



NCL Method STE-2.1

Detection of Microbial Contamination Using Millipore Sampler Devices

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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. The protocol includes tests for yeast, mold and bacteria using Millipore Sampler devices [1]. 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 (GE Life Sciences, SH 30256.01)
- 2.1.2 Yeast and mold sampler (Millipore Corp., MY0010025)
- 2.1.3 HPC Count sampler (Millipore Corp., MHPC10025)
- 2.1.4 Bacterial cell culture for positive control (ATCC, 25254)
- 2.1.5 Yeast cell culture for positive control (ATCC, MYA 774)
- 2.1.6 Test nanomaterial
- 2.1.7 Buffer used to reconstitute test nanomaterial
- 2.1.8 Sodium Hydroxide (NaOH) (Sigma, S2770)
- 2.1.9 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

- 2.3 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.

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 or yeast cell cultures (ATCC #25254 and MYA774, respectively) at a dilution which will allow at least 10 CFU/mL. If standard cultures are not available, a sample from another source (e.g., rain water, floor swipe, etc.) known to contain bacteria and yeast/mold may be used.
- 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 Sampler from its plastic bag and write the date and the sample reference number on the case with indelible marker.
- 6.2 Using sterile conditions, remove a paddle from the case and apply 1 mL of nanoparticle preparation (or dilution) onto the surface of the filter. Allow liquid to absorb, then recap the paddle. To prevent the paddle from drying out during incubation, it should be seated firmly in the case to form an air-tight seal. Prepare 2 paddles per each sample.
- 6.3 Incubate for 72 h at a nominal temperature of 37°C.
- 6.4 Remove paddle from case and examine for appearance of colonies. Count colonies.
- 6.5 Report results according to the following formula:

$$\# \text{ Colonies} \times \text{Dilution Factor} = \text{CFU/mL}$$

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 PC.
- 7.5 A ≥ 2 -fold decrease in the number of colonies in the inhibition control 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. References

1. Samplers, Dilution kits and swab test kits user guide. P15325, Rev. D., 8/99 Millipore Corp.

9. Abbreviations

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