Detection of Bacterial Contamination by Agar Plate Test

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1.0	13.07.2016	All	Initial Document	Rainer Ossig
1.1	01.10.2016	Chapter 7	The acceptance criteria are now in line with the acceptance criteria of NCI-NCL	Matthias Rösslein

Table of Content

1 Introduction

This protocol describes a procedure for the determination of microbial growth contamination in a nanoparticle containing preparation.

2 Principle of the Method

Nanoparticles to be tested are reconstituted or diluted in sterile buffered saline (PBS).

Nanoparticle samples along with controls are spread on the surface of growth media agar plates and formation of bacterial colonies is monitored after 72 h of incubation. Each agar plate is prepared in duplicate.

3 Applicability and Limitations (Scope)

The intended purpose of this assay is to avoid the 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 tested material as sterile.

4 Related Documents

Table 1:

Document ID	Document Title
NCI-NCL_STE2.1	*Detection of Microbial Contamination*
NCI-NCL_STE2.2	*Detection of Bacterial Contamination by Agar Plate Test*
EUNCL-NCL_STE2.1	*Detection of Microbial Contamination*

5 Equipment and Reagents

5.1 Equipment

5.1.1 Sterile Micro-Pipettes with tips or plastic pipettes, range 0.01 to 10 mL

5.1.2 Sterile incubation tubes, 15 mL

5.1.3 Sterile centrifuge tubes (1.5 mL or larger volumes)

5.1.4 Incubator at 35°C

5.1.5 Vortex

5.1.6 Sterile work bench (laminar flow)

5.1.7 Petri dishes

5.1.8 Bacterial spreader

5.1.9 Photometer at 600 nm

5.1.10 Autoclave

5.1.11 Bunsen burner

5.1.12 Magnetic stirrer

5.1.13 Water bath (60°C)

5.2 Reagents

5.2.1 Test nanomaterial

5.2.2 Sterile PBS (Sigma, D8537)

5.2.3 Buffer used to reconstitute test nanomaterial

5.2.4 *E. coli* K12 Bacterial cell culture for positive control (e.g. New England Biolabs, strain JM101, JM109)

5.2.5 Sodium Hydroxide (NaOH) 1.0 N, sterile (Sigma, S2770)

5.2.6 Hydrochloric acid (HCl) 1.0 N, sterile (Sigma, H9892)

5.2.7 Lysogeny Broth (LB) -Medium, Luria/Miller (e.g. Carl Roth, X968)

5.2.8 Agar (e.g. Carl Roth 2266)

5.2.9 purified water, (e.g. Milli-Q® water, Resistivity at 25 °C* 18.2 MΩ•cm r), sterile

5.3 Reagent Preparation

5.3.1 Sodium Hydroxide (sterile)

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

5.3.2 Hydrochloric Acid (sterile)

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

5.3.3 Lysogeny Broth (LB), Luria/Miller

Components of this fluid medium are:

10 g/L Tryptone

5.0 g/L Yeast Extract

10 g/L NaCl, pH 7.0

LB can be purchased as a powder mixture, but also is supplied as a ready-to-use liquid. If you use sterile liquid LB media, it does not require any additional manipulation. Powdered media has to be reconstituted in water.

Follow the instruction from the manufacturer of the powdered media. Sterilize the media you prepare from powdered formula by autoclave 20 minutes at 121 °C in an adequate glas bottle with lid (do not close the lid of the bottle tightly to allow excessive pressor to escape). Afterwards cool fluid media to room temperature and either use fresh or store in refrigerator.

5.3.4 Lysogeny Broth (LB) Agar Plates:

Components of the LB agar are:

10 g/L Tryptone

5.0 g/L Yeast Extract

10 g/L NaCL, pH 7.0

25 g/L Agar

LB Agar Plates can be prepared by dissolving 25 g of Agar in 1 L of LB fluiid medium described or as additive to the powdered formula containing all components in a dry form. For example, the product listed in the reagents section is supplied as a powder to prepare the LB medium. For LB Agar Plates add 25 g/L agar and a magnetic stirring bar to the medium bottle before autoclave (do not close the lid of the bottle tightly to allow excessive pressor to escape). Afterwards remove from autoclave, mix the medium on a magnetic stirrer, and cool down slightly (to approximately 55-65 °C), e.g. by placing in a water bath set to 60 °C. Pour LB agar medium into petri dishes under sterile conditions and allow agar to solidify. Approximately 15 to 20 mL of LB agar medium is sufficient volume to prepare one agar plate. Freshly prepared agar plates are wet, therefore allow plates to dry during 1 to 3 days of incubation at room temperature. The dried LB plates can be stored at 4 °C or used freshly.

Please note that at least 24 agar plates will be needed to test a single nanomaterial in 4 different concentrations in this test procedure, including positive and negative controls as well as the inhibition controls. Calculate additional agar plates for eventually repeats and further controls.

6 Procedure

6.1 General remarks

Perform all test procedure under sterile conditions. The assay requires at least 0.65 mL of the highest test nanomaterial stock concentration to be tested.

The concentration of nanoparticles in this formulation is case-specific depending on the intended application. When such detailed information is not available, for example when a test nanomaterial is received 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 diluted (and reconstituted) in sterile PBS or water, or in another appropriate vehicle. A respective vehicle negative control should be included in the Microbial Growth Contamination test. The samples can be tested directly from the prepared stock concentration and at several dilutions (e.g. no dilution, 1:10, 1:100, 1:1000). Prepare a serious of 1:10 dilutions by adding 1/10 volume of the prior nanomaterial suspension into 9/10 volume sterile PBS or water. Each dilution should be vigorously mixed before proceeding with the next step of the dilution series.

The pH of the study sample should be controlled 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 during measurement procedure, always remove a small aliquot of the sample for the determination of pH.

For the verification of assay results it is necessary to prepare samples containing a defined amount of microbial contamination to determine potential growth inhibition processes of the sample material. Therefore the same amount of bacterial positive control suspension are added to the nanoparticle suspension of the sample, resulting in a spiked bacterial concentration identical to the positive control The concentration of nanoparticles should be equal to one assayed in test-sample. You will need to prepare a growth inhibition control for each dilution of the nanomaterial assayed in this test. A positive control using the same level of microbial contamination in PBS will be used as Quality Control for the reaction. Sterile PBS or the respective diluent buffer is used as a negative control reference.

6.2 Preparation of Controls

6.2.1 Positive control:

Use bacterial cell cultures (e.g. *E. coli* strain JM101) at a dilution, which will allow detection of at least 10 CFU per LB agar plate. (For example, when you calculate 500 CFU per 1 mL of positive control sample and apply 100 µL for seeding the expected number per plate is 50 CFU.) 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.

6.2.2 Negative control:

Use sterile PBS or sterile water as a negative control.

6.2.3 Vehicle control:

If vehicle is not PBS or water, use sample of vehicle which has been used for reconstitution/dilution of nanomaterial as an additional control.

6.2.4 Inhibition Control:

To understand if nanoparticles can inhibit bacterial growth, a bacterial sample at the same final dilution as described for the Positive control is spiked into a test nanoparticle sample. For example, when you spike 200 CFU per 0.4 mL of nanoparticle solution at a given dilution the final inhibition

control contains the same concentration of nanoparticles as the unspiked nanoparticle sample and the same concentration of bacteria as in the positive control (or this example 500 CFU/mL).

6.3 Flow chart

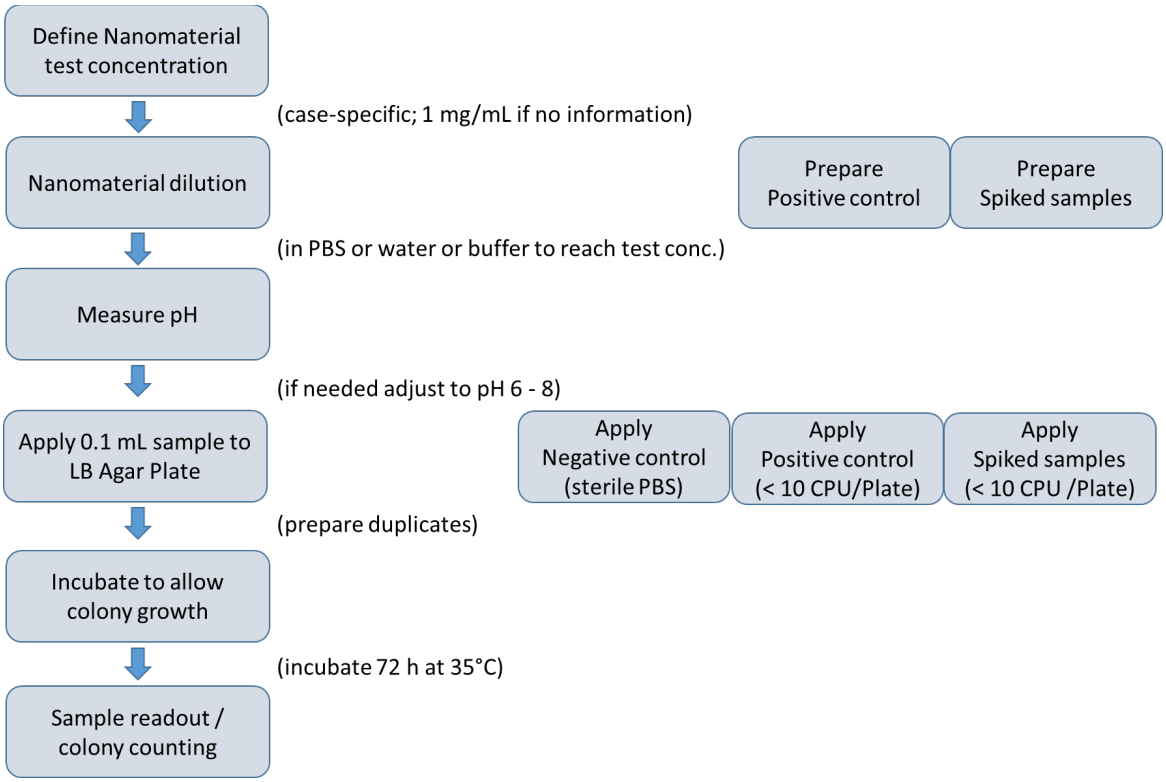

Figure 1: Brief outline of the workflow.

6.4 Material spreading on LB agar plates:

6.4.1 Remove required number the LB plates from refrigerator and let them warm up to room temperature. For freshly prepared wet agar plates allow more time at room temperature to dry.

6.4.2 Prepare two plates for each nanoparticle sample dilution and 4 plates for negative and positive controls. Under sterile conditions apply 100 µL of controls or nanoparticle sample preparation (at each dilution) or spiked nanoparticle preparation onto the surface of the agar and evenly distribute the sample using sterile disposable bacterial spreader.

6.4.3 Recap the Petri dish, allow liquid to absorb, then turn it upside down to prevent condensation and place into the incubator.

6.4.4 Incubate for 72 h at a nominal temperature of 35°C.

6.4.5 Control for appearance of microbial colony growth after one and two days. After indicated incubation time remove Petri dishes from incubator.

6.4.6 Examine agar plate surface for appearance of growing colonies. Perform colonies count.

Note: appearance and size of the microbial colonies will vary, depending on the type growing organisms and conditions. In case of high numbers / large size of colonies on plates of positive controls and spiked nanomaterials both can be subjected to colony counting already after one or two days of incubation to facilitate accurate counting. Final inspection and counting of test samples will be performed after indicated incubation time of 3 days.

6.4.7 Analyse and report results according to the following formula:

Colony counts on agar plate x Dilution Factor x Sampling Factor= CFU/mL

Note: To estimate sampling factor consider what proportion of the test sample is represented by the volume of the test aliquot spread on the plate (e.g. 100 µL). As the final result refers to 1mL volume of non-diluted stock, the sampling factor in case of 100 µL test aliquot is 10. The dilution factor for a non-diluted stock is 1, if the original sample concentration is diluted 1:10, 1:100, 1:1 000 for testing, the dilution factor is 10, 100, 1 000, respectively. Alternatively, the number of CFU can also be referred to the amount (weight) of either active pharmaceutical ingredient or to total construct contained in 1 mL of non-diluted stock of test nanomaterial.

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.
6. If any positive, potentially inconsistent, result is observed then the test procedure should be repeated in an independent laboratory.

8 Health and Safety Warnings, Cautions and Waste Treatment

Inform yourself about the content and sample material and all relevant safety issues concerning the samples before unpacking and handling of any received sample.

Always wear adequate personal protective equipment, in particular protective laboratory coat, gloves and safety glasses and take all necessary precautions to protect yourself and others. Gloves must be inspected prior to use. Use proper glove removal technique (without touching glove's outer surface) to avoid skin contact with the products. Use respiratory protection whenever advisable. Open the sample vials only under sterile conditions of the sterile work bench in order to avoid

sample spilling and contamination. Take all necessary precautions that further sample spilling is avoided in case of damaged sample container. Waste disposal has to be proceeded in a proper form using means adequate for the material specifications, in compliance with the laboratory regulations and general regulatory conditions according to applicable legal regulations.

Avoid any spilling of samples, decontaminate all surfaces and instruments after use.

All used disposable material in contact with samples and controls should be autoclaved after use to decontaminate, then discard properly as described

9 Abbreviations

CFU: colony forming units

HCl: hydrochloric acid

LB: Lysogeny Broth

NaOH: sodium hydroxide

PBS: phosphate buffered saline

MIC: minimal inhibitory concentration

10 Reference