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# CHAPTER VIII

## A simple and sensitive assay for mycoplasma detection in mammalian cell culture

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## ABSTRACT

Mycoplasma contamination in mammalian cell cultures is often overlooked yet is a serious issue, which can induce a myriad of cellular changes leading to false interpretation of experimental results. Here we present a simple and sensitive assay to monitor mycoplasma contamination (mycosensor) based on degradation of the *Gaussia* luciferase reporter in the conditioned medium of cells. This assay proved to be more sensitive as compared to a commercially available bioluminescent assay in detecting mycoplasma contamination in seven different cell lines. The *Gaussia* luciferase mycosensor assay provides an easy tool to monitor mammalian cells contaminants in a high-throughput fashion.

## INTRODUCTION

Mycoplasmas, in the class mollicutes, are the smallest free-living organisms known. Small size combined with the absence of a rigid cell wall allows them to pass through most bacterial filters making them common pathogens in mammalian cell culture. Many contaminations go unnoticed, as there are often no overt signs of mycoplasma presence. This is in contrast to fungal or bacterial contamination where changes such as media pH, turbidity, odor, and direct microscopic visualization of organisms are observed. Mycoplasma contamination rates have been reported to be between 15-35% and could be as high as 70%[1-3] and are resistant to commonly used antibiotics such as Penicillin and Streptomycin. Several antibiotics which are known to kill mycoplasma are mostly effective in the extracellular media with intracellular mycoplasmas being somewhat sequestered from drugs[1]. The effect of mycoplasma contamination depends on the particular species of mycoplasma as well as the contaminated cell type. Whereas some species may have no apparent effect on cell function, many others can produce severe cytopathic changes[3]. Due to a competition with their host cells for medium nutrients, mycoplasma can lead to significant changes in cell metabolism, proliferation, gene expression and function, leading to false data interpretation[2,4-7].

The ideal detection method for mycoplasma contamination would be simple to perform, sensitive, specific, rapid and inexpensive and can be used to test numerous cell cultures simultaneously and on a regular basis. Unfortunately, current mycoplasma assays have some but not all of these characteristics. For instance, classical microbiology culture assays are lengthy and can be unreliable due to the fastidious culture requirements for some species[8]. Polymerase chain reaction (PCR)-based detection of mycoplasma-specific nucleic acids is known to be the most reliable assay, however it is complex, time consuming and suffers from false positive and negative results if performed inadequately[9]. Other assays include fluorescent DNA staining, enzyme-linked immunosorbent assays and immunoblotting, with each having significant drawbacks[10-12].

Here, we describe a simple assay to monitor mycoplasma contamination in mammalian cell cultures based on degradation of the *Gaussia* luciferase (Gluc) reporter in the conditioned medium. This assay proved to be more sensitive in detecting mycoplasma as compared to a commercially available bioluminescent-based assay and is amenable to high-throughput applications.

## EXPERIMENTAL SECTION

**Cell culture.** 293T human kidney fibroblast cells, HeLa human cervix adenocarcinoma cells and U87 human glioma cells were obtained from American Type Culture Collection (Manassas, VA). Gli36 human glioma cells were a kind gift from Dr. Anthony Capanogni, UCLA, CA. GBM11/5 and GBM20/3 glioblastoma cells were kindly provided from Dr. Xandra Breakefield, Massachusetts General Hospital, Boston, MA. MDA-MB-231 were obtained from ATCC. All cells were cultured in high glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Sigma, St Louis, MO) and 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen) in a humidified atmosphere supplemented with 5% $CO_2$ at 37°C. 293T cells were engineered to stably express the naturally secreted Gluc by infecting these cells with a lentivirus vector expressing Gluc and GFP under the control of CMV promoter at a multiplicity of infection of 10

transducing units/cell in the presence of 10 µg/ml Polybrene® (Sigma) as we previously described[13].

**Standard mycoplasma contamination assays.** The status of mycoplasma contamination of cultured cells was determined by MycoAlert® (Lonza Rockland, Rockland, ME) and where indicated by PCR PromoKine Mycoplasma Test KIT I/C (PromoCell, Heidelberg, Germany) according to the manufacturer's standard protocol. Mycoplasma contamination of cells was established by transferring media from cells which were tested positive by both MycoAlert® and PCR PromoKine to clean cells. Cells were intentionally contaminated 3 days prior to use in most experiments unless otherwise stated. Mycoplasma contamination status was monitored on a weekly basis for all cells using Lonza MycoAlert®. Cells treated with Plasmocin™ (InvivoGen, San Diego, CA) were cultured for 2 weeks in the presence of this drug according to the manufacturer's instructions, followed by 1 week without the drug and confirmed to be mycoplasma negative using MycoAlert®.

**Recombinant Gluc protein.** Recombinant Gluc protein was purified from bacterial extracts using a 6-histidine tag as we previously described[14]. Enzyme purity was determined using Commassie staining of purified Gluc after being resolved on SDS-PAGE Gel.

**Gluc mycoplasma sensor assay.** $1x10^5$ mycoplasma clean or contaminated cells were plated in a 24 well plate. 16-24 h later, conditioned medium from cells expressing Gluc (previously aliquoted and frozen) or purified Gluc (35 ng/ml) was added to the media of these cells. Immediately following mixing Gluc with the media on these cells (time 0h) and 24 h later, 50 µl aliquots were collected and frozen at -80 $^0$C. Twenty µl of these samples (in duplicates) were than transferred into a black 96 well microtiter plate (Greiner bio-one, Frickenhausen, Germany) and assayed for Gluc activity after the addition of 80 µl 20 µM coelenterazine (Nanolight, Pinetop, AZ) and acquiring photon counts for 10 sec using the Dynex MLX microtiter plate luminometer (McKinley Scientific, Sparta, NJ). Gluc stability is calculated as the RLU at time point B (e.g. 24 h)/ RLU at time point A (e.g. zero h). For the cell-free assay (Fig. 2a), conditioned media was removed from cells and cell debris removed by a 5-minute centrifugation at 300 x g. Cell-free media was then mixed in a 1:1 ratio with

Gluc-containing media and aliquots of media were collected at several time points before performing the Gluc bioluminescence assay.

**Western blot analysis.** Twelve microliter of conditioned medium from mycoplasma positive and negative cells containing Gluc were electrophoresed on 4-12 % gradient SDS-polyacrylamide gels (NuPAGE Bis-Tris, Invitrogen) and transferred to nitrocellulose membranes (Bio-Rad). Blots were stained with Ponceau to verify equal protein loading and blocked overnight at 4 $^{0}$C using 10% non-fat dry milk in TBS-T. The membrane was then incubated with polyclonal rabbit anti-Gluc primary antibody (Nanolight technology, Pinetop, AZ) diluted 1:2000 in 2% milk/TBS-T for 1h at room temperature followed by washing using TBS-T. The blot was incubated with horseradish peroxidase (HRP) conjugated secondary anti-rabbit antibody (1:10,000; GE Healthcare; Little Chalfontbuckinghamshire, UK). Blots were developed using SuperSignal West Pico Chemiluminescent Substrate™ (Pierce, Rockford, IL) followed by exposure to HyBlot CL autoradiography film (Denville Scientific, South Plainfield NJ).

**Purified mycoplasma experiments.** The following mycoplasma species were purchased from Leibniz Institute DSMZ-German Collection of Microorganims and Cell Cultures (Braunschweig, Germany): *Acholesplasma laidlawii, Mycoplasma arginini, Mycoplasma fermentens, Mycoplasma hominis, Mycoplasma hyorhinis, and Mycoplasma orale*. Lyophilized mycoplasma pellets were sterilely rehydrated for 30 minutes in 500 µl of cell culture media. Next, 50 µl was mixed with 3 ml of complete cell culture media and transferred to a 10 cm$^2$ tissue culture well containing 3x10$^5$ Gli36 cells. Successful mycoplasma growth was determined by Lonza MycoAlert® at 10 days post-infection. Cells were then plated into 24 well plates and mixed with Gluc-containing media and Gluc stability was calculated from 0 h and 72 h time points.

**DNA sequence analysis of mycoplasma in contaminated media.** Conditioned cell culture media (1.5 ml) from a contaminated HeLa culture was centrifuged at 500x g to remove cellular debris and then 15,000x g for 15 minutes to pellet mycoplasma. The pellet was resuspended in 100 µl of nuclease-free water and

mycoplasma DNA was amplified using PromoKine PCR Mycoplasma test kit I/C. The PCR reaction was desalted using Qiagen gel extraction columns (Qiagen, Valencia, CA) and specific amplification of the mycoplasma-specific PCR product was confirmed by agarose gel electrophoresis and ethidium bromide staining. The PCR product (~270 b.p.) was ligated into a subcloning plasmid (PCR®4-TOPO®) using the TOPO TA Cloning kit for Sequencing (Invitrogen) and transformed into electrocompetent DH10B bacteria (Invitrogen). Single bacteria colonies were isolated, cultured and plasmid DNA was purified using a QIAprep Miniprep kit (Qiagen). DNA sequencing was performed at the Massachusetts General Hospital DNA core facility using M13 forward and M13 reverse primers provided in the TOPO TA Cloning kit. The nucleotide sequence of each clone was compared to the DNA database on the National Center for Biotechnology Information website using basic local alignment search tool (BLAST®).

## RESULTS AND DISCUSSION

We incidentally observed that the bioluminescent signal in conditioned media of different cell types expressing the naturally secreted *Gaussia* luciferase (Gluc)[15] varied in stability over time (originally reported to be ~6 days in media[13]). Further analysis using commercially available tests revealed that cells with a decreased Gluc-catalyzed bioluminescent signal were contaminated with mycoplasma, while those with a more stable signal were mycoplasma-free. To further explore this phenomenon, we determined the stability of Gluc activity added to conditioned medium of seven different cell types. Initially, we confirmed that these cell lines were mycoplasma-free using both PCR and bioluminescence-based commercially available assays (see Experimental Section). Next, each cell line was divided into two groups in which one was maintained in mycoplasma-free environment and the other intentionally infected with mycoplasma-contaminated tissue culture media. Gluc-containing conditioned medium (from mycoplasma negative cell cultures) were added to these cells and the Gluc activity was measured immediately and twenty-four hours later. A decrease in Gluc signal (2-30 fold; $p<0.0005$) was observed only in mycoplasma-contaminated cells as compared to mycoplasma-free cells (Fig. 1a).

To investigate the means by which Gluc enzymatic activity declines over time in mycoplasma-contaminated cells, we analyzed Gluc protein levels immediately and 24 h post-transfer of Gluc-containing medium on three mycoplasma-free and positive cells by Western blotting. While the level of Gluc protein remained stable in conditioned medium from mycoplasma-free cells over 24 hours, Gluc protein was undetectable in mycoplasma-contaminated conditioned medium from the same cell type (Fig. 1b). Equal loading of media was confirmed by Ponceau staining of transferred proteins (Supplementary Fig. 1). These data indicate that the decline in Gluc activity over time in mycoplasma-contaminated cells is likely caused by protease degradation of Gluc and not another mechanism of enzymatic inhibition.

To extend these findings and develop an assay to detect mycoplasma contamination (mycosensor), we purified 6xHistidine-tagged recombinant Gluc from bacteria and used it on 293T, U87, and HeLa cells. Recombinant Gluc (35 ng/ml) was added to cultured medium of mycoplasma-free and contaminated cells ($1x10^5$ cells plated in a 24 well plate) and the Gluc activity was analyzed immediately and twenty-four hours later in an aliquot of the conditioned medium. Again, a significant (8-100 fold; $p<0.0005$) decrease in Gluc activity was observed only in mycoplasma-contaminated cells and not in uncontaminated cells confirming that recombinant Gluc can be used as a reporter to monitor mycoplasma contamination in mammalian cells (Fig. 1c).

In order to verify that the mycoplasma strain(s) we are using are one of the six most commonly (90-95%) found in contaminated mammalian cells[1], we performed PCR using primers specific to a region of DNA within the 16S rRNA operon coding region of these strains on U87, 293T, and HeLa cell lines and analyzed them by agarose gel electrophoresis. Specific bands of approximately 250 bp were observed in all mycoplasma-contaminated cultures while no bands were observed in negative cells (Fig. 1d). To ascertain which mycoplasma strain(s) were in our cultures, the PCR products were cloned into a bacterial plasmid and 10 individual clones were sequenced and their sequences were compared to entries in the public nucleotide database. Six clones aligned with 100% homology to *Acholeplasma laidlawii,* three clones aligned with sequence data from *Mycoplasma bovigenitalium,* and one clone which had highest sequence identity aligned with *Mycoplasma faucium.* This analysis indicated that our contaminated cell culture contained a mixture of

mycoplasma species, with the highest represented clone (*A. laidlawii*) being a common cell culture contaminant.

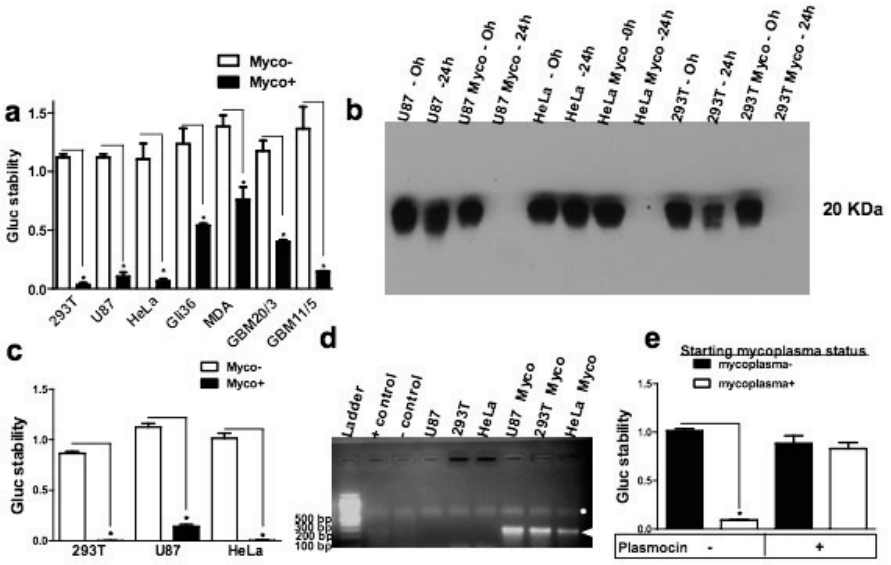


**Figure 1.** ***Gaussia*** **luciferase (Gluc) reporter as a measure of mycoplasma contamination in mammalian cell culture**. (**a-b**) conditioned medium from cells expressing Gluc were added on different mycoplasma clean or contaminated cells ($1x10^5$ cells plated in a 24-well plate) and the Gluc activity was detected immediately (0 h) and 24 h later either using a luminometer after addition of coelenterazine (a) or by Western blot analysis using anti-Gluc antibody and horse radish peroxidase-conjugated secondary antibody (b). (**c**) Purified recombinant Gluc (35 ng/ml) was added to mycoplasma positive or negative cells and Gluc activity was detected immediately and 24 h later using a luminometer. **(d)** A PCR assay, which specifically detects the most common strains of mycoplasma, was performed on all cell lines used. The smaller band (265-278 b.p., arrow head) results from the amplification of mycoplasma DNA. The larger band (479 b.p., asterisk) is an internal control indicating successful PCR amplification; it is not mycoplasma-specific. (**e**) Mycoplasma positive or negative cells were treated with Plasmocin for two weeks or left untreated. Three weeks post-treatment, a subculture of these cells was assayed using the Gluc mycoplasma assay. Activity data are presented as the average (from 3 independent experiment) of RLU ratios of 24 h over 0 h ± standard deviation (*P<0.005).

As the sequencing data revealed that our culture media was contaminated with at least three different types of mycoplasma, the decline in Gluc signal observed in the mycosensor assay could be due to one or more of these species. To ascertain that

the mycosensor assay is applicable for detection of individual and different mycoplasma species, we infected separate wells of uncontaminated Gli36 with three species of mycoplasma commonly found in cell culture contaminations (*Mycoplasma fermentens, Mycoplasma hominis, and Mycoplasma orale*). Infected cells were incubated with mycoplasma for several days. The presence of mycoplasma was initially confirmed using the MycoAlert® test. Next, cells were plated and the Gluc mycosensor assay was performed. Calculating Gluc stability from the 0 h and 72 h time points revealed that the Gluc mycosensor assay detected all three types of mycoplasma (Table I). The decline in Gluc sensitivity ranged from 14% (*M. fermentens*) to 81% (*M. orale*), with *M. hominis* in between at 28% decline.

**Table 1. Gluc Mycosensor Assay Detection of Purified Strains of Mycoplasma**

| mycoplasma strain | Gluc mycosensor assay[a] | MycoAlert assay |
|---|---|---|
| uncontaminated cells[b] | – | – |
| *Mycoplasma fermentens* | + | + |
| *Mycoplasma hominis* | + | + |
| *Mycoplasma orale* | + | + |

[a]A sample was considered to be mycoplasma positive when the Gluc signal declined by a statistically significant amount at 72 h as compared to time zero. [b]Cells used were Gli36 human glioma cells. All mycoplasma strains in Table 1 were cultured in these cells.

To further confirm that the Gluc degradation was caused by mycoplasma, we treated both contaminated and uncontaminated 293T cell cultures with a well described anti-mycoplasma agent that contains both a macrolide and a quinolone (Plasmocin™). We also maintained the original mycoplasma-free or contaminated 293T cells without drug treatment. Three weeks later, the MycoAlert® mycoplasma assay was performed which showed the successful treatment of these cells (i.e. the treated cells tested negative for mycoplasma). The Gluc mycosensor assay was then performed as above. As expected, a significant decrease in Gluc activity over 24 h was observed in conditioned medium from contaminated/non-treated cells compared to mycoplasma-free cells (Fig. 1e; $P<0.0001$). More importantly, the anti-

mycoplasma treatment of contaminated cells restored Gluc stability to levels similar to mycoplasma-free cells showing that this assay can be used to monitor response of mycoplasma to different treatments. These results also confirm that the Gluc decline in these cells is indeed linked to mycoplasma contamination and not other cell culture artifacts.

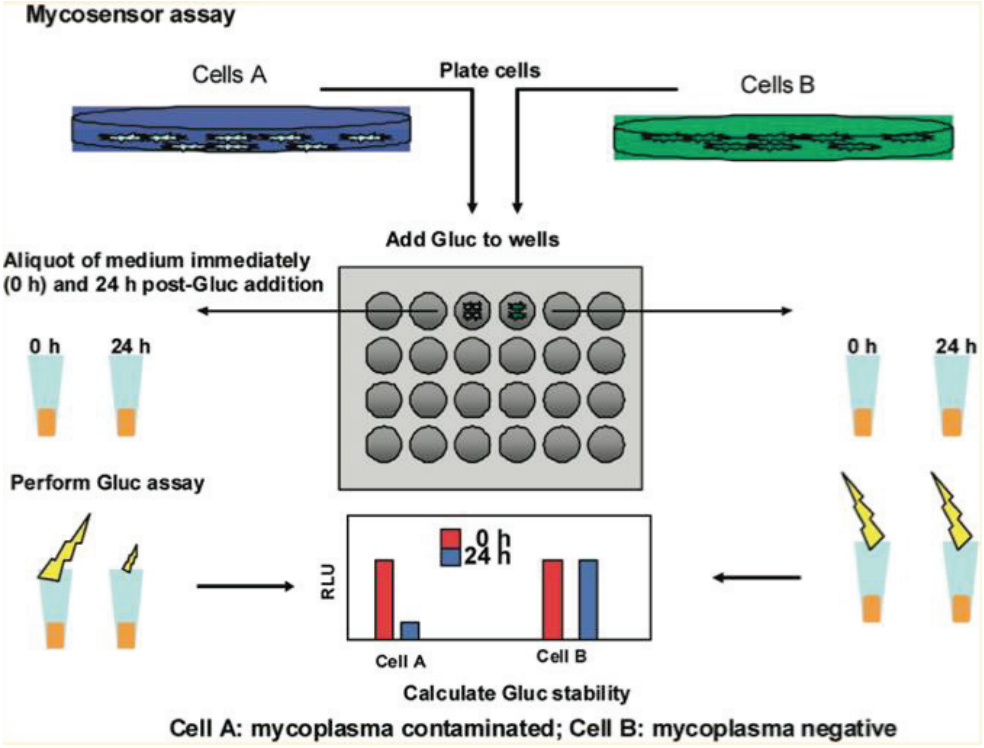


**Image 1.** Setup of the mycoplasma detection assay

As many mycoplasma are found in the extracellular conditioned media, we performed the Gluc mycoplasma assay in a simpler cell-free format. Conditioned medium was obtained from either mycoplasma-free or mycoplasma-contaminated HeLa cells and mixed with purified recombinant Gluc and activity was detected at different time points as above. As expected, no decline in Gluc activity was detected in medium from mycoplasma-free cells while a 38%, 81%, and 91% decline in Gluc activity was observed in medium from mycoplasma contaminated cells at 24 h, 48 h, and 72 h post-Gluc addition, respectively (Fig. 2a).

To evaluate the optimum time to perform the Gluc mycoplasma assay, highly-contaminated and non-contaminated 293T cells were plated in a 24-well plate and incubated with recombinant Gluc as above. Immediately and at different time points, the Gluc activity was measured on an aliquot of the conditioned medium. A linear decrease in Gluc activity was observed over time showing that highly-mycoplasma contaminated cells can be evaluated within one hour of incubation, being most sensitive at 24 hours (Fig. 2b).

To determine the linearity of Gluc mycosensor with respect to contaminated cell number, different amounts of mycoplasma-contaminated 293T cells were plated and analyzed using purified recombinant Gluc as above. Gluc stability was inversely proportional to cell number. The highest decline in activity over 24 h was observed with the highest concentration of cells ($2x10^5$ cells, 50-fold), followed by $1.0x10^5$ cells (7-fold) and $5.0 \times 10^4$ cells (1.5-fold; Fig. 2c). No decline was observed at the lowest number of plated cells ($1.0 \times 10^4$). These data indicate that at least $5.0 \times 10^4$ contaminated cells are required for the Gluc mycoplasma assay under the tested conditions.

To compare the sensitivity of our Gluc mycosensor to a commonly used commercially available bioluminescent-based assay (Lonza MycoAlert®), we performed dilutions of mycoplasma contaminated conditioned medium and added them to clean 293T cells. For a negative control, mycoplasma free 293T cells were used. One day post-contamination, equal amounts of cells were plated in a 24 well plate and recombinant Gluc was added to their conditioned medium 24 hrs later. At different time points (24, 48 and 72 hours), aliquots of conditioned medium were either assayed for Gluc activity or using the commercially available bioluminescent assay (Lonza MycoAlert®). For the Gluc-based mycoplasma assay, a significant decrease in Gluc activity was observed at all dilutions and all time points when compared to the respective negative control sample (Fig. 2d). In contrast, the commercially available assay was not able to detect any of the contaminated cells at the highest dilution (1:1600) at any of the time points (Fig. 2d, using a cut off of 2 as per manufacturer's instructions). This assay did detect mycoplasma lower dilutions of 1:200 (48h and 72h) and 1:800 (only at 72 h). These results indicate that the Gluc

mycoplasma assay is much more sensitive in detecting mycoplasma contamination and at earlier time point compared to the commonly used bioluminescent assay.

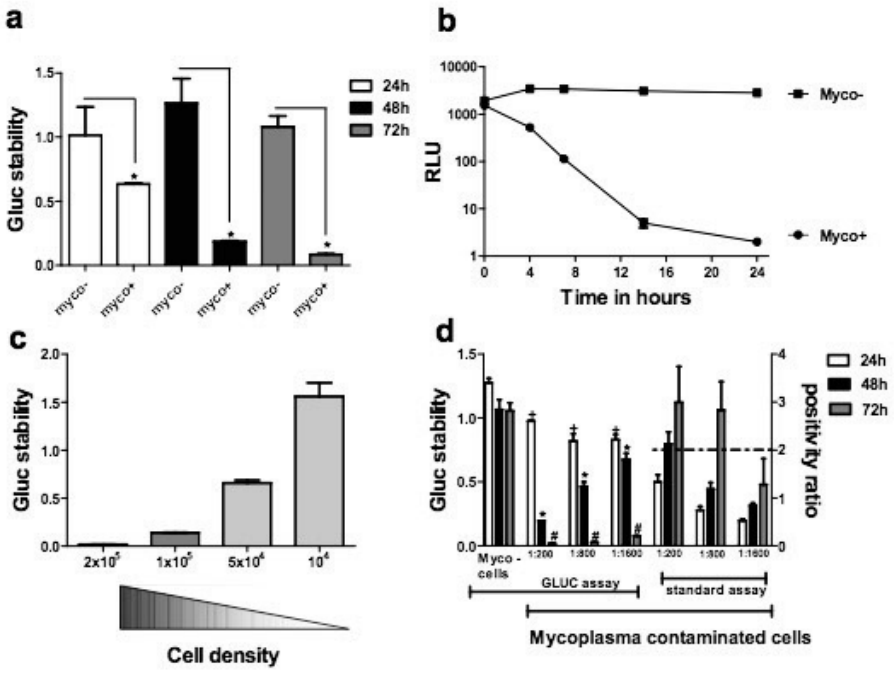


**Figure 2. Gluc mycosensor assay optimization and sensitivity comparison.** (**a**) Cell-free conditioned medium from mycoplasma free or contaminated HeLa cells were incubated with recombinant Gluc (35 ng/ml) and assayed immediatey (0 h) and 24 h, 48 h, and 72 h later for Gluc activity using a luminometer. (**b**) Mycoplasma contaminated and non-contaminated 293T cells were plated in a 24 well plate and incubated with recombinant Gluc. Immediately (time zero) and at different time points, an aliquot of the conditioned medium were assayed for Gluc activity. (**c**) Recombinant Gluc was added to different amounts of cells plated in a 24 well plate. Immediately and 24 h later, aliquots of conditioned medium were assayed for Gluc activity. (**d**) Mycoplasma contaminated conditioned medium were diluted (1:200, 1:800, 1:1600) and added to clean 293T cells, which were plated the next day in a 24 well plate. Twenty-four h later, recombinant Gluc was added to each well. Immediately and at different time points, aliquots of conditioned medium were assayed for mycoplasma contamination using both the Gluc mycosensor assay and the commercially available MycoAlert® bioluminescent-based assay. The left y-axis corresponds to the Gluc assay results and the right y-axis corresponds to the commercially available assay results.

A cutoff ratio of 2 for mycoplasma contamination using the commercially available assay was used as per manufacturer's instructions.

**In conclusion**, we have developed a facile and sensitive assay for detection of mycoplasma contamination in mammalian cell cultures using *Gaussia* luciferase. Either conditioned medium from uncontaminated cells expressing Gluc or purified recombinant Gluc (which is commercially available) can be used for mycoplasma detection, making this assay simple and cost-effective. While the Gluc mycosensor assay detected mycoplasma from three separate types of mycoplasma, it remains to be seen if it can detect all strains of mycoplasma commonly found as cell culture contaminants. As recommended by Young *et al.,* screening of mycoplasma should be performed using two independent techniques to reduce the rate of false positives or false negatives[8]. We recommend using the Gluc mycosensor assay for screening purposes followed by PCR-based detection (which directly detects mycoplasma genomic DNA) for conformational analysis. Since mycoplasma contamination degrades Gluc in the conditioned medium of cells, it could lead to false data interpretation when using Gluc as a reporter. These observations highlight the need for strict and frequent monitoring of mycoplasma contamination of all mammalian cell cultures.

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