

Project:



SOP EUNCL-STE-003

Detection of Mycoplasma Contamination

Subtitle

AUTHORED BY:	DATE:
Rainer Ossig	03-05-2016

REVIEWED BY:	DATE:
Matthias Roesslein	14-05-2016

APPROVED BY:	DATE:
Matthias Roesslein	14-05-2016

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1 Introduction

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

2 Principle of the Method

Mycoplasma species are the smallest known types of bacteria. These self-replicating prokaryotes have a unique deformable cell membrane but lack a solid cell wall. Thus they are not affected by many common antibiotic agents. They often infect humans, animals or plants as parasites, can live intracellular as well as extracellular (often attached to the cell membrane). They are also able to grow in nutrition supplemented cell-free media, but they are sensitive to dehydration. Nanoparticles submitted to the EUNCL may be subjected to testing for mycoplasma contamination when deemed necessary. The types of nanoparticle formulations generally tested for mycoplasma contamination include those that incorporate a component derived from a bacterial culture, animal or hybridoma.

3 Applicability and Limitations (Scope)

The intended purpose of this assay is to avoid introduction of mycoplasma contamination into *in vitro* cell cultures and *in vivo* animal studies utilizing the test-nanomaterial, as such contamination will confound the results of these tests. This method is not intended to certify the nanoformulation as free of mycoplasma. It is used to ensure that no living mycoplasma is introduced into cell culture and transmitted to *in vitro* or *in vivo* (in case of xenograft studies) assays.

4 Related Documents

Table 1:

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5 Equipment and Reagents

5.1 Equipment

- Pipettes covering range from 0.01 to 10 mL
- Sterile pipet tips, 0.01-10 mL
- Sterile micro tubes, 1.5 mL, 2 mL, 5 mL
- Sterile polypropylene tubes, 50 and 15 mL
- T25 culture flasks or cell culture dishes

- Cell culture CO₂-Incubator at 37°C, 95% humidity
- Centrifuge
- Vortex
- Sterile work bench (laminar flow)
- Autoclave

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5.2 Reagents

- Test nanomaterial
- Sterile Phosphate buffered saline (PBS) (Sigma, D8537)
- Buffer used to reconstitute test nanomaterial if not PBS
- Standard Cell line in culture ((e. g. A549, NCI H460 or equivalent cell line)
- Fetal bovine serum (FBS)
- Standard cell culture medium with FBS and supplements (e.g. DMEM for A549)
- L-Glutamine
- Sodium Hydroxide (NaOH) (Sigma, S2770)
- Hydrochloric acid (HCl) (Sigma, H9892)

5.3 Reagent Preparation

Sodium Hydroxide (sterile)

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

Hydrochloric Acid (sterile)

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

6 Procedure

- The assay requires at least 2.2 mL of test nanomaterial final formulation for each approach, additional material should be calculated to measure and adjust pH.
- A negative and a positive control is always applied

The concentration of nanoparticles in this formulation is case-specific. 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 stock solution at a concentration of 1mg/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 or diluted in sterile PBS or water or in appropriate vehicle. To prepare the stock dispersion in a final concentration of 1 mg/mL (Note: the concentration may vary depending on the specific nanoformulation) use sterile centrifuge tubes and perform under sterile conditions. Vortex the prepared diluted samples thoroughly for at least 5 min and incubate at room temperature for 30 min to allow potentially surface-adsorbed mycoplasma to be detached from nanomaterials.

If necessary the pH of the study sample should be 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. In order to

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avoid contamination of the test-sample during pH determination, always remove a small aliquot of the sample for use in measuring the pH.

- Use sterile PBS or respective dilution buffer as a negative control.
- Test each sample in duplicate.

Preparation and cultivation of cells for mycoplasma testing:

1. For amplification of potential mycoplasma in the test nanomaterial use a quickly proliferating cell line (e.g. A549), and cultivate under standard conditions as appropriate for these cells in complete cell culture medium without application of an antibiotic additives.

2. Grow the cells in a T25 cell culture flask (25cm² growth area) or 10 cm cell culture dish until they are approximately 80% confluent.

3. Add 1 mL of test nanoparticle stock preparation to 10 mL cell culture media (to a final concentration of 0.1 mg/mL. Note: the final concentration may vary depending on the specific nanoformulation) and apply to the growing cells.

- As negative control, add an identical volume of sterile PBS to a parallel cell cultured flask .
- Test each sample in duplicate.

4. Incubate cells for 24 hours, then wash and replace growth medium with fresh complete standard medium appropriate for the used cell line, without antibiotic supplement.

5. Split cells using the standard procedure and dilute as needed before re-plating to a fresh T25 culture flask or cell culture dish.

- Passage the nanomaterial exposed cell line at least 25-times using fresh culture media without antibiotic supplement for all passages.

6. After 3 days of cell culture of the last passage, collect 2x 1 mL cell culture supernatant of each nanomaterial exposed and control cell culture.

7. Firmly close the vial and heat the supernatant at 100 °C for 5 minutes using a heating block and let cool down.

8. Centrifuge supernatant at 250 x g for 5 min to remove debris, and collect appr. 0.5 mL of the upper supernatant and transfer to a fresh sterile micro tube.

9. The prepared cell culture supernatant can be used immediately for mycoplasma detection using a nested PCR approach.

- The PCR based detection method includes a positive control for mycoplasma as well an additional negative controls for cell culture medium and vehicle (e.g. PBS)
- If not tested immediately, cell culture supernatant samples can be stored frozen at -20°C until usage.

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6.1 Flow chart

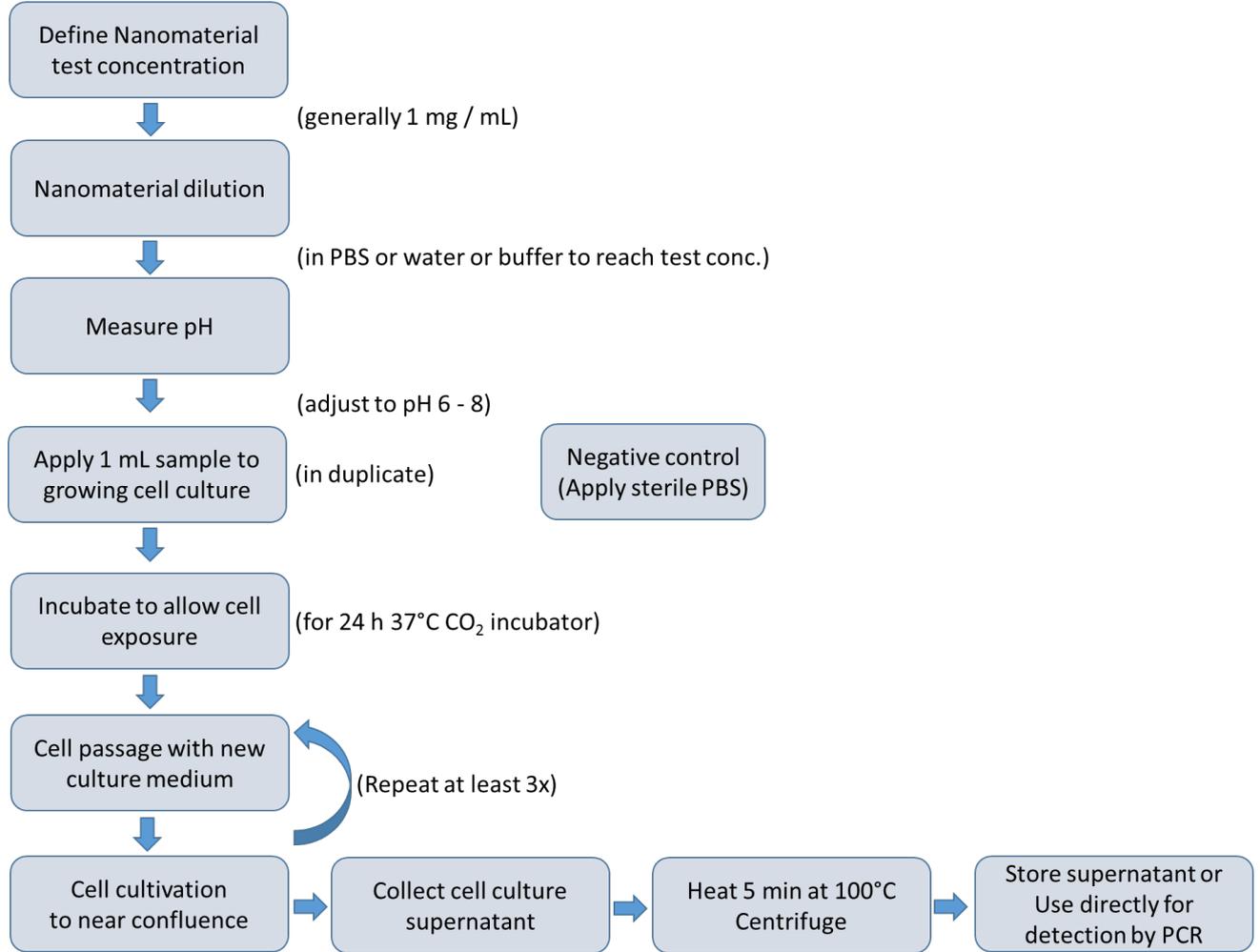


Figure 1: Brief outline of the workflow.

7 Quality Control, Quality Assurance, Acceptance Criteria

- The assay is acceptable if both the positive and negative controls are acceptable
- The negative control is acceptable, if no specific amplificate is observed upon completion of the test.
- A positive control is acceptable, if a specific amplificate is clearly identified with 300 bp, (note: an additional approx. 1kbp product may also occur with positive results)

Sample Acceptance Criteria:

- Test nanomaterial is acceptable with demonstrated negative results in both,
 - negative control and
 - test sample and
 - if a specific 300 bp amplificate is clearly identified with the positive control.

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8 Health and Safety Warnings, Cautions and Waste Treatment

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

bp: base pair

HCl: hydrochloric acid

NaOH: sodium hydroxide

PBS: phosphate buffered saline

10 References

11 Annex

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