

Prevention, Diagnosis and Eradication of Mycoplasma Contamination in Cell Culture

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ABSTRACT

Mycoplasmas, which are the smallest and simplest prokaryotes, lack a cell wall but possess the ability to undergo self-replication. Mycoplasma contamination is a common problem for laboratories engaging in cell culture. Due to their small size, Mycoplasmas can easily permeate filters designed to prevent bacterial and fungal contamination in cell culture. Although Mycoplasma contamination usually does not result in cell death, it can significantly affect cell proliferation, metabolism, and cause chromosomal aberrations. Therefore, it is crucial to detect and eliminate Mycoplasma contamination in cell culture. This step-by-step protocol presents a comprehensive approach to prevent Mycoplasma contamination in cell culture, as well as to detect and eradicate Mycoplasma to ensure accurate experimental and sequencing results.

Keywords: Mycoplasmas; contamination; diagnostic; eradication; cell culture

BACKGROUND

Mycoplasmas, which measure between 0.3 and 0.8 μm in diameter, are unique prokaryotic microorganisms that lack a cell wall. They are one of the smallest microorganisms capable of surviving independently, residing between bacteria and viruses. More than 200 species of mycoplasma have been identified so far, and new species continue to be discovered annually[1]. Mycoplasma can infect various parts of the body such as lungs, skin, urinary tract, and bloodstream, resulting in a range of symptoms[2].

Apart from the detrimental effects of mycoplasmas on human health, other aspects, especially their impact on cell culture, are also targeted by biological research [3]. Mycoplasmas can infect a wide array of eukaryotic cells, including mammalian, avian, and insect cell lines. Mycoplasma contamination in cell culture can occur through various routes, including contaminated cell lines, reagents, equipment, or personnel. Mycoplasmas have the ability to pass through the filter (0.22 μm) used for decontam-

ination of bacteria, thus posing a high risk of contamination in laboratory cell culture. The contamination of Mycoplasmas can persist and is difficult to detect by means of visual inspection using a conventional microscope. Moreover, while antibiotics like penicillin and streptomycin kill many kinds of bacteria to prevent bacterial contamination, Mycoplasmas are highly resistant to them. As a result, preventing mycoplasma infections via antibiotic use turns out to be a tough challenge.

It has been well-known that the presence of mycoplasma contamination significantly impacts virtually all aspects of cell biology and pathogenesis[4]. The insidious nature of mycoplasma contaminants tends to make contamination go undetected and unnoticed, consequently jeopardizing the reliability of research results and conclusions. For instance, (1) Mycoplasma orale impedes host cell growth by competing for arginine in culture media. This can lead to inconsistencies and less convincing interpretations of experimental findings. This is particularly true of long-term experiments or those requiring precise cell

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counting [5]; (2) Mycoplasma-related endonucleases can degrade internucleosomal DNA in cell culture, thus altering intracellular signaling pathways, enzymatic activities, and metabolic fluxes [6]; (3) Mycoplasma infection can contaminate genomic DNA, leading to sequencing failure or misalignment in genomic DNA sequencing [7]; (4) Mycoplasma contamination has been shown to interfere with the immune response of infected cells [8]. The contaminations can modulate host immune signaling pathways, thereby potentially confounding studies investigating immune-related processes or therapeutic interventions [9]. (5) Infection of Mycoplasma can potentially dysregulate hundreds of host genes and thus affect the interpretation of gene expression studies and disrupt the reproducibility of experiments. Differential gene expression between infected and uninfected cells may inadvertently be attributed to experimental conditions or treatment effects, leading to incorrect conclusions [10].

Since the infection of mycoplasmas triggers global changes in gene expression and chromatin state in host cells, it is crucial to place greater emphasis on the RNA sequence-related studies. Currently, high-throughput sequencing techniques are growing fast, such as transposase accessible chromatin sequencing (ATAC-seq) and RNA sequencing (RNA-seq), which allow for the characterization of chromatin accessibility in an unprecedented manner. However, unlike RNA-seq samples that effectively mitigate the impact of mycoplasma contamination through poly(A) enrichment of RNA [7], ATAC-seq employs Tn5 transposase to detect chromatin accessibility on a genome-wide scale, which renders the sequencing results substantially subject to mycoplasma contamination [11]. Consequently, routine and frequent testing, rigorous cell culture techniques, accident prevention, maintaining a clean laboratory environment, and discarding infected cell culture are the most viable options to minimize contamination [12,13].

The objective of this protocol was to design and develop a reliable, rapid and standardized assay for preventing, detecting, and eradicating mycoplasma contamination in cell culture. Additionally, we compared the ATAC-seq results of samples before and after mycoplasma contamination to provide a valuable reference for studying epigenetic regulation.

MATERIALS AND METHODS

Reagents

- ✓ DMEM (Sigma, Cat#C11995500BT)
- ✓ Phosphate-buffered saline (PBS) (Sigma, Cat#D8537-500ML)
- ✓ RPMI 1640 (Sigma, Cat# R8758-500ML)
- ✓ FBS (Life Technologies, Cat#04-001-1ACS)
- ✓ Horse serum (Life Technologies, Cat#04-004-1A)
- ✓ α -MEM (Life Technologies, Cat#C12571500BT)
- ✓ GlutaMAX™-I (Gibco, Cat#35050-061)
- ✓ I-inositol (Sigma Aldrich, Cat#I7508)

- ✓ Folic acid (Sigma-Aldrich, Cat#F8758)
- ✓ 2-ME (Gibco, Cat#21985-023)
- ✓ Human rIL-2 (PeproTech, Cat#200-02-100UG)
- ✓ 2 × Taq Plus Master Mix (Dye Plus) (Vazyme, Cat#P212-01)
- ✓ Trans 2 kb Plus DNA marker (Trans, Cat#BM111)
- ✓ Zell Shield® (MB, Cat#13-0050)
- ✓ MycoStrip™ (Invivogen, Cat#rep-mys-20)

Recipes

- ✓ TAE buffer (40 mM Tris-Acetate, 1 mM EDTA, pH 8.3)
- ✓ 1.5% agarose-TAE gel containing 1× Gel stain (Trans, Cat#BS101-02)

Equipment

- ✓ Weighing balance (Mettler Toledo)
- ✓ Conical flask (TianBo)
- ✓ Microwave (Midea)
- ✓ Thermal cycler (BioRad)
- ✓ Gel preparation tank (BioRad)
- ✓ Electrophoresis tank (BioRad)
- ✓ UV transilluminator Camera (BioRad)

Cell culture

- ✓ Adherently growing cells or suspension cell culture, and appropriate culture medium: The 293T cells were thawed and cultured in 10-cm dish at 37°C under 5% CO₂. The cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and 1% Penicillin/Streptomycin (P/S). Myla cells and Jurkat cells were maintained in 1640 medium supplemented with 10% heat-inactivated FBS and 1% Penicillin/Streptomycin (P/S). NK92 cells were maintained in α -MEM supplemented with 2 mM L-glutamine (Life Technologies), 0.2 mM I-inositol (Sigma Aldrich), 0.02 mM folic acid (Sigma-Aldrich), 0.1 mM 2-ME (Invitrogen), 12.5% FBS (Life Technologies), 2.5% horse serum (Life Technologies) and 500 U/mL human rIL-2 (PeproTech).

PCR primers

- ✓ Mycoplasma-F: GGGAGCAAACAGGATTAGTATC-CCT;
- ✓ Mycoplasma-R: TGCACCATCTGTCACTCTGTTA-ACCTC.
- ✓ Primers were stored at -20 °C in small aliquots. Primers are stable for several years at -20 °C. Multiple freeze-thaw cycles should be avoided.

Transposase-accessible chromatin with high-throughput sequencing (ATAC-seq)

- ✓ Myla cells (~50,000) were treated according to the Omni-ATAC-seq protocol from the Kaestner Lab (<https://www.med.upenn.edu/kaestnerlab/protocols.html>).

Bowtie2 with “-very sensitive” mapping parameters. PCR duplicates were removed by using Samtools and only uniquely mapped reads were considered for further analysis. The signal BigWig files were visualized using computeMatrix, plotHeatmap in DeepTools (2.4.2). The distribution patterns of ATAC-seq fragment size of each sample were analyzed with qplot in R studio.

ATAC-seq data analysis

- ✓ Raw sequencing reads were filtered for low-quality ones after adapter removal and aligned on hg38 using

PROCEDURES

Prevention of mycoplasma contamination in cell culture

To prevent mycoplasma contamination, the key strategy is to avoid introduction of mycoplasma in the first place.

1. Wear appropriate personal protective equipment (PPE), including gloves and a clean lab coat, at all times. It is important to change the lab coat at least once a week.
2. Strictly following sterile techniques is vital for safeguarding your cells and preventing contamination. Here are some practical measures:
 - 2.1. Maintain a clean and well-organized cell culture hood to ensure unobstructed airflow.
 - 2.2. Spray with 75% alcohol before placing items in the hood.
 - 2.3. Ensure proper covering of plates and bottles.
 - 2.4. Refrain from swinging your hands and arms over open dishes and flasks.
3. Promptly clean up any spills.
4. Keep the incubator clean and avoid any splashes or spills. Adhere strictly to the cleaning schedule. Regularly disinfect the incubator with bleach. Change or clean the catch-up dish once a week to prevent internal cell contamination.
5. Isolate all new or previously untested cell lines in a designated incubator. Mycoplasmas are particularly prone to spread from one area to another. Whenever possible, don't place new or questionable cell lines near other cells until they have been tested to ensure they are free from contamination.

Diagnosis of mycoplasma contamination in cell culture

(TIMING results are obtained within 3-4 hours.)

A multitude of methods have been developed for detecting Mycoplasma contamination in cell culture, such as direct culture method, DNA staining method, Enzyme-Linked Immunosorbent Assay (ELISA)-based method and PCR assay[14]. PCR tests are extremely sensitive, specific and rapid, allowing researchers to respond quickly to eliminate mycoplasma contamination

when it's detected. Presented here is a diagnostic procedure of mycoplasma contamination in cell culture by using the PCR method.

6. Cell culture supernatants can be tested or prepared for future use after at least 12 hours of cell culture. Transfer 200 μ L of supernatant into a sterile 1.5-mL safe lock tube and incubate the sample at 95°C for 5 minutes.

NOTE: The sample can be stored at 2-8°C for up to one week and at -20°C for a few months.

7. Prepare the negative control, positive control and samples. Add 1 μ L of DNA-free water to the negative control and add 1 μ L of the sample to each of the sample tubes and label them. Positive controls were obtained from MycoStrip™. The reaction tubes provided in the kit include the nucleotides, primers and internal control DNA.
8. Prepare the samples for PCR and determine the total volume of Taq DNA polymerase buffer required for the reactions.

The components of PCR reaction are as follows:

- 5 μ L 2 \times Taq (Roche Expand Long Template PCR System)
- 1 μ L 10 μ M Mycoplasma-F primer (1 μ M final concentration)
- 1 μ L 10 μ M Mycoplasma-R primer (1 μ M final concentration)
- 1 μ L Cell culture supernatants or defrosted samples for storage at 2-8°C
- 2 μ L ddH₂O

NOTE: Gently mix the contents by flicking the tubes. Do not vortex the contents.

PCR thermal cycle profile:

- 1 \times (5 min at 94 °C)
- 40 \times (30 s at 94°C, 30 s at 56 °C; 30 s at 72 °C)
- 1 \times (7 min at 72 °C)

NOTE: Upon completion of the PCR cycles, the samples should be cooled to 4-8 °C by pausing or placing them on ice.

9. Following the PCR reaction, use 10 μ L of the produced sample, and perform 1.5% agarose gel electrophoresis at 120 V until the electrophoresis exceeds two-thirds of the gel (refer to the example gel in **Fig. 1**). Use 5 μ L of Trans 2 kb DNA ladder to mark the DNA fragments. Store the remaining samples at -20°C for future use.

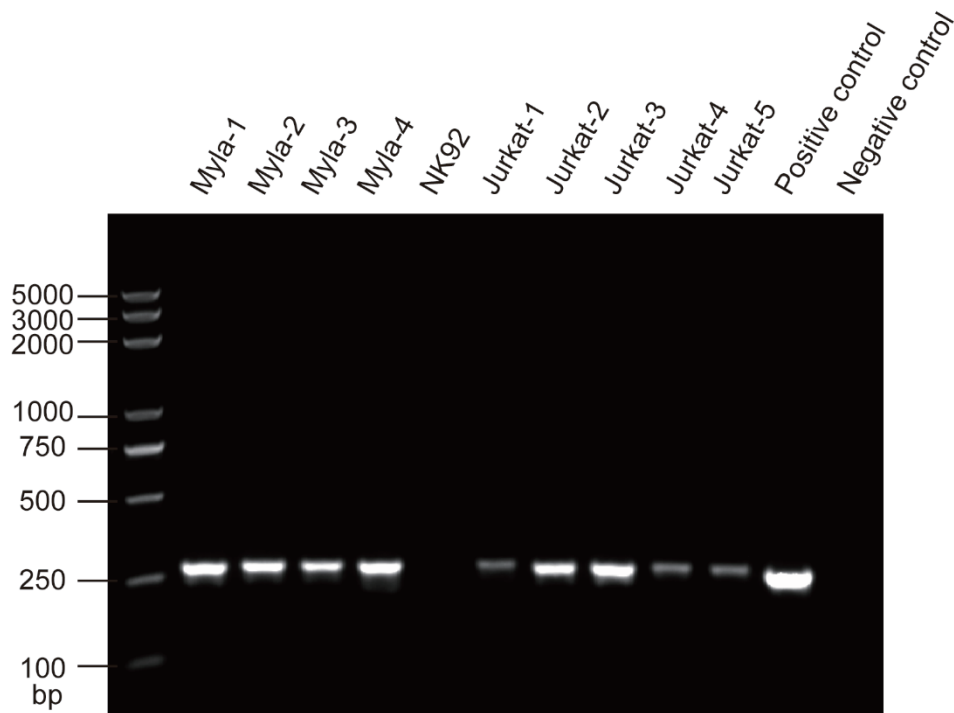


Figure 1 PCR analysis of mycoplasma contamination samples. From left to right: lane 1, Trans2K DNA ladder; line 2-5, Myla cell samples; lane 6, NK92 cell samples; lane 7-11, Jurkat cell samples; lane 12, positive sample; lane 13, negative sample (PBS).

Eradication of mycoplasma contamination in cell culture

(TIMING results are obtained within 1-2 weeks.)

Once mycoplasma is detected, researchers can either eliminate the contamination from the cultures or start over. Due to the importance or scarcity of cell lines, researchers often prefer eliminating the mycoplasma contamination. Zell Shield® is a commercial agent that eliminates a broad range of contaminants such as bacteria, fungi, yeast and mycoplasmas. Here we described how to eradicate mycoplasma contamination by using Zell Shield®.

10. Following reagent manufacturer’s protocol of Zell Shield®, put 0.5 mL Zell Shield® (100×) to a 45 mL cell medium.

NOTE: Zell Shield® is a novel, ready-to-use, multi-agent antibiotic with minimal side effects. It effectively eliminates mycoplasma, bacteria, fungi, and yeast contamination in cell culture. Zell Shield® can be directly added to the culture medium.

11. When the cells in the 10-cm dish reach 90% confluency, use trypsin to digest the 293 T cells, or directly transfer NK92 and Myla cells into a 15-mL centrifuge tube.

12. Centrifuge the cells at 1,000 rpm for 5 min at room temperature. Remove the supernatant and resuspend the cells in 5 mL 1 × PBS.

NOTE: Take 200 µL of supernatant from each sample (the supernatant must be processed for

PCR). Perform the eradication efficiency quality control.

13. Carefully discard the supernatant and resuspend the cells with another 5 mL PBS. Gently pipette up and down to mix cells in all plates. Centrifuge all samples for 5 min at 1,000 rpm at room temperature.
14. Wash the cell plates three times with 5 mL 1×PBS as described in eradication step 13 (step4 in Fig. 2).

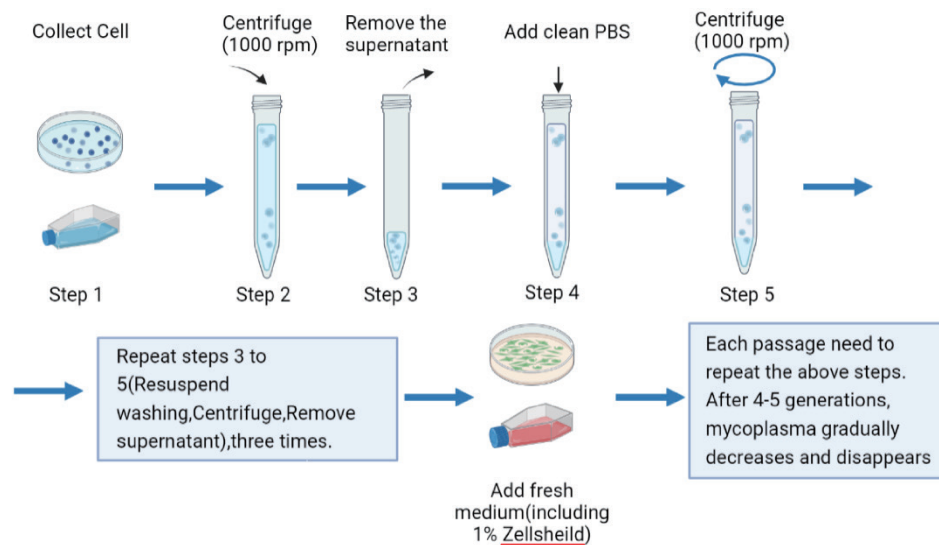


Figure 2 The process of removing mycoplasma.

15. Remove the supernatant and resuspend the cells in fresh medium containing Zell Shield®.
16. According to cell density, repeat steps 12 to 15 (step 2 to 6 in Fig. 2) four times (treatment for a total of 10 days).
17. Collect four supernatants and perform mycoplasma testing as done in PCR procedure.
18. Test the cultures for mycoplasma contamination (refer to the example gel in Fig. 3). Cells cultured without Zell Shield® for an additional 7 days. Afterwards, they should be checked again and then frozen in liquid nitrogen.

Results

The interpretation of testing results involves comparing the presence and size of the PCR bands in samples with those appearing in the positive control reaction. Both the positive control and inhibition sample display a 270 bp band, while the negative controls exhibit no observable bands (refer to Fig. 1). A test is considered valid only if the negative controls yield negative results, and

both the positive control and inhibition sample produce positive results [15].

The results of mycoplasma eradication, as determined by PCR, are evaluated by comparing the presence and size of the PCR bands from test samples that involve collecting supernatant during cell transfer with those of the positive control reaction. The results demonstrate that as cells are repeatedly washed and exposed to Zell Shield[®], the 270-bp band observed through gel electrophoresis becomes faint and eventually disappears (example gel in **Fig. 3A**)

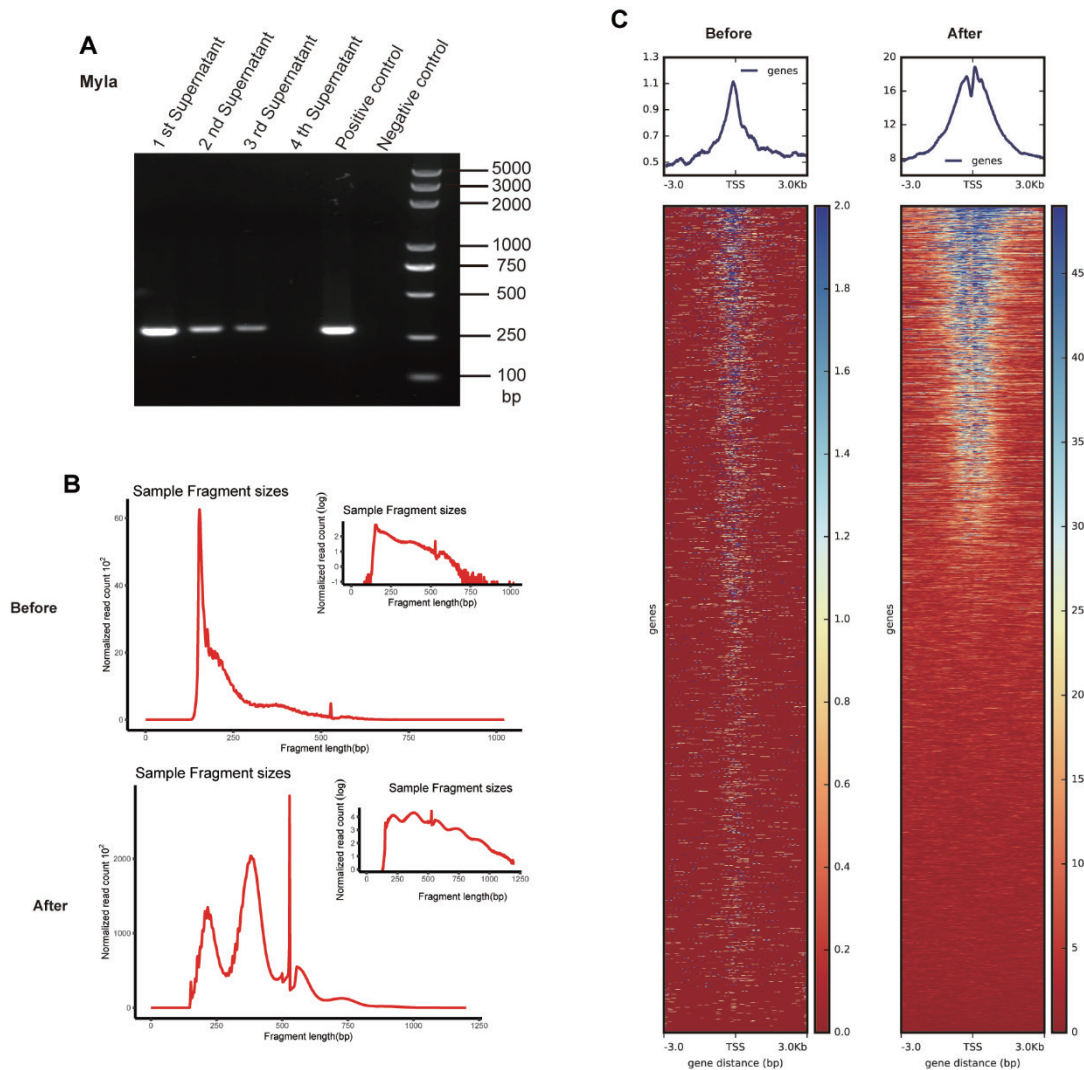


Figure 3 Results before and after removal of mycoplasma. (A) Representative gel electrophoresis image of PCR during Mycoplasma clearance process. From left to right: lane 1, the first collected supernatant; lane 2, second collected supernatant; lane 3, third collected supernatant; lane 4, fourth collected supernatant; lane 5, positive sample. line 6, negative sample (PBS). (B) Fragment lengths within a representative ATAC-seq library before and after removal of mycoplasma. The small fragments represent sequence reads in open chromatin, while the peak at ~150 bp results from sequence reads that span one nucleosome, and larger peaks represent progressively more compact chromatin. (C) Heatmap displaying changed ATAC-seq signals before and after removal of mycoplasma. Each row denotes a 3-kb window centered at TSS. N=2 biological replicates before and after ATAC-seq.

Since the infection of mycoplasmas triggers global changes in gene expression and chromatin state in host cells[16], ATAC-seq results not only indicate changes in chromatin accessibility before and

after Mycoplasma contamination but also provide an illustrative example of how Mycoplasma contamination can impact experimental results. All ATAC-seq libraries exhibit the expected distribution of fragment lengths, with the majority of fragments being small, representing internucleosome open chromatin, and progressively fewer fragments of larger size spanning nucleosomes. However, in the sample with mycoplasma contamination, we found only a 0.49% mapping rate of contaminated ATAC-seq library and most of the fragments were predominantly concentrated at 150 bp (**Fig. 3B**). Additionally, the heatmap displaying ATAC-seq signals is perturbed to an extent that it interfered with the analysis of chromatin accessibility (**Fig. 3C**). In summary, ATAC-seq data showed higher data alignment and sequence SNR (Signal Noise Ratio) after mycoplasma removal.

TROUBLESHOOTING

Due to the limited scope of our investigation, we did not conduct a comprehensive screening of all commercial mycoplasma clearance agents, and we only performed a partial screening (results not shown). Among the mycoplasma clearance agents we tested, Zell Shield® has proven to be more effective without causing any damage to cells. For previous the steps of mycoplasma clearance methods, some cell lines infected with Mycoplasma will experience changes in cell size and morphology, and some will even suppress cell proliferation due to drug toxicity. In this study, we reduced the concentration of mycoplasma clearance agents and minimized the number of PBS washes during the process. Finally, we successfully eliminated mycoplasma within 4-5 cycles, and demonstrated that the cell state and biological function remained unaffected. In conclusion, the removal of Mycoplasma enhances cell proliferation, and ensure the accurate experimental results in the research of chromatomics.

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