



The University of New Mexico ♦ Health Sciences Center
COLLEGE OF PHARMACY



:::VOLUME 14, LESSON 5:::

***Bacterial Endotoxin Test and Sterility Test for
Radiopharmaceuticals***

Continuing Education for Nuclear Pharmacists
and
Nuclear Medicine Professionals

By

James F. Cooper, Pharm.D., FAPhA

And

Joseph C. Hung, Ph.D., BCNP, FASHP, FAPhA

Professor of Pharmacy and Radiology

Director of Nuclear Pharmacy Laboratories

PET Radiochemistry Facility Mayo Clinic



The University of New Mexico Health Sciences Center College of Pharmacy is accredited by the Accreditation Council for Pharmacy Education as a provider of continuing pharmaceutical education. Program No. 039-000-08-006-H04-P and 039-000-08-006-H04-T. 4.0 Contact Hours or .4 CEUs.

-- Intentionally left blank --

Bacterial Endotoxin Test and Sterility Test for Radiopharmaceuticals

By
James F. Cooper and Joseph C. Hung

Editor, CENP

Jeffrey Norenberg, MS, PharmD, BCNP, FASHP, FAPhA
UNM College of Pharmacy

Editorial Board

Stephen Dragotakes, RPh, BCNP, FAPhA
Neil Petry, RPh, MS, BCNP, FAPhA
James Ponto, MS, RPh, BCNP, FAPhA
Tim Quinton, PharmD, MS, FAPhA
S. Duann Vanderslice, RPh, BCNP, FAPhA
John Yuen, PharmD, BCNP

Advisory Board

Dave Abbott, RPh., BCNP
Mark Gurgone, BS, RPh.
Vivian Loveless, PharmD., BCNP, FAPhA
Lisa Marmon, RPh, BCNP
Michael Mosley, RPh, BCNP
Janet Robertson, BS, RPh, BCNP
Brantley Strickland, BCNP
Scott Knishka, RPh, BCNP
Dave Engstrom, PharmD., BCNP
Brigette Nelson, MS, PharmD., BCNP
Samuel Ernesto, RPh, MBA

Director, CENP

Kristina Wittstrom, RPh, FAPhA, BCNP
UNM College of Pharmacy

CE Administrator & Web Publisher

Christina Muñoz, B.S.
UNM College of Pharmacy

While the advice and information in this publication are believed to be true and accurate at the time of press, the author(s), editors, or the publisher cannot accept any legal responsibility for any errors or omissions that may be made. The publisher makes no warranty, expressed or implied, with respect to the material contained herein.

Copyright 2008
University of New Mexico Health Sciences Center
Pharmacy Continuing Education
Albuquerque, New Mexico

BACTERIAL ENDOTOXIN TEST AND STERILITY TEST FOR RADIOPHARMACEUTICALS

STATEMENT OF LEARNING OBJECTIVES:

Upon successful completion of this CE course, the participant should be able to discuss the general concepts and processes associated with the bacterial endotoxin test (BET) and sterility test, and apply them in their daily practice. Specifically, the participant should be able to:

1. Distinguish the requirements for BET and sterility test.
2. Understand the basic principles and procedure for BET and sterility test.
3. Observe, report, and interpret the results of BET and sterility test.
4. Develop corrective action(s) for out-of-specification finding(s) of BET and sterility test.
5. Identify the personnel and facility requirements for the performance of BET and sterility test.
6. Define the term bacterial endotoxin and describe its toxic effects.
7. Identify the sources of bacterial endotoxin in the preparation of injectable radiopharmaceuticals such as an F-18 product.
8. Describe the reagents, materials and equipment needed for the BET.
9. Understand how to select and establish a BET method.
10. Define volumes, solutions and containers needed to prepare a radiopharmaceutical for a BET.
11. Explain the calculations for the endotoxin limit and limit of detection (LOD).
12. Identify the advantages of the photometric-BET in contrast with the gel-clot BET.
13. Describe the procedure for qualifying a LAL reagent and an analyst for the BET.
14. Describe how glassware is rendered endotoxin-free for PET drug preparation and endotoxin testing.
15. Identify the principle cause of invalidity in a photometric or gel-clot BET.
16. Discuss the limitations of sterility test.
17. Compare and contrast various regulations and standards associated with sterility test requirements.
18. Select and validate media for sterility test.
19. List the unique aspects of sterility test for radiopharmaceuticals.
20. Provide the rationale for the filter membrane integrity test.

COURSE OUTLINE

OVERVIEW: ENDOTOXIN DETECTION	8
NATURE OF PYROGENS	8
PRINCIPLE OF THE BACTERIAL ENDOTOXINS TEST (BET)	9
<i>LIMULUS</i> AMEBOCYTE LYSATE (LAL) REAGENT	10
ENDOTOXIN AND ENDOTOXIN STANDARDS	11
ENDOTOXIN LIMITS	11
ENDOTOXIN TEST METHODS	12
PHARMACOPEIAL REQUIREMENTS	12
LAL REAGENT CARTRIDGE METHOD	13
GEL CLOT METHOD	14
CREATING A VALID BET PROCEDURE	16
BET VERIFICATION	16
TEST FOR INTERFERING FACTORS AND MAXIMUM VALID DILUTION (MVD)	17
SPECIFYING A TEST DILUTION AND LIMIT OF DETECTION	18
STANDARD OPERATING PROCEDURE (SOP) AND VALIDATION REPORT	19
DEPYROGENATION AND VALIDATION OF DEPYROGENATION PROCESSES	19
INVALIDITY AND OUT-OF-SPECIFICATION (OOS) INVESTIGATIONS	20
RISK ASSESSMENT	21
CALCULATIONS	21
ENDOTOXIN LIMIT	21
MAXIMUM VALID DILUTION (MVD)	21
LIMIT OF DETECTION (LOD)	22
DEPYROGENATION	22
THE FORMULA FOR DETERMINING LOG REDUCTION VALUE (LRV) FOR A DEPYROGENATION PROCESS IS AS FOLLOWS:	22
STERILITY TEST	23
INTRODUCTION	23
ISSUES RELATED TO STERILITY TESTING	24
ABSOLUTE STERILITY	24
UNDETECTED MICROORGANISMS	24
RADIOPHARMACEUTICAL UNIQUENESS	24
MINIMAL TESTING	25
USP GENERAL CHAPTER <823> “RADIOPHARMACEUTICALS FOR POSITRON EMISSION TOMOGRAPHY – COMPOUNDING”	26
USP GENERAL CHAPTER <797> “PHARMACEUTICAL COMPOUNDING – STERILE PREPARATIONS”	26
<i>High-Risk Level CSPs</i>	27
TESTING PREREQUISITES	28
TESTING ENVIRONMENT	28
PERSONNEL QUALIFICATION	28
TYPES OF MEDIA	29
FLUID THIOGLYCOLLATE MEDIUM (FTM)	29
SOYBEAN-CASEIN DIGEST MEDIUM (SDM)	30
STORAGE AND SHELF-LIFE OF THE CULTURE MEDIA	30
COMPOSITION OF FTM AND SDM	30
GROWTH PROMOTION TEST	30

STERILITY TESTING PROCEDURES	31
MEMBRANE FILTRATION	31
DIRECT INOCULATION	32
NEGATIVE CONTROL	32
VALIDATION TEST	33
<i>Membrane Filtration</i>	33
<i>Direct Inoculation</i>	33
INITIATION TIME	34
POOLED SAMPLES	34
QUANTITY TO BE TESTED	34
NUMBER OF ARTICLES TO BE TESTED	35
OBSERVATION AND INTERPRETATION OF RESULTS	35
FIRST STAGE	36
SECOND STAGE	36
OUT-OF-SPECIFICATION INVESTIGATION	37
MEMBRANE FILTER INTEGRITY	38
BUBBLE-POINT TEST	38
<i>"Limitus Test"</i>	40
<i>Reprocessing</i>	40
SUMMARY	40
APPENDIX	42
REFERENCES	46
GLOSSARY	48
ABBREVIATIONS AND ACRONYMS	49
ASSESSMENT QUESTIONS	50

-- Intentionally left blank --

BACTERIAL ENDOTOXIN TEST AND STERILITY TEST FOR RADIOPHARMACEUTICALS

James F. Cooper, Pharm.D., FAPhA
And
Joseph C. Hung, Ph.D., BCNP, FASHP, FAPhA
Professor of Pharmacy and Radiology
Director of Nuclear Pharmacy Laboratories
PET Radiochemistry Facility Mayo Clinic

OVERVIEW: ENDOTOXIN DETECTION

Pyrogen (fever-inducing agent), principally known as bacterial endotoxin, is one of the most potent bacterial toxins. Its only source is Gram (-) bacteria (GNB), where endotoxin comprises about 75% of the GNB cell wall. Bacterial contamination (bioburden) in water and on surfaces normally contains GNB, so endotoxin is ubiquitous in nature.^{1,2} This unit describes how to apply the Bacterial Endotoxins Test (BET) to assure the absence of unsafe levels of bacterial endotoxin pyrogen in compounded or manufactured radioactive drugs.

The first test for endotoxin pyrogen was an expensive, time-consuming rabbit fever assay. The rabbit test was replaced in recent years by the Bacterial Endotoxins Test, an in vitro test that uses a highly sensitive reagent called *Limulus* amoebocyte lysate (LAL).¹ Chapter <85> of the U.S. Pharmacopeia describes how to conduct the BET with LAL reagent and qualify an analyst for routine testing.³ The Food and Drug Administration (FDA) requires radiopharmaceutical producers to test all injectable products with FDA-licensed LAL reagent to meet predetermined endotoxin limits.^{4,5} The BET is relatively simple and does not require highly specialized equipment. However, the preparation of controls for some BET methods is tedious and demands attention to detail in order to complete the test accurately and reproducibly. This CE unit emphasizes a simplistic, automated BET method.

NATURE OF PYROGENS

Due to its potency and ubiquity in nature, bacterial endotoxin is the only significant pyrogen of concern to the parenteral drug industry.^{6,7} Endotoxins are large complexes from GNB bacterial cell wall that are constantly shed into the environment when the bacteria disintegrate or multiply. Endotoxins contain lipid, carbohydrate, protein, and are composed of a common structure of a

hydrophilic polysaccharide covalently bound to a hydrophobic region known as Lipid A. The latter component causes the toxicity and LAL activation related to endotoxin. Endotoxins are negatively charged macromolecules that are not removed by sterilizing membrane filters, such as those used in aseptic processing of a Positron Emission Tomography (PET) drug. It is quite possible to have a sterile solution that has sufficient endotoxin to be pyrogenic.

The most likely sources of endotoxins in PET radiopharmaceuticals are containers, tubing and non-sterile water and chemicals used in the preparation of the product. Endotoxin is difficult and expensive to eliminate after the product is made. The only practical way to avoid endotoxin contamination is to eliminate it at the outset by using endotoxin-free materials and aseptic technique for all critical steps in the production of a Compounded Sterile Preparation (CSP) in the form of a radiopharmaceutical or PET drug.

The skin and gastrointestinal tract are barriers to endotoxin. However, when endotoxin gains access to blood or tissues by injection, nanogram (parts per billion) quantities can cause fever and hypotension while large amounts can lead to irreversible shock and death.^{6,7} Signs of pyrogenic reactions caused by endotoxin contamination include chills, fever, rigors, tachycardia, hypotension and respiratory distress. Although advances have been made in parenteral drug production, pyrogenic reactions still occur, as evidenced by a pyrogen outbreak to endotoxin-contaminated gentamicin in 1998 and 1999 that produced at least 155 patient reactions.⁷ Signs of intrathecal toxicity are more serious with morbid symptoms of aseptic meningitis.^{2,7}

PRINCIPLE OF THE BACTERIAL ENDOTOXINS TEST (BET)

The Atlantic horseshoe crab (*Limulus polyphemus*) has an enzymatic blood coagulation system that is specific and highly sensitive to endotoxin. The development of the in vitro endotoxins test began when Levin and Bang⁸ proved that the blood cells (amebocytes) from the horseshoe crab responded to endotoxin on the cell wall of Gram-negative bacteria. Cooper, Levin, and Wagner⁹ expanded this concept into an endotoxin test for radiopharmaceuticals and other drugs. The new test was known as the LAL test until it was adopted into the USP. The name was changed to the Bacterial Endotoxins Test (BET) to reflect its purpose. The industry uses the terms BET and LAL test interchangeably, but BET is the official acronym.

The development of radiotracers and endotoxin testing has remarkable parallels. The need for an alternative to the rabbit pyrogen test for radiotracer development led us to define the usefulness of LAL for testing injectables for endotoxin. The FDA began approving LAL reagents in the 1970s about the time radiopharmaceuticals came under the purview of the FDA. Aseptic meningitis caused by endotoxin in cerebrospinal-fluid imaging agents led to a strict USP endotoxin limit for intrathecal drugs. Replacement of the pyrogen test with the BET began in 1983 with introduction into the USP 29 monographs for radiopharmaceuticals and for five USP pharmaceutical waters. The innovation of the LAL cartridge system provides a realistic way to test PET products for endotoxin.

***Limulus* Amebocyte Lysate (LAL) Reagent**

FDA regulated parenteral products must be tested by FDA-approved biological *Limulus* amebocyte lysate (LAL) reagent, before released for patient use.^{4,5} The BET can be conducted either by the traditional gel-clot method or photometric tests done with spectrophotometric readers.^{2,3,6} The basis of the endotoxin-LAL reaction is that endotoxin activates an enzymatic cascade in LAL that modifies a clotting protein to yield a turbid gelatinous endpoint. The BET is standardized for incubation of equal parts of test solution and LAL. Optimum reactivity requires pH neutrality, optimum levels of divalent cations (Mg and Ca) and sodium, low salt strength and generally less-than-milligram concentrations of drug products. Therefore, compatibility testing is required to identify valid test conditions.^{6,7} The basic test in the BET is termed a limit test because it is designed to reveal when an endotoxin limit is exceeded. Positive controls are included to assure that results are valid. The test may be done on a clean bench top; a laminar air flow hood is not necessary. Touch contamination of LAL solution, LAL vial or rehydration water is a principal source of false positives.^{2,3} The reagent is somewhat labile in solution, and as such requires limited storage at ambient temperature and only one freeze-and-thaw cycle.

A unique terminology for BET applications has emerged (see attached glossary). Some of the prominent terms deserve an explanation. The first LAL document was the FDA's LAL-test Guideline for injectables.¹⁰ This guide defined the symbol lambda (λ) as the LAL reagent sensitivity in EU/mL. It introduced the concept of the Endotoxin Limit (EL), based on patient dose, to define a safe level of endotoxin. It also provided a formula for the use of dilution (MVD, maximum valid dilution) to overcome interference mechanisms for the LAL-endotoxin reaction. The first USP gel-clot test required an endotoxin dilution series, from 2λ through $\frac{1}{4}\lambda$, to bracket an expected endpoint of the LAL

reagent and confirm that a test was in control. An operator is deemed to be “qualified” to conduct the BET assay by preparing and testing dilutions of an endotoxin standard with LAL and achieving recovery of positive controls at the expected levels.³

A revision of the BET chapter was introduced into the USP in 2001 that simplified procedures and introduced photometric methods. The BET is now the minimum standard for endotoxin testing, so standard procedures must reference *USP <85> Bacterial Endotoxins Test*.

Endotoxin and Endotoxin Standards

Environmental (unpurified) endotoxins contain lipid, carbohydrate, protein, and are readily dispersed in water; they are remarkably stable. However, the purified endotoxins that are used for standards and positive controls are quite different. Purification processes remove protein units from endotoxin to yield lipopolysaccharide (LPS), which is poorly dispersible and less stable in potency. The Control Standard Endotoxins (CSE) that are supplied by LAL reagent vendors serve as positive controls for routine endotoxin tests. Alas, the low-concentration CSE solutions are susceptible to absorption and poorly understood aggregation phenomena. With time, activity of endotoxin standards, such as CSE, seem to disappear unless subjected frequently to vortex mixing. The most common problem in routine gel-clot methods is loss of endotoxin potency and invalidity due to failure to recover the positive controls. The potency of a CSE preparation is specified in Endotoxin Units (EU) when assigned to a specific lot of LAL reagent through a Certificate of Analysis (CoA). The LAL-reagent suppliers calibrate the sensitivity of their reagents using an international reference standard endotoxin (RSE).

Endotoxin Limits

An endotoxin unit (EU) is a unit of biological activity established by the USP Endotoxin RS (Reference Standard). The adverse effects of endotoxin are dependent on dose, route and rate of administration. A general endotoxin limit of 5 EU/kg/hr or 350 EU per adult (70 kg) was scientifically established to avoid the fever and hypotension from IM or IV injection of endotoxin contamination.⁶ Time is a factor because there are mechanisms in the liver and blood that neutralize endotoxin. However, there are no clearance mechanisms in intraspinal spaces, so the IT (intrathecal) endotoxin limit is much more stringent.^{2,11} If an IV drug is infused over a period of time that exceeds one hour, the dose may be divided by the number of hours to determine the dose-per-hour value. (See example in the section on [calculations](#)).

When endotoxin limits for radiopharmaceuticals were first approved in 1983 as a replacement for the rabbit test, the limit was arbitrarily set at half the general limit. Therefore, the radiopharmaceutical limit was set at 175 EU per adult dose for IV or IM injection and 14 EU/dose for intrathecal administration. Validation of BET methods, including extent of pre-test dilution, is performed after endotoxin limits are established, based on factors such as dose, infusion rate and route of administration. For traditional drugs, endotoxin limits may be expressed in EU/mg of a specific medication or EU/mL for infusion solution or a mixture of medications in solution. The term pyrogen free may be applied to a CSP or finished manufactured pharmaceutical product that contains endotoxin less than the endotoxin limit as specified for each product.

ENDOTOXIN TEST METHODS

Pharmacopeial Requirements

As introduced above, endotoxin activates a cascade of serine-protease enzymes in LAL that alters the clotting protein. Macromolecules of this protein aggregate and cross-link to eventually produce a reversible, opaque gel. Spectrophotometric measurement of increasing optical density (OD) with time and endotoxin concentration describes endotoxin analyses by kinetic LAL methods. A photometer with endotoxin-specific software passes a optical signal through the sample at one-minute-or-less intervals to generate a reaction curve for each sample. The software assigns a reaction time when the optical density of a sample exceeds a preset OD. Then, reaction times of unknowns and positive controls are interpolated against a standard curve that is generated by at least three endotoxin standard concentrations, made at 10-fold intervals. Increases in OD produced by absorption and light-scattering may be monitored with time to produce kinetic turbidimetric assays (KTA). Increases in OD, due to cleavage of a colorless substrate to yield a yellow color, may be monitored with time to yield kinetic chromogenic assays (KCA). The parenteral drug industry primarily uses kinetic LAL methods, with the aid of an incubating 96-well microplate reader, to conduct up to 20 assays of test samples, simultaneously.

The two types of endotoxin tests that are described in the BET require substantially different reagents and equipment. Gel-clot methods require a dry-heat block, calibrated pipettes and thermometer, vortex mixer, freeze-dried LAL reagents, LAL Reagent Water (LRW) for hydrating reagents and depyrogenated assay tubes. In the Gel-clot method test, diluted sample and liquid reagents require about an hour for sample and positive-control preparation and an hour's incubation in a heat block;

results are recorded manually. In contrast, photometric tests require a more highly-processed reagent, a spectrophotometer, endotoxin-specific software and printout capability. In its most simplistic form, the simplest photometric system exists as a handheld unit employing a single-use, LAL cartridge that contains dried, pre-calibrated reagents; no liquid reagents or standards are needed. A FDA-approved hand held system unit was recently introduced under the trade name of Endosafe PTS™ (Charles River Laboratories, Charleston, SC). The device requires about 15 minutes to analyze small amounts of sample, a 100-μL aliquot from a Compounded Sterile Product (CSP) diluted with sterile, pyrogen-free water. The simplicity and speed of this new automated system make it ideally suited for PET drug products.

LAL Reagent Cartridge Method

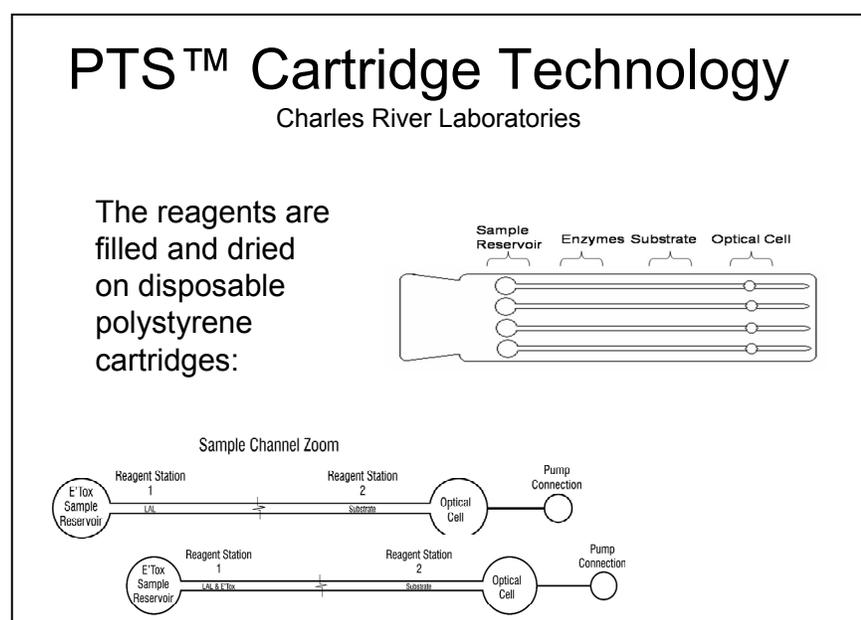


Figure 1. LAL Reagent Cartridge. Kinetic chromogenic reagents and endotoxin controls are dried in the channels of a polystyrene cartridge. Diluted product pumped into the channels where it is mixed with reagents, monitored for changes in optical density and results are interpolated against an archived endotoxin standard curve.

The Endosafe PTS™ (Portable Test System) is a handheld spectrophotometric reader with endotoxin specific software that is designed to measure a kinetic chromogenic assay that occurs within a uniquely designed polystyrene cartridge. (Figure 1) The cartridge has four channels with kinetic LAL reagents dried in place. Two channels have LAL reagent and color substrate; the other two channels have LAL reagent.

The archived curve for each batch of cartridges is embedded in a calibration code that is entered into the software upon first use of the batch by an analyst. The dry cartridges are thus pre-calibrated so that there is no need for handling liquid reagent or standards. An endotoxin test is conducted on a verified test dilution by entering identifying test and product information into the hand held unit, inoculating each of the four cartridge reservoirs with 25 μL of diluted product, incubation for 15 minutes and printing of results by a suitable means. (See procedure in the [Appendix](#)). A study is valid if the

positive control is recovered within a range of 50-200% and there is agreement between each pair of channels.

Gel Clot Method

The traditional gel clot method is more complicated, time-consuming and technique-dependent than other methods. In its simplest form, a gel-clot BET is conducted by mixing 100 µL (0.1 mL) of sample or positive (endotoxin) control with 100 µL of LAL reagent, using aseptic technique, and incubating the mixture undisturbed at 37°±1C for one hour (60±2 minutes). If endotoxin is present in a concentration greater than the labeled LAL sensitivity, an opaque gel will form that remains firm when the assay tube is carefully inverted. Gel-clot reagents are available in single and multi-test vials; single test vials eliminate handling liquid LAL reagent. The labeled sensitivity of the LAL reagent, lambda (λ), is the minimum concentration of endotoxin that will produce a gel endpoint under standardized test conditions; a 0.06 EU/mL reagent is most frequently used. The assay is time and temperature sensitive, so attention to test conditions is important to achieve meaningful results.

Table 1

COMPONENTS OF A GEL-CLOT LIMITS TEST BY USP <85>				
Solution	Endotoxin	Sample Solution	No. Replicates	Expected Result
A (Product)	None	Diluted PET drug	2	Negative
B (PPC)	~2λ	Diluted PET drug	2	Positive
C (PWC)	~2λ	LAL Reagent Water	2	Positive
D (NC)	None	LAL Reagent Water	2	Negative

Note: Lambda (λ) is the LAL Reagent sensitivity; PPC is the positive product control, PWC is a Positive Water Control; NC is a negative control. The PPC and PWC tubes contain co-lyophilized LAL and endotoxin control. A result is reported as positive if the gel holds firm through a 180° rotation, whereas all other results are negative.

The gel-clot test has four components tested in duplicate, as listed in Table 1. The test sample is drug product that has been diluted to avoid interfering test conditions and unnecessary radiation exposure. Component A is diluted product and B is diluted product with a positive control. Component C is sterile, pyrogen-free water with the positive control and D is a negative control; C and D tubes are optional, but should be done once daily with the first set of tests to show that the positive controls have sufficient potency and that sterile water used for dilution is not contaminated. Figure 2 illustrates preparation of reagents and assay tubes for a BET.

The most challenging part of this method is preparation of the solutions for the Positive Product Control (PPC). The components needed to make positive controls are 1) a CSE vial that is rehydrated and stored according to instructions, and 2) a 20- λ CSE tube for preparation of 2 λ positive controls *in situ* for the BET limits test. (Figure 2) The key to efficiency in this method is use of a 20- λ CSE tube that allows the positive controls to be made in the assay tubes rather than making a separate tube; this approach is known as the ‘hot spike’ technique. The USP allows the positive water controls (PWCs) to substitute for an endpoint dilution series in a limit test. The purpose of the PWC is to indicate that the PPC was made properly.

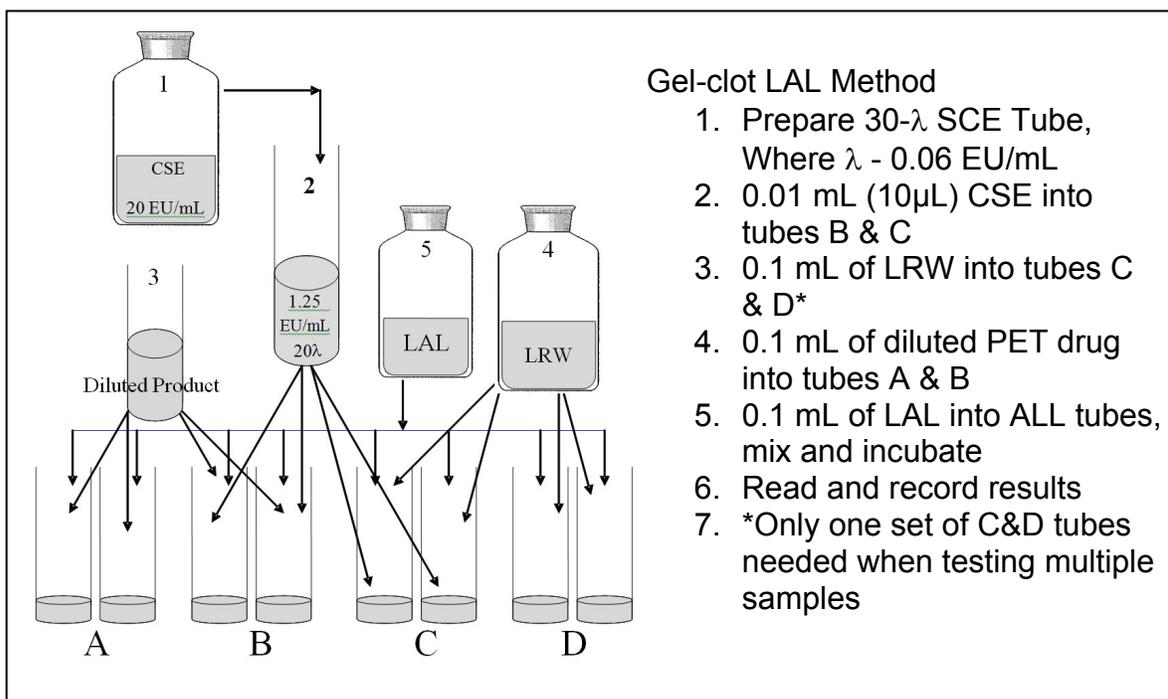


Figure 2. Scheme for USP Bacterial Endotoxins Test (BET) by a gel-clot method. Test preparation for a BET requires dilutions of endotoxin control (2) and PET drug (4). Four sets of solutions are made for testing in duplicate. Two sets contain the diluted product either alone (A) or with endotoxin control (B), and water tubes (3) either with endotoxin control (C) or without (D). A 10- μ L inoculum of endotoxin (4) produces the positive product control (B) and positive water control (C). LAL reagent (5) is added to all tubes prior to incubation in a heating block for 60 ± 2 minutes at $37 \pm 1^\circ\text{C}$. Results are read and recorded after an hour. The drug is within the endotoxin limit and the test is valid if tubes A & D are negative (no gel) and B & C are positive (gel). Figure only applies to BET where λ equals 0.06 EU/mL.

The 20- λ CSE solution is made at least weekly in order to prepare the positive controls in the limit test. As an example, for an LAL reagent with a sensitivity of 0.06 EU/mL, the 20- λ tube is 1.25 EU/mL, which is twenty times lambda. (Figure 2) Preparation of the 20- λ tube, in polystyrene, requires vortex mixing and hydration of a CSE vial according to the vendor’s direction to make 20 EU/mL CSE.

Vigorous and frequent vortex mixing of the tube is needed to maintain its potency. Inadequate vortex mixing is the principal cause of invalidity, which is a failed positive control (no gel). A different dilution scheme is required for LAL reagent with sensitivity other than 0.06 EU/mL.

Potency of a 20- λ CSE tube is verified on the day it is made by preparing a CSE dilution series to assay its potency. The assay is an endpoint test with four sterile polystyrene tubes consisting of a serial two-fold dilution of 2λ , λ , $1/2\lambda$ and $1/4\lambda$ that bracket labeled sensitivity of the LAL reagent. The 2- λ tube is made by creating a 1:10 dilution from 20- λ tube, and the other tubes are made by two-fold dilution. (Figure 3) The 20- λ CSE tube should be able to produce an endpoint (potency) of between 2λ and $1/2\lambda$ after serial dilution. Any other result suggests preparation of a new 20- λ CSE tube and re-assay for 20- λ CSE potency verification. This assay is also an alternative for the PWC tubes (Tubes C in Table 1) in the limit test. This same endpoint assay is used to qualify an analyst for the BET.

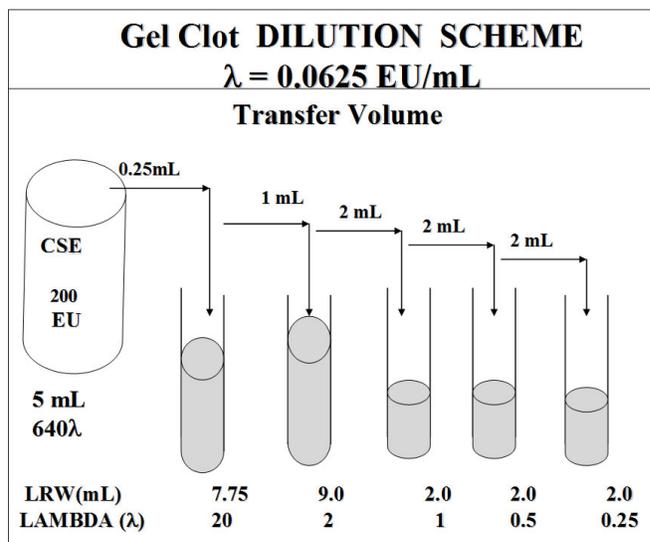


Figure 3. Endpoint Assay for Endotoxin in a 20- λ positive control tube. Ideally, potency is confirmed by endpoints at the λ and $1/2\lambda$ endotoxin levels. Scheme will differ for LAL reagents other than 0.06 EU/mL.

CREATING A VALID BET PROCEDURE

BET Verification

Validation is a term often used to describe the creation of a BET method for a specific drug. For USP methods such as the *Chapter <85> BET*, one is not required to validate the accuracy and reliability of the method. Rather, one is expected to verify the suitability of a test method under actual conditions of use. Verification conditions are described under “Interfering Factors Test” in *USP <85> BET*. The objective of method development and subsequent verification is to generate a standard procedure that has the following attributes:

1. The product under test conditions described in a test procedure is non-interfering (positive controls are positive and negatives are negative);

2. A suitable product dilution or solute concentration is determined by using a published or calculated endotoxin limit;
3. The assay is unaffected by significant batch or laboratory test variability, as demonstrated by the requirement for verification using three batches of product;
4. The combination of LAL and prepared product will have a neutral pH (6-8);
5. The laboratory processes are supported by appropriate user procedures and training, instrument qualifications, and preventive maintenance.

There are questions that should be addressed before developing methods for a new or investigational radiopharmaceutical. For example, what is the maximum human dose, route of administration, product pH and formulation? A unique property of radioactive drugs is that the dose volume increases with time in order to deliver a prescribed dose of activity. Therefore, the dose volume at time of expiration is used for calculations. An unusual route of administration, such as intrasynovial spaces, may need a stricter limit, similar to IT administration. Understanding and knowledge of the final product formulation may lead to anticipation of interference conditions, such as pH.

Test for Interfering Factors and Maximum Valid Dilution (MVD)

The four principal causes of inhibitory conditions for a BET concern divalent cations, pH, enzyme modifiers and loss of potency in endotoxin standards.⁶ Non-neutral pH is a common problem because most LAL reagents have modest buffer capacity in their formulation; a mixture of LAL and diluted drug product should have a pH very near 7. Also, the presence of divalent-cation chelating agents, such as citrate, can deplete the magnesium and calcium ions that are needed for the LAL reaction with endotoxin. Chemicals that denature or alter enzyme function, such as ethanol or chromic ion, will inhibit the enzymatic cascade. Finally, inadequate vortex mixing or prolonged storage of CSE endotoxin standards results in loss of potency and failure of positive controls. Sources of enhancement or false positives include LAL reactive glucans and exposure to cellulose.⁶

Dilution should always be applied to resolve the first three inhibitory factors. The extent of dilution is defined by the formula for MVD in the BET chapter of the USP. MVD is calculated by dividing the endotoxin limit of a drug by the sensitivity (λ) of the endotoxin test method or reagent. (See section on [calculations](#) for examples).

Specifying a Test Dilution and Limit of Detection

The low administration volume of imaging agents invariably yields an MVD calculation that is quite high. It is prudent to assign a valid test dilution for a product that is less than the maximum but sufficiently high to avoid the risk of interfering conditions. For example, it is commonplace for the MVD for Fludeoxyglucose ^{18}F (^{18}F -FDG) to be at least 1:350, as shown in the calculations below. On the other hand, inhibitory conditions can be detected at 1:10 dilutions of ^{18}F -FDG due to the presence of citrate and other factors associated with a given synthesis unit. Therefore, the choice of 1:50 as the test dilution for this product is advisable.

A survey of radiopharmaceuticals, including selected PET drugs, found very little dilution was needed for the BET because their formulations were generally neutral and contain little solute.¹¹ Table 2 shows validated test dilutions for I 123 MIBG and radiotracers labeled with ^{18}F , ^{11}C , and ^{13}N .

After the test dilution is selected, the Limit of Detection (LOD) is determined for the purpose of uniformly reporting the results. The LOD is the sensitivity of the BET method times the extent of dilution, as further described in the section of calculations, below. Invariably, the BET for a radiopharmaceutical will be negative, so the analysis is reported as less than the LOD.

Table 2

VALIDATION OF SHORT-LIVED RADIOPHARMACEUTICALS WITH THE LAL CARTRIDGE			
PET Medication	Dilution	LOD (EU/mL)	PC Recovery (Mean %)
F 18 FDG	10	1	91
F 18 Fluoride	10	1	120
F 18 FLT	10	1	103
C 11 Acetate	20	2	150
C 11 Methionine	20	2	95
N 13 Ammonia	20	2	108
I 123 MIBG	50	5	90

Note: LOD is the Limit of Detection when using cartridges with a sensitivity (λ) of 0.1 EU/mL. PC is positive control recovery of 1 EU of endotoxin standard in the cartridge.

Standard Operating Procedure (SOP) and Validation Report

No test method is complete until there is a validation report and operating procedure approved and placed in document control. As in potty training, the job isn't done until the paperwork is completed. The SOP should be sufficiently clear and complete to serve as a training document for operators as well as conducting tests and interpreting results. The SOP should describe how the results are recorded, including the LOD, and how validity and invalidity is handled in practice. (Refer to the [Appendix](#) for a representative SOP).

The validation report documents the suitability of written test conditions for each radiopharmaceutical. The report should include calculation of the endotoxin limit and MVD, pH measurement, and the results of valid recovery of positive controls for three batches of product. The report may show validation with both gel-clot and photometric methods.

DEPYROGENATION AND VALIDATION OF DEPYROGENATION PROCESSES

Elimination of endotoxin by separation or destruction is called depyrogenation. Distillation, copious rinsing of plasticware with SWI and passage of solutions through certain surface-active materials, such as alumina and positive-ion filters, are common separation methods. Incineration of endotoxin in dry-heat ovens is the definitive method for depyrogenating glassware used in production and for gel-clot assay tubes. Chemical depyrogenation is accomplished by basic hydrolysis through exposure to sodium or potassium hydroxide, or by oxidation with peroxide.⁶

Endotoxin Indicators (EI) are vials that contain known amounts of LPS, such as 2,000 or 10,000 EU per vial, in a dry vial. An EI is exposed to a depyrogenation method, such as a dry-heat sterilizing oven, to document effectiveness. Endotoxin assays of an EI are made before and after a depyrogenating step to determine if there was at least a 3-log reduction in endotoxin content, the criterion for depyrogenation. An oven can be qualified by exposing four or more EI vials, embedded in a typical load of glassware, and quantifying the recovery of endotoxin residues.⁶ When the recovery is non-detectable, lambda is used for the exposed EI value (See section on [calculations](#) below).

It is ironic that the FDA places such emphasis on testing ¹⁸F-FDG because the AI-N cartridge in a typical ¹⁸F-FDG synthesis unit completely removes endotoxin during processing. In our experiments, the contents of a 2000 EU vial were passed through an AI-N cartridge and analyzed in a system with a

limit of detection at 0.05 EU/mL. The depyrogenation effect of alumina was confirmed when no endotoxin was recovered in the eluate of the cartridge. Note that the cartridge eluate was passed through a sterilizing membrane filter to trap trace alumina particles, which could inhibit the recovery analysis by absorbing the endotoxin in the positive control for the eluate. In Europe, there is a more realistic, skip-test policy of endotoxin tests of ^{18}F -FDG, such as testing for one-in-ten preparations.

INVALIDITY AND OUT-OF-SPECIFICATION (OOS) INVESTIGATIONS

Invalidity occurs when positive and negative controls do not react as expected. In photometric assays, there must be recovery of the positive control between 50-to-200% of positive control. The principal cause of invalid recovery in a LAL cartridge system is pipetting inaccurate volumes into the cartridge. Over-pipetting can cause inhibition (<50% recovery) and under-pipetting can cause enhancement (>200% recovery). For example, a 20- μL inoculation of SWI into a LAL cartridge produced only 46% recovery, which is out of range and mimics inhibition. The remedy requires addressing causes for inaccurate volumes, such as training and re-calibration of the pipettor. The most common invalidity in gel-clot method tests is failure to observe a gel in the PPC and PWC tubes (Table 1), which invariably is due to loss of CSE potency associated with inadequate vortex mixing of the standard solutions. The remedy for positive-control failures is usually preparation of new CSE standards. In contrast, gelation in the NC and diluted product tubes suggests that the reagent is contaminated; the remedy here is new LRW and LAL reagent.

In the event of an OOS result, where the endotoxin limit of a test was exceeded, it is prudent to conduct an investigation in a timely fashion in order to determine if the result was a true positive, an invalid test or a false-positive where contamination was introduced by the analyst. For example, if all eight tubes in a gel-clot limit test were positive with firm gels, then the test was invalid because the positive result in the negative control indicated that either the water diluent or LAL reagent was contaminated by the analyst.

There was an incident where an ^{18}F -FDG batch was recalled due to endotoxin contamination near the endotoxin limit. The investigation revealed that the operator inadvertently delivered the batch to a non-sterile beaker instead of the final product vial. The recovered solution was passed through a sterilizing filter to yield a sterile solution, but of course, endotoxin was not removed. This incident reaffirms that risk of contamination is extremely low and only occurs when there is a catastrophic failure in procedure, process or operator error.

RISK ASSESSMENT

The greatest risk for pyrogenic reactions involves the intrathecal (IT) route of administration.^{2,7} In the early 1970s, endotoxin-contaminated imaging agents for radionuclide cisternography caused a large outbreak of aseptic meningitis.¹² Radioiodinated albumin was contaminated by passing the product over an anion exchange column that had not been depyrogenated; the corrective action evolved to treatment of in-process columns with concentrated sodium or potassium hydroxide to render them pyrogen free. Also, In 111 Indium drug was contaminated by pH measurement when using non-sterile, endotoxin-laden buffer solutions for meter calibration. There were frequent reports of pyrogenic reactions in the early days of nuclear pharmacy when imaging agents were produced in house. The use of FDA-approved radiopharmaceuticals and better awareness and control of bioburden in compounding areas has virtually eliminated pyrogenic reactions in current practice.

CALCULATIONS

Endotoxin Limit

The endotoxin limit is calculated from the K/M formula in the BET, as such:

$$\text{Endotoxin Limit (EL)} = \frac{\text{K (tolerance limit)}}{\text{M (maximum dose/kg)(hr)}}$$

$$\text{Radiopharmaceutical EL} = \frac{175 \text{ EU}}{\text{V (mL)}}$$

A unique feature of radiopharmaceuticals is that the dose-volume increases with time because of radioactive decay. Therefore, M is generally the volume of imaging agent at the expiration time that is required to administer the prescribed dose of radioactivity. Another approach often used for ¹⁸F-FDG is to assign M to the maximum volume of administration. For example, a firm had a procedure that limited a patient dose to 10 mL. In this case, the endotoxin limit was 17.5 EU/mL when the radiopharmaceutical limit of 175 EU/dose was divided by the 10 mL/dose.

Maximum Valid Dilution (MVD)

The MVD is used to determine the extent of dilution that may be used to avoid an inhibitory condition and reduce radiation exposure to the analyst.

$$\text{Maximum Valid Dilution (MVD)} = \frac{\text{EL}}{\lambda}$$

The MVD is calculated by dividing the endotoxin limit by the sensitivity (λ) of the BET method. For example, in the paragraph above, the endotoxin limit was determined to be 17.5 EU/mL. An LAL cartridge system with a labeled sensitivity of 0.05 EU/mL was used as the test medium. Therefore, the MVD is 350, expressed as 1:350, when the endotoxin limit of 17.5 EU/mL is divided by the cartridge sensitivity of 0.05 EU/mL. If a 0.06 EU/mL gel-clot LAL reagent was used instead, the MVD would be 290.

Limit of Detection (LOD)

The LOD represents the lowest concentration of endotoxin that may be detected under specific test conditions and specific method. The sensitivity of a BET, or limit of detection (LOD), for a pharmaceutical is the dilution of the drug multiplied by the labeled sensitivity (λ) of the LAL reagent.

$$\text{Limit of Detection (LOD)} = \text{Lambda } (\lambda) \times \text{Dilution Factor}$$

For example, the LOD of a BET is 1.25 EU/mL when the product was diluted 1:20 and tested with a gel-clot reagent with a λ of 0.0625 EU/mL. In the PTS™ system, the LOD is 1 EU/mL, where labeled sensitivity (λ , lowest point on the archived standard curve) is 0.05 EU/mL with a 1:20 dilution.

Depyrogenation

The formula for determining log reduction value (LRV) for a depyrogenation process is as follows:

$$\text{Log reduction value (LRV)} = \frac{\log_{10} \text{unexposed EI}}{\log_{10} \text{exposed EI}}$$

As an example, EIs with approximately 1000 EU was exposed to a 250°C cycle for one hour, in a representative oven load of glassware, to yield EI vials that had non-detectable endotoxin assay on the contents of the vials post pyrogen burn. The standard curve for the photometric assay had a λ of 0.1 EU/mL, so the difference of 1000 and <0.1 EU per vial indicated an LRV of at least 4, where $\text{LRV} = 3 - (-1)$.

STERILITY TEST

Introduction

Sterility is the most important quality of injectable because these drugs bypass most of the body's defense mechanisms. If radiopharmaceuticals are formulated from raw materials, sterile glassware, syringes, and other components should be used to lessen the chance of introducing microorganisms and pyrogenic material into the product. A sterile solution is one that contains no living organisms, pathogenic or nonpathogenic.

All injectable products must be sterilized, and there are five methods of terminal sterilization (i.e., steam sterilization, dry-heat sterilization, gas sterilization, sterilization by ionizing radiation, and sterilization by filtration) described in USP General Chapter <1211> "Sterilization and Sterility Assurance of Compendial Articles".¹³ Sterilization with a 0.22- μ m membrane filter is frequently employed for sterilization of compounded radiopharmaceuticals due to its ease of use. Sterilizing membrane filtration is also the method of choice for heat-labile drug products.

Terminal sterilization of the final dosage form is generally a preferred step to ensure the minimal risk of microbial contamination in the finished drug preparation. However, it is vital that, prior to the terminal sterilization process, the parenteral drug is prepared by trained operating personnel who are adequately cleansed and gowned, in a certified aseptic processing facility, and via a validated aseptic SOP. These "aseptic processing" steps (i.e., trained personnel, certified facility, and validated SOP for aseptic processing) are good practice to minimize the bioburden of the finished lot of final dosage form. Coupling aseptic processing with the terminal sterilization would further minimize the risk of microbial contamination in the finished drug product. Aseptic processing is also a "must" for those products that cannot be terminally sterilized. These "aseptically processed" products can be prepared within a controlled environment by a series of aseptic steps using the component(s) and container(s) that have been sterilized by one of the five terminal sterilization methods mentioned earlier in this section.

A Sterility test is performed to determine whether a product (especially a drug listed in the USP) purporting to be sterile complies with the requirements as stipulated in the test for sterility in the individual USP monograph. The test for sterility should be carried out in accordance with USP General Chapter <71> "Sterility Tests".¹⁴ The incubation and observation process of the USP sterility

test takes 14 days.¹⁴ Due to the short physical half-life of radionuclides used in nuclear medicine, the release of prepared radiopharmaceuticals for parenteral administration in human subjects is not hinged on the completion of the sterility test.¹⁵⁻¹⁸ Nevertheless, the sterility test must still be performed especially for positron emission tomography (PET) radiopharmaceutical that is intended for parenteral administration.¹⁵⁻¹⁸

ISSUES RELATED TO STERILITY TESTING

Absolute Sterility

Within the strictest definition of “sterility”, a specimen would be deemed “sterile” only when there is complete absence of viable microorganisms from it. However, this absolute definition cannot be applied to an entire lot of finished drug products since the specified number of units to be tested only a small percentage of entire lot of the finished drug products. The “absolute sterility” confirmation cannot be practically achieved without complete destruction of every finished drug product. Currently there is no non-destructive technology for the sterility testing. To significantly increase the number of specimens for testing in order to augment the probabilistic prediction of the test outcome is not a feasible option.

Undetected Microorganisms

Sterility test is also limited in that it can only recognize organisms that are able to grow under the conditions (e.g., medium, incubation temperature, and incubation time, etc.)^{19,20} There are a large number of microorganisms that are unable to replicate under standard testing conditions.²¹⁻²³ Even with the longer incubation period there is no assurance that all microorganisms can grow under these conditions, but are still metabolically active.

Radiopharmaceutical Uniqueness

Special difficulties arise with radiopharmaceutical preparations because of the short half-life of some radionuclides (especially those used for diagnostic purposes), small size batches, low production volume, and radiation hazards. The short half-lives of most radiopharmaceuticals used in nuclear medicine studies prohibit completion of the sterility testing before the release of radiopharmaceutical products. In addition, when the half-life of the radionuclide is very brief (e.g., less than 20 minutes), administration of the radiopharmaceutical preparation to the patient is generally on-line with a

validated production system. It is justifiable to dispense radioactive drug products before completion of the sterility test if the radiopharmaceutical is prepared by a validated aseptic process.

Sterility testing can usually be conducted in a hospital's microbiology laboratory. For safety reasons (i.e., high levels of radioactivity), it may be not possible to use the quantity of a radiopharmaceutical preparation stipulated in the USP sterility testing chapter.¹⁴ To limit radiation exposure of personnel, the product should be diluted or allowed to decay to a safe working level before sterility tests are conducted.

There is another aspect of the prepared radiopharmaceutical that is quite different from the conventional drug product. The preparation of radiopharmaceuticals (e.g., ¹⁸F-fludeoxyglucose [FDG] injection) usually results in one lot of a multiple-dose vial containing a homogeneous solution of the radiopharmaceutical. Quality control (QC) results from end-product testing (including sterility test) of samples drawn from the above-mentioned multiple-dose vial have the maximum probability of being representation of all doses to be administered to patients from that vial. Thus, the referee sterility testing (as well as other QC tests) performed on prepared radiopharmaceutical would undoubtedly offer a near "absolute sterility" confirmation.

Minimal Testing

"The extensive aseptic manipulations required to perform sterility testing may result in a probability of non-product-related contamination of the order of 10^{-3} , a level similar to the overall efficiency of an aseptic operation and comparable to the microbial survivor probability of aseptically processed articles. This level of probability is significantly greater than that usually attributed to a terminal sterilization process, namely, 1 in 1 million or 10^{-6} microbial survivor probability."¹³ These facts as stated in the USP General Chapter <1211> "Sterilization and Sterility Assurance of Compensial Articles" seems to suggest that the lower microbial survival probability from an effectively terminally sterilized products may preclude altogether the necessity for performing sterility testing. However, a successful terminal sterilization process depends mostly on whether the "microorganism-elimination" capability of the above sterilization process can effectively handle the bioburden level of the finished drug product. Thus, the reliability of sterility assurance of terminal sterilization heavily hinges on a properly validated and documented aseptic technique and sterilization process.

“If data derived from the manufacturing process sterility assurance validation studies and from in-process controls (e.g., membrane filter integrity test – to be discussed later) are judged to provide greater assurance that the lot meets the required low probability of containing a contaminated unit (compared to sterility testing results from finished units drawn from that lot),” USP General Chapter <1211> “Sterilization and Sterility Assurance of Compendial Articles” indicates that “any sterility test procedures adopted may be minimal, or dispensed with on a routine basis.”¹³ With regard to radiopharmaceutical products intended for parenteral usage, USP General Chapter <1211> “Sterilization and Sterility Assurance of Compendial Articles” further indicates that due to “rapid radioactive decay, it is not feasible to delay the release of some radioactive pharmaceutical products in order to complete sterility tests on them. In such cases, results of sterility tests provide only retrospective confirmatory evidence for sterility assurance, which therefore depends on the primary means thereto established in the manufacturing and validation/certification procedures.”¹³

USP General Chapter <823> “Radiopharmaceuticals for Positron Emission Tomography – Compounding”

As per the statements described in the previous paragraph, the frequency of sterility testing may be reduced if there is adequate accumulated data to support a conclusion that the current preparation process yields satisfactory sterility assurance, as well as an in-process control (e.g., membrane filter integrity test) is included as one of the acceptance criteria of the finished sterile drug product. The reduced testing frequency for sterility is particularly meaningful for radiopharmaceutical labeled with a radionuclide having a short physical half-life. The above-mentioned reasons are probably why USP General Chapter <823> “Radiopharmaceuticals for Positron Emission Tomography – Compounding” has a less-demanding testing frequency for sterility.¹⁵ It requires “only the first lot prepared each day shall be subject to a sterility test” “after a record of successful sterility tests is established for a particular PET drug.”¹⁵ However, there are two situations that a new sterility test must be carried out the PET drug that meets one or both conditions:

- When a different PET drug is made at the facility
- A new lot of sterile components (e.g., filter or final product container) is substituted

USP General Chapter <797> “Pharmaceutical Compounding – Sterile Preparations”

Along the same line of rationale for minimal testing frequency for sterility, the recently released USP General Chapter <797> “Pharmaceutical Compounding – Sterile Preparations” states that only high-

risk level compounded sterile preparations (CSPs) that “are prepared in groups of more than 25 identical individual single-dose packages (e.g., ampuls, bags, syringes, vials) or in multiple-dose vials (MDVs) for administration to multiple patients or that are exposed longer than 12 hours at 2-8°C and longer than 6 hours at warmer than 8°C before they are sterilized shall meet the sterility test (see [Sterility Tests <71>](#)) before they are dispensed or administered.”^{14,24} Additionally, the membrane filtration method is the method of choice for performing the above-mentioned sterility test as per USP General Chapter <797> “Pharmaceutical Compounding – Sterile Preparations”.²⁴

High-Risk Level CSPs

According to USP General Chapter <797> “Pharmaceutical Compounding – Sterile Preparations”²⁴ “CSPs compounded under any of the following conditions are either contaminated or at a high risk to become contaminated.

High-Risk Conditions—

1. Nonsterile ingredients, including manufactured products not intended for sterile routes of administration (e.g., oral), are incorporated or a nonsterile device is employed before terminal sterilization.
2. Any of the following are exposed to air quality worse than ISO Class 5 (see [Table 1](#)) for more than 1 hour (see *Immediate-Use CSPs*):
 - sterile contents of commercially manufactured products,
 - CSPs that lack effective antimicrobial preservatives, and
 - sterile surfaces of devices and containers for the preparation, transfer, sterilization, and packaging of CSPs.
3. Compounding personnel are improperly garbed and gloved (see *Personnel Cleansing and Use of Barrier Protective Equipment*).
4. Nonsterile water-containing preparations are stored for more than 6 hours before being sterilized.
5. It is assumed, and not verified by examination of labeling and documentation from suppliers or by direct determination, that the chemical purity and content strength of ingredients meet their original or compendial specifications in unopened or in opened packages of bulk ingredients (see *Ingredient Selection* under [Pharmaceutical Compounding—Nonsterile Preparations <795>](#)).

TESTING PREREQUISITES

Since sterility testing is a very exacting procedure, it is critical that testing environment and personnel qualification must be carefully evaluated to ensure a successful sterility testing process.

Testing Environment

The draft guidance of current good manufacturing practice (CGMP) for PET drug products issued by the Food and Drug Administration (FDA) indicates that “testing be conducted in a controlled area such as a laminar airflow workbench (LAFW) with clean-room apparel.”¹⁷ However, a more appropriate site for handling sterility testing is a biological safety cabinet (BSC). A BSC is a ventilated cabinet that has an open front with inward airflow for personnel protection, downward high-efficiency particulate air (HEPA)-filtered laminar airflow for product protection, and HEPA-filtered exhausted air for environmental protection. The facility used for sterility testing should be designed, operated, and monitored in such a manner that it offers “no greater a microbial challenge to the articles being tested than that of an aseptic processing production facility.”¹³ Precautions must be taken to avoid any contamination that may affect any microorganisms to be revealed in the test. The working conditions of the test facility should be monitored regularly by carrying out appropriate controls (e.g., pressure differential monitoring) and by appropriate sampling of the working area and (e.g., viable and non-viable environmental sampling, surface cleaning and disinfection sampling and assessment).²⁴

Personnel Qualification

As per the draft guidance of CGMP for PET drug products, “[t]he greatest risk of false-positive results arises in the sampling and transfer of the test aliquot from the vial to the media.”¹⁷ An Individual who performs the sterility testing procedure should pass the routine competency evaluation of garbing and gloving,²⁴ he/she should also have a high level of aseptic technique proficiency. The competency evaluation of aseptic work practices and manipulation should be regularly conducted via glove fingertip sampling and media-fill test procedure, respectively.²⁴ It is also important that personnel be properly trained and qualified for the interpretation of test results. To achieve this objective, these individuals should have formal training in microbiology, having knowledge of industrial sterilization, and the statistical concepts involved in sampling. Additionally, they should be knowledgeable in the environmental control program established for the test facility so that the microbiological quality of air and critical work surface are consistently acceptable. The test facility should maintain an updated file (e.g., certificate of training) for each individual who performs the sterility test.¹³

USP General Chapter <1211> “Sterilization and Sterility Assurance of Compendial Articles” suggests that “known-to-be-sterile finished articles should be employed periodically as negative controls” to check the reliability of the test procedure.¹³ It is equally suitable to utilize the above process to evaluate the reliability of the technician who performs the sterility test. In both situations, it is preferable that “the technicians performing the test should be unaware that they are testing negative controls”.¹³ USP General Chapter <1211> “Sterilization and Sterility Assurance of Compendial Articles” indicates that a false-positive frequency should not exceed 2% of these tests.¹³

TYPES OF MEDIA

Microbes vary greatly in their requirements for growth including temperature, food/nutrient source, pH and oxygen requirement. No single growth medium satisfies the optimum conditions for all microorganisms. The vast majority of microbial contamination found in pharmaceutical manufacturing operations are mesophiles (thrive at 10-40 °C), heterotrophs (require preformed organic compounds for metabolism), and aerobes (need oxygen for growth).

For sterility testing, two media are required which support the growth of human pathogens and a broad spectrum of microbes.¹⁴

Fluid Thioglycollate Medium (FTM)

Fluid thioglycollate medium is designed to support the growth of both aerobes and anaerobes in a single medium. An FTM tube has a yellow and pink (top) layer. The aerobic phase has an oxygen-sensitive dye that is pink when oxygen is present. The bottom yellow phase has residual oxygen scavenged by antioxidant. When incubated at 30-35 °C, FTM supports the growth of heterotrophic and facultative (aerobic or anaerobic) microorganisms (Table 3).

Table 3

PROPERTIES AND USAGES OF STERILITY TESTING MEDIA				
Medium	pH	Phases	Storage Temperature (°C)	Incubation Temperature (°C)
FTM	7.1±0.2	2	2-25	32.5±2.5
SDM	7.3±0.2	1	2-25	22,5±2.5

Soybean-Casein Digest Medium (SDM)

Soybean-Casein Digest Medium (also known as trypticase soy broth [TSB] or trypticase soy agar [TSA]) is devised to support heterotrophic and aerobic organisms at 20-25 °C; its higher pH facilitates the growth of fungi (Table 3).

Storage and Shelf-Life of the Culture Media

“If prepared media are stored in unsealed containers, they can be used for 1 month, provided that they are tested for growth promotion within 2 weeks of the time of use and that color indicator requirements are met. If stored in tight containers, the media can be used for 1 year, provided that they are tested for growth promotion within 3 months of the time of use and that the color indicator requirements are met.”¹⁴

Composition of FTM and SDM

Please refer to USP General Chapter <71> “Sterility Tests” for the composition of FTM and SDM.¹⁴ The culture media for sterility testing can be prepared in-house as per the formulation listed in the above-mentioned USP. Media shall be sterilized using a validated and documented process.

Each ready-to-use (ready-prepared) medium or medium reconstituted from dehydrated medium must be tested to verify that it meets the requirements of the USP “Growth Promotion Test of Aerobes, Anaerobes, and Fungi” (to be referred as “growth promotion test” henceforth).¹⁴ It may be acceptable to use a certificate of analysis (COA) obtained from the manufacturer or distributor of the ready-prepared culture medium in lieu of an actual performance of the growth promotion test for the medium if the COA indicates that the growth promotion test performed for the specific medium meets the USP specification.

Growth Promotion Test

To demonstrate that medium will support microbial growth, a separate portion of FTM is incubated with a small number of each of the three strains of microorganism *Clostridium sporogenes*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Table 4) – the number of inoculated microorganism should not be more than 100 colony-forming units (cfu).¹⁴

Table 4

TYPES AND STRAINS OF TEST MICROORGANISMS USED IN THE GROWTH PROMOTION AND VALIDATION TESTS		
Medium	Types of Microorganisms	Strains of Microorganisms
FTM	Aerobic	<i>Bacillus subtilis</i> <i>Pseudomonas aeruginosa</i> <i>Staphylococcus aureus</i>
	Anaerobic	<i>Clostridium sporogenes</i>
SDM	Aerobic	<i>Bacillus subtilis</i> <i>Pseudomonas aeruginosa</i> <i>Staphylococcus aureus</i>
	Fungi	<i>Aspergillus niger</i> <i>Candida albicans</i>

Inoculate portions of SDM with a small number (not more than 100 cfu) of the microorganisms - *Aspergillus niger*, *Bacillus subtilis*, and *Candida albicans* (Table 4). Using a separate portion of medium for each of the above-mentioned species of microorganism.¹⁴

Incubate for not more than 3 days in the case of bacteria (i.e., growth promotion test for FTM) and not more than 5 days in the case of fungi (i.e., growth promotion test for SDM).² The media are suitable for sterility testing if a clearly visible growth of the microorganisms occurs within the specified time period for each organism.¹⁴

STERILITY TESTING PROCEDURES

According to USP General Chapter <71> “Sterility Tests”, articles listed in the USP “are to be tested by the *Membrane Filtration* method ... If the membrane filtration technique is unsuitable, use the *Direct Inoculation of the Culture Medium* method ...”¹⁴

Membrane Filtration

Membrane filters with a nominal pore size of not greater than 0.45 µm must be used.¹⁴ “Cellulose nitrate filters, for example, are used for aqueous, oily, and weakly alcoholic solutions;” as per USP General Chapter <71> “Sterility Tests”, “and cellulose acetate filters, for example, are used for strongly alcoholic solutions. Specially adapted filters may be needed for certain products (e.g., for antibiotics).”¹⁴

The test article is filtered through a pre-rinsed sterile size exclusion membrane whose effectiveness to retain bacteria and fungi has been established. The filter is then rinsed with the appropriate sterile diluent (see “Diluting and Rinsing Fluids for Membrane Filtration” under USP General Chapter <71> “Sterility Tests”),¹⁴ and the membrane is aseptically cut into two equal parts and transferred each one of them into a different medium (i.e., FTM or SDM).¹⁴ The media is then incubated for not less than 14 days.¹⁴

Direct Inoculation

This method is also referred to as the “immersion” method in which it requires the test article be inoculated directly into test media (i.e., FTM and SDM). Similar to the membrane filtration method, the media inoculated with the test article is incubated for 14 days.¹⁴ The volume of the test product should not be more than 10% of the volume of the medium.¹⁴

If the drug product to be tested has antimicrobial activity, this has to be neutralized with a suitable neutralizing substance (e.g., β -lactamase – see “Media for Penicillins or Cephalosporins” section under USP General Chapter <71> “Sterility Tests” for more information about the “neutralization” procedure).¹⁴ The antimicrobial activity in the test product can also be neutralized by dilution technique. If the subsequent dilution of the test sample results in a large volume of product, it may be preferable to add the concentrated medium directly to the diluted product in its container.¹⁴ Another option for perform a sterility test on a test article that contains bacteriostatic substance is to use the membrane filtration method which is a more suitable technique especially for test articles with large volumes.

The majority of drug sample will be tested using the direct inoculation due to its ease of use. However, for test articles with large volumes in which the entire contents must be tested or there is a substance within the test article that is known or determined to be bacteriostatic, the membrane filtration method may be a more suitable choice.

Negative Control

Known-to-be-sterile finished drug products or sterile diluting fluid (e.g., Fluid A, D, or K)¹⁴ should be used periodically or regularly as negative controls to serve as a check on the reliability of the test procedure and/or the validity of the test outcome.¹⁴

Validation Test

Similar to the use of the inhibition/enhancement test to check any possible interference from the test sample to the final outcome of the USP bacterial endotoxins test,²⁵ the validation test is employed to determine whether the test product contains any antimicrobial activity that may adversely affect the outcome of sterility testing.

Membrane Filtration

“After transferring the content of the container or containers to be tested to the membrane, add an inoculum of a small number of viable microorganisms (not more than 100 cfu) to the final portion of sterile diluent used to rinse the filter.”¹⁴

Direct Inoculation

“After transferring the contents of the container or containers to be tested (for catgut and other surgical sutures for veterinary use: strands) to the culture medium, add an inoculum of a small number of viable microorganisms (not more than 100 cfu) to the medium.”¹⁴

In both cases use the same microorganisms as those described in Table 2. Perform a growth promotion test as a positive control (without test product). Incubate all the containers containing medium for not more than 5 days. If clearly visible growth of microorganisms in test sample, as well as in the positive control (i.e., the growth promotion test) after the incubation, either the product possesses no antimicrobial activity under the conditions of the test or such activity has been satisfactorily eliminated. The test for sterility may then be carried out without further modification.¹⁴

If clearly visible growth is not obtained in the presence of the product to be tested, but the positive control (i.e., the growth promotion test) group shows visible growth of microorganisms, then the test product possesses antimicrobial activity that has not been satisfactorily eliminated under the conditions of the test. Modify the conditions in order to eliminate the antimicrobial activity, and repeat the validation test.¹⁴ The antimicrobial activity in the product can be neutralized by using a suitable neutralizing substance (e.g., using β -lactamase) or dilution in a sufficient quantity of culture medium.¹⁴

According to USP General Chapter <71>“Sterility Tests”, the validation test “is performed (a) when the test for sterility has to be carried out on a new product; and (b) whenever there is a change in the

experimental conditions of the test.”¹⁴ The validation test may be performed simultaneously with the sterility test in accordance with USP General Chapter <71> “Sterility Tests”.¹⁴

Initiation Time

There is no mention, in USP General Chapter <71> “Sterility Tests”, of the initiation time for performing the sterility test.¹⁴ But, USP General Chapter <823> “Radiopharmaceuticals for Positron Emission Tomography – Compounding” states that sterility test be started no later than 24 hours after compounding of PET radiopharmaceuticals for parenteral administration.¹⁵ The “24-hour” initiation time was changed to “30-hour” in the proposed rule and draft guidance (also issued by the FDA) of CGMP for PET drugs.^{16,17} The 30-hour requirement may be exceeded if the test sample for sterility testing has to be held over the weekend or holiday.^{16,17} However, if the sample for sterility testing is held longer than indicated (i.e., 30-hour), the PET drug producers should “demonstrate that the longer period does not adversely affect the sample and the test results obtained will be equivalent” to test results that would have been obtained if the test had been started within the 30-hour time period.”^{16,17} During the 30-hour waiting time frame, the test samples should be stored appropriately (e.g., under refrigeration).¹⁷

Pooled Samples

Test sample from each lot of drug product shall be tested individually and must not pooled with other lot(s) of product(s).^{15,16}

Quantity to be Tested

Please refer to Table 4 in the USP General Chapter <71> “Sterility Tests” for the minimum quantity of the article to be used for each test medium.¹⁴ For most in-house prepared radiopharmaceuticals, the minimum test volume is generally 1 mL, and the sample volume should not exceed 10% of total volume of the medium. Due to the limited volume in the finished product and/or radiation safety, the test sample can be diluted with sterile solution (e.g., sterile saline or water). However, the most suitable diluting fluid for the sterility testing should be Fluid A, D, or K as described in USP General Chapter <71> “Sterility Tests”.¹⁴

Number of Articles to be Tested

Please refer to Table 3 in the USP General Chapter <71> “Sterility Tests” for the minimum number of the article to be used for sterility testing.¹⁴ Since a lot of the majority prepared radiopharmaceuticals equals to one multiple-dose vial, this requirement should not be applicable to radiopharmaceuticals.

OBSERVATION AND INTERPRETATION OF RESULTS

The USP sterility testing method requires observation of the tubes of media over a 14 day incubation period¹⁴ unless otherwise specified elsewhere. It is common to observe the test samples at days 3, 7, and 14.¹⁷ However, it is prudent to observe the media more often during the first week of incubation.¹⁷ Bacteria usually grow out quickly in the first few days, as evidenced by turbidity or precipitation at the bottom of the tube, but the 14-day incubation facilitates the detection of slow-growing microbes and germination of spores.

Turbidity in the media would be indicative of the presence of a microbial contaminant. Identification (at least the genus level) of the microorganisms should be performed by a certified laboratory in order to take appropriate corrective actions (with the assistance of a competent microbiologist, infection control professional, or industrial hygienist) to remedy the identified problem..

If no evidence of microbial growth is found, the product to be examined complies with the test for sterility. However, if evidence of microbial contamination in the product is obtained by the appropriate USP method, the product to be examined fails to comply with the USP sterility test. According the USP General Chapter <71> “Sterility Tests”, the outcome of a sterility test may be considered invalid only if one or more of the following four conditions are met:¹⁴

- a. The data of the microbiological monitoring of the sterility testing facility show a fault.
- b. A review of the testing procedure used during the test in question reveals a fault.
- c. Microbial growth is found in the negative controls (although USP General Chapter <1211> “Sterilization and Sterility Assurance of Compendial Articles” indicates that “Finding of microbial growth in negative controls need not be considered the sole grounds for invalidating a *First Stage* test.”¹³ – to be discussed later).
- d. After determination of the identity of the microorganisms isolated from the test, the growth of this species (or these species) may be ascribed unequivocally to faults with respect to the material and or the technique used in conducting the sterility test procedure.

If the test is declared to invalid, the test is repeated as per USP General Chapter <71> “Sterility Tests”¹⁴ – therefore it may be prudent to save a “backup” sample for a possible retest. If no evidence of microbial growth is found in the repeat test, the product under the evaluation complies with the test for sterility.¹⁴ Otherwise, the product being tested does not comply with the sterility test.¹⁴

USP General Chapter <1211> “Sterilization and Sterility Assurance of Compendial Articles” describes a two-stage quality control sterility test in order to “rule out false positive results”.¹³

First Stage

Regardless of the sampling plan used, if no evidence of microbial growth is found, the results of the test may be taken as indicative of absence of intrinsic contamination of the lot. If microbial growth is found, proceed to the *Second Stage* (unless the *First Stage* test can be invalidated – see conditions for an invalidate test as described previously).

Second Stage

When proceeding to the *Second Stage*, particularly when depending on the results of the test for lot release, initiate and document concurrently a complete review of all applicable production and control records. In this review, consideration should be paid to the following items:

- a. A check on monitoring records of the validated sterilization cycle applicable to the product.
- b. Sterility test history relating to the particular product for both finished and in-process samples, as well as sterilization records of supporting equipment, containers/closures, and sterile components, if any.
- c. Environmental control data, including those obtained from media fills, exposure plates, filtering records, any sanitization records and microbial monitoring records of operators, gowns, gloves, and garbing practices.
- d. The current microbial profile of the product should be checked against the known historical profile for possible change.
- e. Records should be checked concomitantly for any changes in source of product components or in-processing procedures that might be contributory.

Depending on the findings, and in extreme cases, consideration may have to be given to revalidation of the total manufacturing process. For the *Second Stage*, it is not possible to specify a particular number of specimens to be taken for testing.¹⁴ It is usual to select double the number specified for the *First*

Stage under USP General Chapter <71> “Sterility Tests”,¹⁴ or other reasonable number. The minimum volumes tested from each specimen, the media, and the incubation periods are the same as those indicated for the *First Stage*.¹⁴

If no microbial growth is found in the *Second Stage*, and the documented review of appropriate records and the indicated product investigation does not support the possibility of intrinsic contamination, the lot may meet the requirements of a test for sterility.¹⁴ If growth is found, the lot fails to meet the requirements of the test.¹⁴ As was indicated for the *First Stage* test, the *Second Stage* test may similarly be invalidated with appropriate evidence, and, if so done, repeated as a *Second Stage* test.¹⁴

In case a different result is obtained by an alternative procedure for the sterility test, the final decision is based on the result obtained from the testing procedure in compliance with the USP General Chapter <71> “Sterility Tests”.¹⁴ If the test medium with the product to be examined is so turbid that the presence or absence of microbial growth cannot be readily determined by visual examination, portions of the product being examined should be transferred to fresh vessels of the same medium.¹⁴ The original and transfer vessels are then incubated for not less than 4 days.¹⁴

Out-of-Specification Investigation

According to the Proposed Rule § 212.70 of CGMP for PET drug, “If the product fails the sterility test, all receiving facilities must be notified of the results immediately. The notification must include any appropriate recommendations. The notification must be documented.”¹⁶ A complete investigation shall be conducted immediately to identify the cause(s).¹⁵⁻¹⁷ The investigation must include, but is not limited to, as per Proposed Rule § 212.71 of CGMP for PET drug, “examination of processes, operations, records, complaints, and any other relevant sources of information concerning the non-conforming product.”¹⁶ The results of the investigation must be documented, and corrective actions based on the above-mentioned results must be implemented promptly in order to prevent similar recurrence of another PET drug product failed the sterility test.¹⁶⁻¹⁷

Membrane Filter Integrity

A membrane filter assembly should be tested for initial integrity prior to use,¹ provided that such test does not impair the validity of the system, and should be tested after the filtration process is completed to demonstrate that the filter assembly maintained its integrity throughout the entire filtration procedure.^{15,17} For each batch of PET radiopharmaceutical intended for parenteral administration, USP General Chapter <823> “Radiopharmaceuticals for Positron Emission Tomography – Compounding” stipulates that a membrane filter integrity test be performed immediately after completion of product filtration.¹⁵ The batch of PET radiopharmaceutical cannot be released for human use until the post-filtration integrity test is completed and passed.¹⁵ The only sterile PET radiopharmaceutical that does not need to comply with this requirement is ¹⁵O water due to its very short physical half-life.¹⁵ According to USP General Chapter <823> “Radiopharmaceuticals for Positron Emission Tomography – Compounding”, the batch of ¹⁵O water may be released prior to completion of the post-filtration integrity test; however, the integrity test must be completed as soon as possible after release of the batch.¹⁵

Membrane integrity testing can be accomplished by the bubble-point test, the diffusive airflow test, the pressure hold test, and the forward flow test to demonstrate that the integrity of the filter was not compromised during or before use.¹³ All these tests are based on the same physics in which the flow of a gas passes through a liquid-wetted membrane under applied gas pressure. They differ in which part of the flow/pressure spectrum they examine. In this article, we will focus on the bubble-point test since it is specifically mentioned in the USP General Chapter <823> “Radiopharmaceuticals for Positron Emission Tomography – Compounding”, the FDA’s draft Chemistry, Manufacturing, and Controls Section for ¹³N ammonia injection, ¹⁸F FDG injection, and ¹⁸F sodium fluoride injection, as well as the FDA’s draft guidance of CGMP for PET drug products.^{15,18}

Bubble-Point Test

The bubble-point test is the most commonly used method for the membrane integrity testing. A bubble point is the measure of the amount of air pressure required to force an air bubble through a wetted pore. It is based on the fact that, for a given fluid and pore size of a membrane filter with a constant wetting, the pressure required to force an gas bubble through the pore is inversely proportional to the size of the hole. This is sometimes referred to as “capillary theory.” The theory of capillarity states that the height of a water column in a capillary is indirectly proportional to the capillary diameter.

Surface tension forces held up the water in the capillary and as the diameter of the capillary gets smaller, the weight in the water column get heavier. Water can be pushed back down by a pressure which has the same equivalent height as that of the water column. Thus, by determining the pressure necessary to force water out of the capillary, the diameter of the capillary can be calculated. Similarly, the pore size of the membrane filter can be established by wetting the membrane filter and measuring the pressure at which the first stream of bubbles is emitted from the upper surface of the filter when air is introduced to an open end. The bubble-point rating is determined when the largest pore yields a bubble; the larger the pore, the less pressure required to form the bubbles. Bubble point is usually expressed in unit of pounds per square inch (psi).

To perform a bubble-point test, a source of gas pressure (e.g., compressed air) equipped with a pressure gauge is connected to the non-sterile side of a uniformly pre-wetted filter membrane (Figure 4). The filter outlet (the sterile side of the membrane filter) can be either submerged in a bucket of water, or alternatively, a tubing can be connected to the filter outlet and extended so that the opposite-end of the tubing is below liquid level (Figure 4). Gas pressure is gently increased and the formation

of non-continuous bubbles on the liquid side may be noted (residual air from outlet side of the filter disk and/or the tubing being pushed out). At pressures below the bubble point, gas passes across the filter only by diffusion. But, when the pressure is high enough to dislodge liquid from the pores, a steady stream of bubbles is observed exiting the submerged membrane filter disk or tubing (Figure 4). The pressure at which this steady stream is noticed is referred to as the bubble point and it should be a standard value (e.g., ≥ 50 psi) for a specific pore-size of a membrane filter.

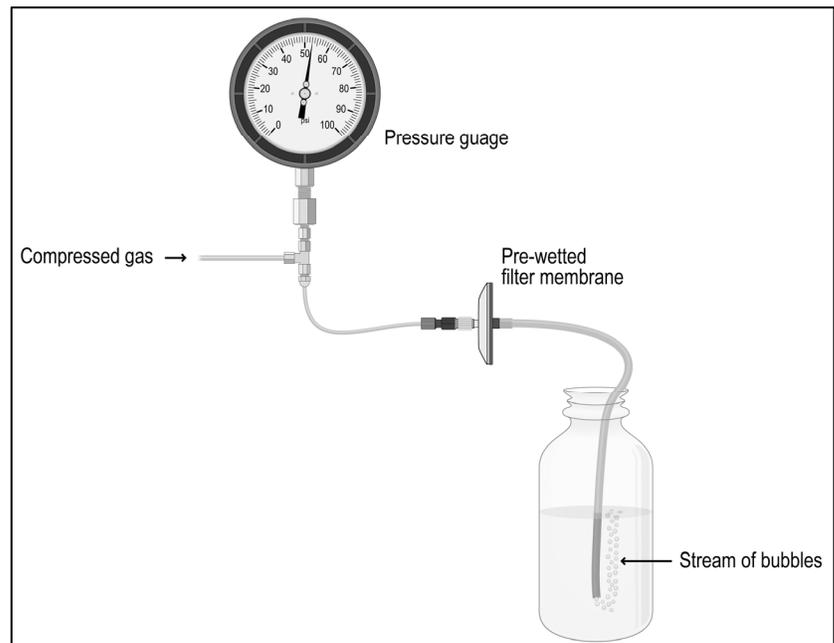


Figure 4. The setup for a bubble-point test. The bubble point is indicated by vigorous bubbling from the tubing. For a 0.22- μm membrane filter that is commonly used in a nuclear pharmacy setting, the typical bubble point is 50 psi (pound per square inch) or psig (pound-force per square inch gauge). Intact membrane filters will not allow a continuous stream of bubbles to appear on the outlet side of the filter. A few air bubbles (a discontinuous stream) are possible due to air infusion.

“Limitus Test”

Since the sterility test is completed retrospectively almost for all radiopharmaceuticals, the membrane filter integrity test should be used as a “limitus test” for the microbiologic purity of the product. Thus, the post-filtration integrity test (e.g., a bubble-point test) can also serve as an in-process rapid sterility “control test.”

Reprocessing

Under proposed § 212.70 (d) for CGMP on PET drug products, “a drug product can be reprocessed if pre-established procedures (set forth in production and process controls) are followed and the finished product conforms to specifications before final release. Examples of reprocessing could include ... a second passage through a filter if the original filter failed the integrity test.”¹⁶ When the option for reprocessing is exercised, FDA recommends that “the event be documented and conditions described in a brief deviation report.”¹⁶

SUMMARY

Microbial contamination is difficult to control because of its ubiquity in nature. Removal of contamination from a process is costly and often incomplete. The best approach for making compounds free of microbiological contamination is to avoid its introduction. Emphasis on prevention of microbiological impurities must be directed toward the synthetic process. The compounding environment, selection of reagents, assembly of equipment and training of operators must unequivocally create a synthesis process that prevent or eliminates microbial contamination. Procurement of sterile, endotoxin-free starting materials and proper sterilization of other in-process materials and equipment are cornerstones to responsible compounding in the hospital or pharmacy setting.

With emphasis on the compounding process, validation of the compounding and QC procedures (including BET and sterility testing) prior to production activities provides the needed assurance that products will have the required quality attributes. After a demonstration of successful validation of these procedures, the frequency for performing BET and sterility testing may be substantially reduced (e.g., only the first batch prepared each day) or these two tests may be replaced with the in-process tests (i.e., 20-minute endotoxin “limit test” for BET and the membrane filter integrity test for sterility test.)

The BET is required for parenteral radiopharmaceuticals. The BET requires an FDA licensed form of LAL reagent, describes necessary conditions for photometric and gel-clot tests, and gives criteria for interpretation of results. The technique dependence and liquid handling requirements make the gel-clot problematic in the hands of unskilled analysts. A new pre-calibrated LAL cartridge and reader allows unprecedented accuracy, simplicity and rapidity for endotoxin testing of short-lived radiopharmaceuticals.

In accordance with USP General Chapter <797> “Pharmaceutical Compounding – Sterile Preparations”, sterility testing is required to be carried out only for certain high-risk level CSPs.²⁴ Since USP General Chapter <797> “Pharmaceutical Compounding – Sterile Preparations” is not applicable to compounded PET parenteral radiopharmaceuticals, the performance of the sterility test on these drugs is subject to USP General Chapter <823> “Radiopharmaceuticals for Positron Emission Tomography – Compounding”¹⁵ (it is not required to be performed on every batch of compounded PET sterile radiopharmaceutical) and FDA’s regulation (sterility testing is required to be carried out for each batch of compounded PET sterile radiopharmaceutical).^{16,17} Nevertheless, radiopharmaceutical preparations to be tested for sterility should meet the requirements stated in USP General Chapter <71> “Sterility Tests”,¹⁴ with the exception that radiopharmaceuticals may be distributed or dispensed before completion of the tests for sterility. Thus, sterility test only provides retrospective information for radiopharmaceuticals.

APPENDIX

Standard Operating Procedure for Photometric BET by Endosafe® PTS™ System

1 Purpose and Scope

This procedure describes how to test PET radiopharmaceuticals for endotoxin using the Endosafe®-PTS Portable Test System, which is based on a kinetic chromogenic BET, as described in the USP chapter <85>. This procedure explains how to prepare samples, set up the PTS unit for analysis, print the test results and interpret the findings.'

2 References

- 2.1 U.S. Pharmacopeia, General Chapter <85> Bacterial Endotoxins Test, current revision
- 2.2 Charles River Laboratory, User's Guide, Version 7.10, Endosafe®-PTS Portable Test System
- 2.3 Operation Manual for Epson TM-U220D Printer, or equivalent
- 2.4 PTS Logger software CD

3 Safety and General Methods

Use appropriate cautions when handling radioactive materials

4 Terms

- 4.1 CoA = Certificate of Analysis, obtained from reagent vendor.
- 4.2 EU = Endotoxin unit, a unit of biological activity of the international reference standard endotoxin.
- 4.3 Lambda, λ = the lowest point of the endotoxin standard curve.
- 4.4 LRW = LAL reagent water, non-reactive with LAL.
- 4.5 PPC = Positive product control for BET interference detection.
- 4.6 MVD = Maximum valid dilution, endotoxin limit/lambda.

5 Equipment and Materials

- 5.1 Endosafe®-PTS Reader
- 5.2 Endosafe®-PTS Cartridge with dried reagents with Certificate of Analysis, in either a range such as 5-to-0.05 EU/mL. where lambda = 0.05 EU/mL.
- 5.3 LAL Reagent Grade Water (LRW) or freshly opened Water for Injection, USP, for sample dilution.
- 5.4 Sterile, disposable polystyrene tubes for sample collection or dilution.
- 5.5 Mechanical pipettor with sterile pipette tips.
- 5.6 Pipettor (Endosafe®-PTS 400 or equivalent) and sterile pipette tips, which are used for sample preparation (dilution) and loading a sample into the PTS.
- 5.7 Computer system and Printer

6 Procedures

6.1 Storage Conditions and Precautions

PTS cartridges should be stored at 2 – 25°C. Keep cartridges within the sealed pouch until ready for use. Allow a cartridge to reach room (ambient) temperature in the pouch before opening and initiating a test.

6.2 Initial Qualification of a new lot of cartridges or new analyst

Each new lot of cartridges must be qualified upon receipt; one cartridge is required with LRW as a sample, as analyzed in steps below. In this qualification, answer the prompt for product ID by entering “Initial Qual”, then enter the lot No. for LAL reagent water. The evaluation must demonstrate no detectable endotoxin and acceptable spike recovery (50-200%). Conduct the same procedure for a new analyst. A negative result with SWI used in this qualification also qualifies the SWI as LRW, as non-reactive in the system.

6.3 Instrument (Endosafe®-PTS Reader) Setup and Operation

6.3.1 Press the menu key on the PTS keypad to turn on instrument (Menu 5 turns it off). The reader then initiates a “SYSTEM SELF TEST” as it warms to 37 °C in about 5 minutes.

6.3.2 The reader displays “SELF TEST OK”, then “INSERT CARTRIDGE.”

6.4 Sample Preparation

6.4.1 Apply aseptic technique, use LRW or SWI for all dilutions and use pyrogen-free containers for preparing and testing samples with PTS®.

6.4.2 Make a sample dilution in accordance with validation information. For example, make a 1:50 dilution by transferring 0.1 mL of FDG F 18 to 4.9 mL of LRW or SWI that is contained in a sterile polystyrene container.

6.4.3 Before adding radioactivity, pre-label the container with the product code, lot number and other pertinent information, as needed.

6.5 Instrument Setup for Sample Analysis

6.5.1 Only when the reader is flashing “Insert Cartridge,” remove a cartridge from a pouch and insert it firmly with the sample reservoirs facing up into slot at the front of the PTS reader. DO NOT TOUCH sample reservoirs or optical cells.

6.5.2 Press cartridge firmly into the slot.

6.5.3 Answer the prompts correctly for “Enter OID” and “Enter Cartridge Lot #.” If entering a new lot number for cartridges, also refer to the Certificate of Analysis for the Calibration Code and answer the prompt for “Enter Calibration Code.”

6.5.4 Enter product information by answering the prompts for Sample Lot #, Sample ID and Dilution Factor. As an example: for FDG F 18, enter Lot #, FDG and 1:20, respectively.

6.5.5 Dispense the diluted test sample only when the reader displays: “ADD SAMPLE PRESS ENTER.” Pipette exactly 25 µL of sample into all 4 sample reservoirs of the inserted cartridge and press Enter on the reader keypad. Do NOT pipette straight down into reservoir.

6.6 Reviewing and Recording Results

6.6.1 The PTS reader signifies completion of the analysis with an audible notification; this occurs about 15 minutes after initiating the test. Review the results for endotoxin measurement and assay acceptance criteria that are displayed on the screen.

6.7 Reporting of Results

6.7.1 Download the results of the test to a printer or computer.

6.7.2 Attach the results to the Batch Record or other suitable document.

6.7.3 Enter results into the appropriate database, if indicated.

6.8 Acceptance Criteria

6.8.1 Validity. The test is valid if the CV (Coefficient of Variation) for the sample and spike are <25% and Spike Recovery is within 50 to 200%.

6.8.2 Invalidity. The test is invalid if one or more of the conditions specified in 6.8.1 are not met. Proceed to a new test if invalidity occurs.

6.8.3 Invalid Spike Recovery. If the invalid Recovery occurred because it was <50% and Spike %CV was valid (<25%), product inhibition may exist. Dilute the sample another 1:4 or 1:10 with LRW or WFI and conduct a new test. Contact the PTS vendor for advice if invalidity persists.

6.8.4 Pass/Fail. The test passes if Sample Value is <14 EU/mL, the endotoxin limit, when there is an assumption of a maximum dose of 12.5 mL of an F 18 drug. The PTS software corrects for dilution. For other medications, the test passes if Sample Value is less than the specific endotoxin limit for the sample being tested. The test fails if a Sample Value is equal to or greater than 14 EU/mL for an F 18 drug or greater than the specific endotoxin limit for other medications tested. Immediately contact the Pharmacist in Charge if a product fails.

6.9 Validation of a Drug Product for Testing by the PTS System

6.9.1 Select samples from three lots of a specific drug product to be validated for the PTS System.

6.9.2 Prepare a test sample from each lot of drug product (non-radioactive) according to Sample Preparation (6.4) described above.

6.9.3 When entering a sample for product validation, modify the Sample ID by enter the product code followed by "VAL." For example, enter FDG VAL when validating samples of Fludeoxyglucose F 18 Injection.

6.9.4 The samples are **valid** for testing by the PTS system if sample and spike CV values are valid ($\leq 25\%$) and the Spike Recovery for each lot is within 50-to-200%. Enter the validated dilution factor into the appropriate files.

6.9.5 Should invalid recovery occur, such as a recovery of <50% (indication of inhibition), repeat the validation tests at a greater dilution provided that the MVD is not exceeded.

6.9.6 In this scenario, where the maximum dose is arbitrarily 12.5 mL, the endotoxin is 14 EU/mL (175 EU/12.5 mL). The MVD is 280, where the endotoxin limit is divided by lambda (14 EU/mL/0.05 EU/mL).

6.10 Validation of Dry-heat Oven Cycle

- 6.10.1 In a typical oven load, expose 2-to-4 (EI) Endotoxin Indicators (2000 EU/vial) to at least 210°C for at least 2 hours.
- 6.10.2 Recover residual endotoxin from the EI vial with 1 mL LRW.
- 6.10.3 Confirm that the recovered endotoxin is less than 1 EU/mL, to give a >3-log reduction of endotoxin. Increase the time of temperature should an exposure experiment fail to yield a 3-log reduction in the EIs.

REFERENCES

1. Williams K. *Endotoxins. 3rd Edition*. Informa Healthcare, New York. 2007:27-90.
2. Cooper JF, Thoma LA. Screening extemporaneously compounded intraspinal injections with the bacterial endotoxins test, *Am J Health-Syst Pharm*, 2002;59:2426-2433.
3. U. S. Pharmacopeial Convention. <85> *Bacterial Endotoxins Test*. Current revision of USP.
4. Food and Drug Administration. Guidance for industry: Sterile drug products produced by aseptic processing. CDER, September 2004.
5. Food and Drug Administration. Guidance: PET drug products – Current Good Manufacturing Practice (CGMP), CDER, September 2005.
6. Cooper JF. Bacterial endotoxins test. In: Prince R, ed. *Microbiology in Pharmaceutical Manufacturing, 2nd Edition*. Bethesda, MD, Par. Drug Assoc., 2008; Vol 2:245-274.
7. Cooper JF, Williams K. Pyrogenicity Case Studies. In: Williams K, ed. *Endotoxins, 3rd Edition*, New York: Maracel Dekker, 2007:532-540.
8. Levin J, Bang FB. The role of endotoxin in the extracellular coagulation of *Limulus* blood. *Bull. Johns Hopkins Hosp.* 1964; 115(3): 265-274.
9. Cooper JF, Levin J, Wagner HN, Jr. Quantitative comparison of in vitro (*Limulus*) and in vivo (rabbit) methods for the detection of endotoxin. *J. Lab. Clin. Med.* 1971; 78:138-148.
10. FDA. *Guideline on validation of the Limulus amoebocyte lysate test as an end-product endotoxin test for human and animal parenteral drugs, biological products and medical devices*. December, 1987.
11. Dragotakes SC, Cooper JF, Hubers D. A new system for the rapid detection of endotoxin in PET radiopharmaceuticals. (abstract) June 19, 2005, Society of Nuclear Medicine. Toronto
12. Cooper JF, Harbert JC. Endotoxin as a cause of aseptic meningitis after radionuclide cisternography. *J Nucl Med.* 1975;16:809-813.
13. Sterilization and sterility assurance of compendial articles (General Chapter <1211>). In: *The United States Pharmacopeia 31st ed.*, and *The National Formulary 26th ed.* Rockville, MD: United States Pharmacopeia Convention, Inc.; 2008:670-675.
14. Sterility tests (General Chapter <71>). In: *The United States Pharmacopeia 31st ed.*, and *The National Formulary 26th ed.* Rockville, MD: United States Pharmacopeia Convention, Inc.; 2008:85-91.
15. Radiopharmaceuticals for positron emission tomography: compounding (General Chapter <823>). In: *The United States Pharmacopeia 31st ed.*, and *The National Formulary 26th ed.* Rockville, MD: United States Pharmacopeia Convention, Inc.; 2008:347-351.

16. US Food and Drug Administration. *Current Good Manufacturing Practice for Positron Emission Tomography Drugs* [proposed rule]. <http://www.fda.gov/OHRMS/DOCKETS/98fr/cd0279.pdf> (accessed on 08/27/08).
17. US Food and Drug Administration. *Guidance: PET Drug Products - Current Good Manufacturing Practice (CGMP)* [draft]. <http://www.fda.gov/cder/guidance/5425dft2.pdf> (accessed on 08/27/08).
18. US Food and Drug Administration. *Attachment 1 Sample Formats – Application to Manufacture Ammonia N 13 Injection Fludeoxyglucose F 18 Injection (FDG F 18) and Sodium Fluoride F 18 Injection Chemistry, Manufacturing, and Controls Section*. <http://www.fda.gov/cder/guidance/cmcsample.pdf> (accessed on 08/27/08).
19. Bryce DM. Tests for the sterility of pharmaceutical preparations. *J Pharm Pharmacol*. 1956;8:561-572.
20. Ernst RR, West KL, Hoyle JE. Problem areas in sterility testing. *Bull Parent Drug Assoc*. 1969;23:29-39.
21. Rappe M, Giovannoni. The uncultured microbial majority. *Annu Rev Microbiol*. 2003;57:369-394.
22. Hughes JB, Hellam JJ, Ricketts TH, Bohannon BJM. Counting the uncountable: statistical approaches to estimating microbial diversity. *Appl Environ Microbiol*. 2001;67:4399-4406.
23. Dixon B, Viable but nonculturable. *ASM News* 1998;64:372-373.
24. Pharmaceutical compounding – sterile preparations (USP General Chapter <797>). In: Second Supplement to *The United States Pharmacopeia* 31st ed., and *The National Formulary* 26th ed. Rockville, MD: United States Pharmacopeia Convention, Inc.; 2008:3700-3734.
25. Bacterial endotoxins test (USP General Chapter <85>). In: *The United States Pharmacopeia* 31st ed., and *The National Formulary* 26th ed. Rockville, MD: United States Pharmacopeia Convention, Inc.; 2008:98-102.

GLOSSARY

Endotoxin - A large toxic molecule derived from the outer cell wall of gram-negative bacteria. It is a complex molecule of polysaccharide, lipid A and other cell-wall components.

Endotoxin Limit (EL) - The maximum amount of endotoxin allowed in a dose of a parenteral product or on a medical device. The limit for radiopharmaceuticals is 175 EU per dose.

Endotoxin Unit (EU) or International Unit (IU)- A standardized amount of endotoxin based on its reactivity in the LAL test. A vial of RSE contains 10,000 endotoxin units.

Lambda (λ) - The symbol for LAL reactivity stated in EU/mL. For gel-clot assay, lambda is the labeled lysate sensitivity; for kinetic assays, lambda is the lowest point on the endotoxin standard curve for PTS.

Limulus Amebocyte Lysate (LAL) - An aqueous extract of the circulating blood cells (amebocytes) of the Atlantic horseshoe crab, *Limulus polyphemus*.

Lipopolysaccharide (LPS) - Highly purified bacterial endotoxin, free of protein; examples include CSE.

Maximum Valid Dilution (MVD) - A calculation used to determine how much a drug sample can be diluted and still detect the endotoxin limit. The MVD is the endotoxin limit divided by lambda, the LAL system sensitivity for endotoxin.

Positive Product Control (PPC) - An aliquot of test sample spiked with a known amount of endotoxin. In the kinetic assay the PPC serves as a control for inhibition and enhancement.

ABBREVIATIONS AND ACRONYMS

BSC	biological safety cabinet
cfu	colony-forming units
CGMP	current good manufacturing practice
CSP	compounded sterile preparation
EL	endotoxin limit
EU	endotoxin unit
FDA	Food and Drug Administration
FDG	fludeoxyglucose
FTM	fluid thioglycollate medium
HEPA	high-efficiency particulate air
IU	international unit
LAFW	laminar airflow workbench
LAL	<i>Limulus</i> Amebocyte Lysate
LPS	lipopolysaccharide
MVD	maximum valid dilution
PET	positron emission tomography
PPC	positive product control
psi	pound per square inch
psig	pound-force per square inch gauge
QC	quality control
SDM	soybean-casein digest medium
SOP	standard operating procedure
TSA	trypticase soy agar
TSB	trypticase soy broth
USP	United States Pharmacopeia

ASSESSMENT QUESTIONS

1. Endotoxin contamination is caused by growth of:
 - a. Gram-positive bacteria.
 - b. Gram-negative bacteria.
 - c. Mold spores.
 - d. Yeast spores.

2. All of the following correctly describe an LAL reagent for the gel-clot BET, except:
 - a) It is an FDA-approved and licensed biological product.
 - b) It may be frozen and used for testing no more than 3 times.
 - c) It has a sensitivity labeled in EU/mL.
 - d) It must be reconstituted with LRW or equivalent SWI.

3. If one or both of the PWC and PPC tubes (positive controls) do not gel in the BET limits test, the first step is to invalidate the test and the most pragmatic second step is to:
 - a) Double the number of hot spikes and repeat the test.
 - b) Increase the heat-block temperature and repeat the test.
 - c) Make a new 20-lambda tube and new LAL and repeat the test.
 - d) Reconstitute a new LAL with new LRW and repeat the test.

4. The best way to depyrogenated glassware is to:
 - a) Apply sterile 70% IPA for a 5-minute contact time.
 - b) Heat in an oven at 250° C for at least 1 hour.
 - c) Rinse with copious amounts of LAL Reagent Water for 5 minutes.
 - d) Soak in SporeCleans for 30 minutes.

5. Which of the following is true about the gel-clot BET limit test?
 - a) No gels in the product tubes indicates less endotoxin than the Limit of Detection.
 - b) The 20-lambda tube serves as the negative control.
 - c) The F 18 FDG is always diluted to the MVD before use in the BET.
 - d) A gel in one of the two “Tube A” test-tubes is acceptable.

6. If all 8 tubes in a gel-clot, limit BET test by give a positive result (gel) after 1 hour, the following should be done:
 - a. Run the test again to confirm the failure results.
 - b. Invalidate the test and remake the 20λ tube for spiking.
 - c. Invalidate the test and do a new test with new LRW and LAL reagent.
 - d. Invalidate the test and select a new batch of glassware for the next test.

7. The most potent route of administration for endotoxin is:
- Intrasynovial
 - Intrathecal
 - Intrathecal
 - Intravenous
 - Subcutaneous
8. The most likely cause of enhanced recovery of positive controls on an LAL cartridge is:
- Glucans
 - Omission of sample
 - Over-pipetting into the reservoir
 - Under-pipetting into the reservoir
9. Endotoxin may be eliminated by all but one of the following processes:
- Absorption by alumina
 - Distillation in a properly baffled still
 - Incineration in a dry-heat oven
 - Removal by sterilizing membrane filters
10. A product of ^{123}I -MIBG has a maximum dose volume of 5 mL at expiration time. If the sensitivity of a LAL cartridge is 0.05 EU/mL, the endotoxin limit and MVD are:
- EL of 5 and MVD of 175
 - EL of 35 and MVD of 700
 - EL of 70 and MVD of 1400
 - EL of 175 and MVD of 2800
11. An ^{18}F -FDG product is validated in a given system as requiring a dilution of 1:40. With an LAL cartridge of 0.01 EU/mL sensitivity, the limit of detection was reported as less than:
- 0.4 EU/mL
 - 1 EU/mL
 - 4 EU/mL
 - 10 EU/mL
12. The BET method embodied in the PTS™ System is which of the following:
- Kinetic chromogenic assay
 - Kinetic turbidimetric assay
 - Endpoint chromogenic assay
 - Endpoint turbidimetric assay

13. The syndrome associated with reactions to endotoxin-contaminated radioiodinated albumin injected into cerebrospinal fluid is known as:
- a) Ankylosing spondylitis
 - b) Aseptic meningitis
 - c) Aseptic cisternitis
 - d) Bacillary meningitis
14. For the validation test as described in USP General Chapter <71> “Sterility Tests”, what is the maximum number of organism cfu to be added to the sterility medium?
- a) 10
 - b) 50
 - c) 100
 - d) 500
15. Which of the following microorganisms can only be detected by the use of the SDM?
- a) Aspergillus niger
 - b) Bacillus subtilis
 - c) Clostridium sporogenes
 - d) Staphylococcus aureus
16. If the sample of a PET radiopharmaceutical cannot be sent to a microbiology lab for sterility testing within the specified time frame, which of the following approaches should be taken?
- a) Pool the sample with the next available sample for sterility testing
 - b) Skip the sterility testing and document this non-compliance event in a logbook
 - c) Store the sample under refrigeration and send it for sterility testing as soon as possible.
 - d) Filter the entire batch of PET drug product with a 0.1- μ m membrane filter prior to the release for human use
17. The most appropriate site for carrying out a sterility test is:
- a) Bench top
 - b) BSC
 - c) Chemical fume hood
 - d) LAFW
18. Under USP General Chapter <797> “Pharmaceutical Compounding – Sterile Preparations”, sterility testing is only required for which of the following categories of compounded sterile CSPs?
- a) Immediate-use exemption
 - b) Low-risk level
 - c) Medium-risk level
 - d) High-risk level

19. For sterility testing as per US P General Chapter <71> “Sterility Tests”, samples are incubated in media for:
- a) 1 day
 - b) 3 days
 - c) 7 days
 - d) 14 days

20. Using the know-to-be-sterile finished drug product as a negative control to evaluate the competency of the technicians performing the sterility testing, USP General Chapter <1211> “Sterilization and Sterility Assurance of Compendial Articles” specifies the false-positive frequency do not exceed _____ %.
- a) 0
 - b) 1
 - c) 2
 - d) 3

21. For the validation test as stated in USP General Chapter <71> “Sterility Tests”, which of the following test results indicates the sterility test may be performed without any further modification:

Test Group	Positive Control	Test Product
1	+	+
2	+	-
3	-	-
4	-	+

- a) 1
 - b) 2
 - c) 3
 - d) 4
22. Filter integrity test is carried out by applying gas pressure to the _____ side for the filter assembly, and the filter integrity is demonstrated to be acceptable in the _____ of a steady stream of bubbles at the specified bubble point for the filter being tested..
- a) nonsterile; absence
 - b) nonsterile; presence
 - c) sterile; absence
 - d) sterile; presence

23. According to USP General Chapter <823> “Radiopharmaceuticals for Positron Emission Tomography – Compounding”, the post-filtration integrity test is to be completed prior to release of a batch of PET radiopharmaceutical intended for parenteral administration in human subject, **except** in the case of:
- a) ^{11}C sodium acetate injection
 - b) ^{18}F FDG injection
 - c) ^{13}N ammonia injection
 - d) ^{15}O water injection
24. The sterility test medium designed to detect both aerobic and anaerobic organisms is:
- a) β -lactamase medium
 - b) Fluid A medium
 - c) FTM
 - d) SDM
25. If the PET drug product fails the sterility test, how soon the PET drug production center must notify all receiving facilities of the results?
- a) Immediately
 - b) Before the end of business day
 - c) Within 24 hours
 - d) Within 30 hours