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## 1.0 Purpose

This procedure describes the assay methodology for determining the percentage of antibiotic-resistant cells (colonies) in bacterial cell bank samples. This procedure may also be used in conjunction with and is designed to follow ***SOP 22153 - Bacterial Cell Bank Viability Assay***.

## 2.0 Scope

This Standard Operating Procedure (SOP) applies to GMP and R&D/FIO QC Test Requests for bacterial cell bank viability testing. Refer to the cell bank Master Specification and/or the Project Scientist for specific antibiotics and concentrations. Some cell banks may require antibiotic media and dilution ranges other than those indicated in this SOP. The use of alternative antibiotics or dilution schemes as directed by the Project Scientist or the Master Specification document must be noted on Attachment but will not require a PA/QC test method deviation.

## 3.0 Authority and Responsibility

3.1 The Director, Process Analytics/Quality Control (PA/QC) has the authority to define and revise this procedure.

3.2 PA/QC personnel are responsible for the training and diligent performance of the procedures established in this SOP.

3.3 PA/QC personnel are responsible for completing required documentation per this SOP.

3.4 PA/QC Managers or designees are responsible for reviewing the data and documentation generated from this SOP.

3.5 Biopharmaceutical Quality Assurance (BQA) is responsible for quality oversight of this procedure.

## 4.0 Reagents and Materials

LB agar plate (100 mm) with cover, without antibiotics, BDP PN **10446**

LB agar plate (100 mm) with cover + Kanamycin (25 μg/mL), BDP PN **10447**

LB agar plate (100 mm) with cover + Chloramphenicol (20 μg/mL), BDP PN **10450**

LB agar plate (100 mm) with cover + Chloramphenicol (25 μg/mL), BDP PN **10449**

LB agar plate (100 mm) with cover + Tetracycline (15 μg/mL), BDP PN **10451**

LB agar plate (100 mm) with cover + Streptomycin (50 μg/mL), BDP PN **10448**

Other defined media or antibiotic plates may be requested for specific projects, refer to project-specific documentation and PA/QC Manager.

Sterile toothpicks, BDP PN **21709** (If received non-sterile, they will need to be autoclaved)

Parafilm, BDP PN **20464**

## 5.0 Procedure and Calculations

**NOTE:** Refer to ***SOP 22153 - Bacterial Cell Bank Viability Assay***, in order to generate sufficient (≥ 200) isolated colonies on "**without antibiotics**" LB agar media to perform this assay.

5.1 Antibiotic Resistance Determinations

5.1.1 At least two (2) "Without antibiotics" LB agar plates are required to complete this assay. For each type of antibiotic requested on QCTR Form 22002-01 or required per the product Master Specification, at least two additional plates of each antibiotic and "without antibiotics" plates will be required.

**NOTE: The *E. coli* controls in Step 5.1.3 and 5.1.4 are only necessary when the BDP Lot# of plates used is different from those used in the Bacterial Cell Bank Viability Assay (SOP 22153) that generated colonies for this assay and it is being performed within 72 hours of SOP 22153.**

5.1.2 **Negative Plate controls** - set aside one of each LB agar plate type required in the assay. Incubate the negative plate controls along with the antibiotic-resistance plates.

5.1.3 **Negative *E. coli* Control Strain plate** – For each antibiotic and no-antibiotic media type used, spread one plate of each media with 100 μL of the *E. coli* DH5α negative control strain to verify the presence of antibiotics in the media.

5.1.4 **Positive *E. coli* Control Strain plate** – For antibiotic media only. Spread one plate of each media type with 100 μL of the appropriate antibiotic-resistant positive *E. coli* control strain to verify the presence of the desired antibiotic.

5.1.5 Pre-warm all agar plates at 37°C ± 2°C until no condensation is present on the agar surface.

5.1.6 Record agar plate part and lot numbers, expiration dates, and number of plates used on Form 22155-01

5.1.7 Using a fresh, sterile toothpick for each colony, pick and transfer 100 well-isolated colonies from the "without antibiotics" LB agar plates generated from ***SOP 22153 - Bacterial Cell Bank Viability Assay***, to new agar plates containing the desired antibiotic. See Figure 1 as a conceptual aide.

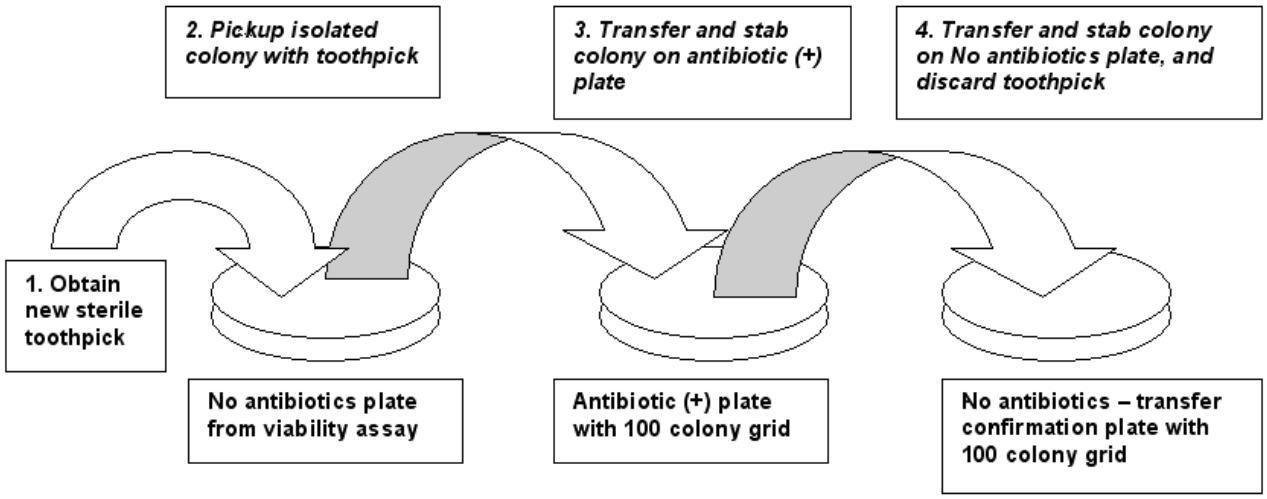


**Figure 1 – Schematic of Colony Transfer Procedure**

5.1.8 Immediately follow with a second stab of the same toothpick onto a new LB agar plate without antibiotics in order to confirm the transfer of bacteria to the plate.

5.1.9 Transfer each colony pick to a defined and numbered location on the antibiotic and "no antibiotics" agar media. See Attachment 1 – 100 Colony Transfer Grid, to help organize stab placement. Alternatively, a gridding unit (such as from VWR) may be used to facilitate the placement of colonies on the antibiotic and the transfer confirming "without antibiotics" plates.

5.1.10 Repeat with a second set of plates for a total of 200 colonies transferred for each antibiotic to be tested.

5.1.11 Following the transfer of the 200 colonies, stab the negative control "no antibiotics plate" plate (see 5.1.2) with 3 additional sterile toothpicks in order to confirm the sterility of the toothpicks used.

5.1.12 Tape plates with Parafilm, invert and incubate plates at 37º ± 2°C for 18 – 32 hours. Record the incubation date and time, and the incubator temperature on Form 22155-01.

5.1.13 Upon completion of incubation, remove the plates from the incubator and record the date and time of removal as well as the incubator temperature on Form 22155-01.

5.1.14 Count all the observed colonies on each plate located at the stab sites and record on Form 22155-01. Note the presence and number of any colonies growing on regions of the plate that have not been stabbed on Form 22155-01 and record in the comments section of Form 22155-01.

5.1.15 Record the position of stabs that show no growth on antibiotic media and compare to the equivalent position (i.e., from the same toothpick) on the confirmatory "no antibiotic" plate – if the stab position on the confirmatory plate shows no growth, then the colony transfer was not successful and should **not** be counted as a antibiotic sensitive colony. Record this observation on the comments section of Form 22155-01.

5.1.16 Subtract the total number of colonies transferred (200) by the number of unsuccessful transfers observed in 5.1.15. **For example** - if 3 colonies were not transferred (did not grow) on the confirmatory plate, reduce the total number of colonies transferred by 3 (i.e., from 200 to 197).

5.1.17 Record the number of failed transfers and the corresponding total number of colonies successfully transferred on Form 22155-01.

5.1.18 Colonies that show growth on the antibiotic plate but show no growth at the corresponding stab on the confirmatory "no antibiotic" plates are considered to be successfully transferred for the purposes of this assay. Record the number of these events for each plate on the comment section of Form 22155-01.

5.1.19 Count all the colonies that show no growth on the antibiotic plate but that do show growth at the corresponding position on the transfer confirmatory plate. These colonies are exhibiting antibiotic-sensitivity, but do not count those colonies that were not successfully transferred as "antibiotic sensitive." Record the total number of antibiotic sensitive colonies for each antibiotic tested on Form 22155-01.

5.1.20 Determine the percentage of antibiotic resistant colonies for each antibiotic according to the following formula.

$$\text{Percent Resistance}(\%) = 100 - 100X\left(\frac{\text{Number of antibiotic sensitive colonies}}{\text{Number of colonies successfully transferred}}\right)$$

5.1.21 Record the Percent Resistance calculated for each antibiotic tested on Form 22155-01, round to the nearest whole percentage value. Refer to ***SOP 22012 - Significant Figures and Rounding of Numbers***.

## 5.2 Auxotrophic Marker Determinations

**NOTE:** When using a minimal (unsupplemented) media to determine the percent of colonies that are auxotrophic, those colonies that **do** exhibit growth (i.e., are not auxotrophic) on unsupplemented media are counted in the assay. This is the reverse of the antibiotic-resistance test in section 5.1 where the number of non-growing (antibiotic-sensitive) colonies are counted by their absence on the plate.

5.2.1 Follow the procedure outlined in 5.1 replacing the antibiotic and "no antibiotics" media plates with plates that are missing the auxotrophic substance (usually minimal media), followed by an agar plate supplemented with the auxotrophic substance (or made with complete media).

5.2.2 Gridding, incubation, and colony-scoring of the auxotrophic plates is performed identically to that used with the antibiotic-resistance method except that the expected outcome is the reverse of antibiotic-resistance testing - i.e., the unsupplemented plate is expected to show no growth if all colonies are auxotrophic.

5.2.3 The determination of the percent auxotrophic colonies is calculated according to the following formula.

$$\text{Percent Auxotrophy (\%)} = 100 - 100 \times \left( \frac{\text{Number of antibiotic sensitive colonies}}{\text{Number of colonies successfully transferred}} \right)$$

5.2.4 Record the Percent Auxotrophy calculated for each phenotypic marker tested on Form 22155-02, round to the nearest whole percentage value.

5.2.5 Dispose of the plates as biohazardous autoclave waste.

## 6.0 Definitions

6.1 **Auxotrophy –** The inability of an organism to synthesize a specific organic compound required for its growth. Prototrophic organisms can synthesize all compounds required for growth from minimal media.

6.2 **Auxotrophic Marker –** The use of a specific auxotrophic compound in a growth media in order to recover cell growth compared to a minimal (simple) media which should exhibit no growth without the auxotrophic compound present.

6.3 **Cam –** Abbreviation for the antibiotic Chloramphenicol.

6.4 **cfu –** Colony Forming Unit.

6.5 **Kan –** Abbreviation for the antibiotic Kanamycin.

6.6 **LB –** Luria-Bertani broth media.

6.7 **RT –** Room Temperature (typically 22°C to 28°C).

6.8 **Str –** Abbreviation for the antibiotic Streptomycin.

6.9 **Tet –** Abbreviation for the antibiotic Tetracycline.

6.10 **TNTC –** Too Numerous to Count (can also be written as TMTC – "Too Many to Count").

## 7.0 Documentation

7.1 Record the reagent information, test sample data, calculations, and comments on Form 22155-01.for antibiotic-resistance assays or Form 22155-02 for auxotrophy assays.

7.2 Document on Forms 22155-01 or 22155-02 (as appropriate) the origin of the isolated bacterial colonies tested per this SOP. If a viability test was used to generate bacterial colonies, indicate and record the QC Test Request number of the bacterial viability assay.

## 8.0 References and Related Documents

8.1 **SOP 22012** *Significant Figures and Rounding of Numbers*

8.2 **SOP 22153** *Bacterial Cell Bank Viability Assay*

8.3 **Form 22155-01** *Bacterial Cell Bank Antibiotic Resistance Calculations and Results*

8.4 **Form 22155-02** *Bacterial Cell Bank Auxotrophy Calculations and Results*

8.5 **Attachment 1** *100 Count Grid Template for Colony Transfers*

[redacted]

### Attachment 1

### 100 Count Grid Template for Colony Transfers

(Some reduction or enlargement may be necessary to fit 100 mm Petri-dishes)