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# Preparation of Biofilm Assay Using 96-Well and 6-Well Microplates for Quantitative Measurement and Structural Characterization: A Review

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ABSTRACT

The term "microbial biofilm" refers to three-dimensional, stationary populations of microorganisms comprising various cell types encased in a matrix primarily produced by the microbial community. In biofilm research, selecting the appropriate experimental platform is crucial, as it determines the data gathered and, consequently, impacts the quality of the studies. Biofilm biomass and viability can be assessed using the crystal violet assay and resazurin assay, respectively. Due to its convenience and adaptability, the 96-well microplate is frequently employed in various scientific and medical applications. Various characterization techniques for biofilm assays using the 6-well microplate include light microscopy, confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM), atomic force microscopy (AFM), and FTIR (Fourier-transform infrared) spectroscopy. This mini review discusses the measurement of biofilm biomass and viability, experimental protocols for crystal violet and resazurin assays, and potential issues in microplate-based biofilm assays.

## INTRODUCTION

The discovery of antibiotics, commercialization, and administration to treat infections has improved therapy and revolutionized modern medicine. Indeed, antibiotic administration has become one of the key medical procedures required for routine clinical interventions such as organ transplantation, surgery, and cancer care. Unfortunately, the significant rise in antibiotic resistance among common bacterial pathogens now threatens this therapeutic achievement, challenging critical patient treatment [1]. Resistance to antibiotics has been described as one of the highest threats to public health in the 21st century [2].

Microbial biofilms are defined as stationary communities of microorganisms in a three-dimensional structure, comprising various cell types enclosed in a matrix primarily synthesized by the microbial community [1-3]. Biofilm formation involves initial microbial adhesion followed by the

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production and buildup of an extracellular matrix consisting of polymers like proteins, polysaccharides, humic substances, and extracellular DNA, along with molecules facilitating cell communication [4,5]. Since the late 1970s, biofilm science and technology have been actively studied, originating from the seminal work of Bill Costerton and colleagues in 1978. Presently, it is widely acknowledged that most microbes in natural settings exist within structured biofilm ecosystems rather than as free-floating entities. Over the past four decades, technological advancements have significantly influenced the understanding of biofilms by developing new imaging techniques, biochemical assays, and tools for molecular ecosystem biology. These advancements have enabled researchers to obtain comprehensive views of biofilm structures in three dimensions and enhanced understanding at the nano-scale level [6,7].

Selecting the right experimental platform for biofilm studies dictates the data that can be obtained, requiring careful consideration to meet experimental needs. Each platform has its pros and cons. Popular choices for biofilm assays encompass microplate, Calgary device, flow chamber, and microfluidics [8-10]. The use of 96-well and 6-well microplates in biofilm research has several advantages that contribute to their importance for high-throughput antibiofilm screening and biofilm characterization, including high-throughput capability, reproducibility, ease of handling and automation, and cost-effectiveness. This mini-review discusses the principles, applications, and potential issues of biofilm assays using both 96-well and 6-well microplates.

## Measurement of Biofilm Biomass and Viability

Various methods are available to measure biofilm biomass and viability, utilizing the biofilm's microbiological, molecular, or physical and chemical properties. The most common method for estimating biofilm cell viability is the colony forming units (CFU) assay on agar media, which is widely accessible in microbiological laboratories. However, this method has drawbacks, including potential misrepresentation of the initial biofilm population and inability to detect viable but non-culturable (VBNC) cells. Alternatively, flow cytometry, though costlier, accurately determines biofilm cell viability, overcoming CFU counting limitations by distinguishing between total, dead, and VBNC cells [11,12]. Quantifying biofilm-viable organisms with qPCR is an alternative to culture but can be overestimated due to extracellular DNA and DNA from dead cells [13]. To avoid this, propidium monoazide (PMA) treatment can used before DNA extraction, selectively entering compromised cells and forming a stable bond with DNA upon exposure to visible light [14].

Biofilm biomass can also be determined through dry or wet weight measurements. The weight difference between a dried slide with biofilm and a cleaned, dried slide with pre-biofilm formation can be utilized. Alternatively, surfaces with attached cells can be vortexed and released biofilm components can be filtered. This approach measured the weight of a dried filter containing biofilm components against a sterile control filter. However, it may underestimate biomass due to incomplete biofilm removal and the passage of small molecules through the filter. This method is limited by time consumption and needs more sensitivity to minor changes in biofilm production. Chemical techniques employ dyes or fluorochromes to bind to biofilm components, indirectly measuring specific elements like EPS. Crystal Violet (CV) staining is commonly used in microtiter plate assays for quantifying total biofilm biomass [15, 16]. However, variations in washing steps can detach sessile bacterial cells, influenced by factors like microbial surfaces and air-bubble velocity. Different staining agents, such as safranin, can be utilized. Concentrated ethanol or a 33% acetic acid solution effectively releases dye. A fixation step with ethanol or methanol at 60°C for 1 hour before staining improves assay reproducibility. Microtiter plate dye-staining offers versatility, avoiding detachment needed for plate counts and enabling high-throughput testing. Limitations include variability due to washing steps and a lack of reproducibility.



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Resazurin, or Alamar Blue, a stable redox indicator, is reduced to resorufin by metabolically active cells, offering advantages such as visual, spectrophotometric, or spectrofluorometric monitoring [17]. Results from resazurin-based quantification correlate well with CFU counts [18]. Limitations include high lower limits of quantification and varied metabolic rates among microorganisms**.** An alternative approach involving fresh growth medium with resazurin reduces the lower limit of quantification, aiding in quantifying anti-biofilm treatments more accurately. Different incubation times for biofilms formed by various species hinder their application to polymicrobial consortia. Colorimetric techniques assess biofilm biomass by quantifying exopolysaccharides, total proteins, and carbohydrates [19]. However, the levels of specific EPS components may not directly correspond to biofilm biomass. To address this, measuring phospholipids, uniformly distributed cellular components expressed consistently across microbial communities, may be useful [20]. Determining phospholipids is constrained by factors such as recovery rate, background lipid contamination, and analytical equipment sensitivity.

## High Throughput Antibiofilm Screening Using 96-Well Microplate

A microplate, also known as a microtiter plate, is a multi-well plate typically made of polystyrene, though other materials like polypropylene or glass can also be used. These plates contain numerous small wells arranged in a grid pattern, with standard formats including 6, 24, 96, 384, or 1536 wells. Microplates are commonly used in various scientific and medical applications due to their versatility and ease of use. The materials used to produce microplates, such as polystyrene, are chosen for their optical clarity, compatibility with automation systems, and inertness to biological samples. Polystyrene microplates are optically transparent, making them suitable for various detection methods in biofilm studies, including absorbance, fluorescence, and luminescence assays [21]. Microplates find wide application in areas such as high-throughput screening in drug discovery, enzyme-linked immunosorbent assays (ELISA), cell culture, DNA and RNA analysis, and microbiological studies [22-24]. Their ability to accommodate small sample volumes, high reproducibility, and scalability make microplates indispensable tools in high throughput screening of a wide range of antibiofilm compounds such as phytochemicals, nanomaterials, and enzymes (Figure 1).

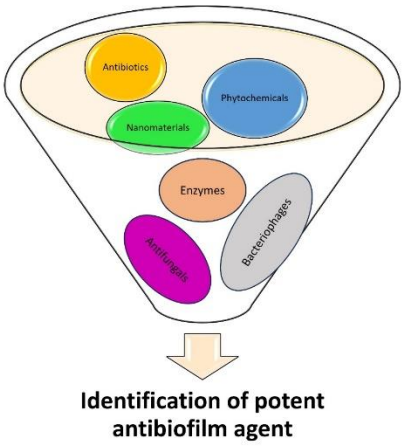


Figure 1: Various types of antibiofilm agents.



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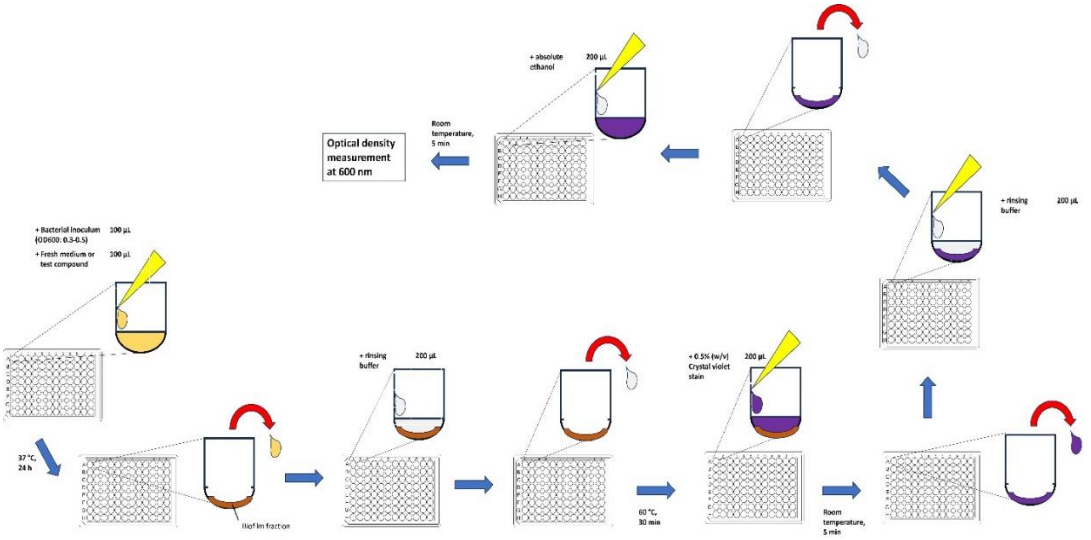
The microtiter plate is effective for studying bacterial attachment and sessile development. In polystyrene microtiter plate wells, bacterial cells proliferate. Planktonic cells are washed out, and the attached biomass is stained [15, 16, 25]. Detachment and plating are steps in quantifying biomass, though biomass from sources other than biofilm formation may be included. This article provides a protocol that outlines simple, repeatable steps for assessing how well novel antibiofilm compounds inhibit and eradicate bacterial biofilms grown in 96-well microtiter plates. It utilizes two cost-effective dyes: crystal violet for staining the attached biofilm biomass (Figure 2) and resazurin for measuring the metabolic activity of biofilm cells (Figure 3).

(A)

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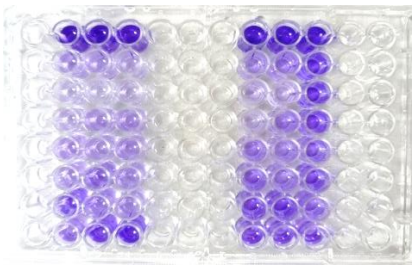

Figure 2: (A) Experimental protocols for crystal violet staining in microplate biofilm assay. (B) Representative microplate image after completion of protocols.

Biofilm formation poses challenges across environmental, industrial, and biomedical domains. Regardless of the context, factors like surface characteristics, temperature, nutrient availability, hydrodynamics, pH, and microbial attributes influence its development [26, 27]. Hu [28] reported an upregulation in biofilm-related genes (*csgD*, *glgA*, *bcsa*, *pdeN*, *dgcC*, *pfs*, and *luxS*) at 25°C compared to 37 °C, whereas other genes experienced downregulation at this lower temperature. However, at 42 °C, the



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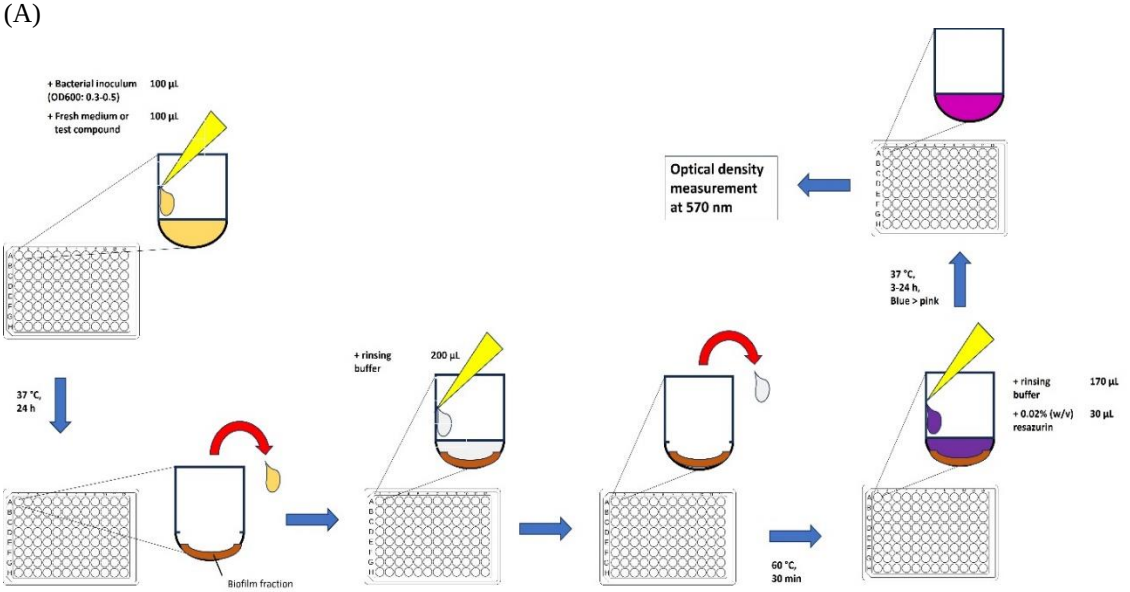
*dgcC* and *pfs* genes showed upregulation while others were downregulated. Furthermore, compared to the gene expression pattern observed in the M9 medium containing glucose, where only *luxS* did not change, the remaining biofilm-related genes were suppressed when the DE17 strain was grown in the M9 medium supplemented with ribose. On the other hand, when cultured in an M9 medium supplemented with fructose, five biofilm-related genes (*csgD*, *glgA*, *bcsA*, *pdeN*, and *pgaA*) were downregulated. In contrast, the expression of other genes increased.

(A)

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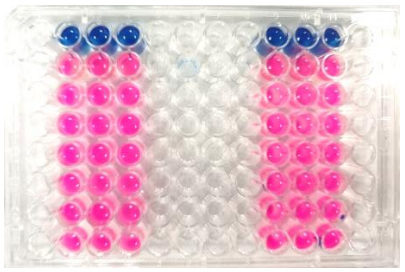

Figure 3: (A) Experimental protocols for resazurin staining in microplate biofilm assay. (B) Representative microplate image after completion of protocols.

## Biofilm Characterization After Biofilm Assay Using 6-Well Microplate

A six-well microplate assay is often used to obtain biofilm fractions for structural characterization [29-31]. Experimental protocols of biofilm assay using a 6-well microplate are shown in Figure 4. Light microscopy remains an essential technique for visually identifying biofilm formation. Practical and economical methods like Hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and Brown and Brenn Gram staining can detect bacterial biofilms in various infection sites. These staining techniques enable a



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quantitative assessment of biofilm biomass and could hold significant prognostic value. The absorption of light by biofilms correlates with their cell and total mass. While light microscopy offers simplicity and affordability, it has limitations such as resolution constraints and difficulty in morphotypic differentiation, especially in thicker samples. Correlative studies combining light microscopy with Confocal Laser Scanning Microscopy (CLSM), Scanning Electron Microscopy (SEM), Atomic force microscopy (AFM), and FTIR (Fourier-transform infrared) provide a comprehensive approach.

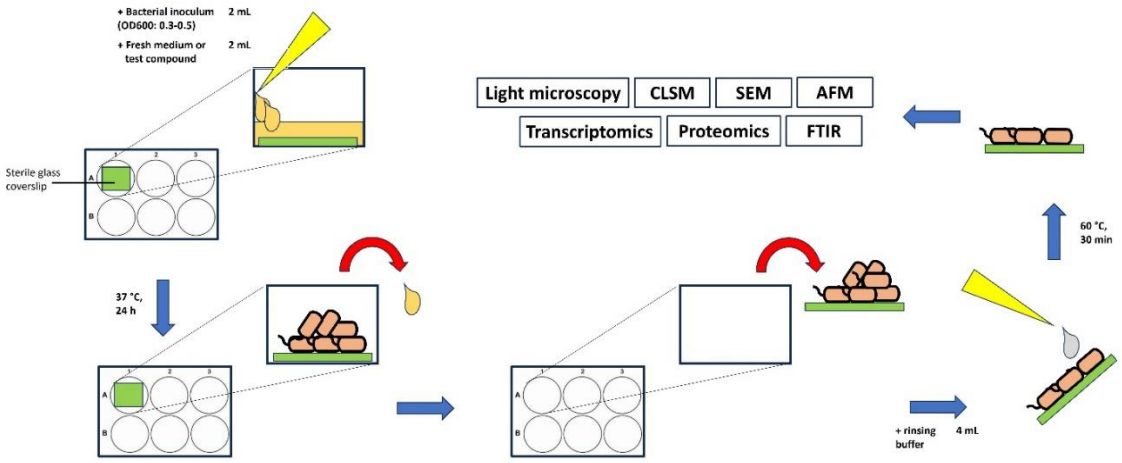


Figure 4: Biofilm assay using 6-well microplate prior to characterization analysis.

Confocal Laser Scanning Microscopy (CLSM) emerged in the early 1990s as a highly versatile and powerful microscopic technique for analyzing biofilms' spatial structure and functions [32]. CLSM eliminates out-of-focus fluorescent signals, allowing the collection of the focal plane with a resolution suitable for single-cell visualization. By acquiring multiple planes at different depths and employing dedicated image analysis, CLSM enables the representation of the sample's 3-D architecture and extraction of quantitative structural parameters such as biofilm volume, thickness, and roughness [33]. It has been successfully utilized across various biofilm types. CLSM imaging of biofilms can utilize various fluorescent probes with specificities. Commonly used stains for labeling microbial cells include cell-permeant nucleic acid dyes like SYTO-9 and SYBR-Green. Specific microorganisms within complex communities can be localized using specific oligonucleotides through fluorescent *in situ* hybridization (FISH) approaches or related methods. Sangha et al. [34] demonstrated that the co-existence of the various bacterial species in the oral biofilm (*S. gordonii* DL1, A. oris MG1, *S. mutans* UA159, *N. subflava* DSM17610, V. *parvula* DSM2008) and the dominance of *S. mutans* at high glucose concentration were successfully confirmed through FISH and visualized through CLSM.

Scanning electron microscopy (SEM) relies on electron surface scattering and absorption. While SEM micrographs offer a broad depth of field, providing a 3-D view beneficial for understanding sample surface structure, they lack vertical resolution. SEM is commonly used to visualize biofilms due to its ability to reveal spatial structure and detect EPS [30]. It is particularly valuable for comparative analysis in biofilm research, especially when assessing the effects of anti-biofilm compounds or treatments [35, 36]. SEM imaging supports findings from other quantification methods, showing high correlation. SEM offers



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advantages such as higher resolution, 3-D data measurement, and a wide range of magnifications. However, SEM's drawbacks include tedious sample preparation processes and potential structural damage or artifact formation. Alternative SEM techniques like cryo-SEM have emerged to address these limitations in biofilm studies [37].

Atomic force microscopy (AFM) is an increasingly robust method for visualizing biological specimens at scales ranging from nanometers to micrometers without causing damage [38]. Its fundamental operation involves scanning a sharp tip across the area of interest while monitoring the interaction between the sample and the tip mounted on a flexible cantilever [39]. When an attractive force is detected, the cantilever deflects, and the force is measured by observing the deflection using a laser beam and photodiode. AFM has evolved into a versatile tool for quantifying adhesion forces between various entities, from living cells to single molecules. Additionally, AFM offers several advantages: it operates under ambient conditions, has uniform resolution in all directions, and generates 3-D topographic images. Initially utilized to visualize biofilms, AFM has elucidated biofilm structure, adhesion mechanisms, and formation processes [40]. It is also valuable for quantitative biofilm analysis, corroborating findings from other quantitative and imaging techniques. AFM provides height and roughness analyses, enabling the quantification of biofilm biomass, including thickness and extracellular polymeric substance (EPS) quantity. Moreover, AFM facilitates the quantitative evaluation of biofilm interactions with surfaces and cohesion. In the study of mechanisms that govern the interactions involved in the biofilm formation performed by Eskhan et al. [41], the adhesion force measured by AFM represents the net of three primary forces: the electrostatic forces, the Lifshitz−van der Waals forces, and the Lewis acid−base forces.

FTIR (Fourier-transform infrared) spectroscopy, a non-destructive method, offers the advantage of rapidly analyzing complex biological samples without the use of reagents or causing destruction. This allows for high-throughput screening and unbiased measurements. It is cost-effective and user-friendly compared to other methods. When molecules absorb infrared radiation, their vibrational modes, involving stretching and bending of the electric dipole, become excited [42]. The different functional groups in organic molecules absorb infrared radiation in relation to their distinct vibrational modes. The FTIR analyzer detects the absorbed infrared radiation to identify the molecular composition of surfaces, determine structural and geometric isomers, analyze the orientation of polymers and solutions, and quantify impurities in the materials. It enables repeated sampling without compromising the integrity of the biofilm or its surroundings, which is essential for tracking temporal changes or assessing treatment responses [15]. The study of biofilm structures using FTIR spectroscopy identified lipids and proteins as critical bands for recognizing structural variations in biofilm formation. Kamaruzzaman et al. [43] demonstrated that Salmonella Typhimurium biofilm treated with chloroxylenol-based disinfectant exhibited changes in FTIR spectral peaks associated with lipids (1460 $cm^{-1}$), proteins (630 $cm^{-1}$, 702 $cm^{-1}$, 1550 $cm^{-1}$ & 1650 $cm^{-1}$), and nucleic acids (1080 $cm^{-1}$ & 1229 $cm^{-1}$). Additional insights into biofilm formation can be obtained in the 750 to 1800 $cm^{-1}$ region, where vibrations of various groups such as C-H, $>CH_2$, and $-CH_3$, as well as amides, carbonyl groups, and polysaccharides, can be observed [44].

Despite significant progress in biofilm research, understanding the mechanisms governing the response of attached or immobilized cells compared to planktonic ones still needs to be completed. Only a handful of studies have delved into the differential expression of proteins in sessile and suspended cells. A deeper understanding of biofilms' overall transcriptomic or proteomic characteristics could facilitate the identification of genetic or proteomic markers with specific functions within the biofilm matrix [45. 46]. This knowledge holds great importance in detecting persistent strains or tracing the contamination route during outbreak investigations. Utilizing -omic methods for biofilm analysis presents a crucial challenge: data originate from harvesting an entire cell population, yet biofilms are inherently heterogeneous [47]. The physiology of bacterial cells varies depending on their location within the biofilm and the stage of development. Consequently, -omic profiling represents an averaged result for a diverse biofilm population



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[48], potentially overlooking unique patterns in underrepresented subpopulations. Thus, physiological or genetic traits derived from -omic profiling must be cautiously interpreted as potentially skewed averages.

## Potential Issues

Figure 5 summarizes the potential issues in performing microplate biofilm assay. During high throughput antibiofilm screening using a 96-well microplate, it is common to observe fluctuations in biofilm biomass and viability. These variations can arise due to several factors, including biofilm dispersion [49] and inconsistent experimental conditions. Several precautionary measures must be followed to ensure consistent and reliable data in microplate biofilm assays. First, meticulous attention to detail when using a micropipette is essential to ensure that the same volume of solutions is loaded into each microplate well. Care should also be taken to avoid the formation of bubbles immediately before measuring the optical density using a microplate reader, as bubbles can interfere with accurate readings. Additionally, it is imperative always to close the lid of the microplate to prevent the introduction of dust particles and to minimize excessive evaporation of solutions, particularly when using absolute ethanol in the crystal violet assay. Finally, all measurements should be conducted in at least triplicate to enhance the reliability and reproducibility of the results.

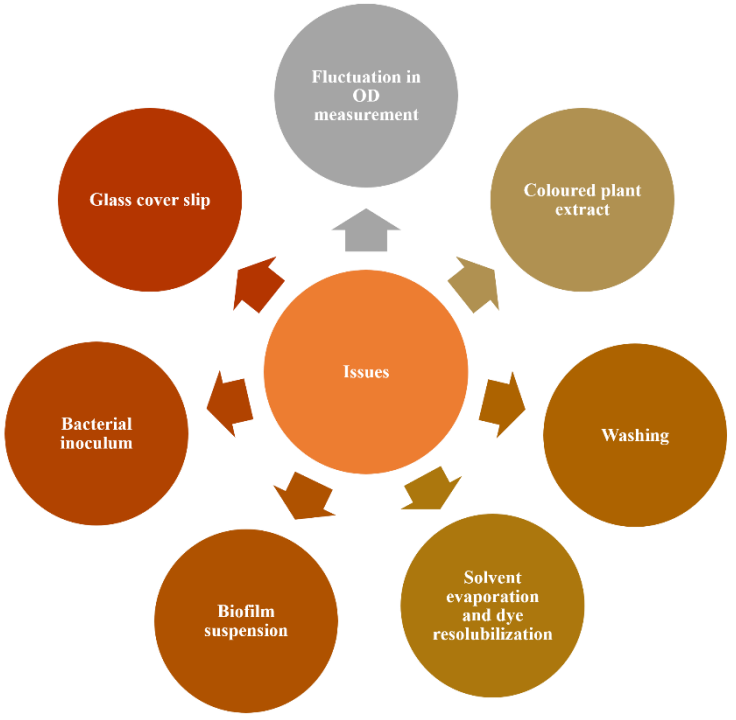


Figure 5: Potential issues in performing microplate biofilm assay.



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In certain cases, high concentrations of plant extracts interfere with the optical density measurement [50]. Interestingly, as the concentration of plant extract increases, the optical density tends to rise, contrary to the expected decrease indicative of reduced biofilm biomass. To address this discrepancy, it becomes imperative to measure the optical density values of each test concentration of plant extract separately and then subtract these values from the actual measurements. This corrective step helps to mitigate the interference caused by the plant extracts, ensuring more accurate and reliable assessments of biofilm biomass. When conducting a 6-well microplate biofilm assay, the glass coverslip utilized is delicate and prone to cracking, requiring careful handling. Typically, sterile forceps delicately remove the coverslip, ensuring minimal disturbance. It is crucial to note that the surface facing upward upon removal is essential for biofilm analysis, as it contains the biofilm of interest [30]. Thus, utmost caution is warranted to ensure the correct surface is utilized for accurate analysis, minimizing errors, and maintaining data integrity.

Washing the biofilm is crucial to remove nonadherent cells and maintain biofilm integrity [51]. Two key aspects of the washing protocol are the number of washings and the technique used. Inadequate washing may yield false positives, while excessive washing may lead to false negatives. The two-step washing protocol is ineffective, while three- and four-step protocols are acceptable. Biofilm can be washed by immersing plates in water or PBS, as pipettes and mechanical washers may disrupt biofilm integrity. Careful pipetting and using micropipettes are recommended. Emptying by flicking is simple and effective, but turning plates upside down will not empty wells due to capillary forces. Splashing content out of plates should be done cautiously to avoid aerosol formation and contamination. Regardless of the method employed, closely observing the integrity of the biofilm during washing is essential. Any wells exhibiting obvious disruption to the biofilm layer should be disregarded in subsequent calculations. Following air-drying of the microplate at room temperature, the dye attached to the cells must be solubilized. It is important to gently introduce the ethanol and then cover the microplate with its lid to prevent evaporation [52], leaving it undisturbed at room temperature for at least 30 minutes without agitation. It is crucial to keep the microplate intact to hasten the solubilization process. Alternatively, 33% glacial acetic acid or methanol can also effectively solubilize the dye from the cells**.**

In the case of a resazurin assay, the biofilm fraction must first be suspended in a rinsing buffer. This aims to ensure that all biofilm cells can be exposed to resazurin solution and metabolize it, changing their blue color to pink. Biofilm fractions that are not first suspended tend to show a slow rate of resazurin color change or do not show any color change at all. Moreover, the color change rate of resazurin also depends on the microorganism species [53]. Different species usually show different resazurin color change rates from each other. The optical density (OD) of crystal violet-stained wells is assessed using a microplate reader. Traditional OD readers pose a challenge as they measure only one point within the well [54], potentially leading to inaccuracies if the biofilm thickness varies. Therefore, ensuring uniform resolubilization of dye bound to bacterial cells is crucial, facilitating indirect yet accurate biofilm production measurement. While some experiments involve transferring decolorized products to a new plate for OD measurement, this costly and time-consuming step is not vital for result accuracy.

Before inoculation, strains under examination must be grown in either broth or solid medium. How the inoculum is prepared, whether from broth or agar, can significantly impact the biofilm of these bacteria because adhesion marks the initial stage of biofilm formation. Typically, infecting bacteria are closely associated with surfaces [55], suggesting that their cell surface is more akin to bacteria cultivated on solid medium rather than those in liquid cultures [56]. Therefore, the inoculum derived from bacteria cultured on agar will likely mirror the *in vivo* conditions more accurately than those grown in broth. In preparing inocula, preventing the inoculation of existing cell clusters is crucial, as they can cause false-positive outcomes [56]. Thus, it is necessary to thoroughly vortex the prepared cell suspensions. Alternatively, any preexisting cell clusters in the testing suspensions can be disrupted using a syringe equipped with a 23-gauge needle, followed by brief vortexing to ensure homogeneity.



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## CONCLUSIONS

The presented protocol integrates accumulated expertise in evaluating biofilm production, drawn from firsthand experience in participating in biofilm research laboratories and a thorough examination of existing methods outlined in the literature. This mini-review meticulously outlines all the steps for quantifying and characterizing biofilm using a 96-well and 6-well microplate, respectively. Additionally, it identifies key challenges encountered during the assay process. These enhancements should facilitate wider procedure adoption, particularly benefiting newcomers to this research field.

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## AUTHOR'S CONTRIBUTION

Siti Sarah Diyana Amran and Anati Abd Rashid Syaida wrote and revised the article. Mohd Taufiq Mat Jalil and Nurul Hidayah Mohamad Nor reviewed the article. Mohd Fakharul Zaman Raja Yahya conceptualized the central research idea and approved the article submission.

## CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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