

Introduction

The Growth Promotion Test (GPT) is an important function in the United States (USP), European (Ph. Eur.) and Japanese (JP) Pharmacopeias. Its purpose is to determine the suitability of media used in pharmaceutical tests. This Technical Information Bulletin (TIB) discusses the basic requirements of the GPT and explains how to perform the test using the EZ-CFU™ and EZ-CFU™ One Step Products. The GPT should be performed on each batch of purchased ready-prepared medium, each batch of dehydrated medium or medium prepared from components in the laboratory (henceforth, any new, untested batch of medium will be called the “new batch of medium”).

The basic requirements for the GPT are as follows:

1. The new batch of medium must be inoculated with a small number of microorganisms.
2. The laboratory should test the medium with the microorganisms required by the pharmacopeias.
3. The microorganisms must not be more than five passages removed from Reference Culture (also called the original master seed lot).

In order for the new batch of medium to be approved for use, growth on the new batch of medium must be comparable to growth obtained on a batch of medium previously approved by the laboratory.

The EZ-CFU™ and EZ-CFU™ One Step microorganisms are standardized, lyophilized preparations designed to deliver an inoculum of less than 100 CFU when used as directed. The microorganisms are only four passages from internationally recognized Reference Culture Collections. Documentation records a chain-of-evidence to ensure that each product is traced to a Reference Culture. See Table 4 for a list of EZ-CFU™ and EZ-CFU™ One Step microorganisms.

The difference between the EZ-CFU™ and EZ-CFU™ One Step products is that the EZ-CFU™ product has a greater microorganism concentration and therefore requires a 1:10 dilution in order to deliver less than 100 CFU per 0.1 mL. The EZ-CFU™ One Step product does not require the dilution step.

MicroBioLogics indicates the average number of CFU per mL on the Certificate of Assay included with every EZ-CFU™ or EZ-CFU™ One Step product. The value on the Certificate of Assay is obtained using a statistically valid method. The laboratory using the product may obtain a different CFU value because different methods can yield different results. MicroBioLogics guarantees that an EZ-CFU™ or EZ-CFU™ One Step inoculum will deliver less than 100 CFU per 0.1 mL when used as directed. The customer should establish the CFU count in the inoculum by plating it on nonselective agar.

The USP, Ph. Eur. and JP have harmonized the Microbial Enumeration Tests and the Tests for Specified Microorganisms. The Sterility Test is scheduled to be fully harmonized by the pharmacopeias sometime in 2009. The GPT varies slightly between the Microbial Enumeration Tests, the Tests for Specified Microorganisms and the Sterility Test. For this reason, the GPT used for each type of test is described in this TIB, in addition to other important considerations for all three tests. This TIB is a guide; please refer to the pharmacopeias for official text.

Microbiologic Examination of Nonsterile Products: Microbial Enumeration Tests

Purpose

The purpose of the Growth Promotion Test (GPT) for Microbial Enumeration Tests is to assure the nutritive properties of a new batch of medium by challenging it with a small number of microorganisms.

Requirements

1. Inoculate agar plates or broth with only a small number of microorganisms (not more than 100 CFU).
2. Use the microorganisms recommended by the pharmacopeias. The microorganisms should be traceable to a Reference Culture Collection, and should be no more than five passages from Reference Culture.
3. Follow USP, Ph. Eur., or JP instructions. The USP, Ph. Eur. and JP are harmonized for this test.
4. Test the new batch of medium and the previously approved batch of medium in parallel.
5. Test all media in duplicate.
6. Test each agar plate or media tube with only one microorganism at a time.

Preparation

1. Determine which EZ-CFU™ or EZ-CFU™ One Step microorganisms are needed to test the medium (See Table 1).
2. For each microorganism to be tested, label two plates or tubes from the new batch of medium and two plates or tubes from the previously approved batch of medium.
3. For each batch of new medium, test a negative control to ensure, the new medium and the diluent (if one is used) are sterile.

Guide for using EZ-CFU™ and EZ-CFU™ One Step for the Growth Promotion Test

If using the EZ-CFU™ One Step product, the negative control is a non-inoculated plate or tube from the new batch of medium. The medium is not inoculated because the microorganism suspension is not diluted. If using an EZ-CFU™ product, the negative control is the diluent used to dilute the microorganism suspension 1:10. Inoculate a tube or agar plate from the new batch of medium with 0.1 mL of the diluent. Incubate the negative control at the same temperature as the microorganisms tested. It may be necessary to incubate the negative control at 20-25°C and at 30-35°C if both bacteria and yeast or fungi are being tested. The negative control must have no growth after incubation, in order for the new batch of medium to be approved. If the new medium is broth and the broth is clear after incubation, it may be subbed to nonselective agar in order to ensure that the broth is sterile.

Test Procedure

1. Prepare an inoculum of each of the test microorganisms by following the directions in the EZ-CFU™ or EZ-CFU™ One Step product inserts.
2. Dispense 0.1 mL of the inoculum per plate of agar or tube of broth. 0.1 mL will deliver less than 100 CFU. Use the same microorganism preparation to inoculate two plates or tubes from the new batch of medium and two plates or tubes from the previously approved batch of medium.
3. If the new medium is broth, also test each microorganism individually on two nonselective control agar plates. Inoculate the nonselective agar plates with 0.1 mL of the *same* inoculum suspension used to inoculate the new and previously approved batches of medium. The purpose of testing the microorganism on nonselective agar is to verify that 0.1 mL of inoculum contains less than 100 CFU. It is possible to determine the CFU concentration in the inoculum by counting the colonies on the agar plates whereas this is not possible after incubating broth.
4. If using solid media, use the Surface-Spread Method. Use a spreader to disperse the inoculum and distribute it over the entire agar plate.
5. Follow pharmacopeia directions for incubation temperature and length of incubation for each microorganism tested (See Table 1). The negative control and the inoculated media should be subjected to the same conditions.
6. Determine if the new medium is suitable for use by using the acceptance criteria listed below.

Acceptance Criteria

1. SOLID MEDIA

Average the number of colonies on the two plates from the new batch of medium and average the number of colonies on the two plates from the previously approved batch of medium. In order for the new batch of medium to be approved, the following acceptance criteria must be met for each microorganism tested:

- A. There must be growth on the agar plates.
- B. There must be no more than 100 colonies on the agar plates.
- C. The average number of colonies on the plates from the new batch of medium must be within a factor of 2 of the average number of colonies from the previously approved batch of medium. For example, if the average number of colonies on the previously approved media is 50, then the average number of colonies on the plates from the new batch of medium must be between 25 and 100.

2. BROTH

Visually compare growth in the two tubes from the new batch of liquid medium to growth in the two tubes from the previously approved batch of medium. In order for the new batch of medium to be approved, the following acceptance criteria must be met for each microorganism tested:

- A. There should be growth in the tubes from both the new and previously approved batches of medium and on the control agar plates.
- B. The control (nonselective) plates should have no more than 100 colonies.
- C. The amount of turbidity in the tubes from the new batch of liquid medium should be comparable to amount of turbidity in the tubes 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.

Growth Promotion Test for Solid Medium Used in Microbial Enumeration Test

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|--|-------------------------------------------------------------------------------------------------|
| | <p align="center">Step 1 Prepare inoculum.</p> |
| | <p align="center">Step 2 Inoculate new and previously approved media.</p> |
| | <p align="center">Step 3 Spread inocula & incubate.</p> |
| | <p align="center">Step 4 Count colonies on previously approved media.</p> |
| | <p align="center">Step 5 Count colonies on new media.</p> |
| | <p align="center">Step 6 Compare results. Counts should be within a factor of 2.</p> |

Growth Promotion Test for Liquid Medium Used in Microbial Enumeration Test

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|--|--------------------------------------------------------------------------------------------------------------------------|
| | <p align="center">Step 1 Prepare inoculum.</p> |
| | <p align="center">Step 2 Inoculate new and previously approved media.</p> |
| | <p align="center">Step 3 Inoculate nonselective agar.</p> |
| | <p align="center">Step 4 Spread inocula & incubate all media.</p> |
| | <p align="center">Step 5 Compare new to previously approved media. Amount of turbidity should be similar.</p> |
| | <p align="center">Step 6 Count colonies on nonselective media. There should be <100.</p> |

Table 1: Growth Promotion Test Requirements for Microbial Enumeration Tests

| Microorganism ^{1,2} | Type of Medium | Temperature | Incubation Period |
|-------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------|---------------------------------------------------------------------------|---------------------------------------------------------------------------------------|
| <i>Staphylococcus aureus</i> EZ-CFU™ One Step 0485Z or 0579Z or EZ-CFU™ 0485C | Soybean-Casein Digest Agar and Soybean-Casein Digest Broth | 30-35° C | ≤3 days (results can be seen in 18-24 hrs.) |
| <i>Pseudomonas aeruginosa</i> EZ-CFU™ One Step 0484Z or 0576Z | Soybean-Casein Digest Agar and Soybean-Casein Digest Broth | 30-35° C | ≤3 days (results can be seen in 18-24 hrs.) |
| <i>Bacillus subtilis subsp. spizizenii</i> ² EZ-CFU™ One Step 0486Z or EZ-CFU™ 0486C | Soybean-Casein Digest Agar and Soybean-Casein Digest Broth | 30-35° C | ≤3 days (results can be seen in 18-24 hrs.) |
| <i>Candida albicans</i> EZ-CFU™ One Step 0443Z or EZ-CFU™ 0443C | Soybean-Casein Digest Agar and Sabouraud Dextrose Agar | Soybean-Casein Digest Agar 30- 35°C Sabouraud Dextrose Agar 20-25°C | ≤5 days (results can be seen at 48 hrs. at 30-35 °C and at 72 hrs. at 20-25°C) |
| <i>Aspergillus brasiliensis</i> ³ EZ-CFU™ One Step 0392Z or EZ-CFU™ 0392C | Soybean-Casein Digest Agar and Sabouraud Dextrose Agar | Soybean-Casein Digest Agar 30- 35°C Sabouraud Dextrose Agar 20-25°C | ≤5 days (results can be seen at 48 hrs. at 30-35 °C and at 72 hrs. at 20-25°C) |

¹ See Table 4 for Cross Reference of MicroBioLogics Products with recommended Reference Cultures

² *Bacillus subtilis subsp. spizizenii* is referred to *Bacillus subtilis* in the pharmacopeias. More information about the name can be found in Table 4.

³ *Aspergillus brasiliensis* has replaced the name *Aspergillus niger* in legacy pharmacopeias. More information can be found in Table 4 footnotes.

Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms

Purpose

The purpose of this growth promotion test is to ensure that the new batch of medium is performing correctly with respect to growth promotion, inhibitory and indicative properties by challenging the medium with a small number of microorganisms.

Requirements

- Inoculate the agar or broth with the number of microorganisms required by the type of test. The types of tests are as follows:
 - Test for Growth-Promoting Properties, Solid Media – Inoculate medium with no more than 100 CFU of the appropriate microorganism.
 - Test for Growth-Promoting Properties, Liquid Media – Inoculate medium with no more than 100 CFU of the appropriate microorganism.
 - Test for Inhibitory Properties, Liquid or Solid Media – Inoculate medium with at least 100 CFU of the appropriate microorganism.
 - Test for Indicative Properties – Inoculate medium with no more than 100 CFU of the appropriate microorganism.
- Use the microorganisms recommended in the pharmacopeias. The microorganisms should be traceable to a Reference Culture Collection, and should be no more than five passages from Reference Culture (original master seed lot).
- Follow USP, Ph. Eur., or JP instructions. The USP, Ph. Eur. and JP are harmonized for this test.
- The new batch of medium may require more than one type of test. All the tests must meet the criteria described in the procedures in order for the new batch to be approved.
- Test the new batch of medium and the previously approved batch of medium in parallel.
- Test all media in duplicate.
- Test such medium with only one microorganism at a time.

Test for Growth-Promoting Properties, Solid Media

Test for Growth-Promoting Properties, Liquid Media

Test for Indicative Properties

Preparation

- Determine which EZ-CFU™ or EZ-CFU™ One Step microorganisms are needed to test the medium (See Table 2).
- For each microorganism to be tested, label two plates or tubes from the new batch of medium and two plates or tubes from the previously approved batch of medium.

Guide for using EZ-CFU™ and EZ-CFU™ One Step for the Growth Promotion Test

- For each batch of new medium, test a negative control to ensure, the new medium and the diluent (if one is used) are sterile.
If using the EZ-CFU™ One Step product, the negative control is a non-inoculated plate or tube from the new batch of medium. The medium is not inoculated because the microorganism suspension is not diluted. If using an EZ-CFU™ product, the negative control is the diluent used to dilute the microorganism suspension 1:10. Inoculate a tube or agar plate with 0.1 mL of the diluent.
Incubate the negative control under the same conditions as the microorganisms being tested. The negative control must have no growth after incubation, in order for the new batch of medium to be approved. If the new medium is broth and the broth is clear after incubation, it may be subbed to nonselective agar to ensure the broth is sterile.







Procedure

- Prepare an inoculum of each of the required microorganisms by following the directions in the EZ-CFU™ or EZ-CFU™ One Step product insert.
- Dispense 0.1 mL of the inoculum per plate of agar or tube of broth. 0.1 mL will deliver less than 100 CFU. Use the same microorganism preparation to inoculate two plates or tubes from the new batch and two plates or tubes from the previously approved batch of medium.
- If the new medium is selective or liquid, also test each microorganism individually on two nonselective control agar plates. Inoculate the plates or tubes with 0.1 ml of the same inoculum suspension that was used to inoculate the new and previously approved batches of medium. This is done because:
 - If testing a solid, selective medium, it is necessary to determine how many microorganisms are in the inoculum since growth-promoting medium can be inhibitory even to microorganisms that should grow on it.
 - If testing a liquid medium, it is necessary to verify that it was inoculated with less than 100 CFU since there are no colonies to count after the liquid medium has been incubated. Inoculate the plates with the same inoculum used on the new and the previously approved batches of medium.
- If using solid media, use the Surface-Spread Method. Use a spreader to disperse the inoculum and distribute it over the entire agar plate.
- Follow pharmacopeia directions for incubation temperature and length of incubation for each microorganism tested (See Table 2). The negative control should be subjected to the same conditions as the inoculated media.
- Determine if the new medium is suitable for use by using the acceptance criteria listed below.

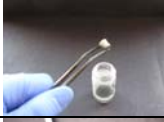





Acceptance Criteria

- SOLID MEDIA**
Average the number of colonies on the two plates from the new batch of medium and average the number of colonies on the two plates from the previously accepted batch of medium. In order for the results to be acceptable, the following acceptance criteria must be met for each microorganism tested:
 - There must be growth on the control (nonselective) agar plates and the growth-promoting agar plates.
 - There must be no more than 100 colonies on the control agar plates or the growth-promoting agar plates.
 - The average number of colonies on the new batch of medium must be “comparable” to the average number of colonies on the batch of previously approved medium. No quantitative definition of “comparable”, such as 50% or 70%, has been established in the USP, Ph. Eur. or JP.
- LIQUID MEDIA**
Visually compare growth in the two tubes from the new batch of liquid medium to growth in the two tubes from the previously approved batch of medium. In order for the results to be acceptable, the following acceptance criteria must be met for each microorganism tested:
 - There should be growth in the tubes from both the new and previously approved batches of liquid medium and on the control agar plates.
 - There must be no more than 100 colonies on the control agar plates.
 - The amount of turbidity in the new batch of liquid medium should be “comparable” to that in the previously approved batch of medium. No quantitative definition of “comparable” has been established by the USP, Ph. Eur. or JP.
- INDICATIVE REACTIONS**
Visually compare the colonies on the agar plates to the colonies on the previously approved batch of medium. The colonies should be similar in appearance. Expected indicative reactions are described in Table 2.

Growth Promotion Test for Solid Media Used in Tests for Specified Microorganisms – Growth Promoting and Indicative Properties

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|  | <p>Step 1 Prepare inoculum.</p> |
|  | <p>Step 2 Inoculate new and previously approved media.</p> |
|  | <p>Step 3 Inoculate nonselective agar. Spread inoculum & Incubate all media.</p> |
|  | <p>Step 4 Count colonies on previously approved media.</p> |
|  | <p>Step 5 Count colonies on new media. Colony count and indicative reactions on new media should be comparable to colony count and indicative reactions on previously approved media.</p> |
|  | <p>Step 6 Count colonies on nonselective media. There should be <100.</p> |

Growth Promotion Test for Liquid Media Used in Tests for Specified Microorganisms – Growth Promoting Properties

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|------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------|
|  | <p>Step 1 Prepare inoculum.</p> |
|  | <p>Step 2 Inoculate new and previously approved media.</p> |
|  | <p>Step 3 Inoculate nonselective agar.</p> |
|  | <p>Step 4 Spread inocula and incubate all media.</p> |
|  | <p>Step 5 Compare new to previously approved media. Turbidity should be comparable.</p> |
|  | <p>Step 6 Count colonies on nonselective media. There should be <100.</p> |

Test for Inhibitory Properties, Liquid or Solid Media

Procedure

This test should be performed at the same time as the Tests for Growth-Promotion and Indicative properties.

Preparation

1. Determine which EZ-CFU™ microorganism is needed to test the medium (See Table 2).

Guide for using EZ-CFU™ and EZ-CFU™ One Step for the Growth Promotion Test

- Label two plates or tubes from the new batch of medium and two plates or tubes from the previously approved batch of medium.
- For each batch of new medium, test a negative control to ensure the new medium is sterile. (This may have already been done during the Tests for Growth Promoting and/or Indicative Properties). The negative control is a non-inoculated plate or tube from the new batch of medium.

Incubate the negative control at the same temperature as the microorganisms being tested.

If the new medium is broth and the broth is clear after incubation, it may be subbed to nonselective agar in order to ensure that the broth is sterile.







Procedure

- Prepare an inoculum of each of the required microorganism. The inoculum must contain at least 100 CFU per 0.1 mL. To prepare the inoculum do the following:
 - Use an EZ-CFU™ product.
 - Rehydrate 2 pellets in 2 mL hydration fluid as described in the EZ-CFU™ product insert.
 - Do not dilute further. The pellet-hydration fluid suspension contains 100 to 999 CFU per 0.1 mL
- Dispense 0.1 mL of the inoculum per plate or agar of tube of broth. Use the same microorganism preparation to inoculate two plates or tubes from the new batch of medium and two plates or tubes from the previously approved batch of medium.
- Also, test two nonselective control agar plates with the test microorganism. This is done in order to verify the agar or broth being tested for inhibitory properties was inoculated with at least 100 CFU since there will be no growth or very little growth on the media that has inhibitory properties. Inoculate the control plates with 0.1 mL of the same inoculum used on the two plates or tubes from the new media and the previously approved media.
- If using solid media, use the Surface-Spread Method. Use a spreader to disperse the inoculum and distribute it over the entire agar plate.
- Follow pharmacopeia directions for incubation temperature and length of incubation for each microorganism tested (See Table 2). The negative and positive control should be subjected to the same conditions.
- Determine if the new medium is suitable for use by using the acceptance criteria listed below.

Acceptance Criteria

- The test microorganism should be inhibited on the plates or tubes from both the new and previously approved batches of inhibitory medium.
- There should be at least 100 colonies on the nonselective control agar plate.

Growth Promotion Test for Solid and Liquid Media Used in Tests for Specified Microorganisms – Inhibitory Properties

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|  | <p>Step 1 Prepare inoculum.</p> |
|  | <p>Step 2 Inoculate new and previously approved media.</p> |
|  | <p>Step 3 Inoculate nonselective agar. Spread inoculum & incubate all media.</p> |
|  | <p>Step 4 Examine previously approved media. Growth should be inhibited.</p> |
|  | <p>Step 5 Examine new media. Growth should be inhibited.</p> |
|  | <p>Step 6 Count colonies on nonselective media. There should be ≥100.</p> |

Guide for using EZ-CFU™ and EZ-CFU™ One Step for the Growth Promotion Test

Table 2: Growth Promotion Test Requirements for Tests for Specified Microorganisms

| Type of Medium | Microorganisms ¹ | Properties | Temp | Incubation Period |
|---------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------|---------|-------------------------------------------|
| Enterobacteria Enrichment Broth Mossel | <i>E. coli</i> EZ-CFU™ One Step 0483Z or 0581Z or EZ-CFU™ 0483C | Growth promoting | 30-35°C | 24 hrs. |
| | <i>P. aeruginosa</i> EZ-CFU™ One Step 0484Z or 0576Z | Growth promoting | 30-35°C | 24 hrs. |
| | <i>S. aureus</i> EZ-CFU™ 0485C | Inhibitory | 30-35°C | 48 hrs. |
| Violet Red Bile Glucose Agar | <i>E. coli</i> EZ-CFU™ One Step 0483Z or 0581Z or EZ-CFU™ 0483C | Growth promoting & Indicative (purplish-red colonies) | 30-35°C | Growth: 18 hrs. Indicative: 18-24 hrs. |
| | <i>P. aeruginosa</i> EZ-CFU™ One Step 0484Z or 0576Z | Growth promoting & Indicative (colorless colonies) | 30-35°C | Growth: 18 hrs. Indicative: 18-24 hrs. |
| MacConkey Broth | <i>E. coli</i> EZ-CFU™ One Step 0483Z or 0581Z or EZ-CFU™ 0483C | Growth promoting | 42-44°C | 24 hrs. |
| | <i>S. aureus</i> EZ-CFU™ 0485C | Inhibitory | 42-44°C | 48 hrs. |
| MacConkey Agar | <i>E. coli</i> EZ-CFU™ One Step 0483Z or 0581Z or EZ-CFU™ 0483C | Growth promoting & Indicative (pink colonies) | 30-35°C | Growth: 18 hrs. Indicative: 18-72 hrs. |
| Rappaport Vassiliadis Salmonella Enrichment Broth | <i>S. enterica</i> subsp. <i>enterica</i> serovar Typhimurium EZ-CFU™ One Step 0363Z or EZ-CFU™ 0363C OR <i>S. enterica</i> subsp. <i>enterica</i> serovar Abony, EZ-CFU™ One Step 0890Z or EZ-CFU™ 0890C | Growth promoting | 30-35°C | 18 hrs. |
| | <i>S. aureus</i> EZ-CFU™ 0485C | Inhibitory | 30-35°C | 24 hrs. |
| | <i>S. enterica</i> subsp. <i>enterica</i> serovar Typhimurium EZ-CFU™ One Step 0363Z or EZ-CFU™ 0363C OR <i>S. enterica</i> subsp. <i>enterica</i> serovar Abony EZ-CFU™ One Step 0890Z or EZ-CFU™ 0890C | Growth promoting & Indicative (red colonies with or without black centers) | 30-35°C | Growth -18 hrs. Indicative 18-48 hrs. |
| Xylose Lysine Deoxycholate Agar | <i>P. aeruginosa</i> EZ-CFU™ One Step 0484Z or 0576Z | Growth Promoting | 30-35°C | 18 hrs. |
| | <i>E. coli</i> EZ-CFU™ 0483C | Inhibitory | 30-35°C | 72 hrs. |
| Mannitol Salt Agar | <i>S. aureus</i> EZ-CFU™ One Step 0485Z or 0579Z or EZ-CFU™ 0485C | Growth promoting & Indicative (yellow or white colonies with yellow zone) | 30-35°C | Growth: 18 hrs. Indicative: 18-72 hrs. |
| | <i>E. coli</i> EZ-CFU™ 0483C | Inhibitory | 30-35°C | 72 hrs. |
| Reinforced Medium for Clostridia | <i>C. sporogenes</i> EZ-CFU™ One Step 0487Z or 0580Z or EZ-CFU™ 0487C OR <i>C. sporogenes</i> EZ-CFU™ One Step 0317Z or EZ-CFU™ 0317C | Growth Promoting | 30-35°C | 48 hrs. Anaerobic conditions |
| | <i>C. sporogenes</i> EZ-CFU™ One Step 0487Z or 0580Z or EZ-CFU™ 0487C OR <i>C. sporogenes</i> EZ-CFU™ One Step 0317 or EZ-CFU™ 0317C | Growth Promoting | 30-35°C | 48 hrs. Anaerobic conditions |
| Sabouraud Dextrose Agar | <i>C. albicans</i> EZ-CFU™ One Step 0443Z or EZ-CFU™ 0443C | Growth Promoting & Indicative (white colonies) | 30-35°C | Growth: 24 hrs. Indicative: 48 hrs. |
| Sabouraud Dextrose Broth | <i>C. albicans</i> EZ-CFU™ One Step 0443Z or EZ-CFU™ 0443C | Growth Promoting | 30-35°C | 3 days. |

¹ See Table 4 for Cross Reference of MicroBioLogics Products with recommended Reference Cultures

Sterility Tests

Purpose

The purpose of the growth promotion test for the Sterility Test is to check the nutritive properties of the new batch of medium by challenging the medium with a small number of microorganisms. The medium being tested is always liquid.

Requirements

1. Inoculate the broth with only a small number of microorganisms (not more than 100 cfu).
2. Use the microorganisms recommended by the USP, Ph. Eur. or JP. The required microorganisms vary in the pharmacopeias since this test is not fully harmonized yet. The microorganisms should be traceable to a Reference Culture Collection and should be no more than five passages from Reference Culture.
3. Follow USP, Ph. Eur. or JP instructions.
4. Test the new batch of medium and the previously approved batch of medium in parallel.
5. Test all media in duplicate.
6. Test each medium with only one microorganism at a time.

Preparation

1. Determine which EZ-CFU™ or EZ-CFU™ One Step microorganisms are needed to test medium (See Table 3).
2. For each microorganism to be tested, label two tubes from the new batch of medium and two tubes from the previously approved batch of medium.
3. For each batch of new medium, test a negative control to ensure, the new medium and diluent (if one is used) are sterile.

If using the EZ-CFU™ One Step product, the negative control is a non-inoculated tube from the new batch of medium. The medium is not inoculated because the microorganism suspension is not diluted. If using an EZ-CFU™ product, the negative control is the diluent used to dilute the microorganism suspension 1:10.

Inoculate a tube from the batch of new medium with 0.1 mL of the diluent. Incubate the negative control tube for 14 days under the same conditions as the microorganisms being tested. If the broth is clear after incubation, it may be subbed to nonselective agar plates in order to ensure that the broth is sterile. The negative control must have no growth after incubation, in order for the new batch of medium to be approved.

Procedure

1. Prepare an inoculum of each of the required microorganisms by following the directions in the EZ-CFU™ or EZ-CFU™ One Step product insert.
2. Dispense 0.1 mL of the inoculum in two tubes of the batch of new media and two tubes of the batch of previously approved media. 0.1 mL will deliver less than 100 CFU. Use the same microorganism preparation to inoculate the new batch and the previously approved batch of medium.
3. Also, test each microorganism individually on two nonselective control agar plates. Inoculate the plates with 0.1 mL of the same inoculum that was used to inoculate the new and previously approved batches of medium. This is done in order to verify 0.1 mL contains less than 100 CFU. It is possible to determine the CFU concentration in the inoculum by counting the colonies on the agar plates whereas this is not possible after incubating broth.
4. Use a spreader to disperse the inoculum and distribute it over the entire plate of nonselective medium.
5. Follow pharmacopeia directions for incubation temperature and length of incubation for each microorganism tested (See Table 3). The negative control should be incubated at the same temperature as the inoculated media.
6. Determine if the new medium is suitable for use by using the acceptance criteria listed below.

Acceptance Criteria

After incubation, visually compare the turbidity in the two tubes from the new batch of medium to the turbidity in the two tubes from the previously approved batch of medium. In order for the new batch of medium to be approved the following acceptance criteria must be met for each microorganism tested:

1. There should be growth in the tubes from both the new and previously approved batches of liquid medium and on the control agar plates.
2. The control plate should have no more than 100 colonies
3. The amount of turbidity in the tubes from the new batch of liquid medium should be “comparable” to that in the tubes from the previously approved batch of medium. No quantitative definition of “comparable” has been established by the USP, Ph. Eur. or JP.

Growth Promotion Test for Media Used in Sterility Tests







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|----------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------|
|  | <p>Step 1 Prepare inoculum.</p> |
|  | <p>Step 2 Inoculate new and previously approved media.</p> |
|  | <p>Step 3 Inoculate nonselective agar.</p> |
|  | <p>Step 4 Spread inoculum and incubate all media.</p> |
|  | <p>Step 5 Compare new to previously approved media. Amount of turbidity should be similar.</p> |
|  | <p>Step 6 Count colonies on nonselective media. There should be <100.</p> |

Table 3: Growth Promotion Test Requirements for the Sterility Test

| Microorganism ¹ | Type of Medium | Temperature | Incubation Period |
|-------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------|-------------|-------------------|
| <i>Clostridium sporogenes</i> EZ-CFU™ One Step 0317Z or EZ-CFU™ 0317C OR <i>C. sporogenes</i> EZ-CFU™ One Step 0487Z or EZ-CFU™ 0487C | Fluid Thioglycollate Medium | 30-35° C | ≤3 days |
| <i>Pseudomonas aeruginosa</i> ² EZ-CFU™ One Step 0484Z or 0576Z | Fluid Thioglycollate Medium | 30-35° C | ≤3 days |
| <i>Staphylococcus aureus</i> EZ-CFU™ One Step 0485Z or 0579Z EZ-CFU™ 0485C | Fluid Thioglycollate Medium | 30-35° C | ≤3 days |
| <i>Clostridium sporogenes</i> EZ-CFU™ One Step 0317Z or EZ-CFU™ 0317C OR <i>C. sporogenes</i> EZ-CFU™ One Step 0487Z or EZ-CFU™ 0487C | Alternative Fluid Thioglycollate Medium | 30-35° C | ≤3 days |
| <i>Aspergillus brasiliensis</i> ³ EZ-CFU™ One Step 0392Z or EZ-CFU™ 0392C | Soybean-Casein Digest Medium ⁴ | 20-25°C | ≤5 days |
| <i>Bacillus subtilis</i> ⁵ EZ-CFU™ One Step 0486Z or EZ-CFU™ 0486C | Soybean-Casein Digest Medium ⁴ | 20-25°C | ≤ 3 days |
| <i>Candida albicans</i> EZ-CFU™ One Step 0443Z or EZ-CFU™ 0443C | Soybean-Casein Digest Medium ⁴ | 20-25°C | ≤5 days |

¹See Table 4 for Cross Reference of MicroBioLogics Products with Reference Cultures.

²*Kocuria rhizophila*, MicroBioLogics EZ-CFU™ One Step, 0688Z, or EZ-CFU™, 0688C may be substituted for *Pseudomonas aeruginosa* (USP).

³*Aspergillus brasiliensis* has replaced the name *Aspergillus niger* in the legacy pharmacopeias. More information can be found in Table 4 footnotes.

⁴Fluid Thioglycollate medium may be used when testing a product containing a preservative that cannot be tested by the membrane filtration method provided the method has been validated.

⁵*Bacillus subtilis* subsp. *spizizenii* is referred to *Bacillus subtilis* in the pharmacopeias. More information about the name can be found in Table 4.

Important Considerations for all Growth Promotion Tests

In order to get consistent results, do the following:

- Use the microorganism cultures recommended by the pharmacopeia. The cultures should be traceable to a Reference Culture Collection, and should be no more than five passages from Reference Culture (original master seed lot).
- If testing selective media, run a control on nonselective media such as Tryptic Soy Agar at the same time because recovery on selective media is not as high as on nonselective media.
- If performing a Growth Promotion Test on liquid medium, run a control on nonselective agar at the same time in order to verify that the correct number of microorganisms was added for the test.
- Don't exchange the rubber stoppers on the hydrating fluid for the rubber stoppers on the pellet vials. The moisture on the hydrating fluid stopper may harm the pellets.
- Let the media and the vial of pellets equilibrate to room temperature.
- The hydration fluid and any dilution fluids should warm for 30 minutes at 35° before use.
- Use a pipettor and calibrate it routinely.
- Vortex suspensions until they are homogenous.
- Use a spreader to disperse the inoculum over the entire plate.
- Follow pharmacopeia directions for temperature, incubation time, and atmosphere.
- After testing EZ-CFU™ One Step suspension, refrigerate it if planning to use it again. With the exception of 0320Z and 0318Z, the refrigerated suspension can be used for up to eight hours.
- Calibrate thermometers and validate incubators yearly.
- Some microorganisms such as Bacillus and Aspergillus produce colonies that spread. These colonies can be hard to count if they become too mature.
- If using the pour plate method, add 0.1 mL of inoculum containing less than 100 CFU to an empty agar plate. Pour molten agar over the inoculum and mix well by swirling the contents in the plate. Since recovery may not be as high as when using the spread plate method, run a spread plate as a control. The molten media must be cooled to 45° Celsius. Invert and incubate the agar plate after it has solidified.
- For optimum results, many experts recommend using parallel testing instead of historical data when comparing new media to previously approved media. When parallel (side by side) testing is used, the new media and the previously approved media are inoculated with the same inoculum by the same technician and are subjected to identical incubation conditions. The only variable is the media.
- A Verification Study is sufficient when using the EZ-CFU or EZ-CFU One Step product for the first time. MicroBioLogics has already validated the procedures and, per the USP, all the customer needs to do is verify that the product works for them using their procedures, equipment etc. MicroBioLogics recommends five repetitive tests, using the same inoculum suspension, should be performed for the verification study. Results should meet the specifications outlined in this TIB.
- In order to ensure the safety of the consumer, a pharmaceutical product may need to be tested for microorganisms other than those mentioned in the Tests for Specified Microorganisms. If microorganism controls other than those mentioned in this TIB are needed, go to www.microbiologics.com to find a complete list of EZ-CFU™ and EZ-CFU™ One Step microorganisms that can be used in Growth Promotion Testing.

Table 4: Cross Reference of MicroBioLogics Products with Reference Cultures

| Microorganism Name | Reference Culture # | EZ-CFU™ One Step and/or EZ-CFU™ Product # ¹ |
|----------------------------------------------------------------|---------------------|--------------------------------------------------------|
| <i>Aspergillus brasiliensis</i> ² | ATCC® 16404™* | 0392Z, 0392C |
| <i>Bacillus subtilis subsp. spizizenii</i> ³ | ATCC® 6633™* | 0486Z, 0486C |
| <i>Candida albicans</i> | ATCC® 10231™* | 0443Z, 0443C |
| <i>Clostridium sporogenes</i> | ATCC® 19404™* | 0317Z, 0317C |
| <i>Clostridium sporogenes</i> | ATCC® 11437™* | 0487Z, 0487C |
| <i>Clostridium sporogenes</i> | NCIMB 12343 | 0580Z |
| <i>Escherichia coli</i> | ATCC® 8739™* | 0483Z, 0483C |
| <i>Escherichia coli</i> | NCIMB 8545 | 0581Z |
| <i>Kocuria rhizophila</i> | ATCC® 9341™* | 0688Z, 0688C |
| <i>Pseudomonas aeruginosa</i> | ATCC® 9027™* | 0484Z |
| <i>Pseudomonas aeruginosa</i> | NCIMB 8626 | 0576Z |
| <i>Salmonella enterica subsp. enterica</i> | NCTC 6017 | 0890C, 0890Z |
| <i>Salmonella enterica subsp. enterica serovar Typhimurium</i> | ATCC® 14028™* | 0363C, 0363Z |
| <i>Staphylococcus aureus subsp. aureus</i> | ATCC® 6538™* | 0485C, 0485Z |
| <i>Staphylococcus aureus subsp. aureus</i> | NCIMB 9518 | 0579Z |

¹ The code Z indicates an EZ-CFU™ One Step product. The code C indicates an EZ-CFU™ product.

² *Aspergillus brasiliensis* is sometimes referred to as *Aspergillus niger* in the pharmacopeias. ATCC® changed the name of ATCC® 16404™ in 2008 because ATCC® determined that this strain belonged to the species *Aspergillus brasiliensis* after performing a polyphasic study in which molecular data was combined with physiological characteristics. More information can be found at the following link. http://www.microbiologics.com/docs/LIT_256.pdf

³ *Bacillus subtilis subsp. spizizenii* is referred to *Bacillus subtilis* in the pharmacopeias. A taxonomic update in 1999 reclassified ATCC® 6633™ as *Bacillus subtilis* subspecies *spizizenii*. The species did not change. Rather, a new subspecies was added. More information can be found in the following reference. Nakamura LK, et al. Relationship of *Bacillus subtilis* clades associated with strains 168 and W23: A proposal for *Bacillus subtilis* subsp. *subtilis* subsp. nov. and *Bacillus subtilis* subsp. *spizizenii* subsp. nov. International Journal of Systematic and Evolutionary Bacteriology 49: 1211-1215, 1999.

Abbreviations and acronyms



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NCIMB is the National Collection of Industrial and Marine Bacteria Ltd. It is located in Aberdeen, Scotland. MicroBioLogics, Inc. is licensed to use these trademarks and to sell products derived from NCIMB cultures.

NCTC™ is the National Collection of Type Cultures. It is registered to PHLS Central Public Health Laboratory in the United Kingdom. MicroBioLogics, Inc. is licensed to use these trademarks and to sell products derived from NCTC™ cultures.

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