Growth Promotion Test Guide for Media Used in Tests for Specified Microorganisms

Tests for Specified Microorganisms

The purpose of the Tests for Specified Microorganisms is to determine if a specified microorganism in a nonsterile pharmaceutical product or component exceeds the limits established by the pharmacopeia. An example of a specified microorganism is *Salmonella enterica* subsp*. enterica* serovar Typhimurium. The test is performed by incubating product samples in enrichment broth and then using growth promoting and/or selective agar to isolate the specified microorganism. Before a product can be tested, the laboratory must know whether the medium it is using for the test will grow the specified microorganism if it is present in small numbers.

The objective of the Growth Promotion Test is to demonstrate the media used to detect 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 tests are designed to isolate specific microorganisms. The media are listed in Table 2.

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 5 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, 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.
5. **Epower™** kits include: 1 vial of a single enumerated microorganism (10 lyophilized pellets per vial). It is available in a variety of strains with concentrations ranging from 10^2 to 10^8 CFU per pellet.

Table 1: Microbiologics Catalog Numbers

Microorganism Name	Microbiologics Catalog Number
Candida albicans derived from ATCC® 10231™*	0443
Clostridium sporogenes derived from ATCC® 19404™*	0317
Clostridium sporogenes derived from ATCC® 11437™*	0487
Escherichia coli derived from ATCC® 8739™*	0483
Pseudomonas paraeruginosa derived from ATCC® 9027™*	0484
Salmonella enterica subsp. *enterica* serovar Abony derived from NCTC 6017	0890
Salmonella enterica subsp. *enterica* serovar Typhimurium derived from ATCC® 14028™*	0363
Staphylococcus aureus subsp. *aureus* derived from ATCC® 6538™*	0485

Requirements of the Test

Perform the Growth Promotion Test on each new batch of purchased ready-prepared medium, dehydrated medium or medium prepared from components in the laboratory.

Inoculate medium with ≤100 CFU for growth promoting and indicative properties. Inoculate medium with ≥100 CFU for inhibitory properties.

Use the microorganism strains recommended by the pharmacopeia. The strains should be no more than 5 passages from the reference culture.

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.

Test each medium with only 1 microorganism strain at a time.

Test in duplicate.

Materials

- **EZ-Accu Shot™**, **EZ-CFU™ One Step**, or **EZ-CFU™** kits. These kits contain lyophilized microorganisms and Hydrating Fluid.[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
- **Epower™** if testing the inhibitory properties of solid and/or liquid medium
- Sterile Phosphate buffer pH 7.2 if testing the inhibitory properties of solid and/or liquid medium

Test Procedure for Growth Promoting and Indicative Properties of Solid Media and Liquid Media

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.[2]
2. Inoculate the new and previously approved batches of the medium with 0.1 ml of the microorganism suspension. A control is recommended to verify the 0.1 ml of microorganism suspension contains ≤100 CFU. The control is the non-selective agar.
3. If the new medium is liquid or selective agar, a control is recommended to verify the 0.1 ml of microorganism suspension contains ≤100 CFU. The control is a non-selective agar.
4. For solid media or the non-selective control agar, use a spreader to disperse the inoculum evenly across the agar.
5. Follow pharmacopeia directions for incubation temperature and length of incubation for each microorganism tested (refer to Table 2).
6. Determine if the new medium is suitable for use by using the acceptance criteria below.

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 new batch of the medium must be “comparable” to the average number of colonies on the previously approved batch. A quantitative definition of “comparable” is not established by the USP, Ph. Eur. or JP.
   - There must be ≤100 colonies on the control (non-selective agar).

[1]Use only the Hydrating Fluid provided with the kit.
[2] **KWIK-STIK™ Plus** is a recommended alternative for testing *Pseudomonas paraeruginosa* on Cetrimide Agar. Inoculate the non-selective agar with the **KWIK-STIK™ Plus** swab, incubate, and use a spectrophotometer or turbidimeter to adjust the CFU concentration to ≤100 CFU per 0.1 ml.

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).

3. **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.

Test Procedures for Inhibitory Properties of Solid Media and Liquid Media

1. Using **Epower**™ E3, prepare an inoculum of each of the required microorganisms.[3]
2. Rehydrate 1 pellet in 1.0 ml hydration fluid as described in the **Epower**™ Instructions for Use document. An **Epower™** E3 pellet contains 1000-9999 CFU. One ml of the **Epower™** microorganism suspension contains 1000-9999 CFU.
3. Inoculate the new and previously approved batches of the medium with 0.1 ml of the microorganism suspension. 0.1 ml contains of the Epower E3 microorganism suspension contains 100-999 CFU.
4. A control is recommended to verify the 0.1 ml of microorganism suspension contains ≥100 CFU. The control is the non-selective agar.
5. For solid media, use a spreader to disperse the inoculum across the agar.
6. Follow pharmacopeia directions for incubation temperature and length of incubation for each microorganism tested (refer to Table 2).
7. Determine if the new medium is suitable for use by using the acceptance criteria below.

Acceptance Criteria

1. The test microorganism should be inhibited on the new batch of agar.
2. There should be at least 100 colonies on the non-selective control agar.

[3] An alternative method is to use colonies grown from **KWIK-STIK™ Plus** to prepare an inoculum of ≥100 CFU.

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

Type of Medium	Microorganism	Properties	Temperature	Incubation Period
Enterobacteria Enrichment Broth Mossel	*E. coli*	Growth Promoting	30°C-35°C	24 hours
	P. paraeruginosa	Growth Promoting	30°C-35°C	24 hours
	S. aureus	Inhibitory	30°C-35°C	48 hours
Violet Red Bile Glucose Agar	*E. coli*	Growth Promoting & Indicative (purplish-red colonies)	30°C-35°C	Growth:18 hours Indicative:18-24 hours
	P. paraeruginosa	Growth Promoting & Indicative (purplish-red colonies)	30°C-35°C	Growth: 18 hours
MacConkey Broth	*E. coli*	Growth Promoting	42°C-44°C	24 hours
	S. aureus	Inhibitory	42°C-44°C	48 hours
MacConkey Agar	*E. coli*	Growth Promoting & Indicative (pink Colonies)	30°C-35°C	Growth: 18 hours Indicative: 18-72 hours
Rappaport Vassiliadis Salmonella Enrichment Broth	*S. enterica* subsp. *enterica* serovar Typhimurium or *S. enterica* subsp. *enterica* serovar Abony	Growth Promoting	30°C-35°C	18 hours
	S. aureus	Inhibitory	30°C-35°C	24hours
Xylose Lysine Deoxycholate Agar	*S. enterica* subsp. *enterica* serovar Typhimurium or *S. enterica* subsp. *enterica* serovar Abony	Growth Promoting & Indicative (red colonies with or without black centers)	30°C-35°C	Growth:18 hours Indicative: 18-48 hours
Cetrimide Agar	*P. paraeruginosa* [4]	Growth Promoting	30°C-35°C	18 hours
	E. coli	Inhibitory	30°C-35°C	72 hours
Mannitol Salt Agar	*S. aureus* subsp. *aureus*	Growth Promoting & Indicative (yellow or white colonies with yellow zone)	30°C-35°C	Growth: 18 hours Indicative: 18-72 hours
	E. coli	Inhibitory	30°C-35°C	72 hours
Reinforced Medium for Clostridia Columbia Agar	*C. sporogenes*	Growth Promoting	30°C-35°C	48 hours Anaerobic conditions
Columbia Agar	*C. sporogenes*	Growth Promoting	30°C-35°C	48 hours Anaerobic conditions
Sabouraud Dextrose Broth	*C. albicans*	Growth Promoting	30°C-35°C	Growth: 3 days
Sabouraud Dextrose Agar	*C. albicans*	Growth Promoting & Indicative (white colonies)	30°C-35°C	Growth: 24 hours Indicative: 48 hours

[4] **KWIK-STIK™ Plus** is a recommended alternative for testing *Pseudomonas paraeruginosa* on Cetrimide Agar. Inoculate the non-selective agar with the **KWIK-STIK™ Plus** swab, incubate, and use a spectrophotometer or turbidimeter to adjust the CFU concentration to ≤100 CFU per 0.1 ml.

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

Control

A negative control (diluent[5]) is recommended for the Growth Promotion Test. Microbiologics has performed a Sterility Test on the Hydrating Fluid included in the kits. 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 kits in packs of 10 (2.0 ml Hydrating Fluid catalog HF0612, 1.2 ml Hydrating Fluid catalog HF0611).

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. It may be necessary to double the inoculum when using selective agar. If this is the case, inoculate non-selective and selective agar in parallel. There must be ≤100 colonies on the non-selective agar.
- **KWIK-STIK™ Plus** is a recommended alternative for testing *Pseudomonas paraeruginosa* on Cetrimide Agar. Inoculate the non-selective agar with the **KWIK-STIK™ Plus** swab, incubate, and use a spectrophotometer or turbidimeter to adjust the CFU concentration to ≤100 CFU per 0.1 ml.

[5] The diluent is the Hydrating Fluid. If using **EZ-CFU™** the diluent is the Hydrating Fluid and phosphate buffer pH 7.2.

Incubation

Due to pour plates requiring longer incubation periods and the variability of colony sizes, the use of a backlit colony counter is recommended.

Other

- Recovery on some types of selective media may be less than on non-selective media.
- Microbiologics has validated the procedures for using **EZ-Accu Shot™**, **EZ-CFU™ One Step,** and **EZ-CFU™** on TSA for the following microorganism strains:
    - *Escherichia coli* derived from ATCC® 8739™*
    - *Pseudomonas paraeruginosa* derived from ATCC® 9027™*
    - *Staphylococcus aureus* derived from ATCC® 6538™*
    - *Salmonella enterica* subsp. *enterica* serovar Abony derived from NCTC 6017
    - *Salmonella enterica* subsp. *enterica* serovar Typhimurium derived from ATCC® 14028™*
- Microbiologics has validated the procedures for using EZ-Accu Shot™, EZ-CFU One Step™, and EZ-CFU™ on Anaerobic Blood Agar:
    - *Clostridium sporogenes* derived from ATCC® 19404™*
    - *Clostridium sporogenes* derived from ATCC® 11437™*
- A customer qualification study is recommended to verify that the product works for the type of selective media, the company procedures, equipment, etc.
- 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 used in the sterility test, 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.

References

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

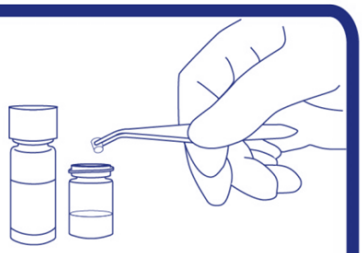
ISO 11133: 2014 Microbiology of food, animal feed and water – Preparation, production, storage and performance testing of culture media.

Acknowledgements

Illustrated Instructions

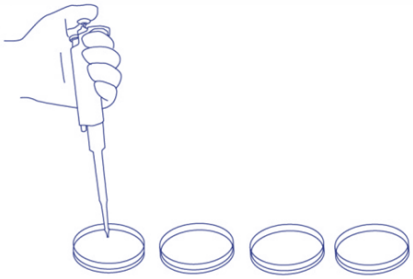
Instructions for testing the growth promoting and indicative qualities of new batches of solid medium to be used in Tests for Specified Microorganisms.

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.

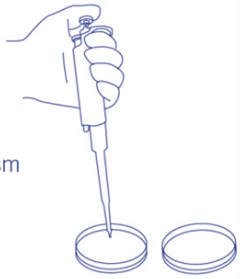
2



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

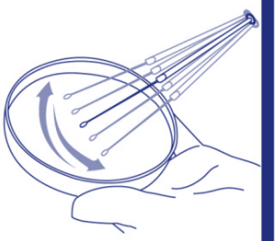
3

If the new medium is selective agar, a control is recommended to verify the 0.1 ml of microorganism suspension contain ≤ 100 CFU. The control is the non-selective agar.



4

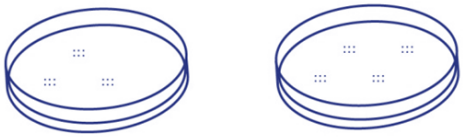
Use a spreader to disperse the inoculum across the agar.



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.

Illustrated Instructions

Instructions for testing the growth promoting qualities of new batches of liquid medium to be used in Tests for Specified Microorganisms.

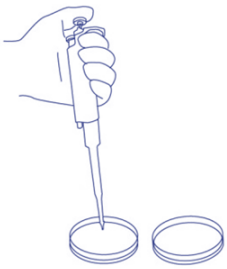
1

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

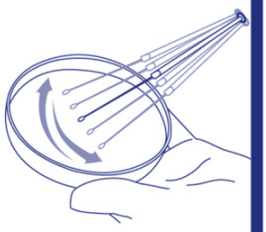
Inoculate the new and previously approved batches of the medium with 0.1 ml of the microorganism suspension.

3



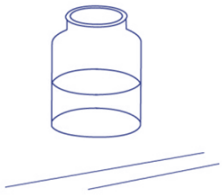
A control is recommended to verify 0.1 ml contains ≤100 CFU. The control is the non-selective agar.

4



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

5



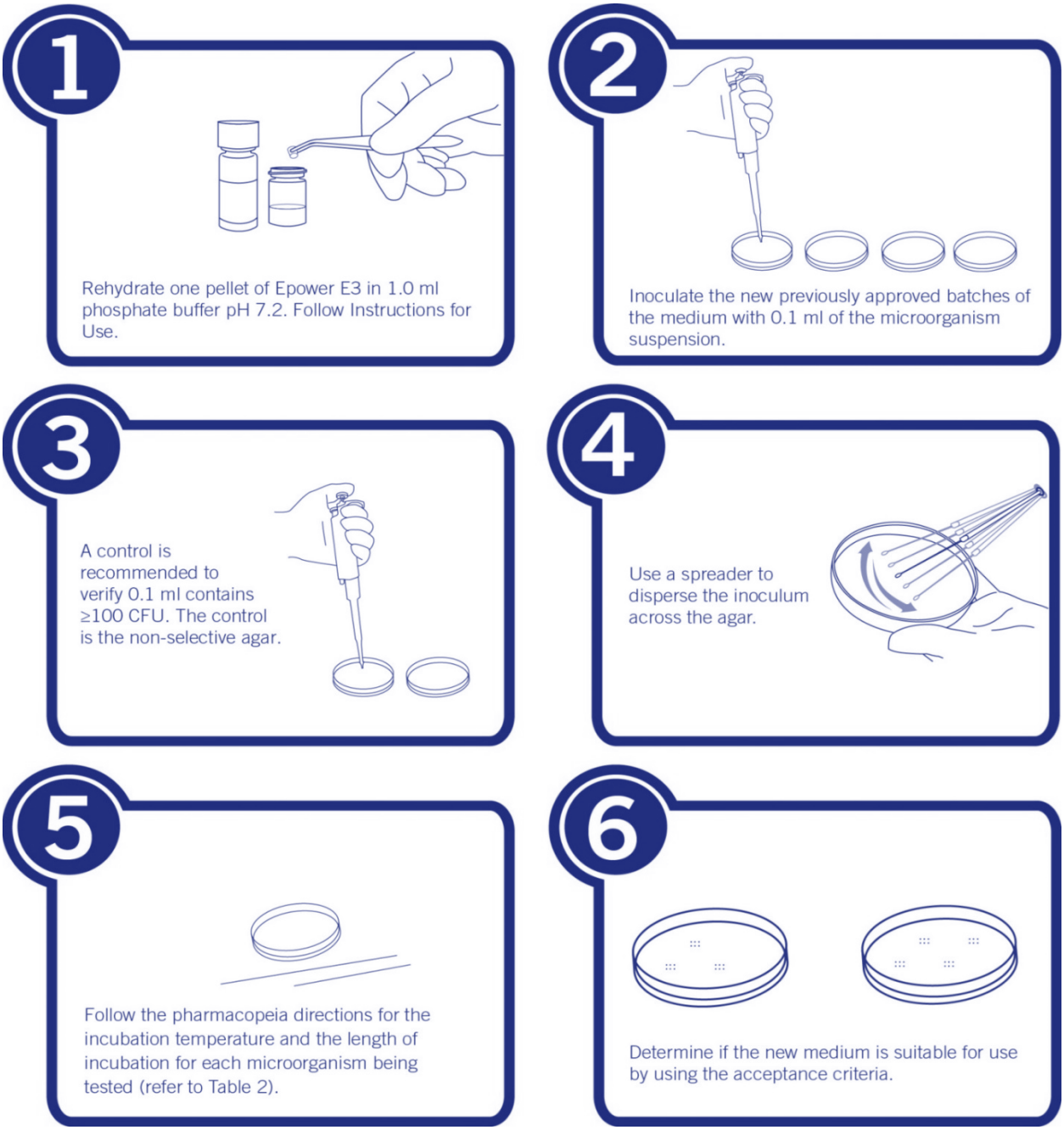
Follow the pharmacopeia directions for the incubation temperature and the length of incubation for each microorganism being tested (refer to Table 2).

6

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

Illustrated Instructions

Instructions for testing inhibitory qualities of new batches of solid medium using Epower E3™. Note: Although the illustrations show solid medium, they can also be used for liquid medium.



*An alternative method is to use colonies grown from **KWIK-STIK™ Plus** to prepare an inoculum of ≥100 CFU.