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PMF NEWSLETTER

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to the continued success of the *PMF Newsletter*

Feedback is Good

Scott Sutton, Ph.D.
Editor, PMF Newsletter

This has been an interesting month. For starters, we have been looking forward to publishing Russ Nyberg's essay on the verification of biological indicators. It is a unique perspective that Russ brings to the discussion as both a vendor and a testing laboratory expert in D-value determination. Secondly, the next installment of Rich Almond's 483/Warning Letter compilations appear in this issue, bringing us up to the beginning of 2006 (and 2006 has already seen some interesting regulatory action!).

One of the goals of this newsletter, as well as the PMFList (see page 7) is to stimulate discussion. Last month's article by Jeff Little certainly did that. In fact, we drafted Jeff and Michael Sherriff (of Qualicon) to write a follow-up article to expand on some of the points raised in that article. Grace Thornhill wrote in on this topic as well. From the newsletter perspective, it is sometimes a challenge to focus on the user at the bench rather than the vendor or the regulator. Grace pointed out that we had perhaps omitted some aspects of the RiboPrinter's daily use that are important to consider. She has graciously contributed her own perspectives on the use of the RiboPrinter.

I had promised to publish an essay this month on the difference between the USP interest in "absence of specified organisms" and the FDA interest in "absence of objectionable organisms." However at 15 pages, this issue is on the verge of becoming unwieldy. Look for that discussion next month.

As always, we welcome your input. Please write to us if you have a comment on an article in the newsletter, or would like to contribute to the publication.

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Follow-up to RiboPrinter Data Analysis Article of April, 2006

Jeff Little Vectech Pharmaceutical Consultants
Michael Sherriff DuPont Qualicon
Scott Sutton Vectech Pharmaceutical Consultants

In his article on data analysis that appeared in last month's newsletter, one of the authors provided an extensive discussion of the operation of the RiboPrinter and some potential issues in the analysis of the data. This article has generated a great deal of interest, and in fact sparked a separate handling of the topic by Dr. Grace Thornhill (in this issue). Based on reader comments on the article there seem to be a few points where clarification is needed.

First of all, it is important that the operator always pick a well-isolated colony from a plate streaked for isolation. While we had thought this self-evident from basic good practices, apparently there are those concerned that a lab might be pressed for time and pick an "apparently pure" colony from a mixed plate. This is bad practice for traditional microbiology, genotypic techniques are not magic – bad practice remains bad practice. We apologize now if this statement causes problems in terms of turn-around time, materials usage or whatever rationale has been put in place to justify it. It is bad practice and should not be done for any identification methodology you are using. Secondly, it is important that the operator pick sufficient amount of colony(s) material to provide enough DNA for camera to see over the amount of the molecular weight standards. Dr. Thornhill provides an excellent discussion of the role of the molecular weight markers in her article.

The nature of the probe is also important. The RiboPrinter is fundamentally an automated Southern Blotting apparatus with a very sophisticated data analysis package. The nature of the probe is critical. The probe used is specific for the entire rRNA operon genes (16S, 23S, 5S, GluT RNA and spacer regions), rather than only the 16S region as implied in last month's article. This larger probe size provides the extra discriminatory power needed for the strain differentiation possible with the RiboPrinter.

A point of clarification is needed on the numbering conventions used to identify samples. There are three types of samples that are allowed for in the software – "S" (sample – the most commonly used designation), "M" (molecular weight marker), or "C" (QC control strain). These designations allow the operator to run controls on the batch (as described last month). It is this number that may eventually get used to identify a new RiboGroup (XXX-YYY-SN where X identified the specific RiboPrinter, Y is the batch ID number and N is the lane number). It should be noted here that the gel automatically runs 5 molecular weight markers to calibrate the batch. This is part of the standard operation, and the material supplied as part of the reagent kit for the batch. It is not under the control of the operator while running the RiboPrinter under validated conditions (see the accompanying article by Thornhill for more detail).

While not necessary, the use of a QC control strain in the batch may make sense from a GMP perspective, allowing a control on the entire process of lysis, DNA extraction, restriction endonuclease digestion of the DNA, separation of the fragments, hydridization and data analysis. If one or all 4 of the DuPont Qualicon supplied QC strains are used the acceptance criteria is a similarity index of 0.85 or higher. If the user chooses to run their own QC organism(s), then these QC sample(s) must have a defined acceptance criteria. Typically, a similarity index of 0.85 or higher is what most users set as their acceptance criteria if they are running user-defined QC organisms.

Now as for the 0.85 similarity index for identification, Dupont Qualicon's proprietary mathematical algorithm is not a percent probability or likelihood (used with phenotypic systems), rather a threshold. The identification algorithm is used to find the closest pattern in the DuPont Identification Database or in a Custom Identification Database. The method is the same for both types of library. When the system compares a sample pattern to the variety of strains within the database it is looking for matches that have a 0.85 or greater similarity index (based on pattern homology which factors in band positioning, band #, and band intensity) in order to assign a Genus species match. So an exact strain match is not the objective of the identification database screen, but rather a similar match (species level). Any match at or above 0.85 similarity is considered a high confidence automated ID (the threshold was set high so that the system would not mis-ID even closely related species) and thus a Genus species name is automatically assigned.

If no matching patterns (> or = 0.85 sim) are found within the ID database, the system will bring up the most similar patterns (below this 0.85 sim threshold) within the ID database

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DNA Fingerprinting: Basic Principles of Data Analysis

Grace Thornhill, Ph.D.
Technical Director
P3 Scientific

Introduction

Traditional ribotyping (DNA fingerprinting) is a molecular sub-typing method that was first used to study the genetic, taxonomic and evolutionary relationships between strains of microorganisms. It has emerged as a powerful tool to determine the relatedness of microorganisms and has been used by the clinical and food industries to characterize pathogens, detect outbreaks and contamination patterns as well as track a contaminant or pathogen to its source. Although ribotyping is not new to the food industry or to molecular epidemiologists it is new to the pharmaceutical industry. Even with the support of the FDA the adoption of genetics-based microbial identification methods has been slow. There are many hindrances to the adoption of new technology in the pharmaceutical industry, but lack of familiarity with the technology and the data is a prime reason. The introduction of DNA sequencing and automated ribotyping to the microbiology lab has required that some practioners of microbial identification stretch their technological sophistication especially when compared to the familiar phenotypic methods. As is true for any new technology, to fully utilize the capability of the RiboPrinter one must understand the scientific basis for the test as well as the data generated from the test.

Traditional, manual ribotyping methods had some drawbacks (labor intensive, lack of standardized methods, ease of performance and throughput) that, in the past, limited its use to the well-equipped clinical or research laboratory. (Pfaller and Hollis) Dupont Qualicon developed a fully automated ribotyping system, the RiboPrinter® Microbial Characterization system, which addressed the major drawbacks of manual ribotyping thus making the technology widely available. The basics of the test were describe in last month's newsletter (Little, 2006) and the follow-up in this issue.

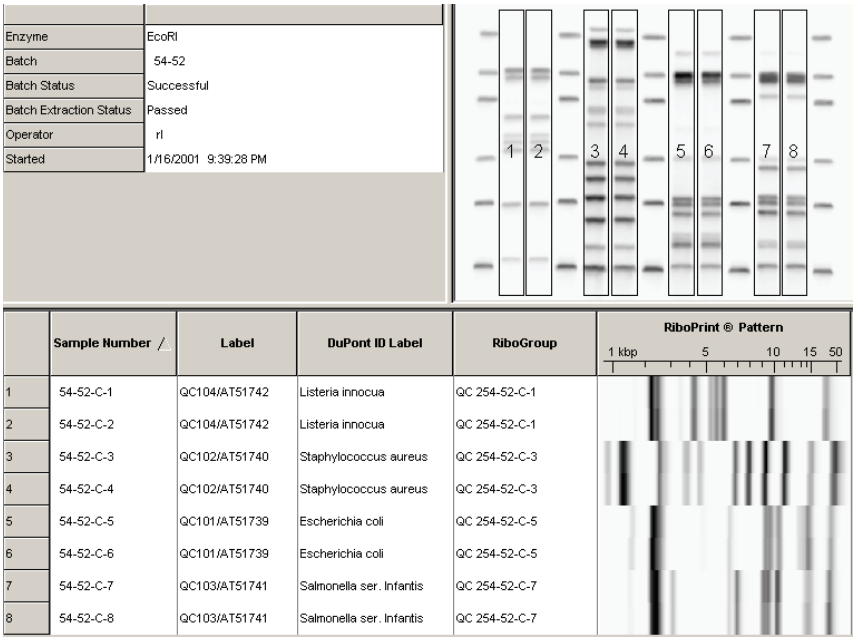
The goal of this article is to provide the reader with an overview of the basics of data analysis.

Background

Ribotyping is based on restriction fragment length polymorphism (RFLP) analysis of the highly conserved rRNA genes and is used to study the taxonomy of bacteria and other microorganisms. It uses a universal probe targeted at specific conserved domains of ribosomal RNA coding sequences. Southern blot hybridization methods generate DNA band patterns (DNA fingerprints) that can be compared with known species and strains of organisms to determine genetic and evolutionary relationships.

Data analysis

All samples are automatically identified, characterized and summarized in a batch report. (Fig 1).The RiboPrinter® system simultaneously provides a species level identification as well as a sub-species level characterization that is able to discriminate between clonally different strains. The various aspects of data analysis with respect to raw data, identification and characterization are discussed below with special attention given to common problems experienced by RiboPrinter® users.



Enzyme	EcoRI
Batch	54-52
Batch Status	Successful
Batch Extraction Status	Passed
Operator	rl
Started	1/16/2001 9:39:28 PM

	Sample Number /	Label	DuPont ID Label	RiboGroup	RiboPrint ® Pattern (1 kbp 5 10 15 50)
1	54-52-C-1	QC104/AT51742	Listeria innocua	QC 254-52-C-1	
2	54-52-C-2	QC104/AT51742	Listeria innocua	QC 254-52-C-1	
3	54-52-C-3	QC102/AT51740	Staphylococcus aureus	QC 254-52-C-3	
4	54-52-C-4	QC102/AT51740	Staphylococcus aureus	QC 254-52-C-3	
5	54-52-C-5	QC101/AT51739	Escherichia coli	QC 254-52-C-5	
6	54-52-C-6	QC101/AT51739	Escherichia coli	QC 254-52-C-5	
7	54-52-C-7	QC103/AT51741	Salmonella ser. Infantis	QC 254-52-C-7	
8	54-52-C-8	QC103/AT51741	Salmonella ser. Infantis	QC 254-52-C-7	

Figure 1. RiboPrinter System Batch Report.

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Biological Indicators and Third Party Verification
'Problems That Lay Ahead'

Russ Nyberg BS, BSEd, MAM
Director Tech Support/Biological Indicators
Raven Biological Laboratories

End users of Biological Indicators are sending out samples of BI's to Third Party Laboratories for both D-Value and population verification in a larger percentage than ever before. On an initial validation or on an annual revalidation, cycles are challenged with Biological Indicators to demonstrate actual microorganism lethality produced during the sterilization a cycle. These 'resistant microorganisms' must be tough enough to meet specific standard requirements as set out by AAMI, ISO or USP. For example, AAMI, ISO and USP all state that if a BI is being used for a Validation, the minimum acceptable D-Value or Resistance for that BI is 1.5 minutes for Steam Sterilization. Thus the need may be present for resistance verification prior to BI use. *Be prepared for the problems that may lie ahead.*

According to *USP User's Responsibility- "The user may consider conducting a D value assessment before acceptance of the lot. Laboratories that have the capability of performing D value assays could conduct a D value determination using one of the three methods cited in the general test chapter Biological Indicators- Resistance Performance Tests <55>and in the appropriate USP monographs for specific biological indicators."*[1]
It is important to note that the wording says ' *..may consider'* and '*Laboratories that have the capability… could conduct…'.* The *may consider* does not make this mandatory but many facilities have adopted the protocol of 'testing for resistance verification prior to acceptance'. In this case, it must now be done. Most facilities do not have the capability of conducting D value assessments so they send their BI samples out to independent laboratories that do have the ability to do D-Value verification. This is usually a very costly undertaking and costs can range from $1,500.00 to $2,500.00. One main area of concern is how competent is your Laboratory choice for accurately conducting D-Value verification according to Testing Standards? Just because the *service* is offered does not mean that either 'equipment used' or 'methods' are ISO, ANSI/AAMI, and USP compliant. It should also be the user's responsibility to verify that the contract Laboratory has an ISO, ANSI/AAMI compliant BIER Vessel and follows ISO, USP procedures without variation.

As part of resistance verification, the Lot of BI's being tested are expected to meet specific requirements as set out in ISO or USP as to the accuracy of the 'label claimed' Resistance or D-Value and population of the BI's. However, for verification, 'What is my *range of acceptance* going to be so that the verification can be considered acceptable'?

If one were expecting to comply with USP 28 Official Monograph/ Biological Indicator, *Resistance Performance Tests*- "*The requirements of the test are met if the determined D value is within 20% of the labeled D value for the selected sterilizing temperature and if the confidence limits of the estimate are within 10% of the determined D value."*[2] This acceptance range found on page 258 of USP is part of the Official Monograph. Need I be held to this tight Manufacturer's range since I'm not a manufacturer and I am the 'user'? I would be of the opinion that you do and acceptance needs to be within the + or – 20% acceptance criteria stated. If one considers verification and expects to have a 'USP Grade' BI and chooses to run one of the Test Methods cited in the Official Monograph, one also needs to accept the *acceptance criteria* of the method used. This would be the same for *total viable spore count.* If the USP method, *Total viable spore count,*[3] is used by the contract lab for determining population verification, along with the method used goes the acceptance criteria of not more than 300% and not less than 50% of the label claim.

To obtain a label claim D-Value verification, *USP <55> Resistance Performance Tests, ISO 11138 series*[4], allows for the use of three methods. One may use the **Most Probable Number** method by direct enumeration, a **Fraction Negative** method (such as Spearman/Karber) or assess the D-Value accuracy by using the USP **Survive/ Kill** calculated cycles. According to ISO 1138-1 "Sterilization of Healthcare Products Biological Indicator Systems, General Requirements" (Section 6.4.1 Resistance Characteristics) two of the three methods must be used for initial determination of D-value. Verification of D-value may be determined using only one. Regardless of which combination of the three methods used, one piece of equipment that will be needed is a **Resistometer.** A Resistometer, also known as a BIER Vessel (Biological Indicator-Evaluator Resistometer), is a piece of test

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Raw data

The raw batch image of the gel (Figure 2) should be visually inspected for the presence of artifacts that may impact the identification and characterization of the sample. Artifacts may include a bubble in the gel, narrowing or "coning" of a sample lane, smeared DNA due to the presence of extra-cellular polysaccharides, sheared DNA or uncut DNA due to over-loading. Underloading of a sample may generate a raw data pattern that is too faint to allow band detection; under these circumstances a RiboPrint™ pattern will not be generated. In many of these instances rerunning the sample is the best option. One should also compare the raw image pattern to the normalized RiboPrint™ pattern for the presence/absence of very light minor bands (ghost bands) resulting from light sample load, heavy sample load, and/or the presence of high in-lane background. The RiboPrinter® system software is able to correct for the presence of many of these artifacts and problems therefore sample reruns are often not necessary. Note that each sample lane is flanked by molecular weight marker controls. Each gel has 13 wells, eight wells for samples and five wells reserved for the molecular weight markers. Molecular weight markers are required for proper data processing and analysis and therefore are an integral part of the DNA preparation reagents supplied by Dupont Qualicon. In addition to these controls, it can also be useful to include a QC strain in one of the eight analysis lanes as a control on the overall process.

Identification

The identification algorithm compares incoming RiboPrint™ patterns to a static DUP ID library that consists of thousands of reference patterns. Using fragment size, number of bands and signal intensity the RiboPrinter® system uses a statistical match at or above a similarity index of 0.85 to assign a species level match. (Sistanich and Sherriff)

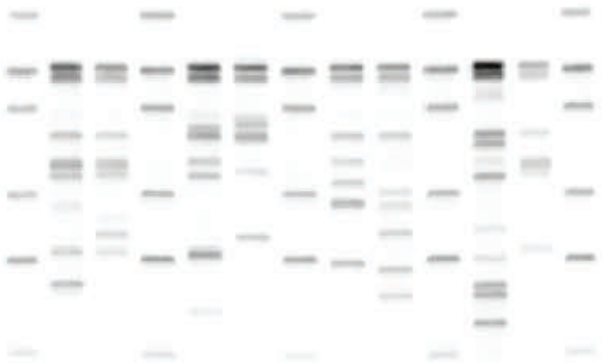

Figure 2. Raw batch image of gel. Note that each lane containing sample DNA is flanked by molecular weight markers run in lanes 1,4,7,10 and 13.

RiboPrint™ patterns whose similarity index falls below 0.85 will not be assigned an ID by the system. In these cases, the user has the option of performing a manual ID using Nearest Neighbors analysis (Fig. 3) The Nearest Neighbors window displays the five most similar patterns. Dupont Qualicon has published a guide (Dupont Qualicon. Guidelines for Pharmaceutical/GMP Applications: Manual Assignment of Taxonomic ID) which lays out seven circumstances under which manual ID's should be made. These guidelines are easy to incorporate into SOP's so that manual IDs are made in a "reasonable and documentable manner."

	Type	Number	Similarity	Label
1	DuPont Manual ID	(DUP-16744)	(0.84)	(Providencia rettgeri)
2	DuPont Neighbor	DUP-12056	0.77	Providencia rettgeri
3	DuPont Neighbor	DUP-13662	0.66	Pseudomonas oryzihabitans
4	DuPont Neighbor	DUP-18004	0.63	Pseudomonas aeruginosa
5	DuPont Neighbor	DUP-16381	0.62	Streptococcus oralis
6	RiboGroup	ECORI 260-44-S-3	1.00	

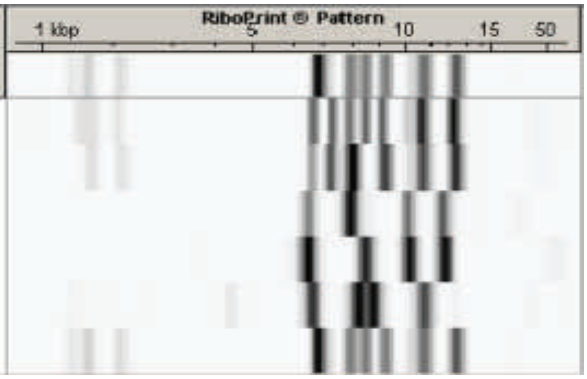

Figure 3. Automated Nearest Neighbor Search

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All manual IDs are bracketed indicating that the user has intervened in the identification process . There may be exceptions to the guidelines due to the fact that some Genus/species exhibit greater diversity than others, reinforcing the need for a competent microbiologist to exercise technical expertise in the identification.

Even with the aid of the Nearest Neighbors window it may not be possible to assign a manual ID. In these cases the strain can be identified using 16S RNA sequencing or other alternate technology. This externally generated ID can be entered into a Custom ID library; a dynamic user-generated library that may be comprised of proprietary strains or strains that were identified externally (Sistanich and Sherriff). All incoming RiboPrint™ patterns are compared to the separate DUP ID library as well as the users Custom ID libraries.

For manual ribotyping methods, the choice of restriction endonuclease used to cut the genomic DNA will affect the pattern and thus the identification and characterization of the sample. For automated ribotyping, Dupont Qualicon uses *EcoR1* as the primary enzyme. *Micrococcus, Kocuria, Pseudomonas, Salmonella and* streptomycetes requires the use of the enzyme *Pvu*II. Both *Eco*RI and *Pvu*II are provided with the RiboPrinter® system, however, users can customize their analyses by using a wide variety of commonly available restriction enzymes such as *Hind*III or*Bam*HI.

Characterization

Independent of the identification process, the RiboPrinter® system automatically characterizes each strain at the sub-strain level (molecular typing) by comparing each RiboPrint™ pattern to a RiboGroup Library – an historical library of all the strains run to date on a specific instrument(s). A ribogroup is a set of statistically identical patterns that are indistinguishable from one another by the system (Pfaller and Hollis). Strains that are sorted into an existing ribogroup must meet a similarity threshold of 0.90 or greater. A strain that does not meet the similarity threshold of 0.90 will be sorted into a newly created ribogroup. Each ribogroup is given a unique identifying number. Because identification is not required to sort a strain into a specific ribogroup, a strain may be monitored and tracked to its source even if a taxonomic identification is not available.

For some organisms, the discriminatory power of automated ribotyping using *Eco*RI is comparable to that of pulse-field gel electrophoresis (PFGE) (Pfaller and Hollis). Strains that fall into different ribogroups using *Eco*RI can, in most cases, be called different strains. For strains that fall into the same ribogroup, the level of discrimination using one enzyme may not be sufficient to discriminate between the ribogroup member strains. It is highly recommended that strains that fall into the same ribogroup be analyzed with at least one additional enzyme to confirm strain identity among the isolates as shown below (Figure 4).

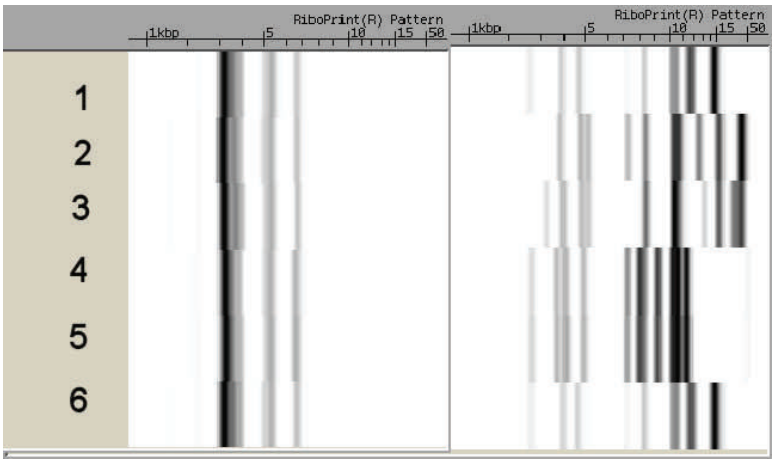

Figure 4. Strain analysis of *Paenibacillus glucanolyticus* (DUP 6046) using two restriction endonuclease enzymes - EcoRI on the left, and PvuII on the right.

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Internet Address	Description
FDA Bad Bug Page http://vm.cfsan.fda.gov/~mow/intro.html	The U.S. Food and Drug Administration's handbook which provides basic facts regarding food borne pathogenic microorganisms and natural toxins. Good information and a lot of good content.
Bugs in the News http://people.ku.edu/~jbrown/bugs.html	A great microbiology site. It contains a "What the Heck is...?", a general interest, and other bug bytes sections. Good information.
If you have found an Internet site that contains information of relevance to pharmaceutical microbiology, please let us know.	

Upcoming Events

May

- 3rd - 5th **Environmental Monitoring and Stability Technical Seminar & Workshop**
  Location: New York, NY
  WebSite: http://www.novaseminars.com/
- 18th - 19th **Microrite's Training in Contamination Monitoring and Control**
  Location: Crowne Plaza, Minneapolis North
  Email Contact: info@microrite.com
- 21st - 25th **ASM Annual Meeting**
  Location: Orange County Convention Center, Orlando, FL
  Web Site: http://gm.asm.org/

June

- 5th - 6th **PMF Microbiology GMP Conference**
  Location: Philadelphia, PA
  Web Site: http://www.highpeaks.us/2006/GMP/
- 26th - 29th **Aseptic Processes and Sterile Processes**
  Location: Amsterdam, The Netherlands
  Web Site: http://www.ivthome.com/shop/Scripts/prodList.asp?idcategory=2&sortField=STARTDATE

Offering In-House Courses on Microbiology/ Aseptic Processing:

- The Microbiology Network/High Peaks Associates http://www.highpeaks.us/in-house.htm
- USP
  Contact Steven Paul (stp@usp.org) for information on the course "Fundamentals of Microbiological Testing"

USP Corner

Any questions concerning USP documents should be sent to Radhakrishna (Radha) Tirumalai, Ph.D. You can reach Dr. Tirumalai at: (706) 353-4514, via mail at United States Pharmacopeia, 126 Twinbrook Parkway, Rockville, MD 20852 or via e-mail at RST@USP.org. You can write representing your company, or as an individual scientist.

483's December 2004 – December 2005

Richard Almond
Synthon Pharmaceuticals

Device

Failure to adequately validate manufacturing processes with a high degree of assurance, approve, and document the results of the validation activities to ensure that product specifications can be consistently met, as required by 21 CFR 820.75(a) [FDA-483 Item 3]. Your devices contact blood and are labeled as' sterile devices, but neither your firm nor the foreign contract sterilizer has validated and documented [redacted] the sterilization process. Further, your firm failed to ensure that certain significant manufacturing processes used by the foreign contract manufacturer are identified and validated or provide the justification for not conducting any manufacturing process validation. For example, in your May 23, 2005 e-mail to the foreign contract manufacturer, internal or external leaks appear to be critical defects identified for your devices, but specific manufacturing processes that could cause leaks were not identified, evaluated, and docu-

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Discussion List Update

PMFList:
Number of Subscribers: 1,514
Number of Countries: 62
Number of Messages Last Month: 241

PSDGList (Pharma Stability Discussion Group):
Number of Subscribers: 784
Number of Countries: 19

Membership is FREE. To **join the PMFList**, visit http://microbiol.org/pmflist.htm and register.

A sister Email is devoted to topics in the **stability testing** of pharmaceuticals, medical devices and personal products. To **join the PSDGList**, visit http://microbiol.org/psdglist.htm and register.

You can ask, answer, or read questions and comments from your colleagues. Archives of the lists are available at:

- http://lists.microbiol.org/archives/PMFLIST.html
- http://lists.microbiol.org/archives/PSDGLIST.html

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mented. Warning Letter to Estill Medical Technologies, Inc. August 2005

Failure of the Management Representative to ensure that quality system requirements are effectively established and effectively maintained in accordance with 21 CFR 820 as required by 21 CFR 820.20(b)(3)(i). Specifically, sterility procedures have not been updated in over 5 years; and chemical specification sheets, raw material certifications, and non-conforming material reports are not adequately reviewed.

Failure to establish and maintain procedures to adequately control environmental conditions that could adversely effect product quality, as required by 21 CFR 820.70(c). For example:

- Filling room operators have exposed facial skin surfaces.
- Product contact surfaces are only sanitized, not sterilized.
- Sterile forceps used to manipulate sterile container components were placed on exposed surge tank surface when not in use.
- Smoke studies were performed only under static conditions.
- Differential pressure between filling room and adjacent controlled environment is only monitored once daily.
- No documented action was taken when out of specification pressure differentials occurred.
- Surface monitoring procedures fail to provide quantitative assessment of the Class 100 and controlled environment surfaces.
- Action levels for the RCS air sampling and the purified water bioburden allow for multiple days of air sampling or multiple unlimited bioburden counts before corrective action is taken.
- There are no established personnel environmental monitoring specifications or limits for filling room operators.
- Four out of five Alert/Action Level Notifications issued from January 31, 2001, until the present were identified as alert notifications, although the results indicate all five met the criteria for action level notifications.

You failed to establish and maintain procedures for monitoring and control of process parameters for validated processes to ensure that the specified requirements continue to be met, as required 21 CFR 820.75(b). For example:

- The vent filters for the formulation/sterilization tank and the surge tank are ' not integrity tested before or after product filling operations.
- In-process product bioburden is not always assessed immediately prior to filtration sterilization.
- No specification has been established for product bioburden.
- The size of media fill runs performed for revalidation of the aseptic filling process is insufficient to simulate production conditions.
- Your acceptance criteria of no more than one microbial positive unit per 1000 units filled without determination and rectification of the cause allows for recurrent contamination.

Failure to establish and maintain procedures for finished device acceptance to ensure that each production run, lot, or batch of finished devices meets acceptance criteria, as required by 21 CFR 820.80(d). For example:

- Your sterility, bacteriostasis and fungistasis test methods reference and follow obsolete USP 23 methods. The current official USP methods are referenced in USP 28.
- Bacterial inoculum levels for stasis tests do not always meet the USP requirement of less than 100 colony forming units.
- Your procedure for Antimicrobial Preservative Effectiveness Tests, SOP No. 40-015-00, references and follows obsolete USP 23 methods.
- Your "Sterility Test: Membrane Filtration" procedure, dated 7/26/04 allows for additional testing following a failed initial sterility test.

Warning Letter to Lobob Laboratories, Inc. September 2005

Drug

Failure to have operations performed within specifically defined areas of adequate size as necessary to prevent contamination and to have operations relating to the manufacture, processing, and packing of penicillin and cephalosporin performed in facilities separate from those used for other drug products for human use [21 CFR 211.42(c) and (d)].

For example, penicillin products (amoxicillin and peni-

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cillin VK) and a cephalosporin product (cephalexin) were repacked using the same equipment and/or in the same production area of your facility as other non-penicillin products.

Cephalosporin products, like penicillin products, are categorized as beta-lactam drugs and present a health hazard to consumers with sensitivities to these compounds. Consequently, under 21 CFR 211.42(c) and (d), the Agency requires that the manufacture, processing. and packaging of beta-lactam drugs (e.g., penicillin and cephalosporin) be separate from non-beta-lactam drugs (e.g., ibuprofen). Pursuant to 21 CFR 211.42(d), penicillin and non-penicillin beta-lactam drugs must also be separate from each other. In this case, there is neither separation of the cephalosporin and penicillin drug products repackaging processes, nor separation between the beta-lactam and non-beta-lactam products.

The agency has taken the position that all three of those drug products should be separated from each other. In order to reach the goal of no cross contamination, a system-based approach towards separation should be taken. This entails a complete separation of every aspect of the manufacturing operation. Adequate separation should include physical barriers, air handling systems, personnel, and equipment with well established written procedures and controls. The separation should be verified by testing, auditing, and continuous monitoring if necessary. Warning Letter to Patient First Corporation, March 2005

Failure to assure all production and control records were reviewed and approved by the quality control unit to determine compliance with all established, approved, written procedures before the batch was released or distributed [21 CFR 211.192]. Specifically, the quality control review and approval is performed [redacted], prior to storing them at your firm's office in [redacted] and not after each transfilling at each home healthcare location prior to releasing finished Oxygen, USP. A comprehensive review of your production records dating back to May 12, 2004, when your firm began its operations, has revealed some prefill checks (such as the odor test), in-process checks (such as the heat of compression), and/or finished product tests (such as identity, purity, post-fill leak, and postfill odor) were not performed for over 35 lots of your Compressed Oxygen, USP, drug product. Warning letter to C&M Oxyfill LLC, May 2005

Your firm failed to establish and follow appropriate written procedures designed to prevent microbial contamination of drug products purporting to be sterile and to assure that such procedures include validation of sterilization processes, [21 CFR 211.113(b)] in that final product vials were subjected to the following manipulations during and subsequent to the aseptic fill operations.

- Sterility test samples were collected from stoppered final product vials by removing product from the vials with syringes. The vials from which samples were withdrawn were then subsequently released and distributed.
- Personnel were observed adding product from a graduated cylinder to underfilled final product vials and removing product with a syringe from over-filled final product vials.

Your firm failed to establish an adequate system for cleaning and disinfecting the room and equipment to produce aseptic conditions [21 CFR 211.42(c)(10)(v) and 600.11(a)]. For example your firm's cleaning validation studies demonstrate the selected cleaning agent is not effective on spore forming microorganisms. However, spore forming microorganisms have been detected in the environmental monitoring samples, personnel monitoring samples, and sterility test samples of final product.

Your firm failed to establish an adequate system for monitoring environmental conditions of aseptic processing areas [21 CFR 211.42(c)(10)(iv)]. For example:

- There is no documentation that monitoring covers all production shifts and is performed during active operations.
- There is no assurance that monitoring is at the locations where critical operations are performed.

Your firm failed to establish the control systems necessary for aseptic processing operations to prevent contamination [21 CFR 211.42(c)(10) and 600.11(a)]. For example, studies to determine airflow patterns have not been conducted in the sterile filtration room where aseptic connections are made. Warning letter to Greer Laboratories, June 2005

Sterility Complaint investigations failed to:

- Address whether fundamental equipment or processing design issues might be a cause. 21 CFR 211.198
- Discuss or extend to other batches that may have been associated with a failure. 21 CFR 211.198

Sterility complaint investigation reports failed to identify and discuss any possible correlation of the sterility test isolate with microorganisms found in your firm's environmental and personal monitoring. 21 CFR 211.198. Warning Letter, ACS Dobfar, July 2005

(Continued from page 6)

The RiboGroup library is a dynamic library that changes as more isolates are analyzed As bacterial strains naturally and rapidly evolve forming new strains they may split off one ribogroup, merge into another or form a new ribogroup. The system software can automatically perform these merging and splitting functions or a "No Merge/No Split" option is available for users that require GMP compliant documentation and audit trail functions. The "No Merge/No Split" option allows the user to manually refine (merge and split) ribogroups as necessary. It is highly recommended that the ribogroup library be regularly maintained by the user to avoid dilution of ribogroups with strains that should be split or merged into different ribogroups. Assignment of a new pattern to an existing or new ribogroup may also be affected by under/over loading of sample DNA resulting in a right or left pattern shift with respect to the reference pattern. This situation is easy to recognize with any impact on identification and characterization corrected by performance of a manual ID and/or manual refinement of ribogroups.

Conclusions

While automated ribotyping is highly standardized, interpretation of identification and characterization data still includes a subjective component. It is important to consider all of the available data when deciding if an identification is acceptable. Computer-assisted analysis, while a powerful tool, may not always provide the correct analysis (Pfaller and Hollis). Although it is not necessary, nor recommended, that one perform additional testing to verify every ID generated by the RiboPrinter®, in some cases it might be prudent to perform a gram stain, 16S RNA sequencing and/or biochemical tests to gather further information on the isolate in question.

Once the user learns the principles of riboprinting and the specifics of data analysis, riboprinting data analysis is as easy to interpret as the more familiar phenotypic methods. One is not left to fend for oneself in learning automated riboprinting; Dupont Qualicon offers basic and advanced training in data analysis as well as support in writing GMP compliant documentation.

It must be said that even in the face of powerful riboprinting technology users will still end up with a "no ID" result. As discouraging as this is it truly reflects the fact that we are working with living organisms that do not conform to our rules or expectations. The advantage of riboprinting over other ID systems is that one still generates a RiboPrint™ pattern that may be used to track and trend the isolate without benefit of a taxonomic ID.

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Acknowledgements
All figures were provided by Dupont Qualicon. Some were modified to fit the space available.

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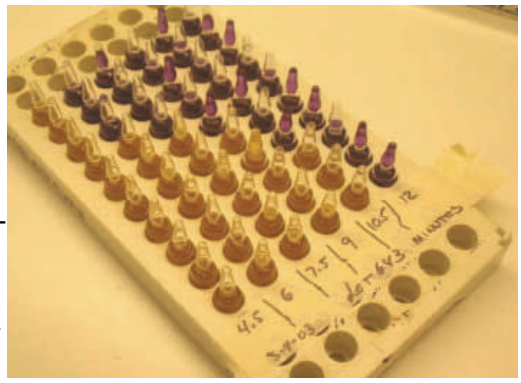
equipment that can very quickly and accurately deliver and control very precise sterilization process parameters that are critical to the process. Various Standards developed by ANSI/AAMI, ISO and USP have very tight equipment or BIER Vessel operational capabilities that must be met. As an example, ANSI/AAMI ST44:2002[5] states that with a Steam BIER Vessel the equipment must be capable of hitting the target temperature set point within 10 seconds or less from the time 'steam charge (100C)' occurs, must maintain that set temperature to within + or – 0.5C and then at cycle end, the post-vacuum time to reach atmospheric pressure must be within 10 seconds or less. ST44:2002 further states that the Steam Resistometer be capable of measuring such conditions as Time (resolution of 00:00:01 and accuracy within + or – 00:00:02), Temperature (resolution of 0.1C and accuracy of + or – 0.5C) and Pressure (range of 0 to 60psia, resolution of 0.1 and accuracy of + or – 0.5psia). The duration time of exposure at a given temperature is thus controlled as exactly as possible for both time and temperature.

One can note that on the pictured Steam BIER Vessel, manufactured by the Steris Corporation, the Vessel Chamber is fairly small as compared to an autoclave chamber. The small chamber is part of the vessel design that allows for an extremely fast steam charge and rapid increase in chamber temperature. Rather than the normal autoclave 'come-up' time needed to reach set temperature, a Steam BIER Vessel should be very capable of hitting set point for temperature in less than 10 seconds and in many cases closer to 6 seconds. This allows for the very exact 'exposure time' needed to determine BI resistance characteristics.

If one were performing a Fraction/Negative method of verifying BI resistance in such a BIER Vessel, one would expose multiple groups of 20 BI's to varying cycle exposure times.



In the example provided, self-contained ampoules were used. There are several manufacturers of such ampoules and most all ampoules are purple in color to start with. Bacterial Spores are suspended in the purple TSB. The color is from an added pH indicator. After exposure and incubation, if the spores in the ampoule survived the exposure and grow, the ampoule will turn a yellow color as an indication of growth. In the photo, one can see that all the ampoules exposed at a time exposure of 4.5 minutes survived and that at the exposure time of 12 minutes, all the BI's were killed and no signs of growth occurred. This gives us 4 exposures where a fraction of the ampoules were killed at each exposure, *a fraction were negative*. As the exposure time increased, more and more BI's were killed. With fractional information such as this, one can use the Limited Spearman-Karber method[6] to determine just how tough the BI's are or what their resistance is to this particular cycle.

Getting the above method accomplished in a BIER Vessel by a 3rd Party Laboratory for Resistance Verification may not look to be very difficult. Again, the main area of concern is 'How competent is the Laboratory you have chosen to perform the D-Value Assessment?' A proper D-Value Assessment is not as straight forward as one may think. There are numerous critical components involved in performing such a test. The following will provide just four areas of concern.

Equipment

Does the facility that you have contracted to do your D-Value Assessment have an ANSI/AAMI, ISO compliant BIER Vessel? This may seem like a moot point, but you would be surprised at the facilities that contract to do D-Value Assessments and do not actually have compliant Vessels. This is an area

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that one should look into prior to contracting with a 3rd Party Lab for work to be done.

Along with an instrument being capable of meeting the above abbreviated equipment specifications, is the instrument capable of accurately documenting that the conditions, phases, time and temperatures that occurred during the cycle actually occurred and were within specifications? Some 'Final D-Value Verification' reports are issued where Time, Temperature and Pressure are 'hand-written' notations on a sheet of paper and not actual Resistometer Data printouts. Where 'equipment' is nothing more than a well maintained pressure chamber where verification of critical cycle parameters simply can not be documented. Critical factors such as prevac depth, come-up time and post-vac may not even be part of the report data. **The final result report from your contract testing laboratory should include documentation to provide evidence that all critical cycle parameters were met.**

Test Method Used

In an effort to reduce variables and allow for the closest duplication of the BI Manufacturers procedure for D-Value certification, it is important that the same Test Method be used for D-Value Verification. If the BI Manufacturers BI Certification states that Direct Enumeration Method was used to establish that BI's resistance, then the method that should be used for Verification would be Direct Enumeration (Survivor Curve). Using different methods for D-Value Certification and for Assessment only allows for the introduction of additional variables that could affect D-Value reproducibility. This strongly applies to situations where one is trying to reproduce the D-Value for verification. If only verification is intended, *USP* allows for the use of the Survive/Kill Method. This is pretty straight forward and will only involve two cycles being run. As per ISO 11138-1[7], one would expose a minimum of 50 replicate samples to each of the USP calculated Survive/Kill time cycles. All samples exposed to the Survive time must survive the exposure and all exposed to the Kill time must not show growth. Using this method may be much quicker and less expensive than running a full D-Value assessment and will provide additional information as to the consistency of a particular Lot of BI's performance.

Recovery Media

Different Brands and different Lots of both TSB and TSA may not have the same degree of 'ability to promote growth of injured spores'. Brands 'X', 'Y' and 'Z' of tryptic soy agar may all perform in a very similar manner for a majority of typical laboratory tasks in obtaining growth for culture streaks, slants, etc. with most common laboratory microorganisms. Quantifying the presence of remaining CFU's from a BI of *Geobacillus stearothermophilus* 'injured spores' is a different matter. Not all Brands or Lots of recovery media have equal ability to accurately promote the growth of such injured spores. Several articles have been published that demonstrate as much as a 'full Log difference' in the recoverability of injured spores when comparing one Brand of TSA to another Brand[8, 9, 10].

The table information shown (*Infection Control Today, January 2000)* demonstrates the variance that is possible in CFU recovery between two different Brands of TSA used in population assays of several different Lots of BI Spore Strips. *USP Total Viable Spore Count*[3] procedure was used to run population assay's on 6 different Lots of Spore Strips containing *Geobacillus stearothermophilus* spores. From the last two dilution tubes in the dilution series while performing the assay, 1ml aliquots were added to 6 separate Petri dishes. To three of the plates TSA Brand A was added and to the remaining three plates, TSA Brand B was added. The only variable in this exercise was the Brand of TSA used for the pour plates. One can easily see the extreme difference in the 'spore recovery ability' between the two Brands used. A D-Value result based upon Direct Enumeration Method using Brand A media for several fractional cycles would provide a much different result than one produced using Brand B media. If one were using Brand A media, one would get the impression of spore reduction occurring much quicker than with Brand B and thus a lower D-Value resulting calculation.

Table 1: The Comparitive CFU Results

Lot # of BIs Tested	Average CFUs (of triplicate plates) Recovered with TSA BRAND A	Average CFUs (of triplicate plates) Recovered with TSA BRAND B
1A	0.3 X 10^6	1.4 X 10^6
1B	0.1 X 10^6	1.0 X 10^6
2A	0.1 X 10^6	0.6 X 10^6
2B	0.1 X 10^6	0.7 X 10^6
3A	0.3 X 10^6	0.9 X 10^6
3B	0.7 X 10^6	1.4 X 10^6

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Technique and Lab Utensils/Personnel

Under this heading one can add a score of additional variables that would make D-Value assessment success even more difficult. Besides recent BIER Calibration, what about utensils such as lab pipettes or repeaters? Are these as well as incubators in good calibration or within acceptable specifications? As most would readily recognize, there can be a wide variability in accuracy between technicians doing serial dilutions or plating techniques. Are all BIER Cycles reviewed for compliance to specifications prior to acceptance? Concerning BI placement into and removal from the BIER Vessel chamber; is placement consistent from one run to another, are the BI's removed immediately upon cycle completion and upon cycle initiation, are the BI's quickly inserted with little warm-up occurring. Does the BI holding rack offer little to no protection to the BI's being tested as compared to the holding rack used by the Manufacturer? All these factors can add up to a test result well outside the accepted + or – 20% variation needed.

Areas addressed above focused on Steam Sterilization. If one is working with VHP or EO even more variables are introduced. Adding concentration, RH and sterilent dispersion factors make verification to within the tight range of + or – 20% even more difficult.

All in all, if one is contracting for a D-Value Verification, some problems may lay ahead. Even with all the possible variations mentioned, some Verifications are successful and well within the + or – 20% allowance. When this occurs and can be repeated with additional Lots of BI's, one can only assume that more than luck is taking place. Both the BI Manufacturer and the Testing Laboratory are running the resistance testing in a very similar manner and both are paying excellent attention to equipment function, test methods, calibration and maintenance.

With this in mind, when trying to verify a D-Value and problems occur, arrange so that the Verifying Lab and the BI Manufacturer start communication to help resolve the problem. Both the BI manufacturer and the contract lab want the verification to go well and should do all that is possible to help find what aspect of the testing is causing a difficulty is such exists. It is possible to get verification within the 20% area. Aspects such as differences in recovery media used, verification method used, equipment calibration, etc. could all be contributors to the problem. This should not be an 'I'm right and you are wrong' issue. Open communication between those directly involved can usually resolve the differences.

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that can be used to manually ID these patterns to the species level if the user so desires. All manual ID's that result from matches that are below this threshold are indicated by the presence of {brackets}. This functionality was built into the software to allow the user to the ability to make IDs on strains that show a bit more variation (either real genetic variation such as the presence of additional bands.....or cell load/ sample processing variation such as pattern intensity differences) than those in the database ...but that still show certain conserved bands that are demonstrated across a species...or Genus.

The system simultaneously compares every sample pattern generated to every other historical pattern that has been run on that system (or imported from any other system) and clusters all statistically identical patterns together in RiboGroups using a similarity threshold of 0.90. The characterization process is based upon the integration of a sample pattern into a Dynamic Recognition Library (**DRL** or **RL**) via a clustering algorithm.

There was some confusion over the use of the term "Internal Reference Library." Although it seemed a good idea at the time to distinguish this concept from the DuPont term "Custom ID Database," upon reflection this was ill-advised. The basic point is that the operator can develop their own identification database of RiboPrint™ patterns if the facility so desires. However, this addition needs to be under some control. The SOP on addition of new patterns should include some of the same safeguards that DuPont Qualicon uses to add new patterns. First of all, the RiboGroup RiboPrint™ pattern is actually the average (composite) of every sample present in the RiboGroup. It is this pattern that will get added to the Custom Identification Database when the user creates a CID (Custom Identification) pattern. Therefore, the user should supply at least three runs to the instrument, derived from batches run on separate days, from separate growths of the unknown organism. This will incorporate the maximum amount of variation into the newly created CID database RiboPrint™ pattern. As noted in last month's article, and in Dr. Thornhill's article, the ability to add patterns to the custom database is a powerful tool, especially when coupled with independent identification techniques for those patterns not in the validated DuPont database.

There was a statement in last month's article that "Differences in peak intensity may also be caused due to the concentration of probe used." This is a true statement. However, it is only applicable if you are supplying your own probe. There are clients who take advantage of the RiboPrinter as an automatic southern blot apparatus and do not use the Dupont reagents. They are not using the equipment necessarily for bacterial identification but to evaluate the presence or absence of a variety of genes of interest to them. For them, the probe concentration is not under GMP control and is a concern. However, this does not apply to the QC pharmaceutical microbiologist who is running the system under validated conditions.

As noted above, Dupont allows for the use of molecular weight markers in the numbering scheme. This is in addition to the automatic MW markers run with the RiboPrinter as part of it's standard operation. While possible, this operation may not be as useful as the inclusion of a QC organism in the batch, especially when you consider the costs involved.

There has been a great deal of discussion off-line about the manual over-ride aspect of the reporting capabilities. On one side we have had comments that the database is validated, and therefore there should be no overrides allowed. On the other side we have heard that the technician must have the flexibility to apply his technical expertise to the identification. For example, if the technician has performed the gram stain, knows the organism is a gram negative but the riboprint pattern most closely represents a gram positive organism the technician must have the flexibility to select a different microorganism from the list provided. DuPont has established a set of conditions on how to make that determination which is available to the end-user for downloading.

We have been gratified by the level of interest last month's article received. The opportunity to introduce genotypic identification techniques into the QC microbiology lab is an exciting one, but one that must be done with an appreciation for good microbiology practice. The principles of single colony isolation, good aseptic technique and good lab practices are not waived by the use of a new technology. Rather this poses a new challenge to both the operator and the supervisor to ensure that adequate controls are in place to ensure the integrity of data that will initially be unfamiliar to many. It is our hope that these three articles in the *PMF Newsletter* will help in that process.

Pharmaceutical Filtration: The Management of Organism Removal

Theodore H. Meltzer and Maik W. Jornitz

DHI Publications, 2006

Scott Sutton, Ph.D.
Vectech Pharmaceutical Consultants

What a pleasant surprise this book was! Not that I expected a work by Drs Meltzer and Jornitz to be anything other that top quality (the authors have collaborated on other books for DHI Publishers). However, to be honest I sometimes find writing on filtration to be somewhat heavy going. This newly published book from DHI is, however, very readable and a solid source of information and insight.

Now, having said that I also have to say that I am always favorably impressed when the author of any book details the role microbiology plays in the process. This book, as the title implies, is fundamentally an in-depth review of the theory and practice of filtration as a means of bioburden reduction in the pharmaceutical industry. However it extends this discussion to the related topics of water microbiology, biofilms, pipe treatment methods and the mechanism of filtration by various means.

The chapter titles provide good idea of the breadth of topics covered:

1. The "Sterilizing" Filter
2. Casting-Process Membranes
3. Membrane Pore Structure
4. Depth-Filter Structure
5. Prefilters and Flow Decay
6. Particles/Organisms
7. Biofilms
8. The Fluid Vehicle
9. The Operational Conditions
10. The Polymer Matrix
11. The Challenge Density
12. Organism Size Alterations
13. Grow-through and Penetration
14. The Air Vent Filter
15. Multifilter Arrangements
16. Cartridge Type Constructions
17. The Stainless Steels and Rouging
18. Passivation and Electropolishing
19. Polymeric Conditions
20. Cartridge Handling
21. Mechanism of Particle Retention
22. Mathematical Modeling of Filter Blockage
23. Adsorption Bonding
24. Electrical Double Layer
25. Hydrophobic Adsorptions

As the chapter titles document, "Pharmaceutical Filtration: The Management of Organism Removal" is a broad treatment of filtration and microbial contamination in pharmaceutical processing and water treatment. The authors are uniquely positioned in terms of experience and training to provide a complete view of the range of topics that touch on pharmaceutical filtration.

The authors did a great job of discussing several topics of particular interest to me. The first was the controversy over the question of 0.22 um sterilizing filters *vs.* 0.1 um rated filters. Dr. Meltzer has lectured frequently on this controversy, and this discussion is informative reading no matter which side of the debate you support. The advantages of enhanced filtration, the disadvantages of reduced flow rate and uncertain ratings, and a realistic appraisal of the need are all addressed.

Another discussion I found particularly interesting involved the mechanism of filtration. While we generally acknowledge that the filter pore size rating is a critical measurement, few understand exactly how that rating is determined. The role of adsorption and secondary interactions is not a revelation (anyone who has tried to filter a BAC-containing solution is familiar with this problem) but the comprehensive nature of the review was extremely useful.

Finally, I especially enjoyed the chapter on challenge density, and the discussion of the questionable regulatory impositions currently placed on products sterilized by filtration. While I understand in theory the concern over this product type, years of practical experience has demonstrated the industry's ability to manufacture using this process. The requirement that the pre-filtration bioburden should not exceed levels set for WFI is more than over-cautious, and as the authors point out is questionable from a scientific perspective.

"Pharmaceutical Filtration: The Management of Organism Removal" will be valuable to anyone associated with filtration in the pharmaceutical, medical device or personal products industries. While many chapters are written from a microbiological perspective, process engineers will also find much of interest in it, as will regulatory affairs professionals and QA reviewers. This book may be the only book most of us will really need to have on filtration and its role in manufacturing.