PMF NEWSLETTER

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Editor's Message	1
Exactly….How Many! Real-Time Quantitative Polymerase Chain Reaction (qPCR) - Janet Barletta	2
Articles of Interest: 2006 - Scott Sutton	3
Upcoming Events	9
Discussion List Update	9
The Open Conference Was a Great Success!	14
PMF's GMP in Microbiology Conference	20

How to Count?

The debate continues. An earlier newsletter looked at some of the issues inherent in the Plate Count method of enumerations. Janet Barletta presents a completely different method in this issue. The technique of the polymerase chain reaction (PCR) can be used in a quantitative manner. This qPCR technology is well-established in the research fields, and in this month's issue Dr. Barletta argues for its adoption in the pharmaceutical arena as well.

Important Links:

Information on the PMFList at
http://www.microbiol.org/pmflist.htm

Past Issues of the *PMF Newsletter* at
http://www.microbiologyforum.org/news.htm

This month also sees a listing of articles from 2006 that might be of interest. As stated in the introduction to the article, this is not a comprehensive list - if your article was omitted please accept my apologies. Be sure to send me a copy of any article you publish in 2007 and I will get it in next year's listing.

We also have a few meetings to talk about. The 2007 Open Conference was very well attended and successful. This will, in one form or another, become a staple for the PMF. Next month we have the GMP in Microbiology Conference, followed in May by a Validation conference and in June by a Cosmetic Microbiology conference. These conferences are provided by request and notice of interest by our membership. If you want a particular topic developed, or want to develop one, please let us know.

I hope you enjoy the issue, and that we have an opportunity to hear from you soon.

Scott Sutton scott.sutton@microbiol.org

Exactly….How Many!
Real-Time Quantitative Polymerase Chain Reaction (qPCR) in the Microbiology Laboratory

Janet Barletta
Peer Consultants, P.C.

Variation inherent in the microbiological enumeration of CFU as described by Dr. Scott Sutton in his article "How Many" (Sutton, 2006) is entertaining as much as it is pathetically true.

Everything Sutton states (with only minor definitional explanations/ expansions about the dilution range) is logical, rational, and scientifically valid. There can be no argument that no matter what method of colony counting is used, the method may only be defined as "semi-quantitative" with high variability. Variation in the procedure begins with an "estimation" of the original stock microbial culture count using turbidity by the McFarland standard with verification by plating, continues with pipetting dilutions of the stock culture of that "estimated" count, and is completed by the visual estimation of counting colonies on a plate where organisms may (or may not) have proliferated, depending on the media, method of sample collection, culture conditions, inhibition by, or adhesion with, other organisms…etc. This method of colony count estimation (particularly for low counts) can never be considered accurate or reproducible by scientific definition and leads one to the question:

> "How can acceptance criteria for collected data be enforced when these data are generated using a method only capable of producing highly variable data points?"

The answer to these persistent problems of quantification of the microbial CFU is the quantitative polymerase chain reaction (qPCR).

Why is the qPCR method the Gold-Standard of Biological Quantification?

The PCR method was first described Dr. Kary Mullis in the mid-1980's (Saiki et al., 1985), and Mullis was quickly awarded the Nobel Prize in Chemistry in 1993 for the discovery. Since the original publication of the method in the literature in 1985, the number of publications with PCR as either the subject heading or major subject heading total 280,000. By 1993, conventional PCR was already an essential, universally accepted tool, which displaced identification based on pathogen **phenotype** rather than **genotype**. PCR (and minor variations of the method) have been well-described and repeatedly validated in the scientific literature hundreds of thousands of times (this estimate is without exaggeration).

The development of real-time qPCR (a refinement of PCR) in 1992 (Higuchi et al., 1992) eliminated the problem of "end-point" quantification inherent in the PCR method, where the final molecular quantification is determined after the completion of the reaction and the products are analyzed by gel electrophoresis. With the development of qPCR, highly efficient detection chemistries, sensitive instrumentation, and optimized assays are able to determine the number of molecules of a particular DNA sequence in a complex sample with unprecedented accuracy and sensitivity sufficient to detect a single molecule.

The widespread use of qPCR in the field of molecular diagnostics has advanced to the point where it is unconditionally accepted as the "gold-standard" for the absolute quantification of nucleic acids in the scientific literature. There is no point to argue this statement. It is a well-established, repeatedly validated fact supported by sound data, published in the scientific literature over the last 21 years (since it's inception)! (Kubista et al., 2006; Mackay IM, 2004).

With this degree of repeated utilization and validation of the QPCR method, there is no rational reason why microbiology laboratories in the pharmaceutical industry are not utilizing these methods to the level and extent academia (and the rest of the scientific world) are using them…now 21 years later.

Real-time qPCR (The Method and its Development from Conventional PCR)
Conventional PCR:

Conventional PCR is performed on a nucleic acid template (typically DNA, but it can be RNA using reverse-transcriptase qPCR: RT-qPCR) which may be single or double-stranded. Also needed are two oligonucleotide primers (small single-stranded pieces of DNA) that flank a target DNA sequence. A specific (known) sequence may be targeted if one is interested in detecting specific viruses/ organisms. But a degenerate (or slightly non-specific) sequence may be targeted to detect a family, class, or genera of viruses/organisms. The reaction is performed by temperature cycling where the target is denatured at a high temperature (95^{o} C) into two individual strands and/or secondary structure (if single-stranded) is minimized. The oligonucleotide primers may now easily hybridize (at a lower temperature: 55-60^{0} C) to the complementary sequence on the target molecule. After hybridization of the primers to the target sequence, the DNA polymerase (Taq polymerase: an enzyme used for its special properties of endurance to high temperatures for long periods of time) moves in to polymerize (synthesize) the DNA strand between the two primers. A new (second) DNA strand is thus synthesized and the cycle is

(Continued on page 5)

Articles of Interest—2006

The following is a listing of some of the articles I found of interest in 2006. This is not a comprehensive listing, and if I left off one of yours that you are particularly fond of, send it to me!

Scott Sutton

Antibiotics

Andersson, DI. 2006. The Biological Cost of Mutational Antibiotic Resistance: Any Practical Conclusions?. Curr Opinion Microbiol. 9:461-465.

Cameron, NL et al. 2006. Bacteria Commonly Isolated from Keratitis Specimens Retain Antibiotic Susceptibility to Fluoroquinolones and Gentamicin Plus Cephalothin. Clin Exp Ophthalmol. 34:44-50.

Connolly, J. 2006. Emerging Resistance. Food Quality. Dec/Jan:49-53.

Dixon, B. 2006. Sulfa's True Significance. Microbe. 1 (11):500-501.

EP. 2006. 2.7.2 Microbiological Assay of Antibiotics. Pharm Eur. 5.0:188-194.

Falagas, M./Bliziotis, I. 2006. Quinolones for Treatment of Human Brucellosis: Critical Review of the Evidence from Microbiological and Clinical Studies. Antimicrob Agents Chemother. 50(1):22 - 33.

FDA. 2006. New Antimicrobial Agents Approved by the U.S. Food and Drug Administration in 2005 and New Indications for Previously Approved Agents. Antimicrob Agents Chemother. 50(5):1912.

Hurd, H. 2006. Assessing Risks to Human Health from Antibiotic Use in Food Animals. Microbe. 1(3):115 - 119.

Khachatryan, AR et al. 2006. Use of a Nonmedicated Dietary Supplement Correlates with Increased Prevalence of Streptomycin-Sulfa-Tetracycline-Resistant Escherichia coli on a Dairy Farm. Appl Environ Microbiol. 72(7):4583-4588.

Nascimento, JS et al. 2006. Bacteriocins as Alternative agents for Control of Multiresistant Staphylococcal Strains. Lett Appl Microbiol. 42:215-221.

Prasad, T et al. 2006. Unexpected Link between Iron and Drug Resistance of Candida spp.: Iron Depletion Enhances Membrane Fluidity and Drug Diffusion, Leading to Drug-Susceptible Cells. Antimicrob Agents Chemother. 50(11):3597-3606.

Stix, G. 2006. An Antibiotic Resistance Fighter. Sci American. Apr:80-83.

Aseptic Processing

Amin, A and AK Bansal. 2006. Formulation Development for Sterile Liquid Products in Blow-Fill-Seal Packs. Pharm Technol. 30(10):142-154.

Akers, J. 2006. The Proper Role of Environmental Monitoring in Aseptic Processing. Amer Pharm Rev. 9(4):24-28.

ASHP. 2006. ASHP Guidelines on Quality Assurance for Pharmacy-Prepared Sterile Products. Guidance.

Austin, P and S Dixson. 2006. Hub Fluid Does not Increase Microbiological Contamination of Prepared and Stored Syringes. Pharm J. 276:47-49.

Battistini, M. 2006. The Role of Glasses in Aseptic Production. Pharm Technol. 30(10):136-140.

Berger-Preiß, E., et al. 2006. Aircraft Disinsection: Exposure Assessment and Evaluation of a New Pre-Embarkation Method. Int J Hyg Environ Health. 209:41 - 56.

Buddemeyer, J. 2006. Gelatin Filters Usher Better Blow Fill/Seal Technology. Contamination Control. Summer:35-36.

Carter, J. 2006. Getting It Right. Contamination Control. Spring:36-38.

Cobo, F et al. 2006. Environmental Monitoring in Stem Cell Banks. Appl Microbiol Biotechnol. 70:651-662.

Corcoan, BM et al. 2006. Enhanced Survival of GroESL-

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(Continued from page 3)

Overproducing Lactobacillus paracasei NFBC 338 under Stressful Conditions Induced by Drying. Appl Environ Microbiol. 72(7):5104-5107.

Doshna, S. 2006. Media Fills: Special Considerations for Clinical Manufacturing. Amer Pharm Rev. 9(6):50-55.

Dutton, RL and JS Fox. 2006. Robotic Processing in Barrier-Isolator Environments: A Life Cycle Cost Approach. Pharma Engin. 26(5)

Elinski, D. 2006. EM of Manufacturing and Storage Areas for Non-Sterile Oral Solid Drugs: A Survey. PDA Letter. 17(7):8-11.

Hodges, LR et al. 2006. Evaluation of a Macrofoam Swab Protocol for the Recovery of Bacillus anthracis Spores from a Steel Surface. Appl Environ Microbiol. 72 (6):4429-4430.

Hubbell, A. 2006. A process-based Miodel for a biopharmaceutical Environmental Monitoring Program. Amer Pharm Rev. 9(6):72-83.

ISO. 2006. ISO/DIS 13408-1 Aseptic processing of health care products — Part 1: General requirements. DRAFT Guidance.

Mach, CJ. 2006. Do You Know What's in the Air?. Contamination Control. Summer:39-40.

Mach, CJ. 2006. Do You Know What's in the Air?. Pharm Formul & Quality. June/July:54-55.

O'Hagan, K. 2006. Evaluating and Implementing; Blow-Fill-Seal Equipment for Aseptic Liquid Filling. Amer Pharm Rev. 9(6):78-83.

PDA. 2006. PDA Tech Report # 28 (Revised) Process Simulation Testing for Sterile Bulk Pharmaceutical Chemicals.

Poisson, P, et al. 2006. Challenges to a Blow/Fill/Seal Process with Airborne Microorganisms having Different Resistances to Dry Heat. PDA J Pharm Sci Tech. 60(5):323-330.

Polen, M. 2006. Particle Monitoring in Phamaceutical Cleanrooms. Contamination Control. Spring:43-47.

Salamán-Byron, AL. 2006. Implementation of Microbial Environmental Monitoring Program for Non-Aseptic Pharmaceutical Processes. Amer Pharm Rev. 9(5):10-15.

Samavedam, R, e tal. 2006. Implmentation of Disposables: Validation and Other Considerations. Amer Pharm Rev. 9(6):46-51.

Smolinski, E. 2006. Mold Mysteries. Contamination Control. Winter:34-35.

(Continued on page 6)

Internet Address	Description
http://www.microbiologytext.com/	An electronic microbiology textbook: *Through the microscope: a look at all things small*.
http://www.microbiologybytes.com/	Microbiology Bytes - A web site that has a large amount of information for training.
If you have found an Internet site that contains information of relevance to professional microbiology in the industrial sector, please let us know.	

(Continued from page 2)

repeated. This process results in a doubling of the PCR product (amplicon) from 1 molecule to 2, 2 molecules to 4, 4 molecules to 8….and so on. By the 30th cycle, optimized reagent and cycle conditions will produce the highest efficiency (typically 99-100%)…e.g., a billion copies are produced from one. When finely tuned, the entire process is complete in a maximum of 20 min to 3-4 hours (depending on the instrumentation and reaction vessels/containers). (You may view an animated version of the PCR reaction at:http://users.ugent.be/~avierstr/principles/pcrani.html).

Conventional PCR is typically only a qualitative assay for the same reasons that microbial colony counting is semi-quantitative…the **end-product** of the reaction is analyzed at the **end-point** of the reaction. This feature of conventional PCR (i.e., analysis of the reaction at end-point) constitutes one of the major differences between conventional PCR and real-time qPCR. This is because the starting number, or concentration of any chemical or molecule cannot be determined by analysis of the final products when the reaction products have accumulated in the final (or plateau) phase.

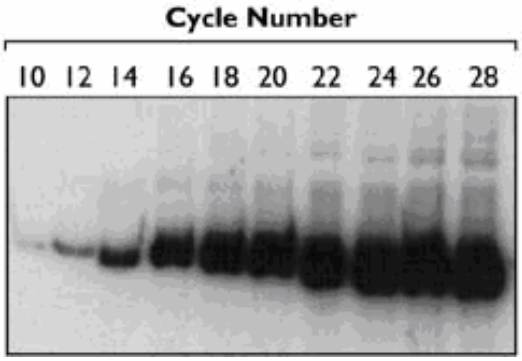
To accurately determine the starting number/ concentration of a molecule, a standard curve must be generated using data points taken during the reaction at the exponential phase of amplification when the reaction kinetics are linear. This approach ensures that the plateau phase is not used in the calculation. At plateau phase, growth or production lags. As the reaction slows down interfering factors accumulate, enzymes are depleted, substrates are expended, pH changes, and toxic end-products accumulate.

Real-time qPCR: There is a straight line (linear) relationship between the amount of new amplicon and the PCR cycle number (Fig. 1). For generation of the standard curve, the data points must be derived from this kinetic analysis. Real-time qPCR is a significant improvement to conventional PCR because amplicon production can be visualized as the amplification proceeds (during the test analysis). Data points are accumulated during the exponential phase (i.e., the optimal phase of amplicon synthesis or target DNA replication) where efficiency approaches 100%. Accurate determination of the microbial colony count, as well as the reaction efficiency cannot be determined when performing microbial CFU, because the determination of CFU is an **end-point** determination .

The monitoring of accumulating PCR product (amplicon) in real-time qPCR is possible by labeling either primers, probes, or amplicons with fluorescent detector molecules (fluorescent dyes or fluorophores). This topic alone would involve additional chapters for adequate discussion and will be simplified here by noting that the use of fluorophores as a signal molecule:

1. Is directly correlated to the amount of amplicon and increases proportionately during the exponential phase as the amount of amplicon increases;
2. is monitored by sensitive instrumentation (again material for another chapter) on a real-time basis as the

(Continued on page 11)



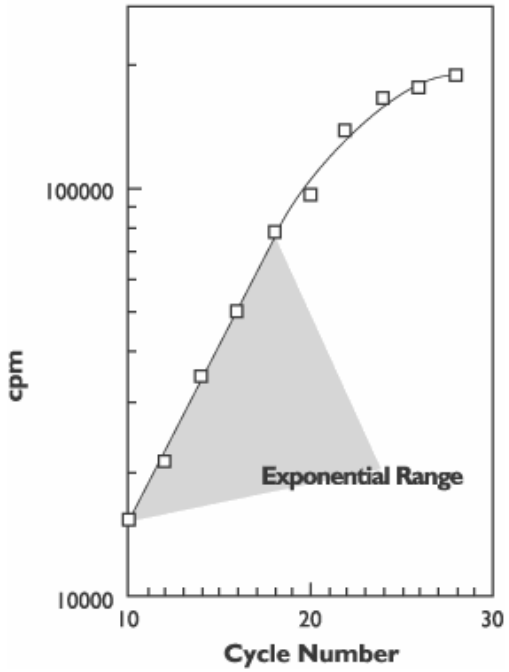


Fig. 1: Exponential Range of the qPCR Reaction:
A series of diluted DNA target templates were amplified by qPCR. Aliquots of PCR products from cycles 10-28 were resolved by gel electrophoresis. Cycle number is plotted against the log of the signal and a straight line is obtained for samples in the exponential phase of amplification. Note how it is impossible to discriminate the relative quantities of product from gel electrophoresis beginning at cycle 22 (which is beyond the linear region of the curve). RFU: relative fluorescence units. (Data reproduced from: Hunt, M. Real-time PCR. Microbiology and Immunology On-Line. University of S. Carolina School of Medicine. http://pathmicro. med.sc.edu/pcr/ realtime-home.htm).

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Soerenson, C. 2006. Monitoring Revisited. Pharm Formul & Quality. Dec/Jan:46-50.

Wherry, R. 2006. Blow-Fill-Seal (BFS) Sterility Assurance VaIidation (SAV) - Saving Time and Pain on Your SAV. Amer Pharm Rev. 9(4):56-60.

Yao, M and G Mainelis. 2006. Investigation of Cut-Off Sizes and Collection Efficiencies of Portable Microbial Samplers. Aerosol Sci Technol. 40:595-606.

Biofilm/Quorum-sensing

Azevedo, N., et al. 2006. Shear Stress, Temperature, and Inoculation Concentration Influence the Adhesion of Water-Stressed Helicobacter Pylori to Stainless Steel 304 and Polypropylene. Appl Environ Microbiol. 72 (4):2936 - 2941.

Banin, E et al. 2006. Chelator-Induced Dispersal and Killing of Pseudomonas aeruginosa Cells in a Biofilm. Appl Environ Microbiol. 72(3):2064-2069.

Barraud, N et al. 2006. Involvement of Nitric Oxide in Biofilm Dispersal of Pseudomonas aeruginosa. J Bacteriol. 186(21):7344-7353.

Bremer, P., et al. 2006. Laboratory Scale Clean-In-Place (CIP) Studies on the Effectiveness of Different Caustic and Acid Wash Steps on the Removal of Dairy Biofilms. Intl J Food Microbiol. 106:254 - 262.

Burmolle, M et al. 2006. Enhanced Biofilm Formation and Increased Resistance to Antimicrobial Agents and Bacterial Invasion Are Caused by Synergistic Interactions in Multispecies Biofilms. Appl Environ Microbiol. 72(6):3916-3923.

Burton, E et al. 2006. Antibiofilm Activity of GlmU Enzyme Inhibitors Against Catheter-Associated Uropathogens. Antimicrob Agents Chemother. 50(5):1835-1840.

Busscher, HJ and HC van der Mei. 2006. Microbial Adhesion in Flow Displacement Systems. Clin Microbiol Rev. 19(1):127-141.

Camilli, A and BL Bassler. 2006. Bacterial Small-Molecule Signaling Pathways. Science. 311:1113-1116.

Chambless, J., et al. 2006. A Three-Dimensional Computer Model of Four Hypothetical Mechanisms Protecting Biofilms from Antimicrobials. Appl Environ Microbiol. 72(3):2005 - 2013.

Choo, JH et al. 2006. Inhibition of Bacterial Quorum Sensing by Vanilla Extract. Lett Appl Microbiol. 42:637-647.

Dubern, J., et al. 2006. The ppul-rsaL-ppuR Quorum-Sensing System Regulates Biofilm Formation of Pseudomonas Putida PCL1445 by Controlling Biosynthesis of the Cyclic Lipopeptides Putisolvins I and II. J Bacteriol. 188(8):2898 - 2906.

Fuqua, C. 2006. The QscR Quorum-Sensing Regulon of Pseudomonas an Orphan Claims Its Identity. J Bact. 188 (9):3196-3171.

Gohl, O., et al. 2006. The Thin Pili of Acinetobacter sp. Strain BD413 Mediate Adhesion to Biotic and Abiotic Surfaces. Appl Environ Microbiol. 72(2):1394 - 1401.

Gonzalez, JE and ND Keshavan. 2006. Messing with Bacterial Quorum Sensing. Microbiol Mol Biol Rev. 70 (4):859-875.

Hallberg, K., et al. 2006. Macroscopic Streamer Growths in Acidic, Metal-Rich Mine Waters in North Wales Consist of Novel and Remarkably Simple Bacterial Communities. Appl Environ Microbiol. 72(3):2022 - 2030.

Hazan, Z et al. 2006. Effective Prevention of Microbial Biofilm Formation on Medical Devices by Low-Energy Surface Acoustic Waves. Antimicrob Agents Chemother. 50(12):4144-4152.

Huang, T, et al. 2006. Differential Biofilm Formation and Motility Associated with Lipopolysaccharide/

(Continued on page 7)

Exopolysaccharide-Coupled Biosynthetic Genes in Stenotrophomonas maltophilia. J Bacteriol. 188(8):3116 - 3120.

Kblankenship, JR and AP Mitchell. 2006. How to Build a Biofilm: a Fungal Perspective. Curr Opinion Microbiol. 9:588-594.

LaFleur, MD et al. 2006. Candida albicans Biofilms Produce Antifungal-Tolerant Persister Cells. Antimicrob Agents Chemother. 50(11):3839-3849.

Langenheder, S et al. 2006. Structure and Function of Bacterial Communities Emerging from Different Sources under Identical Conditions. Appl Environ Microbiol. 72(1):212-220.

Lequette, Y et al. 2006. A Distinct QscR Regulon in the Pseudomonas aeruginosa Quorum-Sensing Circuit. J Bact. 188(9):3365-3370.

Mangalappalli-Illathu, AK and DR Korber. 2006. Adaptive Resistance and Differential Protein Expression of Salmonella enterica Serovar Enteritidis Biofilms Exposed to Benzalkonium Chloride. Antimicrob Agents Chemother. 50 (11):3588-3596.

Nalca, Y et al. 2006. Quorum-Sensing Antagonistic Activities of Azithromycin in Pseudomonas aeruginosa PAO1: a Global Approach. Antimicrob Agents Chemother. 50(5):1680-1688.

Nilsson, L., et al. 2006. Elevated Shear Stress Protects Escherichia Coli Cells Adhering to Surfaces Via Catch Bonds from Detachment by Soluble Inhibitors. Appl Environ Microbiol. 72 (4):3005 - 3010.

Riedel, K., Köthe, M., et al. 2006. Computer-Aided Design of Agents That Inhibit the cep Quorum-Sensing System of Burkholderia Cenocepacia. Antimicrob Agents Chemother. 50(1):318 - 323.

Riedewald, F. 2006. Bacterial Adhesion to Surfaces: The Influence of Surface Roughness. PDA J Pharm Sci Tech. 60(3):164-171.

Romeo, T. 2006. When the Party Is Over: a Signal for Dispersal of Pseudomonas aeruginosa Biofilms. J Bacteriol. 188(21):7325-7327.

Ryan, RP et al. 2006. Cyclic Di-GMP Signaling in Bacteria: Recent Advances and New Puzzles. J Bacteriol. 188(24):8327-8334.

Surdeau, N., et al. 2006. Sensitivity of Bacterial Biofilms and Planktonic Cells to a New Antimicrobial Agent, Oxsil® 320N. J Hosp Infect. 62:487 - 493.

Touhami, A., Jericho, M., et al. 2006. Nanoscale Characterization and Determination of Adhesion Forces of Pseudomonas aeruginosa Pili by Using Atomic Force Microscopy. J Bacteriol. 188(2):370 - 377.

van Merode, A., et al. 2006. Influence of Culture Heterogeneity in Cell Surface Charge on Adhesion and Biofilm Formation by Enterococcus Faecalis. J Bacteriol. 188(7):2421 - 2426.

Wei, J-R et al. 2006. A Mobile Quorum-Sensing System in Serratia marcescens . J Bact. 188(4):1518-1525.

Wopperer, J., et al. 2006. A Quorum-Quenching Approach to Investigate the Conservation of Quorum-Sensing-Regulated Functions within the Burkholderia cepacia Complex. Appl Environ Microbiol. 72 (2):1579 - 1587.

Disinfectants/Biocides

Anon. 2006. SDA and CTFA Defend Antibacterial Products. HAPPI. 43(1):33.

Disinfectants/Biocides

Castillo, JA et l. 2006. Comparative study of the Antimicrobial Activity of bis(Na-caproyl-L-arginine)-1,3-propanediamine Dihydrochloride and Chlorhexidine Dihydrochloride Against Staphylococcus aureus and Escherichia coli. J Antimicrob Chemother. 57:691-698.

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Chelossi, E. and Faimali, M. 2006. Comparative Assessment of Antimicrobial Efficacy of New Potential Biocides for Treatment of Cooling and Ballast Waters. Sci Total Environ. 356:1 - 10.

Cords, BR. 2006. Hazard or Hype? Antimicrobials and the Rise of "Supergerms". Food Quality. June/ July:60-62.

Holah, JT and KE Hall. 2006. The Effect of an Antibacterial Washing-up Liquid in Reducing Dishwater Aerobic Plate Counts. Lett Appl Microbiol. 42:532-537.

Kratzer, C et al. 2006. Validation of AKACID Plus as a Room Disinfectant in the Hospital Setting. Appl Environ Microbiol. 72(6):3826-3831.

Martinez, J. 2006. What is Disinfectant Validation?. Pharm Tech. 30(3):72 - 80.

Mazzola, PG et al. 2006. Chemical Resistance of the Gram-negative Bacteria to Different Sanitizers in a Water Purification System. BMC Infectious Dis. 6:131-141.

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Simões, M., et al. 2006. Comparative Antibacterial Potential of Selected Aldehyde-Based Biocides and Surfactants Against Planktonic Pseudomonas Fluorescens. J Indust Microbiol Biotechnol. pp 1 - 9.
USP. 2006. <1072> Disinfectants and Antiseptics. USP 29. Suppl 2:3759-3765.
Weber, DJ and WA Rutala. 2006. Use of Germicides in the Home and the Healthcare Setting: Is There a Relationship Between Germicide Use and Antibiotic Resistance?. Infect Control Hosp Epidemiol. 27(10):1107-1119.

Finished Product Tests

Campana, R et al. 2006. Microbiological Study of Cosmetic Products During Their Use by Consumers: Health Risk And Efficacy of Preservative Systems. Lett Appl Microbiol. 43:301-306.
Chen, L. 2006. Reckoning Recombinant Factor C. Contamination Control. Spring:24-26.
Chen, L. 2006. Reckoning Recombinant Factor C. Pharm Formul & Quality. Dec/Jan:58-60.
CTFA. 2006. Midwest Chapter Investigates Accelerated Preservative Efficacy Testing. Cosmet Toilet. 121(5):14.
Daufenbach, LZ et al. 2006. Pyrogenic Reactions and Hemorrhage Associated With Intrinsic Exposure to Endotoxin-Contaminated Intravenous Solutions. Infect Control Hosp Epidemiol. 27(7):735-741.
Ebrahimi, M et al. 2006. Preliminary Analysis of the Factors Determining Bacterial Fragment Removal from Water of Different Commercial Ceramic and Polymeric Membranes. Desalination. 200:496-497.
EP. 2006. 2.6.1 Sterility. Pharm Eur. 5.0:145-149.
EP. 2006. 2.6.12 Microbiological Examination of Non-Sterile Products (Total Viable Aerobic Count). Pharm Eur. 5.0:154-156.
EP. 2006. 2.6.13 Microbiological Examination of Non-Sterile Products (Test for Specified Micro-Organisms). Pharm Eur. 5.0:156-161.
EP. 2006. 2.6.14 Bacterial Endotoxins. Pharm Eur. 5.0:161-168.
EP. 2006. 2.6.8 Pyrogens. Pharm Eur. 5.0:152.
EP. 2006. 5.1.3 Efficacy of Antimicrobial Preservation. Pharm Eur. 5.0:447-449.
EP. 2006. 5.1.4 Microbiological Quality of Pharmaceutical Preparations. Pharm Eur. 5:449.
Fox, C. 2006. An Antimicrobial Deodorant. Cosmet Toilet. 121(10):34-40.
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(Continued on page 10)

Upcoming Events

March

- 19th - 23rd **PDA Annual Meeting**
  Location: Las Vegas, NV, USA
  WebSite: www.pda.org

April

- April 1st - 4th **Annual Conference of the Association for General and Applied Microbiology (VAAM 2007)**
  Location: Osnabrück, Germany
  WebSite: www.conventus.de/vaam2007
- April 11th - 12th **AAI Compendial Sterility Testing: Techniques & Technology**
  Location: Wilmington, NC
  WebSite: http://www.aaipharma.com/sterility
- April 12th - 13th **PMF GMP in Microbiology Conference**
  Location: Philadelphia, PA
  WebSite: www.highpeaks.us/2007/GMP
- April 12th - 13th **Auditing Microbiological Aspects of Manufacturing in Pharmaceuticals and Biotechnology**
  Location: Foster City, CA
  WebSite: http://www.microrite.com/Auditing_Microbiological_Aspects_Foster_City.pdf

May

- May 14th—15th **PMF Microbial Validation Conference**
  Location: Forth Worth, TX
  WebSite: : www.highpeaks.us/2007/val
- May 22nd—25th **Annual ASM Meeting**
  Location: Toronto, Canada
  WebSite: www.asm.org

Offering In-House Courses on Microbiology/Aseptic Processing:

- The Microbiology Network/High Peaks Associates http://www.highpeaks.us/in-house.htm
  Experienced in custom-designed courses for the lab or the manufacturing facility, including:
  - GMP for the Microbiology Lab
  - Microbiology for Manufacturing
  - Microbiology for Management
  - Auditing the Microbiology Function
  - Investigating Microbiological Data Deviations
- 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.

Discussion List Update

PMFList:
Number of Subscribers: 2,282
Number of Countries: 64
Number of Messages Last Month: 243

PSDGList (Pharma Stability Discussion Group):
Number of Subscribers: 916
Number of Countries: 23

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
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The 2007 PMF GMP Conference Rapidly Approaching!

This conference looks at the basic requirements of compliance in a science-driven, technical function. The assumption is that all participants have some background in laboratory operations and are interested in developing practices and processes that will ensure success in the microbiology laboratory.

This conference met with great success last year, and we will again provide the text Quality Control Systems for the Microbiology Laboratory by Lucia Clontz text to all participants.

*Don't miss this **unique** conference - your chance to discuss GMP from the Microbiology Perspective!*

More information at http://www.highpeaks.us/

(Continued from page 5)

signal is being produced.

Real-time qPCR does not involve a need for post-reaction manipulations which introduce additional errors (i.e., pipetting errors, loading artifacts in gels, etc.). The reaction is set up in one tube which is then closed. The analysis takes place in this completely closed vessel which is never opened after the placement of all reagents into the qPCR reaction mix. One of the primary arguments against using PCR in the pharmaceutical laboratory is that the billions of amplified products will contaminate the environment. This cannot happen with real-time qPCR because the reaction tube is never opened again after the original reagents are added to the tube (unless sequencing of the products in another facility is required).

Validation of the qPCR Method
Conversion of Manual Counting Methods to qPCR

Validation of the qPCR method has now been performed and published in the scientific literature hundreds of thousands of times for various nucleic acid targets. What would validation of the qPCR method involve in the pharmaceutical microbiology laboratory?

1. Purchase of a PCR reagent kit (manufactured by hundreds of companies). The performance of the kit reagents are validated by the manufacturer using a standard curve (the quantification typically graphs over a dynamic range of 7 orders of magnitude). The testing laboratory must also perform a standard curve using the kit's reagents with the target molecule/organism.
2. Optimized design of sequence primers/probes. Thousands of primers/probes are already designed, validated and published for optimized performance for hundreds of viral and microbial targets (as well as the entire spectrum of eubacterial organisms). There is no reason to re-invent the wheel here! It is sufficient to either select primers/probes and cyling parameters from the scientific literature, or purchase primers/probes/reagents with validated, optimized cycling parameters from many available kit reagent manufacturers.
3. Purchase of a real-time thermal cycler. There are over a dozen models to choose from, ranging in price from 15K-100K. The performance and capabilities of each brand cycler vary minimally. Smaller models are not exorbitant in price and perform all of the data analysis functions as well as detect up to 4 different fluors. The difference in capabilities of the instruments are primarily for mixing multiple fluorescent dyes to derive a much wider range of absorbance/emission spectra in multiplex reactions for greater than 5 target templates.
4. Qualification of a real-time thermal cycler analytical instrument. These instruments are purchased with either service and maintenance contracts, or yearly calibration service may be purchased from the company. Real-time thermal cyclers are qualified by the manufacturer for installation and operation. Little to no maintenance other than occasional (monthly) cleaning of the plate wells is required. Calibration is performed with each run as a prerequisite for performance of the instrument. If the instrument does not internally calibrate successfully with each run, the run will not execute.

   Routine quality control only requires generation of a standard curve performed at the same time as the analysis. Data analysis is programmed by instrument software. Standard curves are interpreted by the software for linearity, efficiency, and quantification of starting molecules. Data documentation is electronically stored by the instrument, keeping manual documentation to a minimum (if any).
5. Validation would involve comparative testing of the target microorganism using both purified target template and the complete microorganism to establish the correlation of qPCR results for the DNA template of the target vs the microorganism.

   Thus, for validation of the qPCR method, two standard curves would be generated for comparison to the compendial method:

   a. **A standard curve using dilutions of exact copy numbers (from 10^6 copies to 10^0 copies) of the extracted nucleic acid template.** The exact amount of starting nucleic acid target is determined by OD (optical density) and copy numbers may be calculated from this quantity.

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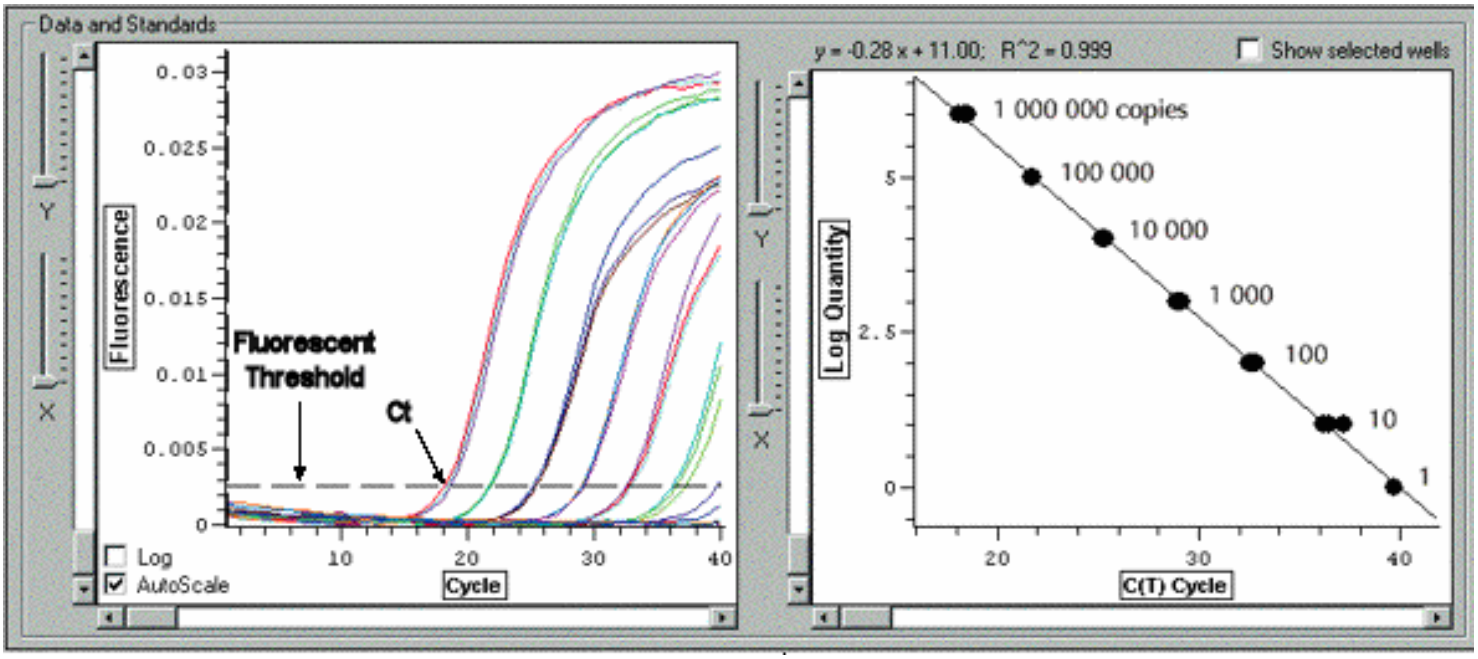
This standard curve is generated for validation in optimized reagents.

b. **A standard curve using dilutions of exact copy numbers (from 10^6 copies to 10^0 copies) of the extracted nucleic acid template spiked into the product sample.** This standard curve validation mimics the actual "real-life" organisms which may be found in the sample product.

Data analysis: The Ct is the cycle at which fluorescent signal detection occurs over background fluorescence (threshold), indicating a positive result (Fig. 2a). The mean Ct value obtained for each dilution is plotted against the $\log_{10}$ of the corresponding initial dilution concentration by the automated analysis software system to generate a standard curve. There is no interaction from the operator for the standard curve generation, nor for the determination of efficiency, correlation coefficient, or quantification of data points. The target quantity is calculated by the instrument software by extrapolation of the mean Ct value obtained on the standard curve (Fig. 2b).

1. **Efficiency**: The efficiency of the reaction is derived from the slope of the standard curve. If the PCR reaction represents exponential amplification of the template, and the efficiency is 100%, the amount of template doubles each cycle. 100% efficiency will generate a linear slope of –3.323. The standard accepted criteria for efficiency is a slope of -3.1 to -3.6 (90-110% efficiency).
2. **Correlation Coefficient:** The correlation coefficient

(Continued on page 13)



2a 2b

Figure 2a : Amplification plots (2a and 2b) showing the linearity of the 10-fold dilutions of target template as cycle numbers vs. fluorescence. (Threshold) indicates the background fluorescence above which a positive signal is detected. (Ct) indicates the cycle number at which a sample reaction crosses over the background fluorescent threshold. The Ct is plotted against the RFU (relative fluorescence units with subtracted baseline fluorescence).

Figure 2b: Dilutions of standard target template with copy numbers ranging from 1 to 1,000,000 were amplified by qPCR. A standard curve was generated by the real-time instrument from Ct values. The linear portion of the standard curve is used to measure unknown amounts of sample target by determining the copy number from the sample Ct. Note that the curve is linear across 7 orders of magnitude with a limit of detection of 1 copy of sample target.

(Data reproduced from: DyNAmo™ SYBRâ Green qPCR Kit with ROX™ Passive Reference Dye. Product Technical Data Sheet for Catalog #F400RS, F400RL).

(Continued from page 12)

(CC)..also described as "R-squared" defines precision…(e.g., how well the data points generated "fit" to the straight line that represents the slope of the standard curve). If the data lie perfectly along a straight line, then the CC will be 1.00 (a perfect line). The standard accepted criteria for CC is $\geq$ 0.985.

3. **Limit of Detection:** Determine the limit of detection (LOD) as the highest dilution (lowest concentration or copy number) of target organism detected by the instrument (Ct at which sample crosses background fluorescent threshold).
4. **Limit of Quantification:** Determine the limit of quantification (LOQ) as the highest dilution (lowest concentration or copy number) of target organism detected by the instrument within the **linear range** of detection. LOQ is a function of both efficiency and precision and defines **sensitivity** of the method.

The data shown in Figs. 2a, 2b are the evidence that the qPCR method displays highly accurate and precise quantitative estimation in contrast to data generated from the microbial colony count (CFU) method. One copy of the nucleic acid target is equivalent to one copy of the microorganism. The ability to equate a "living" organism to a chemical entity (by way of the nucleic acid molecule) eliminates the **"guessing"** property of colony count estimation. There is no "guessing" involved in qPCR analysis. It is, in fact, a highly accurate and precise Analytical Chemistry Method for quantification….where the substrate of the enzyme in the reaction is nucleic acid….which (in itself) defines the presence of life.

qPCR is clearly the equivalent of the analytical quantification of a chemical or protein target using linear regression with extrapolation from a standard curve, and as such, eliminates the variability inherent in the quantification of any form (i.e., microbial colonies) of biological life.

In "How Many" (Volume 12 of the PMF Newsletter, 2006), Sutton cites numerous situations of colony counting discrepancies, confusion and paradoxes in counting dilutions, and discussions of different approaches (recommended by USP, ASTM and FDA) to rounding and averaging of microbial colonies. All of these dilemmas are eliminated with the use of qPCR to replace manual counting of microbial colonies on nutrient agar. PDA Technical Report #33 states…"Because microbiological methods are inherently different than chemical assays, this lack of agreed upon demonstration criteria can present serious obstacles to their (e.g., new microbiological methods) implementation." With qPCR, this situation is **no longer the case. qPCR is an analytical chemical assay.**

In other papers (Sutton, 2005), Sutton succinctly makes the statement "Microbiology is *not* Chemistry." With the use of qPCR, Microbiology *becomes* Chemistry and is readily defined by all of the regulatory guidance criteria previously described. **We should therefore not redefine the scientific criteria to fit the Microbiology data, but instead redefine the Microbiology data to fit the scientific criteria.**

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About the Author

Janet Barletta, Ph.D. is presently a Project Scientist with Peer Consultants, P.C., an international civil and environmental engineering consultancy. Her training includes BS degrees in both Microbiology and Medical Technology from the University of Arizona, an MBA degree in Health Services Administration from Florida International University, and a Ph.D. degree from the University of Miami. Dr. Barletta continues to serve as Adjunct Faculty at the University of Maryland in the Department of Pathology, and she has previously been employed as a Review Microbiologist at the FDA. Her research studies include the design and development of rapid molecular tests for the detection of unculturable microbes and viruses. She has authored several publications and reviews describing rapid molecular methods and the use of real-time PCR for the detection of latent viruses (Epstein Barr virus, Human Herpesvirus Type I, HIV-1), and pathologic prion.

The Open Conference Was a Great Success!

Scott Sutton, Ph.D.
David Porter, Ph.D.
Organizers, *PMF Open Conference on Compendial Issues*

Last month saw a new type of conference for PMF. It was not really a new type of conference in the general sense – USP has run many of these in years past. Rather than a traditional lecture format, this conference stressed interpersonal communication facilitated by a team of moderators. The response was fantastic – over 100 participants came for 2 ½ days of meetings.

The first day of the conference led off with a keynote speech by Dr. Jim Akers, chair of the USP Committee of Experts on Microbiology and Sterility Assurance. This was followed by background presentations on the five main topics of the conference:

- Aseptic Processing - USP <1211>, <1116> and the proposed EU Annex 1 changes (*Ed Balkovic*)
- Validation of Alternative Microbiological Methods (USP <1227>, Pharm. Eur 5.1.6) (*David Porter*)
- Microbiology Best Laboratory Practices (USP <1117>) (*Don Singer*)
- Harmonized Microbial Limits Chapters (*Scott Sutton*)

Water Microbiology (USP <1231>) (*TC Soli*)

After these, Sylvie Guyomond (Sanofi, Ph. Eur) presented on European perspectives on validation and water issues, followed by FDA perspectives (*Dennis Guilfoyle* and *David Hussong*) and a USP wrap-up (*Radhakrishna Tirumalai*). The day ended with a well-attended reception.

The second day of the conference saw everyone break into small groups (~20/room) to discuss the topics in detail. As most of the facilitators were members of the USP committee of experts, there was a great deal of interest in generating feedback from industry on the chapters recently implemented or scheduled for implementation. Each participant rotated through the five topics so that a broad perspective was offered. These discussions were documented by dedicated scribes in each room (thank you again to Vectech for providing the scribes). These meetings are always a struggle – the facilitators have a natural desire to participate in the discussion, but have to guard against the temptation to dominate the discussion. Each session had a list of questions provided to the facilitators to spark discussion (and to help the USP committee on sticky points). Between these questions and the participants' own interests, most groups enjoyed lively discussion.

The final day of the conference started off with a series of

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drawings – participants who visited the exhibitors got a paper initialed and were entered into the pool. The morning session followed, where the discussions from the second day were summarized (after a somewhat grueling evening for the facilitators and scribes!). This also provided the last opportunity for comments from the attendees.

The presentations from the last day and transcripts of the comments from the second day will be collected and sent out as transactions of the meeting.

Comments from Attendees:

"I thought that this was an excellent conference and opportunity to listen and participate in open friendly discussion. All areas covered impact my profession and my company. Congratulations! This reflects the dedication of the people involved in this conference to the forward progress of microbiology in the pharma industry as well as other areas."

"Great opportunity to meet with USP & users. Would have liked more FDA participation. Need more meetings like this."

Editor's note: This sentiment expressed by many.

"Overall, the conference could have been a week in length – provide more time for discussions/networking. Seemed like too much was crammed in to a short period of time. Would like to see a conference that offered true guidance – everyone has a unique situation but perhaps most of us came away with more questions than answers. Fabulous opportunity to have so many microbiologists together!"

Editor's note: Seems like a strong idea for the 2007 PMF Fall Forum – any comments?

"The reception was an excellent networking opportunity! The breakout sessions were extremely helpful in that it allowed for more contact and discussion."

Take-Home Messages for PMF

Strengths of the Open Conference Event:

- Discussion groups
- Reception
- Recognized experts available for discussions
- Strong topics
- New discussions
- Excellent organization

Opportunities for Improvement::

- Provide basic instruction on workings of USP
- Fewer topics, or at least fewer in a day
- Don't rotate moderators
- Allow choice of discussion topics (?)

On behalf of PMF and all the people involved in putting on this ambitious conference, we want to thank the participants and the exhibitors who were ultimately responsible for the success of this first PMF "Open Conference."

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USP Chapter <1010>: The Most Important Unknown Chapter On Interpreting Analytical Data

Dr. David A. Porter
Hilton Raleigh-Durham, Airport at Research Triangle Park - NC 4810 Old Page Road 919-941-6000
April 11, 2007 8:00 AM- 4:30 PM

This one day course is designed for biologists and chemists responsible for the analysis of data arising from the performance of pharmacopeial (USP) methods, or alternative compendial methods, and is based upon chapter <1010> Analytical Data - Interpretation and Treatment, USP 30.

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