NCL Method STE-2.2

Detection of Bacterial Contamination Using Luria Broth Agar Plates

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

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1. Introduction

This protocol describes a procedure for quantitative determination of microbial contamination in a nanoparticle preparation. Nanoparticle samples, along with controls, are spread on the surface of agar and growth of bacterial colonies is monitored after 72 hr of incubation. The intended purpose of this assay is to avoid introduction of microbial contamination into in vitro cell cultures and in vivo animal studies utilizing the test-nanomaterial, as microbial contamination will confound the results of these tests. This assay is not intended to certify the material as sterile.

2. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; these reagents were used in the development of the protocol and their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted. Please note that suppliers may undergo a name change due to a variety of factors. Brands and part numbers typically remain consistent but may also change over time.

2.1 Reagents

- 2.1.1 Sterile PBS (Sigma, D8537)
- 2.1.2 Luria Broth (LB) agar (Sigma, L2897)
- 2.1.3 Luria Broth (Sigma, L1900)
- 2.1.4 Test nanomaterial
- 2.1.5 Buffer used to reconstitute test nanomaterial
- 2.1.6 Sodium Hydroxide (NaOH) (Sigma, S2770)
- 2.1.7 Hydrochloric acid (HCl) (Sigma, H9892)

2.2 Materials

- 2.2.1 Pipettes, 0.05 to 10 mL
- 2.2.2 Sterile pipets, 1-10 mL
- 2.2.3 Sterile tubes, 5 mL
- 2.2.4 Petri dishes
- 2.2.5 Bacterial spreader

2.3 Equipment

2.3.1 Incubator at 37°C

2.3.2 Vortex

3. Reagent Preparation

3.1 Sodium Hydroxide

Prepare from concentrated stock by dilution into sterile water to make a 0.1 N final concentration solution.

3.2 Hydrochloric Acid

Prepare from concentrated stock by dilution into sterile water to make a 0.1 N final concentration solution.

3.3 Luria Broth

Components of this media are:

10 g/L Tryptone

5.0 g/L Yeast Extract

0.5 g/L NaCl

This is supplied as a liquid but can also be purchased as a powder. If you use liquid media, it does not require any additional manipulation. Powdered media must be reconstituted in water. In this case please follow the instruction from the manufacturer of the powdered media. Sterilize the media you prepare from powdered formula 15 min at 121°C. Cool to room temperature and either use fresh or store in refrigerator.

3.4 Luria Broth Agar

Components of the LB agar are:

10 g/L Tryptone

5.0 g/L Yeast Extract

0.5 g/L NaCl

15g/L Agar

This is prepared by dissolving 15 g of agar in 1 L of LB media described in section 3.3 or from the commercial powdered formula containing all components in a dry form. For example, the product listed in the reagents section is supplied as a powder. The manufacturer suggests the following procedure: “add 30.5 g powder to 1 L water; heat to boiling while stirring to dissolve powder; autoclave for 15 minutes at 121°C to sterilize”. Cool the media slightly, pour into petri dishes and allow to solidify. The plates can be used freshly prepared or stored at 4°C.

4. Preparation of Controls

4.1. Negative Control (NC)

Use sterile PBS or water as a negative control. The negative control is acceptable if no colony forming units (CFU) are observed upon completion of the test.

4.2. Positive Control (PC)

For the positive control, use bacterial cultures (e.g. ATCC #25254) at a dilution which will allow at least 10 CFU/mL.

4.3. Inhibition Control

To assess whether nanoparticles can inhibit bacterial growth, a positive control sample at the same final dilution as described in section 4.2 is spiked into the test nanoparticle sample. For example, spike 22 CFU per 2.2 mL of nanoparticle solution at a given dilution and use 1 mL for seeding into a paddle as described in section 6 below. The final inhibition control contains the same concentration of nanoparticles as nanoparticle-unspiked sample and the same concentration of bacteria as in the positive control (10 CFU/mL).

It is important to note, this control will only estimate anti-bacterial properties relevant to bacteria present in the given positive control. The anti-bacterial properties of a nanoparticle formulation are often specific to a given strain or type of bacteria and depend on nuances in the bacterial cell wall organization and other biological processes within bacterial cells. Therefore, if the analysis of anti-bacterial activity is of interest, it is advisable to perform such analysis using bacterial strains that are relevant to the expected type of anti-bacterial activity of

the nanoparticle formulation. For example, if a nanoparticle is prepared to inhibit E. coli growth, then relevant strains of E. coli should be used. If such information is not available, conducting a test using a panel of bacteria representative of gram-positive and gram-negative bacteria is recommended.

5. Study Samples

The assay requires 5 mL of the test nanoparticle in its final formulation. The concentration of nanoparticles in this formulation is case-specific. Samples are typically tested at stock and at several serial 1:10 dilutions, i.e., no dilution, 1:10, 1:100, 1:1000. When the final formulation is not available, for example when a test nanomaterial is received from a commercial supplier in a form not intended for biomedical applications, prepare a stock solution at a concentration of 1 mg/mL. The weight information can refer to either active pharmaceutical ingredient or total construct; it can also represent total metal content or other units. Such information is specific to each nanoparticle and should be recorded to aid result interpretation.

Test nanoparticles should be reconstituted in sterile PBS, water or in appropriate vehicle. If vehicle is a buffer or media other than water or PBS, the vehicle control should be included in the test. The pH of the study sample should be checked using a pH microelectrode and adjusted with either sterile NaOH or HCl as necessary to be within the pH range 6-8. If NaOH or HCl are not compatible with a given nanoparticle formulation, adjust pH using a procedure recommended by the nanomaterial manufacturer. To avoid sample contamination from microelectrode, always remove a small aliquot of the sample for use in measuring the pH.

6. Experimental Procedure

6.1 Remove the LB plates from refrigerator and let them warm to room temperature. Prepare two plates for each sample and four plates for negative and positive control. Plate one set of negative and positive control before plating test samples and the second set after plating test samples.

6.2 Using sterile conditions, apply 50 µL of controls or nanoparticle preparation (at each dilution) onto the surface of the agar and evenly distribute the sample using sterile disposable bacterial spreader. Allow liquid to absorb, then recap the Petri dish, turn it upside down to prevent condensation, and place into the incubator.

6.3 Incubate for 72 hr at a nominal temperature of 37°C.

6.4 Remove dishes from the incubator and examine for appearance of colonies. Count colonies.

6.5 Report results according to the following formula:

Colonies x Dilution Factor x Sampling Factor = CFU/mL

***Note**:* To estimate sampling factor, consider the proportion of the test sample represented by the 50 µL test aliquot spread on the plate. For example, if the final formulation is supplied as a 1 mL aliquot, the sampling factor is 20; if it is 10 mL, the sampling factor is 200; if it is 0.5 mL, the sampling factor is 10, etc.

7. Interpretation of Results and Acceptance Criteria

7.1 A positive control is considered acceptable if it allows identification of at least 10 CFU/mL.

7.2 A negative control is acceptable if no colony is detected.

7.3 A test sample is considered negative if no colony is detected.

7.4 An inhibition control is considered acceptable if it shows no significant difference in CFU number from that observed in the positive control.

7.5 A ≥ 2-fold decrease in the number of colonies in the inhibition control sample versus that in the positive control sample suggests the test nanomaterial has the potential to inhibit bacterial growth. Further investigation including analysis of minimal inhibitory concentration (MIC) is needed to verify such findings.

8. Abbreviations

CFU	colony forming units
HCl	hydrochloric acid
NaOH	sodium hydroxide
PBS	phosphate buffered saline
MIC	minimal inhibitory concentration