Growth Promotion Test Guide for Media Used in Microbial Enumeration Tests

The Microbial Enumeration Test

The purpose of the Microbial Enumeration Test is to estimate the number of microorganisms in a nonsterile pharmaceutical product or component. This test is necessary to determine if a nonsterile pharmaceutical product or component meets specifications for the microbiological quality established by the pharmacopeia.

The objective of the Growth Promotion Test is to demonstrate the media used to enumerate the microorganisms is suitable. Laboratories perform the test by inoculating new batches of media with a small number of microorganisms. The microorganisms will grow if the media is suitable.

The Media

The media used in the Microbial Enumeration Test are Tryptic Soy Agar and Sabouraud Dextrose Agar.

The Microorganisms

Table 1 lists the reference culture strains that are used for the Growth Promotion Test. The list is based on the United States Pharmacopeia (USP), European Pharmacopeia (Ph. Eur.), and the Japanese Pharmacopeia (JP) requirements. The 3 pharmacopeias are harmonized for this test so the requirements are the same.

The Microbiologics products listed in Table 1 are lyophilized microorganism preparations that are 3 passages or fewer from the reference culture. The reconstituted microorganisms deliver ≤100 CFU per 0.1 ml. For more information about the products, visit our website at www.microbiologics.com. The microorganisms are offered in the following 4 formats:

1. **EZ-Accu Shot™** kits include: 5 vials of a single enumerated microorganism (1 lyophilized pellet per vial) and 5 vials of Hydrating Fluid (1.2 ml in each vial). Ten tests can be performed with each pellet for a total of 50 tests per kit.
2. **EZ-CFU™ One Step** kits include: 2 vials of a single enumerated microorganism (10 lyophilized pellets per vial) and 10 vials of Hydrating Fluid (2 ml in each vial). Nineteen tests can be performed from each microorganism suspension for a total of 190 tests per kit.
3. **EZ-CFU™** kits include: 2 vials of a single enumerated microorganism (10 lyophilized pellets per vial) and 10 vials of Hydrating Fluid (2 ml in each vial). **EZ-CFU™** is similar to **EZ-CFU™ One Step**; however, a 1:10 dilution with phosphate buffer is required after reconstituting pellets. Over ninety tests can be performed from each microorganism suspension for a total of over 900 tests per kit.
4. **EZ-Accu Shot™ Select** kits include: 1 vial of each of the 5 compendial strains for the Microbial Enumeration Test as listed in Table 1, plus 1 vial of Microbiologics catalog number 0483 *Escherichia coli* derived from ATCC® 8739™* (1 lyophilized pellet per vial) and 6 vials of Hydrating Fluid (1.2 ml in each vial). Ten tests can be performed with each pellet for a total of 60 tests per kit.

Table 1: Microbiologics Catalog Numbers

Microorganism Name	Microbiologics Catalog Number
Aspergillus brasiliensis derived from ATCC® 16404™*	0392
Bacillus spizizenii derived from ATCC® 6633™*	0486
Candida albicans derived from ATCC® 10231™*	0443
Pseudomonas paraeruginosa derived from ATCC® 9027™*	0484
Staphylococcus aureus subsp. *aureus* derived from ATCC® 6538™*	0485

Requirements of the Test

1. Perform the Growth Promotion Test on each new batch of purchased, ready-prepared medium, dehydrated medium, or medium prepared from components in the laboratory.
2. Inoculate medium with ≤100 CFU.
3. Use the microorganism strains recommended by the pharmacopeia. The strains should be no more than 5 passages from the reference culture.
4. Test new medium in parallel with previously approved medium. Inoculate the previously approved, non-selective agar to determine the number of CFU in the inoculum. The number of CFU in the inoculum is the standard value. When parallel testing is used, the new and previously approved batches of the medium must be inoculated with the same inoculum, by the same technician, and subjected to identical incubation conditions. The only variable is the medium.
5. Test each medium with only 1 microorganism strain at a time.
6. Test in duplicate.

Materials

- **EZ-Accu Shot™**, **EZ-CFU™ One Step**, or **EZ-CFU™** kits[1]
- New batch of medium
- Previously approved batch of medium
- Non-selective agar if testing liquid medium
- Calibrated micropipette
- Spreader for distributing the inoculum
- Vortex mixer

[1] Use only the Hydrating Fluid provided with the kit

Acceptance Criteria

1. **Solid Medium**
   Average the number of colonies from the new batch of medium and the number of colonies from the previously approved batch of medium. For the new batch of medium to be approved, the following acceptance criteria must be met for each microorganism tested:

- The average number of colonies on the plates from the new batch of the medium must be within a factor of 2 of the average number of colonies from the previously approved batch of the medium. For example, if the average number of colonies on the previously approved media is 40, then the average number of colonies on the plates from the new batch of medium must be between 20 and 80.
- There must be ≤100 colonies on the agar.

2. Liquid Medium

After incubation, visually compare the turbidity in the new batch of liquid medium to the turbidity in the previously approved batch of liquid medium. For the new batch of medium to be approved, the following acceptance criteria must be met for each microorganism tested:

- There must be growth in the new and previously approved batches of the medium. The amount of turbidity from the new batch of liquid medium should be comparable to amount of turbidity from the previously approved batch of liquid medium. A quantitative definition of "comparable" is not established by the USP, Ph. Eur. or JP; visual comparability is sufficient.
- There must be ≤100 colonies on the control (non-selective agar).

Table 2: Growth Promotion Test Requirements for Microbial Enumeration Tests

Microorganism	Type of Medium	Temperature	Incubation Period
Aspergillus brasiliensis	Soybean-Casein Digest Agar and Sabouraud Dextrose Agar	Soybean-Casein Digest Agar 30°C-35°C Sabouraud Dextrose Agar 20°C-25˚C	≤5 days (growth can be seen at 48 hours at 30°C-35°C and at 72 hours at 20°C-25°C)
Bacillus spizizenii	Soybean-Casein Digest Agar and Soybean-Casein Digest Broth	30°C-35°C	≤3 days (growth can be seen in 18-24 hours)
Candida albicans	Soybean-Casein Digest Agar and Sabouraud Dextrose Agar	Soybean-Casein Digest Agar 30°C-35°C Sabouraud Dextrose Agar 20°C-25˚C	≤5 days (growth can be seen at 48 hours at 30°C-35°C and at 72 hours at 20°C-25°C)
Pseudomonas paraeruginosa	Soybean-Casein Digest Agar and Soybean-Casein Digest Broth	30°C-35°C	≤3 days (growth can be seen in 18-24 hours)
Staphylococcus aureus subsp. *aureus*	Soybean-Casein Digest Agar and Soybean-Casein Digest Broth	30°C-35°C	≤3 days (growth can be seen in 18-24 hours)

Best Practices for Growth Promotion Tests

Preparation

Use the microorganism strains recommended by the pharmacopeia. The cultures should be traceable to, and no more than 5 passages from, the reference culture.

Equipment

- Use a calibrated micropipette. Routinely verify its accuracy.
- Calibrate thermometers and incubators yearly.

Control

A negative control is recommended for the Growth Promotion Test. If using **EZ-Accu Shot™** or **EZ-CFU™ One Step**, the diluent is the Hydrating Fluid. If using **EZ-CFU™**, the diluent is the Hydrating Fluid and phosphate buffer pH 7.2. Microbiologics has performed a Sterility Test on the provided Hydrating Fluid and a Certificate of Analysis stating the results is available upon request. If additional Hydrating Fluid is required, Microbiologics offers Hydrating Fluid sold separately from the kit.

The Test

- Allow the medium and the vial of pellets to equilibrate to room temperature before use.
- If using **EZ-CFU™ One Step** or **EZ-CFU™** the Hydrating Fluid and any dilution fluids should warm for 30 minutes at 35˚C before use.
- If using **EZ-CFU™ One Step** or **EZ-CFU™** do not exchange the stoppers on the Hydrating Fluid for the stoppers on the pellet vials. The moisture on the Hydrating Fluid stopper may harm the pellets.
- Vortex suspensions until they are homogenous.
- If using the Pour Plate Method, add 0.1 ml of the microorganism suspension to a sterile petri dish. Pour molten agar over the inoculum and mix well by swirling the contents in the plate. Invert and incubate the agar after it has solidified.
  The molten medium must be cooled to 44°C-46°C before it is poured. It should not be left in the molten state for more than 4 hours. Unused medium should not be re-solidified and used again.

Incubation

- Some microorganisms, such as *Bacillus* and *Aspergillus*, produce colonies that spread. These colonies can be difficult to count if they become too mature.
- Due to pour plates requiring longer incubation periods and the variability of colony sizes, the use of a backlit colony counter is recommended.

Other

- Microbiologics has validated the following quantitative microorganism preparations for use in the growth promotion test on Soybean Casein Digest Agar:

Microorganism Strain	Catalog #	EZ-Accu Shot™	EZ-CFU™ One Step	EZ-CFU™
Aspergillus brasiliensis derived from ATCC® 16404™*	0392	X	X	X
Bacillus spizizenii derived from ATCC® 6633™*	0486	X	X	X
Candida albicans derived from ATCC® 10231™*	0443	X	X	X
Pseudomonas paraeruginosa derived from ATCC® 9027™*	0484	X	X	X
Staphylococcus aureus subsp. *aureus* derived from ATCC® 6538™*	0485	X	X	X

A customer qualification study is recommended to verify that the product works for their company procedures, equipment etc. Microbiologics offers an EZ-Accu Shot™ Qualification Program for laboratories using EZ-Accu Shot™ products for the first time. The EZ-Accu Shot™ Qualification Program provides EZ-Accu Shot™ pellets from three different lots of each of the five USP compendial strains plus *Escherichia coli* (18 pellets) as well as a complete guide for qualifying EZ-Accu Shot™ microorganism strains. Contact your Microbiologics sales representative if you would like more information about the program.

- To ensure end-user safety, a pharmaceutical product may need to be tested for microorganisms other than those mentioned in the Tests for Specified Microorganisms. Visit our website, www.microbiologics.com, for a complete list of **EZ-Accu Shot™**, **EZ-CFU™ One Step**, and **EZ-CFU™** microorganisms that can be used in growth promotion testing.
- Wild-type microorganism strains found in the manufacturing environment can contaminate pharmaceutical products. To ensure the environmental strains can grow on new batches of culture media, include them when performing the growth promotion test. Your environmental isolates can be professionally characterized, preserved, and manufactured in a convenient, ready-to-use format using Microbiologics Custom Environmental Isolate Controls. Contact your Microbiologics sales representative if you would like more information about the program.

Reference

United States Pharmacopeia <61> Microbial Examination of Nonsterile Products: Microbial Enumeration Tests, The United States Pharmacopeial Convention. Rockville, MD.

ISO 11133: 2014 Microbiology of food, animal feed and water – Preparation, production, storage and performance testing of culture media.

Acknowledgements

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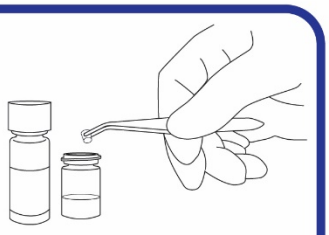
Illustrated Instructions

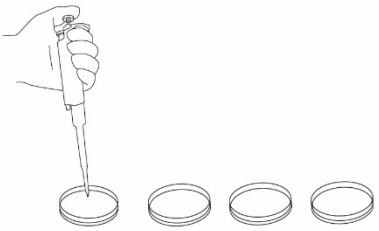
Instructions for performing the Growth Promotion Test on new batches of solid medium to be used for the Microbial Enumeration Test.

1



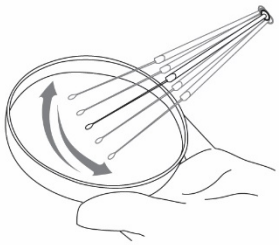
Prepare an inoculum for each of the required microorganisms by following the **EZ-Accushot™**, **EZ-CFU™ One Step** or **EZ-CFU™** Instructions for Use document.

2



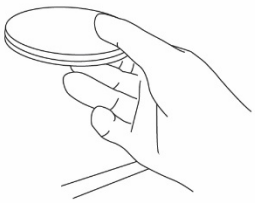
Inoculate the new previously approved batches of medium with 0.1 ml of the microorganism suspension.

3



For solid media, use a spreader to disperse the inoculum across the agar.

4



Follow pharmacopeia directions for incubation temperature and length of incubation for each microorganism tested. (Refer to Table 2).

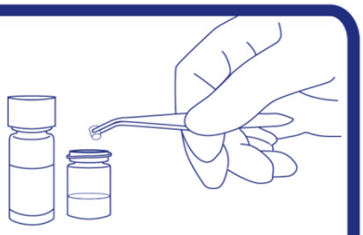
5

Determine if the new medium is suitable for use by using the acceptance criteria.

Illustrated Instructions

Instructions for performing the Growth Promotion Test on new batches of liquid medium to be used for the Microbial Enumeration Test.

1

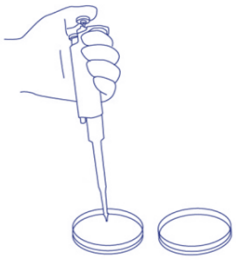


Prepare an inoculum for each of the required microorganisms by following the **EZ-Accu Shot™**, **EZ-CFU™ One Step** or **EZ-CFU™** Instructions for Use document.

2

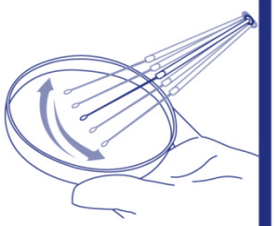
Inoculate the new and previously approved batches of the medium with 0.1 ml of the microorganism suspension.

3



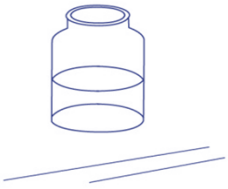
A control is recommended to verify 0.1 ml contains ≤100 CFU. The control is the non-selective agar.

4



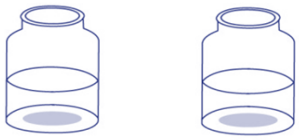
For the non-selective control agar, use a spreader to disperse the inoculum across the agar.

5



Follow the pharmacopeia directions for the incubation temperature and the length of incubation for each microorganism being tested (refer to Table 2).

6



Determine if the new medium is suitable for use by using the acceptance criteria.