NCL Method STE-2.2

Detection of Bacterial Contamination by Agar Plate Test

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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 incubation.

Note: This assay is not intended to certify the material as sterile. The intended purpose 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.

2. Reagents and Equipment

Note: The NCL does not endorse any of the suppliers listed below; their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted.

2.1 Reagents

1. Sterile PBS (GE Life Sciences, SH 30256.01)
2. Luria Broth (LB) agar (Sigma, L3272)
3. Luria Broth (Sigma, L9233)
4. Sodium hydroxide (NaOH) (Sigma, S2770)
5. Hydrochloric acid (HCl) (Sigma, H9892)
6. Test nanomaterial
7. Buffer used to reconstitute test nanomaterial

2.2 Materials

1. Pipettes, 0.05 to 10 mL
2. Sterile pipets, 1-10 mL
3. Sterile tubes, 5 mL
4. Petri dishes
5. Bacterial spreader

2.3 Equipment

1. Incubator, 37°C
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

1. Components of this media are 10 g/L Tryptone, 5.0 g/L Yeast Extract, and 0.5 g/L NaCl.
2. It is supplied as a liquid, but can also be purchased as a powder. Liquid media does not require any additional manipulation. Powdered media has to be reconstituted in water. In this case, please follow the manufacturer's instructions. Sterilize the media prepared from powdered formula, 15 min at 121°C. Cool to room temperature. Use fresh or store at 4°C.

3.4 Luria Broth Agar

1. Components of the LB agar are 10 g/L Tryptone, 5.0 g/L Yeast Extract, 0.5 g/L NaCl, 15 g/L Agar.
2. This can be prepared by dissolving 15g of agar in 1 L of LB media described above in section 3.3, or from the commercial powdered formula containing all components. 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 fresh 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 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 10 CFU/mL into the nanoparticle solution, such that 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).

5. Study Samples

The assay requires 0.5 mL of the test nanomaterial. The concentration of nanoparticles is case-specific. Most samples are tested at the supplied stock concentration, and at several serial 1:10 dilutions, i.e. no dilution, 1:10, 1:100, 1:1000. When such information 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 solution at 1 mg/mL. The weight information can refer to active pharmaceutical ingredient, total construct, 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 other appropriate vehicle. If vehicle is a buffer or media other than water or PBS, the vehicle control should also 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 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

1. Remove LB plates from refrigerator and allow to equilibrate to room temperature. Prepare two plates for each sample and four plates for negative and positive control. Plate one set of negative and positive controls before plating test samples, and the second set after plating test samples.

2. Under sterile conditions, apply 50 µL of controls or nanoparticle (at each dilution) onto the surface of the agar and evenly distribute the sample using sterile disposable bacterial spreaders. Allow liquid to absorb, recap the Petri dish, turn upside down to prevent condensation, and place into the incubator.
3. Incubate for 72 hr at a nominal temperature of 37°C.
4. Remove dishes from the incubator and examine for appearance of colonies. Count colonies.
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 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

1. A positive control is considered acceptable if it allows identification of at least 10 CFU/mL.
2. A negative control is acceptable if no colony is detected.
3. A test sample is considered negative if no colony is detected.
4. An inhibition control is considered acceptable if it shows no significant (≥2-fold) difference in CFU number from that observed in the positive control.
5. A ≥2 fold decrease in the number of colonies in the inhibition control sample versus the positive control sample suggests the 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