal of phospholipids
- 2–3 step generic procedure
- 100 % removal of phospholipids & precipitated proteins
- Minimal to no method development
- Available in 96-well and 1 mL cartridge dimensions
- Patent pending technology

The three most common sample prep techniques used in bioanalytical sample prep are protein precipitation (protein PPT), liquid-liquid extraction (LLE), and solid phase extraction (SPE). Each technique offers unique advantages and disadvantages that are considered during the method development process. For example, protein precipitation methods are simple (2–3 steps), fast, and often require minimal method development. However, the technique offers minimal selectivity as it only removes gross levels of protein from a sample prior to analysis. In contrast, SPE offers significant benefits in terms of selectivity/sample cleanup, but the technique often requires moderate to extensive levels of expertise and time for adequate method development. In addition, SPE often requires multiple steps (5–8), resulting in increased assay time.

In this report, we introduce a new sample prep platform trademarked HybridSPE™-Precipitation (HybridSPE™-PPT) in which we merge two predominate techniques in bioanalytical sample prep: protein precipitation and SPE. The end result is a technique that offers the advantages of both approaches while minimising their disadvantages.

■ Ion-Suppression & Phospholipid Contamination

Excessive background from endogenous matrix components has always been a great concern in quantitative bioanalysis, and has become paramount with decreasing analytical run times. In bioanalytical mass spectrometry, the issue of excessive background contributes to the growing problem of ion-suppression.

Ion-suppression is caused by one or more interfering components or species, that co-elute with the analyte(s) of interest during LC-MS analysis and manifests itself as a loss of analyte response. These co-eluting species can affect droplet formation or ionise concurrently, resulting in an erroneous decrease (suppression) or increase (enhancement) in signal response. Ion-suppression often leads to poor assay reproducibility, accuracy and sensitivity, and such deleterious effects are often most notable at the lower limits of quantitation (LLOQ) (1).

One of the major causes of ion-suppression in bioanalysis is the presence of phospholipids during LC-MS or LC-MS-MS analysis in the positive ion electrospray mode (+ESI) (2). Phospholipids are the second largest lipid component in biological matrices after triglycerides, and are typically present in extremely high concentrations in biological plasma samples. **Figure 1** compares the LC-MS chromatograms of two clonidine spiked rat plasma samples processed by protein precipitation (100 µL spiked plasma + 300 µL 1 % formic acid in acetonitrile) alone and protein precipitation followed by phospholipid removal. The black trace chromatogram shows the response of clonidine after protein precipitation and phospholipid removal. The red trace chromatogram was subjected to protein precipitation only. By removing phospholipid interferences prior to analysis, response for clonidine was nearly doubled.

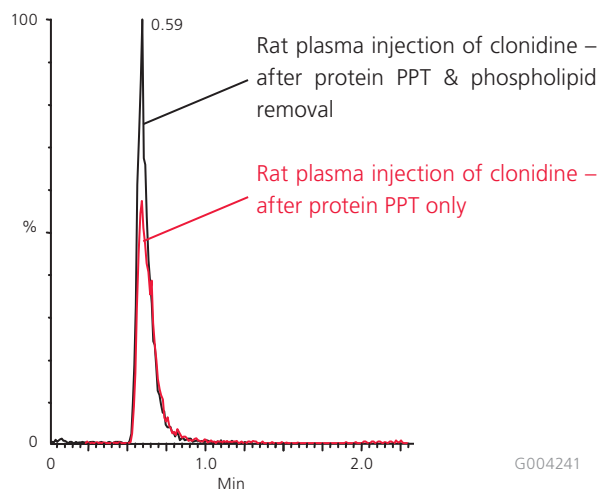


Figure 1: Phospholipid effect on ionisation of clonidine

■ How Does HybridSPE™-PPT Technology Work?

HybridSPE™-PPT technology is a simple and generic sample prep platform designed for the gross level removal of endogenous protein and phospholipid interferences from biological plasma and serum prior to LC-MS or LC-MS-MS analysis. Biological plasma or serum is first subjected to protein precipitation via the addition and mixing of acidified (with formic acid) acetonitrile. Precipitated proteins are then removed by centrifugation and the resulting supernatant is loaded on the HybridSPE™-PPT 96-well plate or cartridge which acts as a chemical filter that specifically targets the removal of endogenous sample phospholipids. The 96-well version contains a series of low porosity hydrophobic filters/frits, the packed-bed filter/frit assembly acts as a depth filter facilitating the concurrent removal of both phospholipids and precipitated proteins during the extraction process. The phospholipid retention mechanism is based on a highly selective Lewis acid-base interaction between the proprietary zirconia ions functionally bonded to the HybridSPE™-PPT stationary phase and the phosphonate moiety consistent with all phospholipids. The resulting eluent is ready for immediate LC-MS or LC-MS-MS analysis.

An alternative “In-Well Precipitation” method is available for the HybridSPE™-PPT 96-well version in which biological plasma/serum is first added to the 96-well plate followed by acidified acetonitrile (precipitation agent). After a brief mixing/vortexing step, a vacuum is applied to the 96-well plate. **Figure 2** visually depicts the HybridSPE™-PPT process (“In-Well Precipitation”) and describes how phospholipids are removed.

■ How are Phospholipids Selectively Removed?

Once the plasma/serum sample is subjected to protein precipitated via the addition of 1 % formic acid diluted in acetonitrile, it is passed through the HybridSPE™-PPT packed bed. The packed bed consists of a proprietary zirconia-coated silica particle. The zirconia sites exhibit Lewis acid (electron acceptor) properties that will interact strongly with Lewis bases (electron donor). Phospholipids structurally consist of a polar head group (zwitterionic phosphonate moiety) and a large hydrophobic tail (two fatty acyl groups that are hydrophobic). The phosphate group inherent with all phospholipids acts as a very strong Lewis base that will interact strongly with zirconia atoms functionalised on the particle surface (**Figure 3**).

1) Precipitate Proteins by adding 100 μ L plasma or serum to the HybridSPE™-PPT plate followed by 300 μ L 1 % formic acid in acetonitrile. Add I.S. as necessary.

2) Mix by vortexing/shaking HybridSPE™-PPT plate or by aspirating/dispensing with 0.5-1 mL pipette tip (e.g. TOMTEC Quadra liquid handler).

3) Apply vacuum. The packed-bed filter/frit assembly acts as a depth filter for the concurrent physical removal of precipitated proteins and chemical removal of phospholipids. Small molecules (e.g. pharma compounds and metabolites) pass through unretained.

4) Resulting filtrate/eluate is free of proteins and phospholipids and ready for immediate LC-MS-MS analysis; or it can be evaporated and reconstituted as necessary prior to analysis.

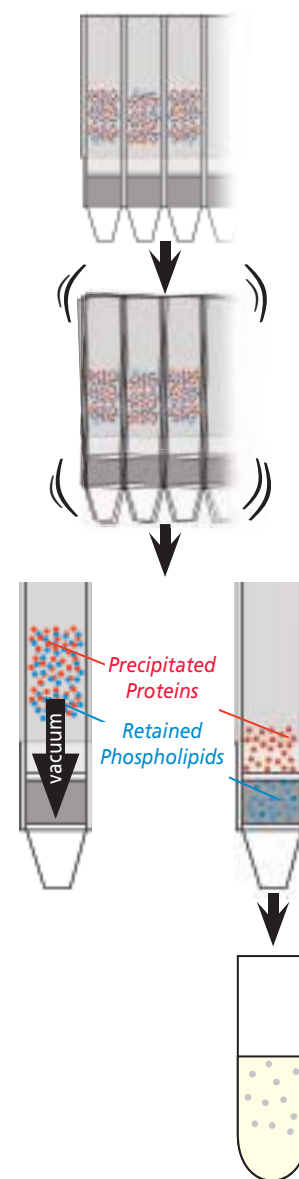
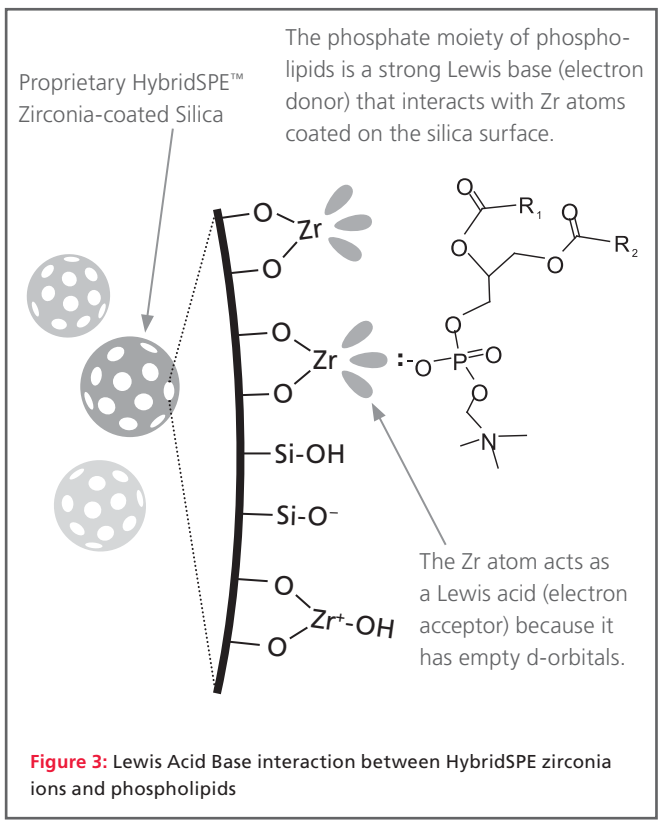


Figure 2: HybridSPE™-PPT “In-Well” 96-well precipitation method and phospholipid removal

Note:

The presence of ≥ 1 % formic acid in the acetonitrile precipitation agent is critical because:

- 1) Formic acid is a stronger Lewis base than most carboxyl (-COOH) groups found in acidic pharmaceutical compounds. As a result, formate ions will tie up the phase's zirconia ions, minimising retention of acidic analytes of interest. Because formate is not a strong enough Lewis base to displace the phosphate moiety found in phospholipids, phospholipids preferentially retain on the HybridSPE™-PPT phase.
- 2) The low pH environment induced by formic acid neutralises residual silanol activity on the silica surface thereby eliminating secondary cation-exchange interaction with basic compounds of interest.

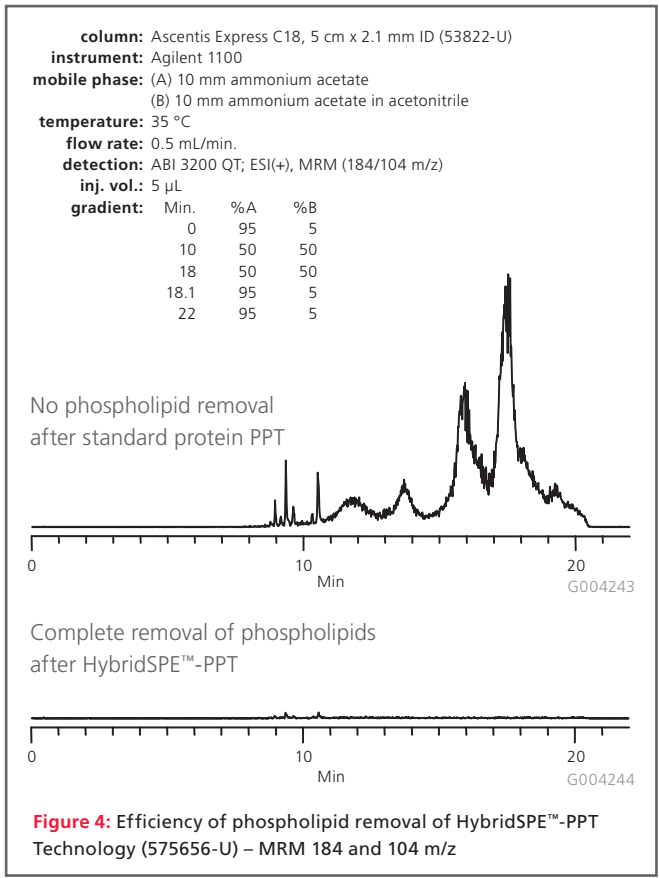


To demonstrate the efficiency of phospholipid removal using HybridSPE™-PPT technology, 100 µL of blank rat plasma was subjected to protein precipitation via the addition of 1 % formic acid in acetonitrile followed by 1 min. of vortex and centrifugation. A second set of rat plasma samples were processed using the HybridSPE™-PPT procedure described in Figure 2. The resulting supernatant (standard protein PPT) and filtrate/eluent (HybridSPE™-PPT) was analysed via LC-MS specifically monitoring for phospholipids (184/104 m/z). These transition ions represent the trimethylammonium-ethyl phosphate MS fragment consistent between the major phospholipids (e.g. phosphatidylcholine) found in plasma (2). In Figure 4, samples processed using HybridSPE™-PPT resulted in 100 % removal of phospholipids from 100 µL of rat plasma. In contrast, standard protein precipitation yielded high levels of phospholipid contamination which can potentially co-elute with analytes of interest or build up on the column and elute uncontrollably during a given injection sequence. This is especially problematic as analysts strive for shorter analytical run times through the use of smaller column dimensions and particle sizes.

■ **Comparison of HybridSPE™-PPT, Protein Precipitation and SPE**

In this application example, rat plasma samples were spiked with clenbuterol (R(-) and S(+)) enantiomers at the level of 10 ng/mL and extracted using three different procedures: HybridSPE™-PPT, Protein PPT, and a 9-step SPE procedure optimised for trace level clenbuterol analysis. The analysis was performed using a chiral stationary phase containing a macrocyclic glycopeptide covalently bound to silica and detection via MS-MS. Comparisons of sample preparation methods were made in terms of the amount of phospholipids in the sample extract and the overall effect on signal response of clenbuterol enantiomers. Absolute recovery was determined against an external standard.

Representative chromatograms of each of the sample prep techniques are depicted in Figure 5. From the results indicated in Figure 5, phospholipid contamination levels were highest for protein precipitation, resulting in signal suppression levels 70 and 25 % for the R(-) and S(+)) enantiomers of clenbuterol, respectively. For the SPE procedure, phospholipid contamination was still evident after multiple wash steps, and overall absolute recovery was less than 50 %. In contrast, HybridSPE™-PPT offered 100 % removal of phospholipids, resulting in absolute recovery levels of 95 %.



column: Chirobiotic T, 10 cm x 2.1 mm, 5 µm (12018AST)
instrument: Agilent 1100
mobile phase: 10 mM ammonium formate in methanol
temperature: 30 °C
flow rate: 0.3 mL/min.
detection: ABI 3200 QT; ESI(+), MRM: 184/104 m/z (phospholipids) and 277.2/203.1 (clenbuterol)
inj. vol.: 10 µL

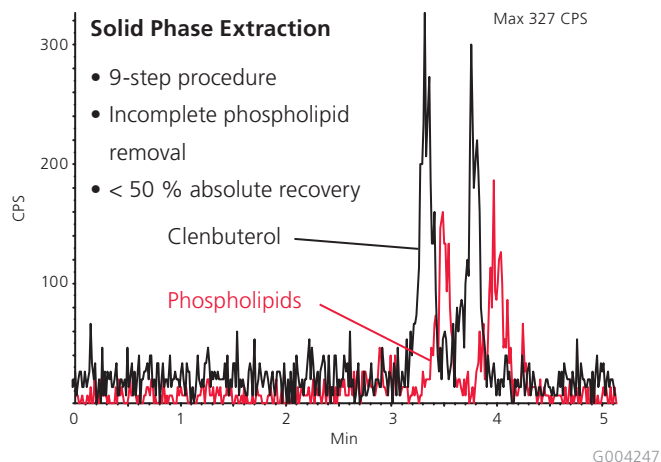
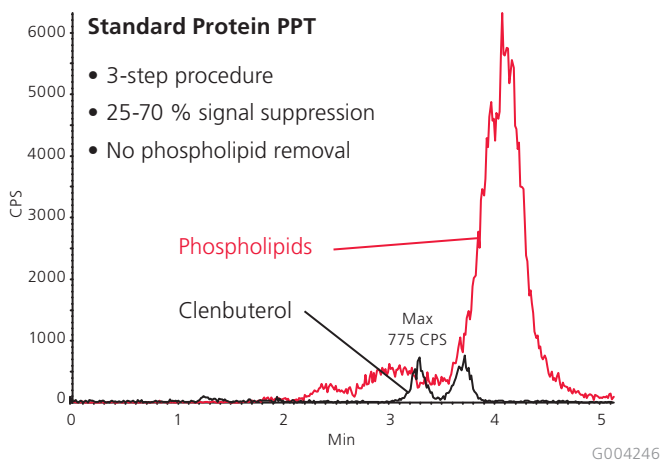
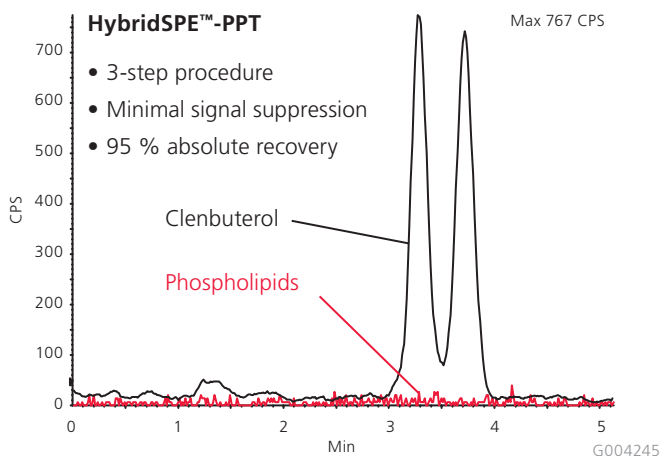


Figure 5: Comparative extraction and LC-MS-MS of 10 ng/mL Clenbuterol (R(-) and S(+)) enantiomers in rat plasma



Figure 6: HybridSPE™-PPT Plate and PlatePrep 96-well Vacuum Manifold

Conclusion

In this report, a new sample prep platform specifically designed for pharmaceutical bioanalysis was introduced. The technique, trademarked HybridSPE™-Precipitation or HybridSPE™-PPT, merges the simplicity of protein precipitation with the selectivity of SPE for the targeted removal of endogenous proteins and phospholipids from biological plasma for subsequent LC-MS analysis. Example applications demonstrate the chromatographic impact of phospholipids and how its presence can result in signal suppression during MS quantitation. When compared with traditional sample prep techniques such as protein precipitation and solid phase extraction for the extraction of clenbuterol enantiomers from rat plasma, HybridSPE™-PPT offered complete phospholipid removal, resulting in excellent recovery, minimal signal suppression and improved S/N ratios. In contrast, lower recovery and higher signal suppression was evident using the traditional sample prep techniques such as protein precipitation and solid phase extraction.

References

- 1] King et al., J Am Soc Mass Spectrom 11 (2000), 942–50.
- 2] Little et al., Journal of Chrom B, 833 (2006), 219–30.

Featured Products

Description	Cat. no.	Price £
HybridSPE™-Precipitation		
96-well Plate, 50 mg/well, pk. 1	575656-U	113.00
SPE Cartridges, 30 mg/1 mL, pk. 100	55261-U	98.00

Related Information

For more information on HybridSPE™-Precipitation technology, please visit sigma-aldrich.com/hybridspe-ppt

Mycobacteria – ongoing interest in an old pathogen

The genus *Mycobacterium* is known and dreaded as the causative agent of serious diseases like tuberculosis (*M. tuberculosis*) and leprosy (*M. leprae*).

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Mycobacterium avium complex (MAC) infection has gained notoriety recently as a significant cause of death in AIDS patients. After a period where *Mycobacterium*-related diseases were considered to be eradicated – at least in countries with high medical standards – the occurrence of multiresistant strains and a worrisome number of problematic infections in immunocompromised individuals have generated a new interest in research on this genus.

Mycobacteria are aerobic, often microaerophilic, and generally nonmotile bacteria that are characteristically acid-alcohol fast [1]. This is due to their distinctive hydrophobic cell wall, comprised of a thick layer of mycolic acid and outer lipids in addition to the normal peptidoglycan, which gives them considerable protection against acids, alkali and certain antibiotics that attack bacterial cell walls. Mycobacteria are classified acid-fast Gram-positive (because they lack an outer cell membrane), although they do not retain the crystal violet stain as typical Gram-positive bacteria do. Many mycobacteria can survive and grow in nutritionally poor environments such as water puddles and even chlorinated tap water. Other species like *M. leprae* are difficult to cultivate and seem to be obligate parasites.

Mycobacteria's exceptional hardiness and low nutritional demands are the principles of their isolation on such media as the Gruft-modified Loewenstein-Jensen medium (Table 1). The supplemented antibiotics are intended to eliminate all Gram-negative and normal Gram-positive germs and spare only the more resistant *Mycobacteria*. Appropriate staining methods include the procedures according to Ziel-Neelson or Kinyoun as well as the auramine fluorochrome method, all of which are available from Sigma-Aldrich (Table 2). The auramine fluorochrome is a specific stain for Acid Fast Bacilli (*Mycobacterium* sp.) in specimens and in culture. This fluorescent method, which is actually considered the best procedure, stains mycobacteria selectively by binding dye to the mycolic acid of the cell wall. The differentiation of the numerous species and subspecies has in the past been based on a variety of physiological tests [2], but molecular biological methods are gaining in importance [3].

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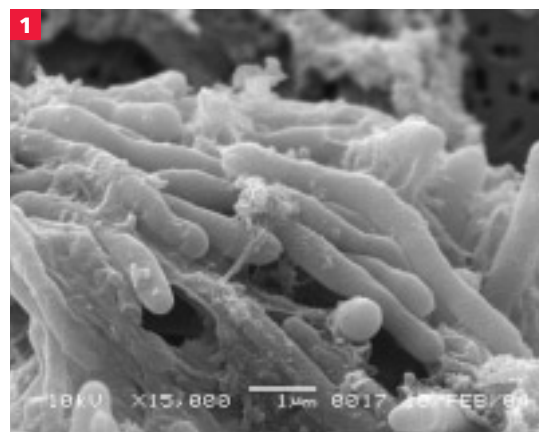


Figure 1: Mycobacterium (Scanning electron microscope image; Photo Mazen T. Saleh, Laurentian University)

Brand	Cat. no.	Media & supplements
Fluka	63237	TB-Medium Base according to Loewenstein-Jensen
Fluka	51803	Gruft Mycobacterial Supplement
Sigma	M0178	Middlebrook 7H9 Broth Base
Sigma	M0303	Middlebrook 7H10 Broth Base
Sigma	M0428	Middlebrook 7H11 Broth Base

Table 1: Media for detection, isolation, differentiation of mycobacteria

Brand	Cat. no.	Media & supplements
Fluka	21820	Carbol-Fuchsin solution according to Ziehl-Neelsen
Fluka	21819	Carbol-Fuchsin solution according to Kinyoun
Fluka	05151	Fluorescent Stain Kit for Mycobacteria
Fluka	56694	Acid Alcohol solution
Fluka	30503	Phenolic auramine solution
Fluka	81199	Potassium permanganate solution

Table 2: Fluka products for staining of mycobacteria

References:

- 1] Ryan, K. J.; Ray, C. G., eds. *Sherris Medical Microbiology*, 4th ed.; McGraw Hill: New York, 2004.
- 2] Koneman, E. W.; Allen, S. D.; Janda, W. M.; Schreckenberger, P. C.; Winn, W. C., Jr. *Diagnostic Microbiology*, 5th ed., Lipincott Williams & Wilkins: Philadelphia, 1997.
- 3] Parish, T. Making Sense of Mycobacteria. In *Mycobacteria: Molecular Biology and Virulence*; Ratledge, C., Dale, J., Eds.; Trends in Microbiology, 2000, 8 (5), p245.

Differentiation of Escherichia Coli from Coliforms

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In August 2008, the discovery of *E. coli*-contaminated beef in the United States prompted a nationwide recall of the meat. The source turned out to be one supplier that had a history of contamination of its beef products. The usual sources of *E. coli* in beef are faeces-contaminated animal carcasses, water supply, and/or other hygiene problems. Even in Switzerland, where drinking water is unusually pure, there are rare cases of faecal contamination by liquid manure. Detection is critical to maintaining hygiene.

E. coli is an aerobe, rod-shaped, motile, Gram-negative intestinal bacterium that ferments lactose and diverse other carbohydrates. Detection is possible because the bacterium ferments dextrose (D-glucose) by producing mixed acids (e.g. lactic, acetic and formic acids) that can then be made visible with the addition of the indicator methyl red. There are many other methods of detection to indicate the presence of *E. coli*. For instance, Voges and Proskauer found a test to detect acetoin and 2,3-butanediol produced when *Klebsiella* and *Enterobacter* ferment glucose. The researchers found that under alkaline conditions, these two compounds oxidise themselves into diacetyl. Diacetyl then reacts with creatine (a guanidine derivative) and appears as a pinkish-red compound, or it reacts with α -naphthol and appears cherry-red in colour.

Some other characteristic enzymes can also be detected by their interactions. Tryptophanase cleaves Tryptophan into pyruvate, indol, and ammonia; by using reagents (Kovac's and DMCA), researchers can detect indole production (Figure 1). β -Galactosidase is detected with ONPG (2-Nitrophenyl β -D-galactopyranoside), a chromogenic substrate that turns yellow after cleavage has occurred. Further, the ability to reduce nitrate to nitrite can be detected with the addition of sulphaniilic acid and β -naphthylamine, which results in a red precipitate (prontosil). Finally, lysine is degraded by *E. coli* to cadaverine by the lysine decarboxylase. Because this is an alkaline reaction, the indicator (bromocresol purple) will change colour from yellow to purple.



Figure 1: Kovac's indole reaction (from left to right: blank, negative, positive)



Figure 2: TSI Agar: From the left, we see the medium without organisms, followed by an extreme reaction in the butt of the tube and on the slant surface; the second tube from left shows the typical reaction when *E. coli* organisms are present.

Interesting differentiation results are obtained with the inoculation of TSI Agar slants. Due to the formation of acid during fermentation of lactose, sucrose and glucose, the pH level usually drops. However, in the case of oxidative decarboxylation of peptone alkaline products, the pH rises. This increase is indicated by phenol red, which changes colour in acidic surroundings from red-orange to yellow; upon alkalisation, it turns deep red. *E. coli* shows an acid reaction (yellow) and gas formation in the butt of the test tube and an acid reaction (yellow) on the slant surface.

An overview of the important biochemical reactions of *E. coli* is included in Table 1.

Biochemical test	Reaction
Catalase	+
Citrate utilisation (Simmon's citrate Agar, Fluka 85463)	-
TSI Agar (Fluka 44940)	AG/A
Gelatin liquefaction (Nutrient Gelatin, Fluka 70151)	-
Indole Production	+
Nitrate Reduction	+
Urease (Urea Broth, Fluka 51463; or Christensen's Urea Agar, Fluka 27048)	-
Voges-Proskaur	-
Methyl Red	+
Presumptive test (Lauryl sulphate Broth, Fluka 17349)	+
Phenylalanine deaminase (Phenylalanine Agar, Fluka 78052)	-
Motility (SIM Medium, Fluka 85438; or Tryptone Agar, Fluka 93655)	+
Lysine (LD Broth, Fluka 66304)	+
ONPG (β -galactosidase)	+
Oxidase	-

Table 1: Biochemical reactions of *E. coli*

Key: AG/A acid (yellow) and gas formation in butt of tube and acid (yellow) on slant surface

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

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