METHODS OF BIOLOGY
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CHAPTER IX

Gaussia Luciferase-based mycoplasma detection assay in mammalian cell culture

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Summary

Mycoplasma contamination in mammalian cell culture is a common problem with serious consequences on experimental data, and yet many laboratories fail to perform regular testing. In this chapter, we describe a simple and sensitive mycoplasma detection assay based on the bioluminescent properties of the *Gaussia* luciferase reporter.

Key words: Mycoplasma contamination, Mycoplasma detection assay, Gluc mycosensor, cell culture contamination, antibiotics, bioluminescence reaction

1. Introduction

Mycoplasmas are common contaminants of mammalian cell culture; due to their small size and the absence of a rigid cell wall, they have the ability to pass through most bacterial filters. In contrast to other contaminants such as fungi and bacteria, mycoplasma contamination is impossible to detect by eye, since the contamination does not influence the color of the cell culture medium, the pH, the turbidity, nor the odor. Mycoplasmas are also not visible under the regular light microscopes in cell culture laboratories. Furthermore, the fact that mycoplasma can grow both intracellular and extracellular greatly contributes to their resistance to several antibiotics in the extracellular cell culture medium, such as penicillin and streptomycin.[1] Due to these issues, mycoplasma contamination rates in mammalian cell culture laboratories have been found to be as high as 70%.[1-3]

Mycoplasma contamination can have a tremendous effect on host cells. For instance, mycoplasma can interfere with numerous cellular processes including cell metabolism, proliferation, gene expression, and function.[2,3,4,5,6,7] Therefore, all cell culture laboratories should test cell cultures for mycoplasma contamination on a regular basis. The ideal detection method for mycoplasma contamination should be simple to perform, sensitive, specific, rapid, inexpensive, and suitable for testing of numerous cell cultures simultaneously.

In this chapter, we describe a simple mycoplasma detection assay based on degradation of the *Gaussia* luciferase (Gluc) reporter protein. Typically, in conditioned medium of cells under regular culture conditions, Gluc has a half-life of >7 days.[8] The half-life of Gluc is tremendously decreased in the presence of mycoplasma contamination, and therefore the level of decline in Gluc activity correlates to mycoplasma infection rate.[9] This mycoplasma-specific decrease has been confirmed by several assays including Western blot analysis, which showed Gluc protein degradation over short period of time (2 - 24 hours) only in the presence of mycoplasma contamination. We reasoned that this phenomenon could be used as a sensitive and specific biosensor to monitor the mycoplasma contamination in mammalian cells (Mycosensor).[9]

Around 90-95% of all mycoplasma contamination in mammalian cell cultures is caused by either *M. orale*, *M. hyorhinis, M. arginini*, *M. fermentans*, *M. hominis* or *A. laidlawii*.[1] The Gluc mycosensor has been confirmed on three of these commonly isolated mycoplasma strains including *Mycoplasma fermentens, Mycoplasma hominis*, and *Mycoplasma orale*. Importantly, this Mycosensor showed to be more sensitive in detecting mycoplasma contamination as compared to a commercially available bioluminescent-based assay and is amenable to high-throughput applications.[9] Alternative Mycoplasma detection methods are available including mycoplasma DNA amplification by polymerase chain reaction, which is sensitive but prone to errors; by traditional bacterial cell culture, which is very time consuming;[10] or by commercially-available kits which have low sensitivity and are costly. The Gluc Mycosensor however, is easy to use, sensitive, and especially suited for testing of numerous cell quantities simultaneously. Nevertheless, we recommend in all cases to combine two different detection methods to achieve and maintain total mycoplasma clearance in the cell culture hoods.

2. Materials

2.1. Gluc Recombinant Protein

1. Gluc recombinant protein (Nanolight)

Or, for the preparation of Gluc recombinant protein:

2. cDNA encoding Gluc (Nanolight), and N-terminal pelB periplasmic signal sequence, and a C-terminal 6-His tag in pET26b (+) vector (Novagen).
3. HMS174 competent bacterial cells and LB containing 30 **µ**g/mL kanamycin.
4. IPTG Bugbuster Master Mix containing Benzonase (Novagen).
5. NJ45 **µ**m syringe filter nickel charged resin column (Novagen).
6. Binding buffer: 0.5 M NaCl, 20mM Tris-HCl, 5 mM imidazole, pH 7.9
7. Wash buffer: 0.5 M NaCl, 20 mM Tris-HCl, 60 mM imidazole, pH 7.9
8. Elution buffer: 0.5 M NaCl, 20mM Tris-HCl, 1 M imidazole, pH 7.9
9. Coelenterazine and a luminometer
10. 3500 MW cut-off dialysis tubing (Fisher Scientific).
11. Bradford assay.
12. SDS-PAGE, NuPAGE„¥ 10% Bis-Tris gel and coomassie blue staining (Invitrogen).

2.2. Gluc-containing medium

1. 293T human fibroblast cells.
2. Lentivirus vector expressing Gluc and the enhanced green fluorescent protein (GFP) under the control of CMV promoter
3. Polybrene.

2.3. Cell culture

1. High glucose Dulbecco's modified Eagle's medium. Supplemented with 10% fetal bovine serum, and 100 U/mL penicillin and 100 µg/mL streptomycin (**see note 1 and 2**).
2. Incubator with a humidified atmosphere supplemented with 5% CO2 at 37 °C.
3. 24-well cell culture plates.

2.4. Bioluminescence reaction

1. A luminometer to measure the bioluminescence activity of Gluc (**see note 3**).
2. 20 **µ**M coelenterazine (Nanolight). Diluted in phosphate buffer saline (PBS; **see note 4)**.
3. Black 96 well microtiter plates (**see note 5**).

3. Methods

Be aware that all materials used for cell culture and the Mycosensor assay should be stored and used under sterile conditions to avoid contamination during the Gluc incubation period. An overview of the complete Gluc Mycosensor assay protocol is presented in Figure 1.

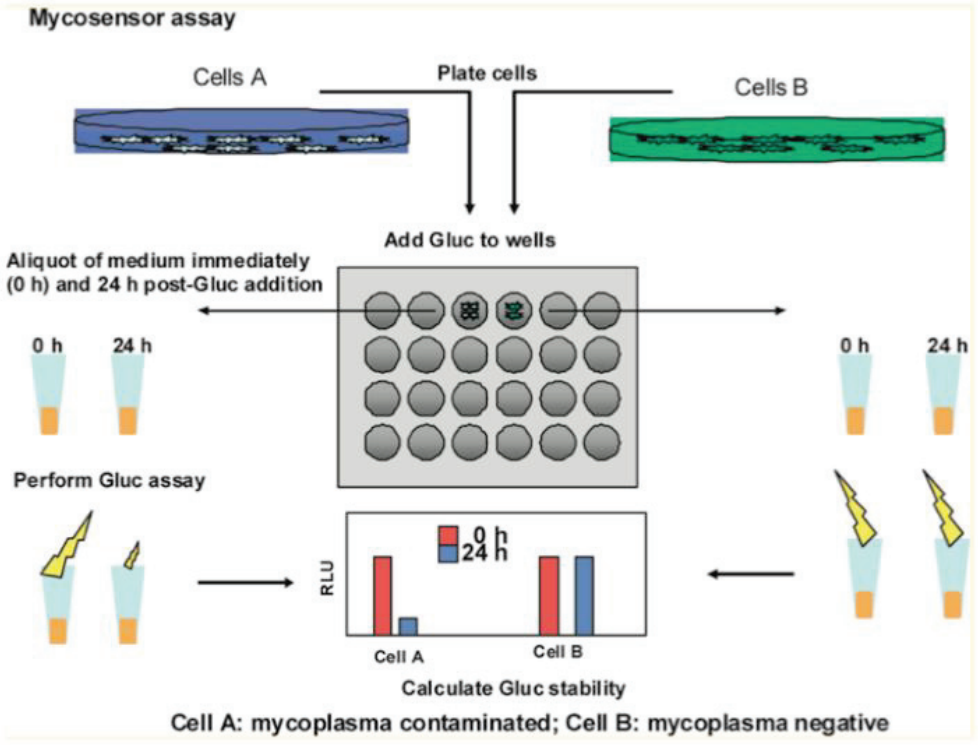

Figure 1. Setup of mycoplasma assay.

3.1. Gluc recombinant protein purification

In brief, the cDNA encoding Gluc is amplified by PCR using specific primers and cloned in-frame between an N-terminal pelB periplasmic signal sequence and a C-terminal 6-His tag in pET26b(+) vector using BamHI and XhoI sites. In this vector, gene expression is under the control of an isopropyl **β-**D-1-thiogalactopyranoside (IPTG)-inducible T7 RNA polymerase promoter. The vector is transformed into HMS174 competent bacterial cells and grown overnight in 200 mL LB containing 30 **μ**g/mL kanamycin. Once the culture reached an

OD_{600} of 0.6, protein expression is induced by adding 20 **µ**M IPTG to the culture which is grown for 18 h at room temperature. Cells should be pelleted by centrifugation for 15 min at 10,000x g and resuspended in 10 mL Bugbuster Master Mix containing Benzonase. Insoluble debris is pelleted by another spin. The clarified lysate is then filtered through a 45 **µ**m syringe filter and loaded onto a nickel charged resin column equilibrated with binding buffer. The column should be rinsed with 20x volumes of binding buffer followed by 18x volumes of wash buffer. His-tagged Gluc is eluted from the column with 1.2 mL of elution buffer collecting 200 µL fractions. The fraction containing the highest Gluc activity (as measured using coelenterazine and a luminometer should be pooled and dialyzed against 30 mM Tris-HCl, pH 8.0 overnight using 3500 MW cut-off dialysis tubing. Glycerol is added to a final concentration of 10% and protein concentration is determined using the Bradford assay. Purity of protein can be analyzed by SDS-PAGE under reducing conditions using a NuPAGE„¥ 10% Bis-Tris gel and coomassie blue staining.

3.2. Gluc-containing medium

As an alternative to recombinant Gluc, conditioned medium from mycoplasma-free cells stably expressing Gluc can be aliquoted and stored at -80 ^{0}C and can be used for the Mycosensor assay. In this chapter, we will take the widely used 293T human fibroblast cells as an example for the Mycosensor assay. 239T cells can be engineered to stably express the naturally secreted Gluc (**see note 1**) by infecting these cells with a lentivirus vector expressing Gluc and the enhanced green fluorescent protein (GFP) under the control of CMV promoter at a multiplicity of infection of 10 transducing units/cell in the presence of 10 **µ**g/mL Polybrene. [8] The next day, cells are washed and maintained in regular growth medium. GFP can be used as a marker to monitor transduction efficiency and therefore Gluc expression.

3.3. Mycosensor assay

1. Culture the test cells for at least 72 hours under normal culture conditions as described above (**see note 2**).
2. Plate cells to be tested in triplicates in a 24 well plate at around 30% confluency; an ideal amount of 293T cells would be 1x10^5 cells/well, however, as low as

$5x10^4$ cells can be used. Cells should be plated in 500 µL of growth medium (**see note 6**). In order to save time, it is also possible to simply assay the cell-free conditioned medium of the test cells by transferring a small aliquot to the 24 well plate instead of re-plating the cells, In this case, the Gluc-containing medium or purified Gluc can be added immediately and step 3 can be skipped. One should take into consideration that this technique is much less sensitive than the standard protocol (**see note 7**).

3. The next day, add purified Gluc to a final concentration of 35 ng/mL to conditioned medium. Alternatively, if one chooses to use Gluc-containing medium, remove 250 µL of medium from cells to be tested and add 250 µL of Gluc-containing media (**see note 8**).
4. Mix by stirring the plate and immediately take a 50 µL aliquot from each well; this will be used as the time point zero. Samples can be frozen at -80 ^{0}C until further analysis (**see note 9**).
5. Return the plate to the cell culture incubator.
6. At 24 hours post-Gluc addition, take another 50 µL aliquot from each well and freeze at -80 ^{0}C. If fast analysis is required, and if mycoplasma contamination is high, it is possible to detect a decrease in Gluc activity (and therefore mycoplasma contamination) after 4 hours. However, if mycoplasma contamination is low, 4 hours will not yield a significant degradation of Gluc and longer incubation time is needed. One may choose to perform time analysis by storing an aliquot of conditioned medium at different time points.
7. Do not discard the cells at the 24-hour time point since very low levels of mycoplasma can be detected after 48h or 72h, even if test results are negative at earlier time points (**see note 9**).
8. Assay these aliquots for Gluc activity using coelenterazine and a luminometer (see below).

3.4. The Mycosensor bioluminescence assay

9. Thaw aliquots from the different time points to room temperature (**see note 10**).
10. While waiting, dilute coelenterazine in PBS to a final concentration of 20 **µ**M and incubate for 20-30 minutes in the dark (**see note 11**).
11. Transfer 20 µL of each aliquot into black 96 well microtiter plate.

12. Using a luminometer with a built-in injector, inject 80 µL of coelenterazine and acquire the signal immediately for 10 seconds (**see note 12**).

3.5. The Mycosensor assay results

Mycoplasma contamination is determined by comparing the Gluc signal at time point B (e.g., 24h) to the signal at time zero. A significant decrease in Gluc activity indicates mycoplasma contamination (**see note 13**). Typical results from a positive and negative mycoplasma assay are presented in Figure 2.

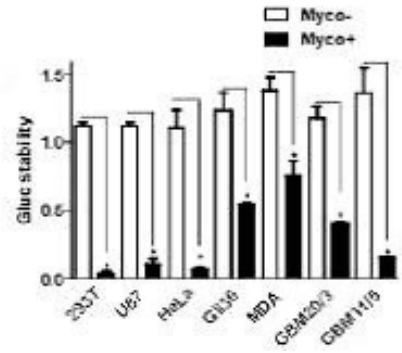

Figure 2. Gaussia luciferase as a measure of mycoplasma contamination in mammalian cell culture. Conditioned medium from cells expressing Gluc were added on different mycoplasma clean or contaminated cells (1*10^5 cells plated on 24-well plate) an the Gluc activity was detected immediately (0h) and 24 h later using a luminometer after addition of coelenterazine.

4. Notes

1. Be alert that the cells used to express Gluc, and from which the conditioned medium is used for the Mycosensor assay, are not contaminated with mycoplasma. We recommend to test these cells on a regular basis with two different mycoplasma detection assays such as: MycoAlert® (Lonza Rockland, Rockland, ME) and PCR PromoKine Mycoplasma Test KIT I/C (PromoCell, Heidelberg, Germany) according to the manufacturer's standard protocol.
2. Mycoplasma needs at least 48 – 72 hours to recover from frozen stocks and therefore cells should be cultured for at least 3 days after thawing from liquid nitrogen before testing for contamination.
3. Any plate reader with a built-in injector that can adequately measure bioluminescence can be used.
4. Other concentrations of CZN can be used, up to 100 **µ**M**;** however, the 20 **µ**M concentration of CZN in combination with the luminometer used in our laboratory,

and the black microtiter plate will lead to the most optimum Gluc value under our assay conditions.

5. This note is also to some extent applicable to notes 3 and 4; be aware that the Gluc signal can reach saturation depending on the amount of Gluc used and the sensitivity of the luminometer. In the case that Gluc-containing conditioned medium is used for the Mycosensor assay, this problem is more likely to occur. The use of black plates could solve this problem as they typically yield up to one log lower signal as compared to white plates. As an alternative, one can dilute the medium before the assay.
6. Keep in mind that plating too many cells will result in overconfluency at 72 hours resulting in cell death and therefore the Mycosensor will not be accurate.
7. As an alternative to testing mycoplasma contamination on plated cells, recombinant Gluc can be added directly to an aliquot of conditioned medium from cells to be tested; however, this strategy is less sensitive as compared to testing the cells themselves. One may choose to do the assay on conditioned medium initially and if the results are negative, it can then be confirmed on plated cells.
8. Be careful when adding Gluc not to disrupt the cells, since some cell types are easily detached from the plate leading to false results.
9. It is of uttermost importance to include a time point zero when performing the Mycosensor at a range of time points. It is recommended to take 2 different aliquots of conditioned medium at time point zero in case one chooses to run the 24 hour time point first. If test results are negative, the 48 and 72 hours time point can then be assayed and compared to the second aliquot of time point zero.
10. We observed that aliquots from the same sample but treated in a different manner (e.g. frozen versus not-frozen) can show some variation in Gluc signal intensity. It is important to treat all aliquots, including the ones from different time points, in the same manner.
11. Coelenterazine purchased should be first reconstituted in acidified methanol (1 drop of HCL to 10 mL of Methanol) to a final concentration of 5 mg/mL. Just before use, it is diluted in PBS to a final concentration of 20 **µ**M. Coelenterazine is prone to auto-oxidation and a decrease in bioluminescence signal over time can simply be observed due to this artifact. Incubating diluted coelenterazine for 20 min in the dark stabilizes the signal and will result in a stable bioluminescent output.

12. A luminometer with built-in injector is required to perform the Mycosensor assay, since Gluc catalyzes a flash-type bioluminescence reaction. Therefore, the reaction starts with a high signal, followed by a rapid decrease over the course of few seconds.[11] If a luminometer with built-in injection is not available, one can dilute coelenterazine in PBS containing 0.1% Triton X-100. We showed that this detergent yields some stability to the Gluc signal, especially when used in combination with GlucM43I mutant.[12] Alternatively, stabilization kits are available from Targeting Systems or Nanolight (GAR-reagents). The substrate should be diluted in the appropriate reagent and can be added manually to the medium and assayed using a luminometer.
13. We recommend testing cells for mycoplasma contamination on a regular basis using the Gluc Mycosensor. Confirm negative results with another type of assay, such as the PCR-based mycoplasma assay.

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